IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

ZYMOGENETICS, INC., a Washington Corporation, Plaintiff, v. BRISTOL-MYERS SQUIBB CO., a Delaware Corporation, and DOES 1 through 100, Defendants.	)	Civil Action No: 06-500-SLR-MPT REDACTED PUBLIC VERSION

DECLARATION OF JOHN W. KIRKLAND IN SUPPORT OF BRISTOL-MYERS SQUIBB COMPANY'S MOTION FOR SUMMARY JUDGMENT OF NONINFRINGEMENT

I, John W. Kirkland, hereby declare the following:

1. I am an associate with the law firm of Fitzpatrick, Cella, Harper & Scinto, attorneys for Defendant Bristol-Myers Squibb Company ("Bristol") in this action. I submit this Declaration in support of Bristol's Motion for Summary Judgment of Noninfringement.

2. Attached hereto as Exhibit 1 is a true and correct copy of U.S. Patent No. 6,018,026 (ZG000001-77).

3. Attached hereto as Exhibit 2 is a true and correct copy of U.S. Patent No. 5,843,725 (ZG000842-919).

4. Attached hereto as Exhibit 3 is a true and correct copy of Plaintiff ZymoGenetics' Responses to Bristol-Myers Squibb Company's First Set of Interrogatories (12/18/2006).

5. Attached hereto as Exhibit 4 is a true and correct copy of Plaintiff ZymoGenetics' Third Supplemental Response to Bristol-Myers Squibb Company's First Set of Interrogatories (9/25/2007).

6. Attached hereto as Exhibit 5 is a true and correct copy of the product label for Orencia® (BMS004286461-485).

7. Attached hereto as Exhibit 6 is a true and correct copy the publication Magistrelli et al., *A soluble form of CTLA4 generated by alternative splicing is expressed by nonstimulated human T cells*, EUR. J. IMMUNOL. 29:3596-3602 (1999).

8. Attached hereto as Exhibit 7 is a true and correct copy of the publication Oaks et al., *A native soluble form of CTLA-4*, CELL. IMMUNOL. 201:144-153 (2000).

9. Attached hereto as Exhibit 8 is a true and correct copy of the publication Saverino et al., *Soluble CTLA-4 in autoimmune thyroid diseases: Relationships with clinical status and possible role in the immune response dysregulation*, CLIN. IMMUNOL. 123:190-198 (2007).

10. Attached hereto as Exhibit 9 is a true and correct copy of the publication Brunet et al., *A new member of the immunoglobuline superfamily -- CTLA-4*, NATURE 328:267-270 (1987).

11. Attached hereto as Exhibit 10 is a true and correct copy of the publication Linsley et al., *Binding Stoichiometry of the Cytotoxic T lymphocyte-associated Molecule (CTLA-4)*, J. BIOL. CHEM. 270:15417-15424 (1995).

12. Attached hereto as Exhibit 11 is a true and correct copy of the publication Linsley et al., *Coexpression and Functional Cooperation of CTLA-4 and CD28 on Activated T Lymphocytes*, J. EXP. MED. 176:1595-1604 (1992).

13. Attached hereto as Exhibit 12 is a true and correct copy of the publication Schwartz et al., *Structural basis for costimulation by the human CTLA-4/B7-2 complex*, NATURE, 410:604-608 (2001).

14. Attached hereto as Exhibit 13 is a true and correct copy of the publication Stamper et al., *Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses*, NATURE 410:608-611 (2001).

15. Attached hereto as Exhibit 14 is a true and correct copy of the publication Schwartz et al., *Structural mechanisms of costimulation*, NATURE IMMUNOLOGY, 3:427-34 (2002).

16. Attached hereto as Exhibit 15 is a true and correct copy an Excerpt of Carl Branden & John Tooze, *Introduction to Protein Structure*, GARLAND PUBLISHING INC. (1991) at 180-181.

17. Attached hereto as Exhibit 16 is a true and correct copy of Section 3.2.S.2.3.3.3, Confirmation of the CTLA4 Ig Gene Sequence in Plasmid pcSDhuCTLA4 Ig, Abatacept Biologics License Application (2005) at BMS001290348-351.

18. Attached hereto as Exhibit 17 is a true and correct copy of Excerpts of Peter Linsley Trial Testimony, *The Regents of the University of Michigan v. Bristol-Myers Squibb Co.*, Case No. 73690 (E.D. Mich. April 9, 2003) at BMS004254737-746, 004254750-751, 004254759-764, 004254769-70; 004254772-777, 004254783-88; 004254835-837.

19. Attached hereto as Exhibit 18 is a true and correct copy of the publication Damle et al., *Differential regulatory signals delivered by antibody binding to the CD28 (Tp44) molecule during the activation of human T lymphocytes*, J. IMMUNOL. 140:1753-1761 (1988).

20. Attached hereto as Exhibit 19 is a true and correct copy of Excerpts of Peter Linsley Notebook No. 1670, BMS 08746-748, 08803, 08858-8859.

21. Attached hereto as Exhibit 20 is a true and correct copy of Excerpts of Peter Linsley Notebook No. 1748, BMS 18955-957, 19104.

22. Attached hereto as Exhibit 21 is a true and correct copy of Excerpt of William Brady Trial Testimony, *The Regents of the University of Michigan v. Bristol-Myers Squibb Co.*, Case No. 73690 (E.D. Mich. April 14, 2003) at BMS004254840, 004254880-881.

23. Attached hereto as Exhibit 22 is a true and correct copy of Excerpts of Nicholas R.J. Gascoigne, Ph.D. Dep. Tr. at 20; 21; 32; 34; 35; 36; 43; 45; 48; 49; 51; 52; 54; 75; 76; 79; 80; 91; 94; 106; 118; 129; 130; 144; 145; 166; 171; 174; 178; 182; 202-205; 210; 217; 219; 220.

24. Attached hereto as Exhibit 23 is a true and correct copy of 5,155,027 Pros. Hist., Office Action (3/2/1990) at BMS004259274-279.

25. Attached hereto as Exhibit 24 is a true and correct copy of U.S. Patent No. 4,816,397 ("Boss (A)").

26. Attached hereto as Exhibit 25 is a true and correct copy of EP 0 120 694 ("Boss (L)").

27. Attached hereto as Exhibit 26 is a true and correct copy of 5,155,027 Pros. Hist., Amendment (6/29/1990) at BMS004259284-290.

28. Attached hereto as Exhibit 27 is a true and correct copy of an Excerpt of Wayne Kindsvogel Ph.D. Dep. Tr. at 91; 92; 217; 218.

29. Attached hereto as Exhibit 28 is a true and correct copy of Excerpts of David A. Ostrov Ph.D. Dep. Tr. at 43; 45; 54; 59; 60; 66; 70; 79; 80; 116; 135; 153; 175; 234; 235; 284.

30. Attached hereto as Exhibit 29 is a true and correct copy of an Excerpt of Paul E. Purdue Ph.D. Dep. Tr. at 114; 115.

31. Attached hereto as Exhibit 30 is a true and correct copy of Linsley et al., *CD28/CTLA-4 receptor structure, binding stoichiometry and aggregation during T-cell activation*, RESEARCH IN IMMUNOL. 146:130-40 (1995).

32. Attached hereto as Exhibit 31 is a true and correct copy of 5,155,027 Pros. Hist., Office Action (8/23/1990) at BMS004259314-16.

33. Attached hereto as Exhibit 32 is a true and correct copy Merriam-Webster's Online Dictionary, http://www.merriam-webster.com/dictionary/moiety, last visited on 5/15/2008.

34. Attached hereto as Exhibit 33 is a true and correct copy of 5,155,027 Pros. Hist. Amendment (11/19/1990) at BMS004259320-22.

35. Attached hereto as Exhibit 34 is a true and correct copy of an Excerpt of Andrzej Sledziewski Ph.D. 30(b)(6) Dep. Tr. at 251; 252.

36. Attached hereto as Exhibit 35 is a true and correct copy of an Excerpt of Gary E. Parker 30(b)(6) Dep. Tr. at 30.

37. Attached hereto as Exhibit 36 is a true and correct copy of Plaintiff ZymoGenetics, Inc.'s Amended and Supplemental Response to Defendant Immunex Corp.'s First Set of Interrogatories, Interrogatory No. 2, *ZymoGenetics Inc. v. Immunex Corp.*, C02-561 (BJR) (D. Wa. Aug. 5, 2002) at (ZG058813-33).

38. Attached hereto as Exhibit 37 is a true and correct copy of Gascoigne Deposition Exhibit 6 (Schwartz et al., *Structural basis for costimulation by the human CTLA-4/B7-2 complex*, NATURE, 410:604-608 (2001)).

39. Attached hereto as Exhibit 38 is a true and correct copy of Gascoigne Deposition Exhibit 14 (Stamper et al., *Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses*, NATURE 410:608-611 (2001)).

40. Attached hereto as Exhibit 39 is a true and correct copy of Gascoigne Exhibit 3 (Brunet et al., *A new member of the immunoglobuline superfamily -- CTLA-4*, NATURE 328:267-270 (1987)).

41. Attached hereto as Exhibit 40 is a true and correct copy of Ostrov Exhibit 3 (Brunet et al., *A new member of the immunoglobuline superfamily -- CTLA-4*, NATURE 328:267-270 (1987)).

I declare under penalty of perjury under the laws of the United States of America that the forgoing is true and correct.

Executed this 16th day of May, 2008, in New York, New York.

John W. Kirkland

UNITED STATES DISTRICT COURT
DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I HEREBY CERTIFY that on May 23, 2008, I electronically filed the foregoing document with the Clerk of Court using CM/ECF and caused the same to be served on the plaintiff at the address and in the manner indicated below:

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EXHIBIT 1

US006018026C1

(12) EX PARTE REEXAMINATION CERTIFICATE (5438th)
United States Patent
Sledziewski et al.

(10) Number: US 6,018,026 C1
(45) Certificate Issued: *Jul. 4, 2006

(54) BIOLOGICALLY ACTIVE DIMERIZED AND MULTIMERIZED POLYPEPTIDE FUSIONS

(75) Inventors: Andrzej Z. Sledziewski, Seattle, WA (US); Lillian Anne Bell, Seattle, WA (US); Wayne R. Kindsvogel, Seattle, WA (US)

(73) Assignee: ZymoGenetics, Inc., Seattle, WA (US)

Reexamination Request:
No. 90/006,971, Mar. 16, 2004

Reexamination Certificate for:
Patent No.: 6,018,026
Issued: Jan. 25, 2000
Appl. No.: 08/980,400
Filed: Nov. 26, 1997

(*) Notice: This patent is subject to a terminal disclaimer.

Certificate of Correction issued Jul. 13, 2002.

Related U.S. Application Data

(60) Division of application No. 08/477,329, filed on Jun. 7, 1995, now Pat. No. 5,750,375, which is a continuation of application No. 08/180,195, filed on Jan. 11, 1994, now Pat. No. 5,567,584, which is a continuation of application No. 07/634,510, filed on Dec. 27, 1990, now abandoned, which is a continuation-in-part of application No. 07/347,291, filed on May 2, 1989, now Pat. No. 5,155,027, which is a continuation-in-part of application No. 07/146,877, filed on Jan. 22, 1988, now abandoned.

(51) Int. Cl.
C07K 19/00 (2006.01)
C07K 16/46 (2006.01)
C07K 14/71 (2006.01)

(52) U.S. Cl. 530/350; 530/387.3; 530/388.24; 530/399; 530/402

(58) Field of Classification Search 530/350, 530/387.3, 388.24, 399, 402; 435/69.7
See application file for complete search history.

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Primary Examiner—Prema Mertz

(57) ABSTRACT

Methods for producing secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof.

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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims 9–11 and 13–29 is confirmed.

Claims **1**, **7** and **12** are determined to be patentable as amended.

Claims **2–6** and **8**, dependent on an amended claim, are determined to be patentable.

New claim **30** is added and determined to be patentable.

1. A biologically active, dimerized polypeptide fusion, comprising:

first and second polypeptide chains, wherein each of said polypeptide chains comprises a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein heterologous to said non-immunoglobulin polypeptide, *wherein the dimerizing protein is selected from the group consisting of yeast invertase, at least a portion of an immunoglobulin light chain comprising at least an immunoglobulin light chain constant region domain, and at least a portion of an immunoglobulin heavy chain comprising at least an immunoglobulin heavy chain constant region domain.*

7. The biologically active, dimerized polypeptide fusion of claim **1** wherein the dimerizing protein *of* one of said polypeptide chains comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

12. A biologically active, multimerized polypeptide fusion comprising: a non-immunoglobulin polypeptide requiring multimerization for biological activity joined to an immunoglobulin heavy chain constant region; and

an immunoglobulin light chain.

30. The polypeptide fusion of claim 1 wherein the dimerizing protein is at least a portion of an immunoglobulin heavy chain comprising an immunoglobulin heavy chain constant region domain joined to an immunoglobulin heavy chain hinge region.

* * * * *

US006018026A

United States Patent [19]

Sledziewski et al.

[11] Patent Number: 6,018,026

[45] Date of Patent: *Jan. 25, 2000

[54] BIOLOGICALLY ACTIVE DIMERIZED AND MULTIMERIZED POLYPEPTIDE FUSIONS

[75] Inventors: Andrzej Z. Sledziewski; Lillian Anne Bell; Wayne R. Kindsvogel, all of Seattle, Wash.

[73] Assignee: ZymoGenetics, Inc., Seattle, Wash.

[*] Notice: This patent is subject to a terminal disclaimer.

[21] Appl. No.: 08/980,400

[22] Filed: Nov. 26, 1997

Related U.S. Application Data

[62] Division of application No. 08/477,329, Jun. 7, 1995, Pat. No. 5,750,375, which is a continuation of application No. 08/180,195, Jan. 11, 1994, Pat. No. 5,567,584, which is a continuation of application No. 07/634,510, Dec. 27, 1990, abandoned, which is a continuation-in-part of application No. 07/347,291, May 2, 1989, Pat. No. 5,155,027, which is a continuation-in-part of application No. 07/146,877, Jan. 22, 1988, abandoned.

[51] Int. Cl.[7] C07K 19/00; C07K 16/46; C07K 14/71

[52] U.S. Cl. 530/350; 530/387.3; 530/388.24; 530/399; 530/402

[58] Field of Search 530/350, 387.3, 530/388.24, 399, 402; 435/69.7, 172.3

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(List continued on next page.)

Primary Examiner—Lorraine Spector
Assistant Examiner—Claire M. Kaufman
Attorney, Agent, or Firm—Townsend and Townsend and Crew

[57] ABSTRACT

Methods for producing secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof.

29 Claims, 21 Drawing Sheets

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1    GGCCCCTCAGCCCTGCTGCCCAGCACGAGCCTGTGCTCGCCCTGCCCAACGCAGACAGCCAGACCCAGG  69
70   GCGGCCCCTCTGGCGGCTCTGCTCCTCCCGAAGGATGCTTGGGGAGTGAGGCGAAGCTGGGCGCTCCTC  138
139  TCCCCTACAGCAGCCCCCCTTCCTCCATCCCTCTGTTCTCCTGAGCCTTCAGGAGCCTGCACCAGTCCTG  207
208  CCTGTCCTTCTACTCAGCTGTTACCCACTCTGGGACCAGCAGTCTTTCTGATAACTGGGAGAGGGCAGT  276
277  AAGGAGGACTTCCTGGAGGGGGTGACTGTCCAGAGCCTGGAACTGTGCCCACACCAGAAGCCATCAGCA  345
346  GCAAGGACACCATGCGGCTTCCGGGTGCGATGCCAGCTCTGGCCCTCAAAGGCGAGCTGCTGTTGCTGT  414
                M  R  L  P  G  A  M  P  A  L  L  A  L  K  G  E  L  L  L  L  S    20
415  CTCTCCTGTTACTTCTGGAACCACAGATCTCTCAGGGCCTGGTCGTCACACCCCCGGGGCCAGAGCTTG  483
       L  L  L  L  E  P  Q  I  S  Q  G  L  V  V  T  P  P  G  P  E  L  V    43
484  TCCTCAATGTCTCCAGCACCTTCGTTCTGACCTGCTCGGGTTCAGCTCCGGTGGTGTGGGAACGGATGT  552
       L  N  V  S  S  T  F  V  L  T  C  S  G  S  A  P  V  V  W  E  R  M  S    66
553  CCCAGGAGCCCCCACAGGAAATGGCCAAGGCCCAGGATGGCACCTTCTCCAGCGTGCTCACACTGACCA  621
       Q  E  P  P  Q  E  M  A  K  A  Q  D  G  T  F  S  S  V  L  T  L  T  N    89
622  ACCTCACTGGGCTAGACACGGGAGAATACTTTTGCACCCACAATGACTCCCGTGGACTGGAGACCGATG  690
       L  T  G  L  D  T  G  E  Y  F  C  T  H  N  D  S  R  G  L  E  T  D  E   112
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       R  K  R  L  Y  I  F  V  P  D  P  T  V  G  F  L  P  N  D  A  E  E  L   135
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Fig. 1A

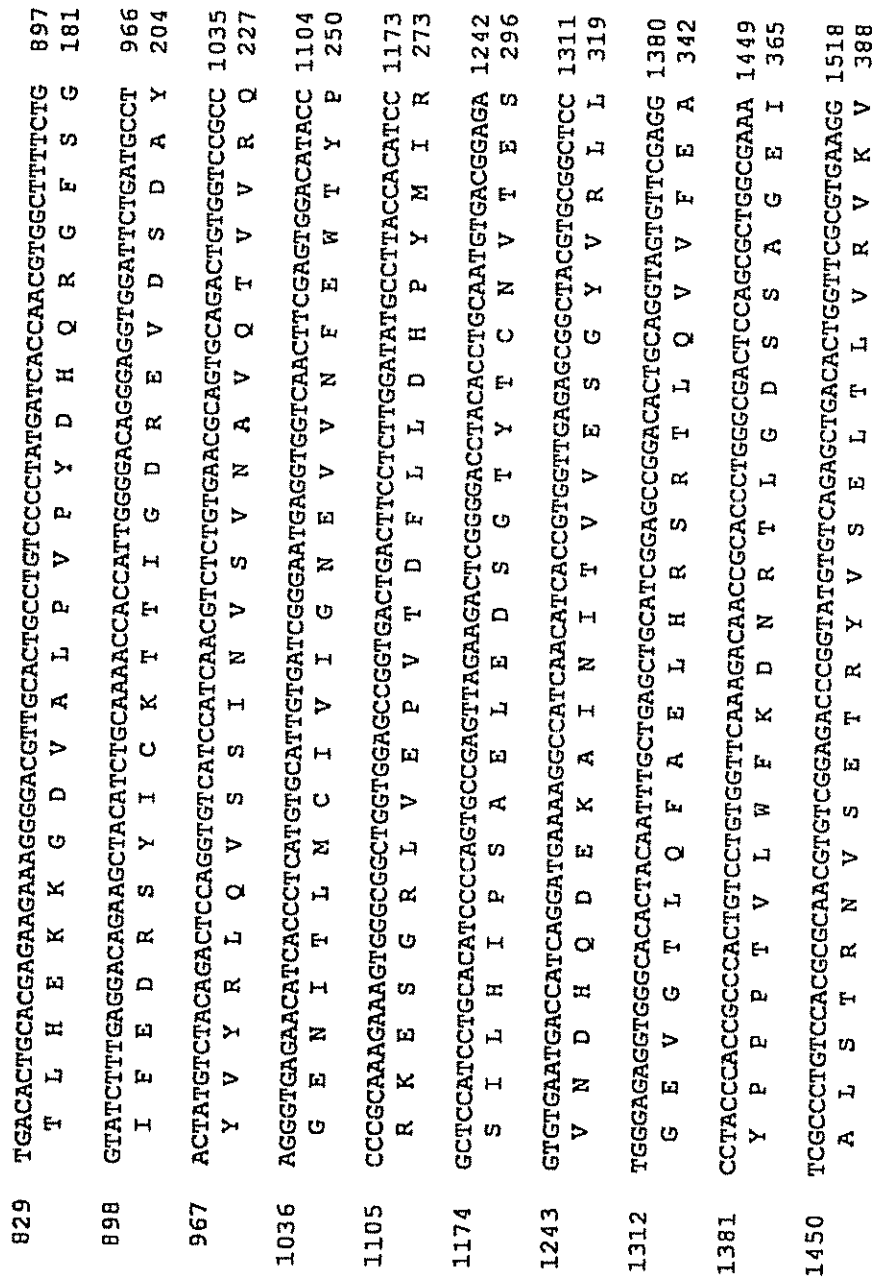

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G E V G T L Q F A E L H R S R T L Q V V F E A 342
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Fig. 1B

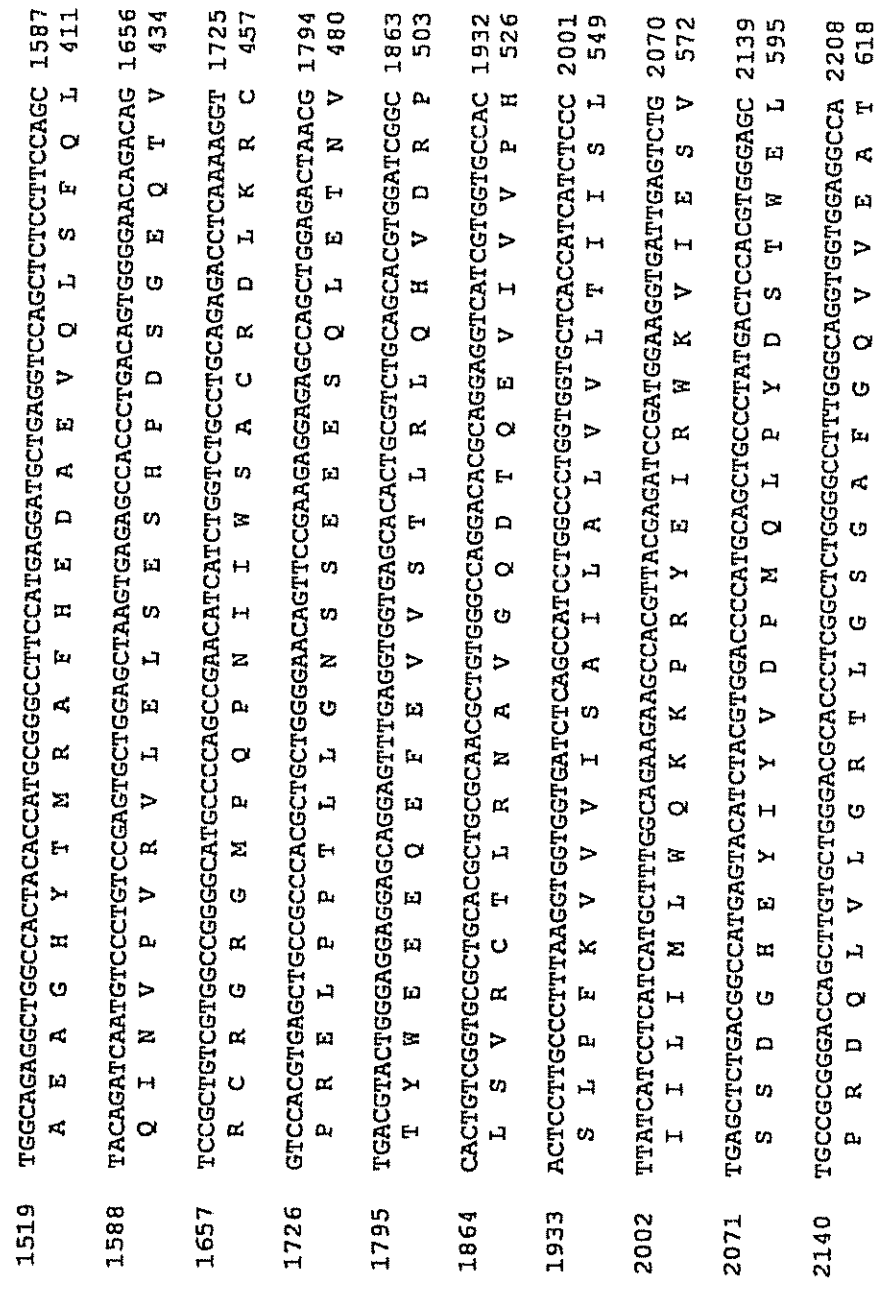

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Fig. 1C

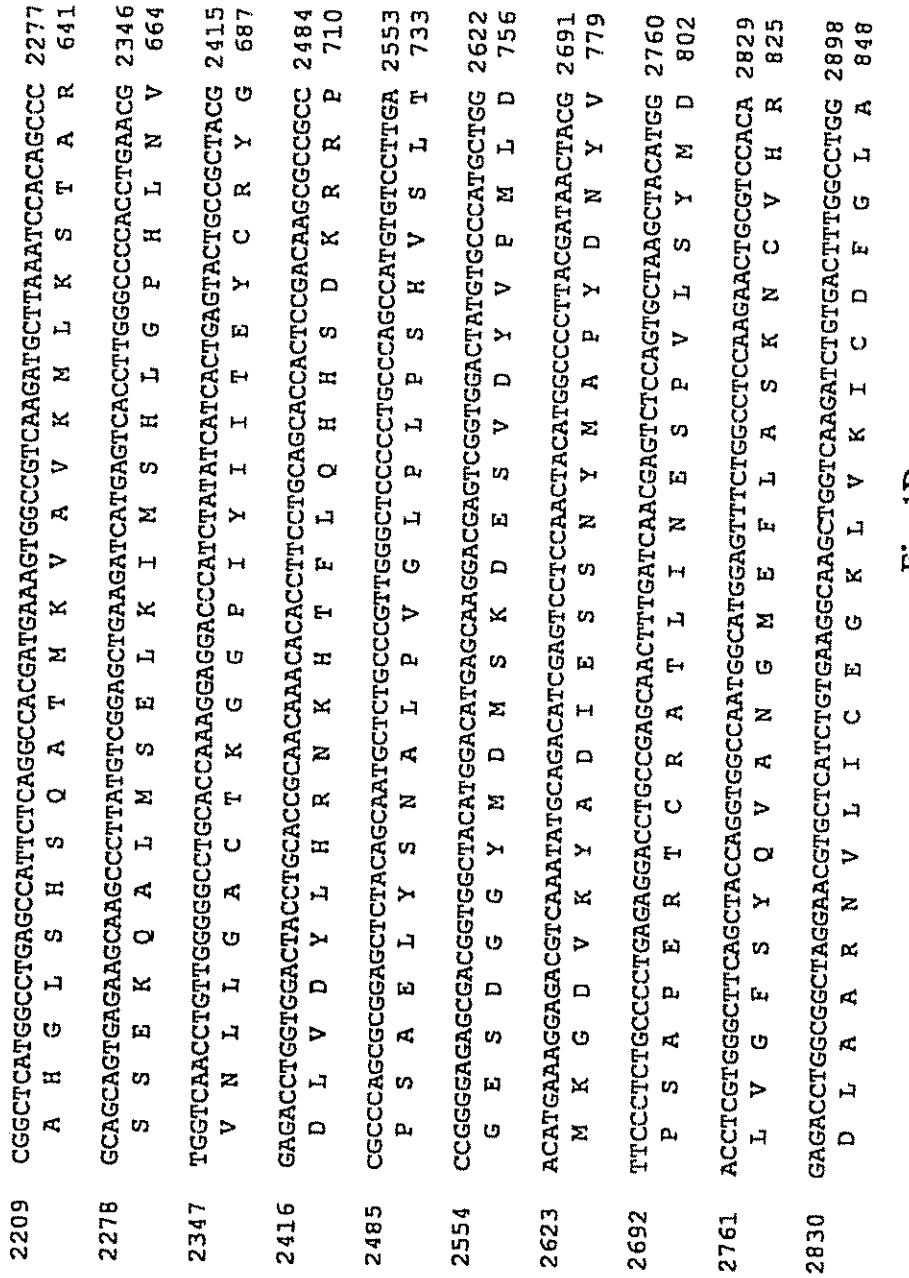

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Fig. 1D

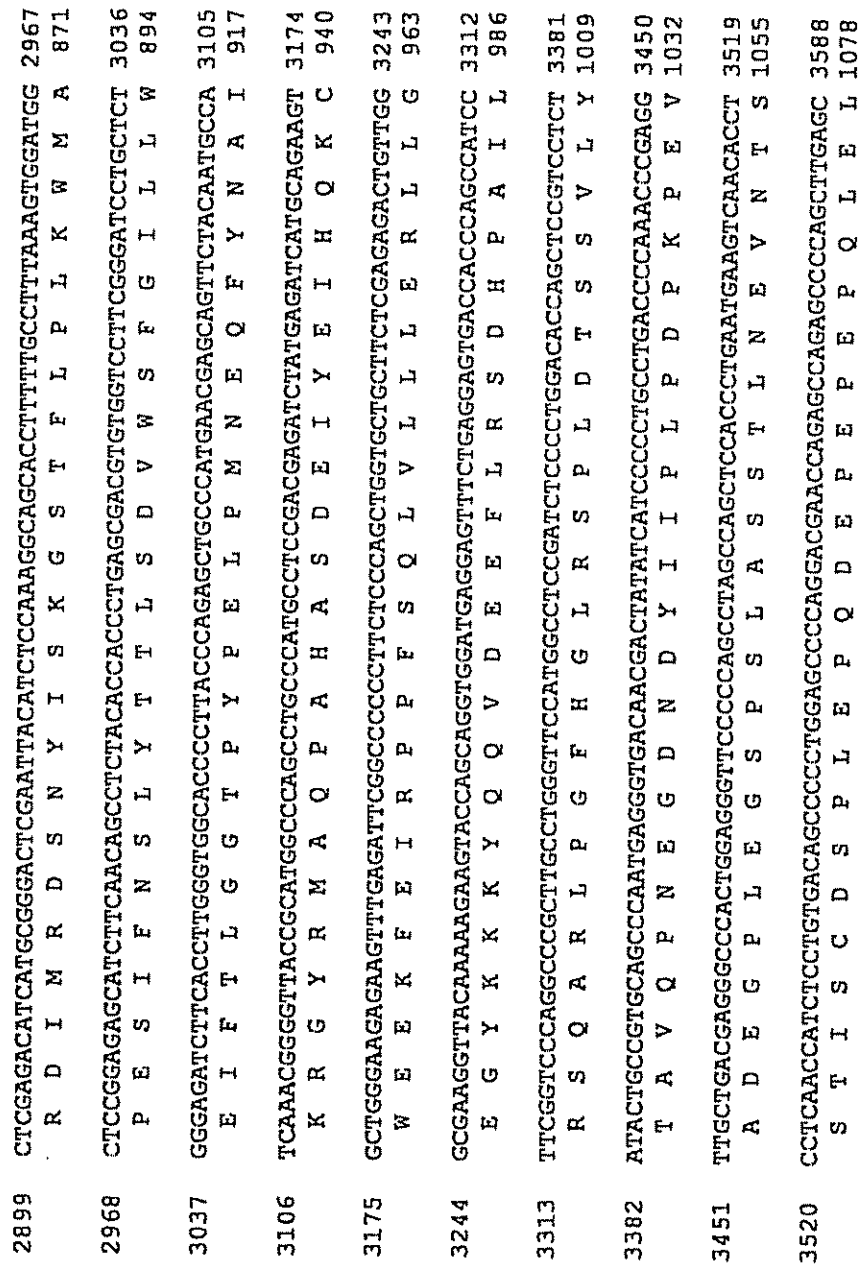

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Fig. 1E

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4417  TGGGGCTTTTTCTTTATCACCCTCAGTCTTAATCCATCCACCAGAGTCTAGAAGGCCAGACGGGCCCCG 4485

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Fig. 1F

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Fig. 1G

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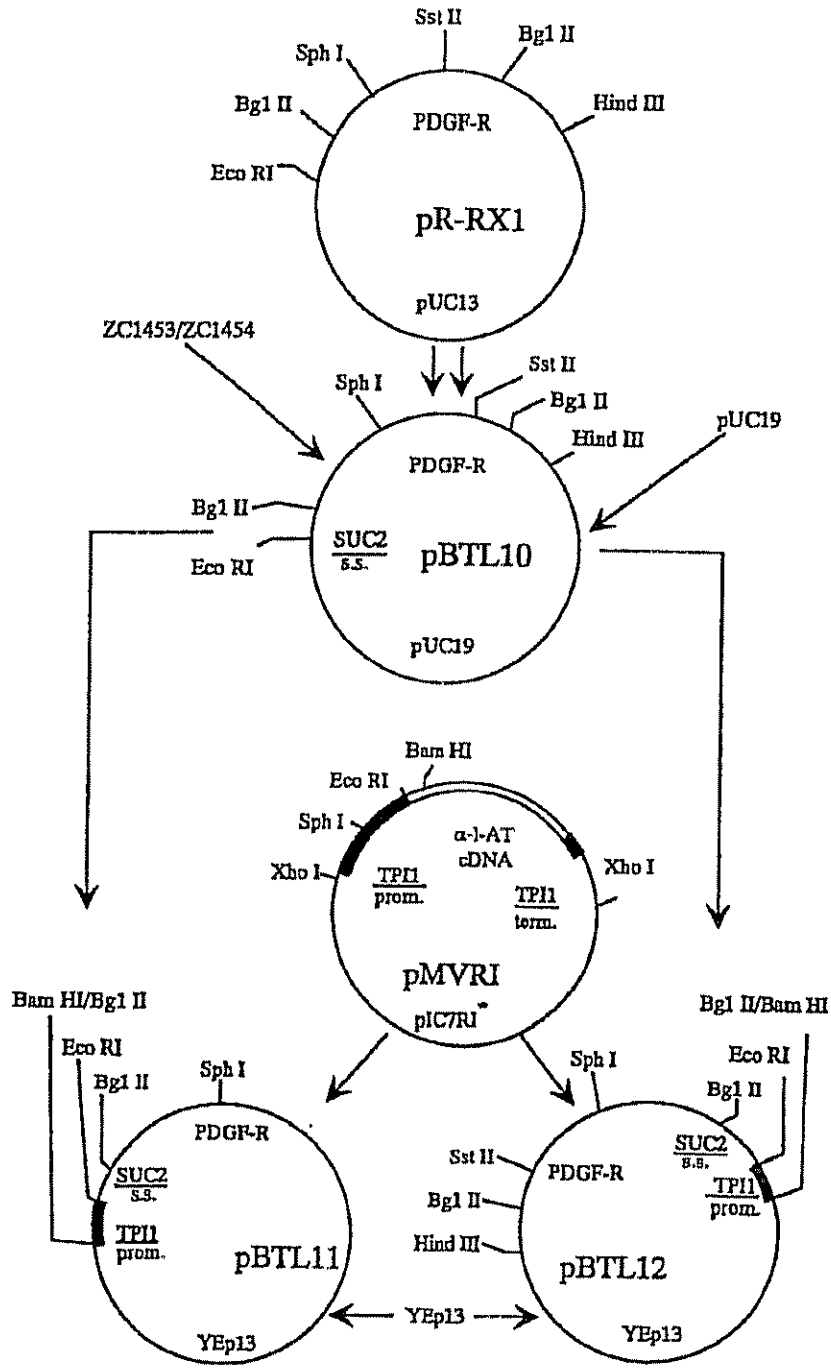

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Sph I
α-1-AT cDNA
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Xho I
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TPI1 term.
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Figure 3

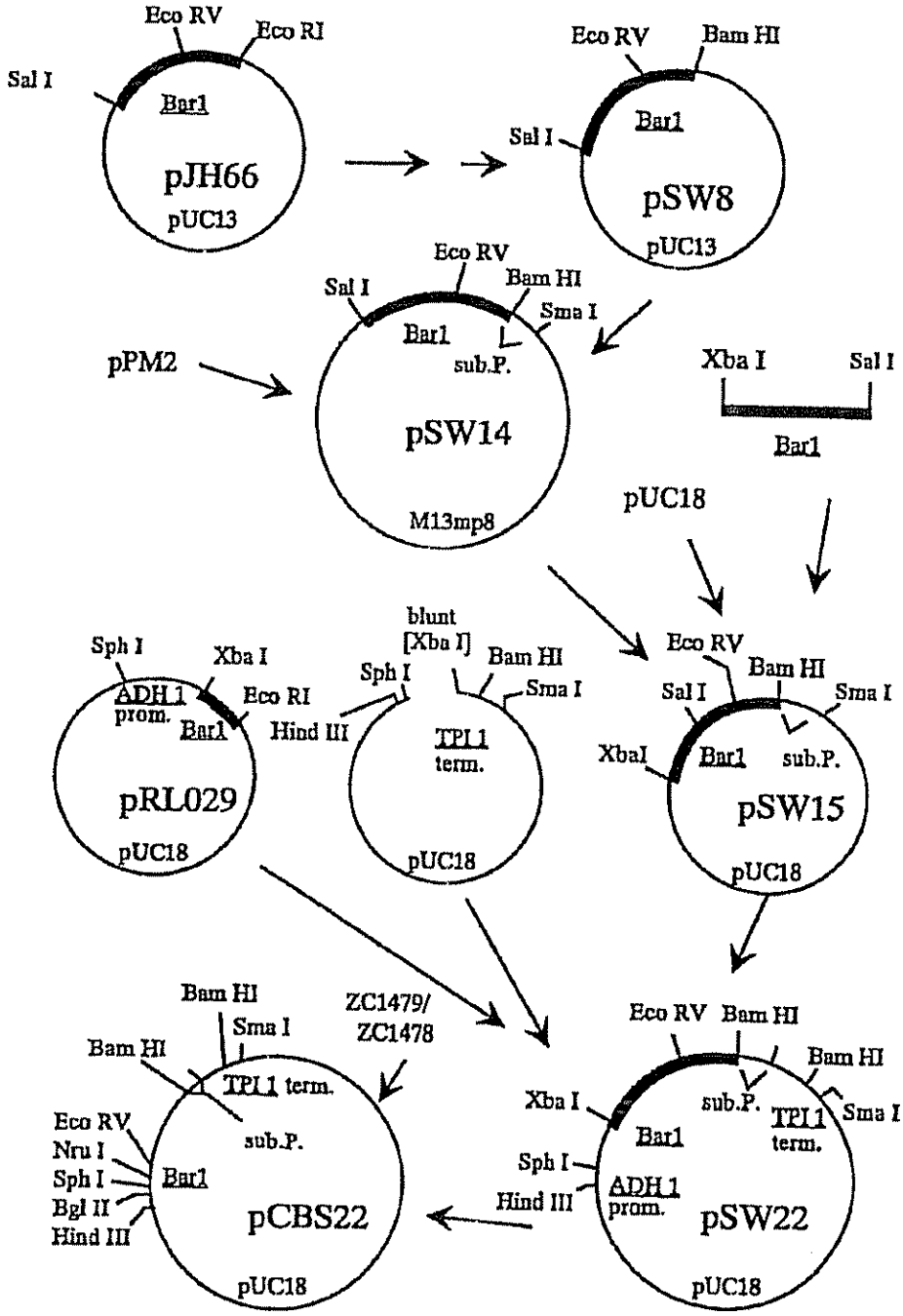

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Figure 4

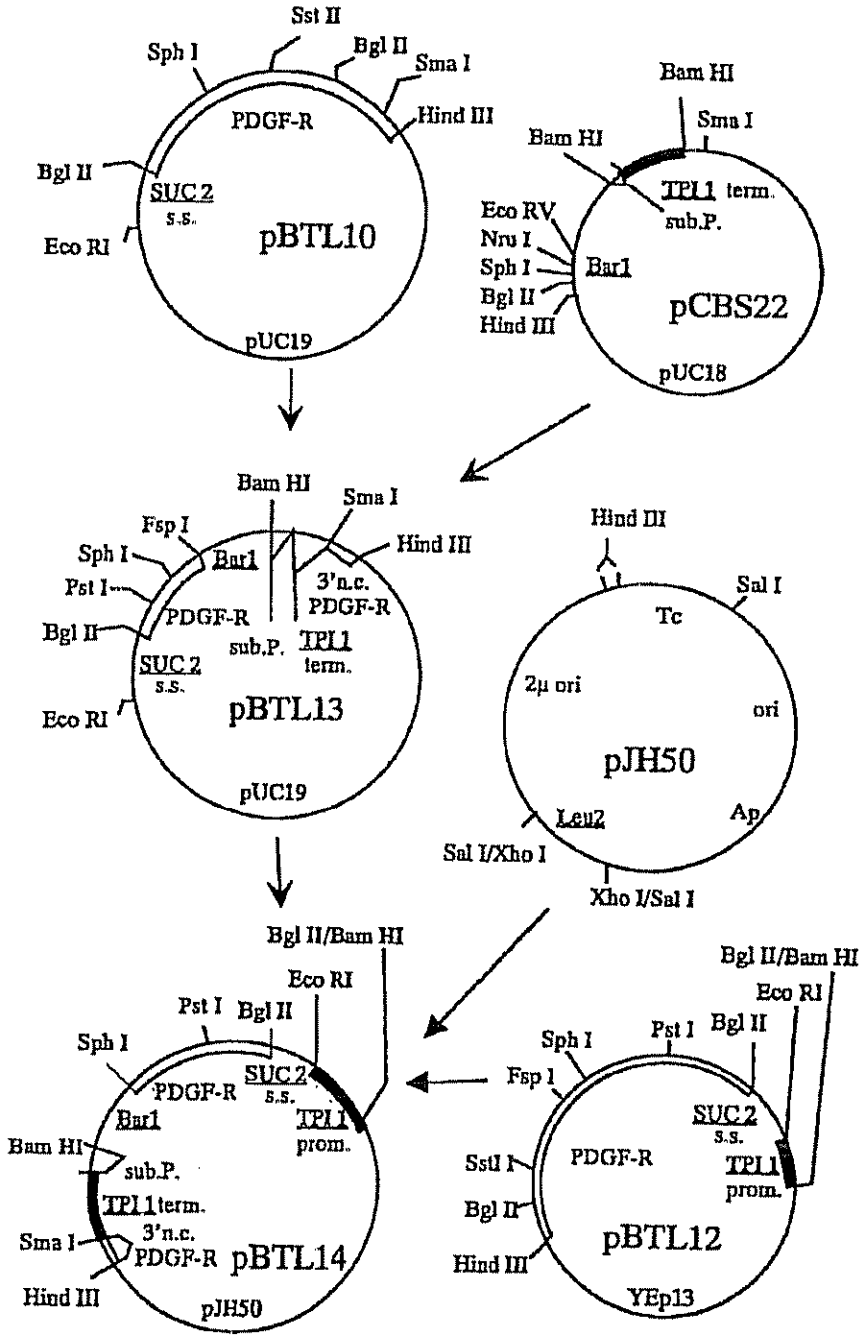

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Figure 5

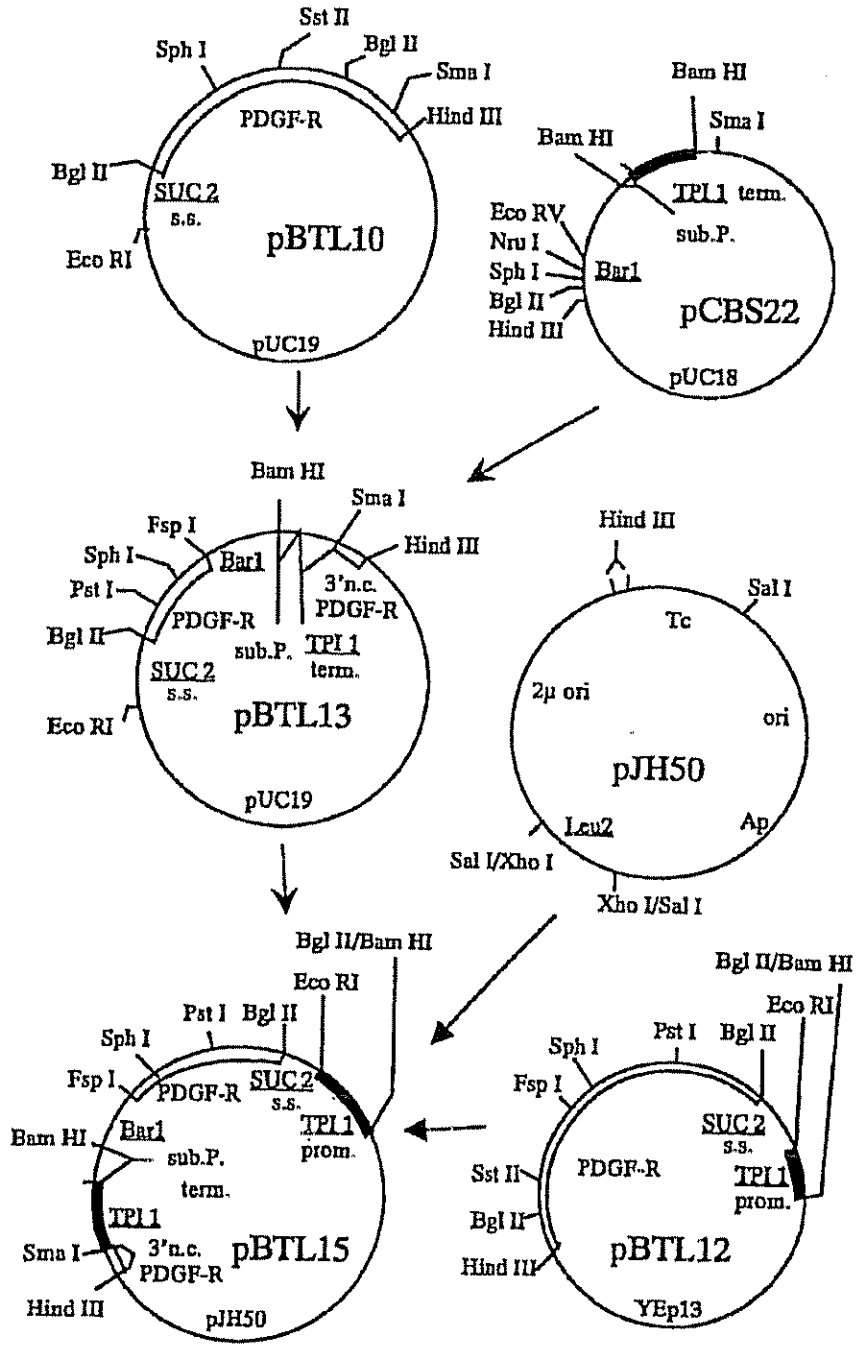

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Figure 6

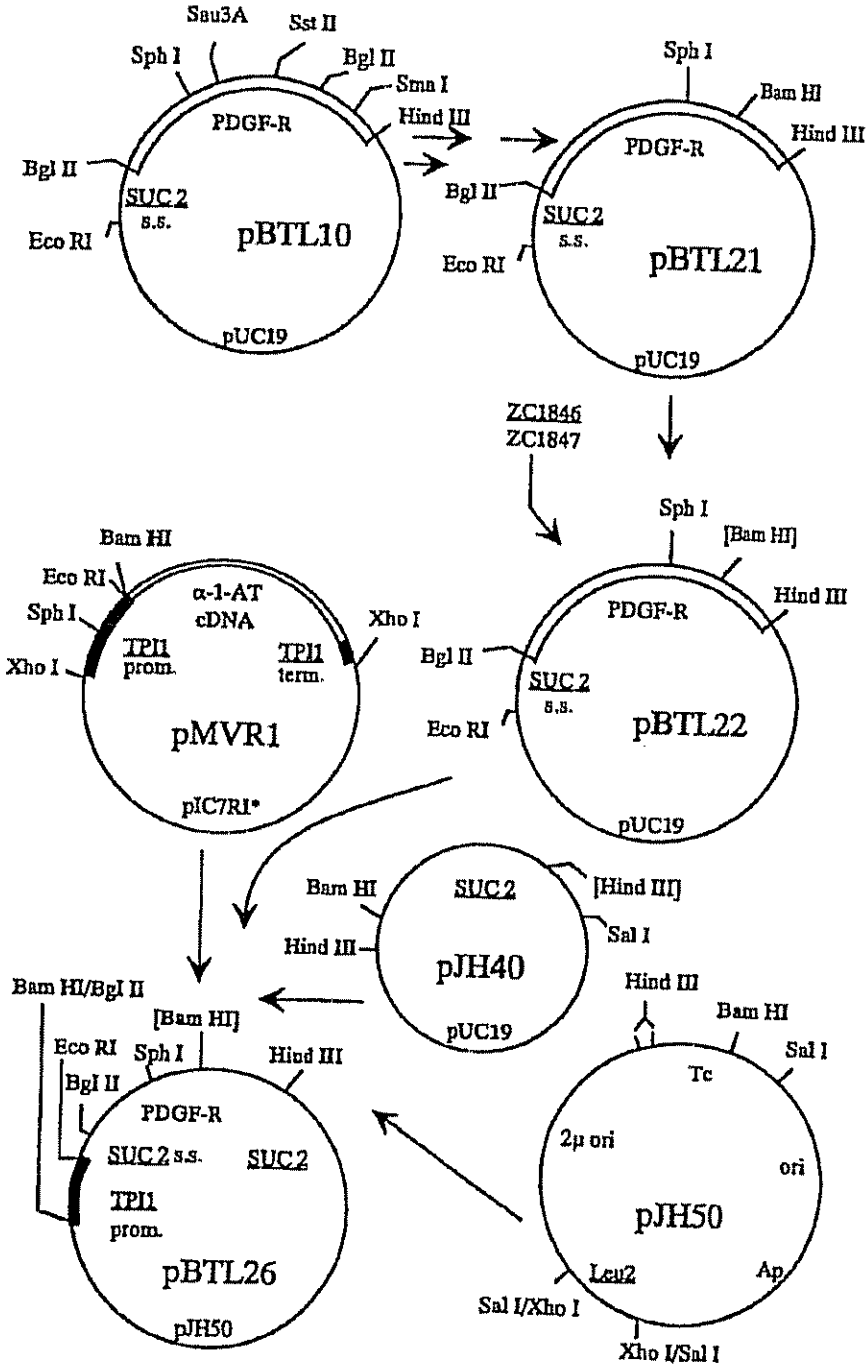

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Xho I/Sal I

Figure 7

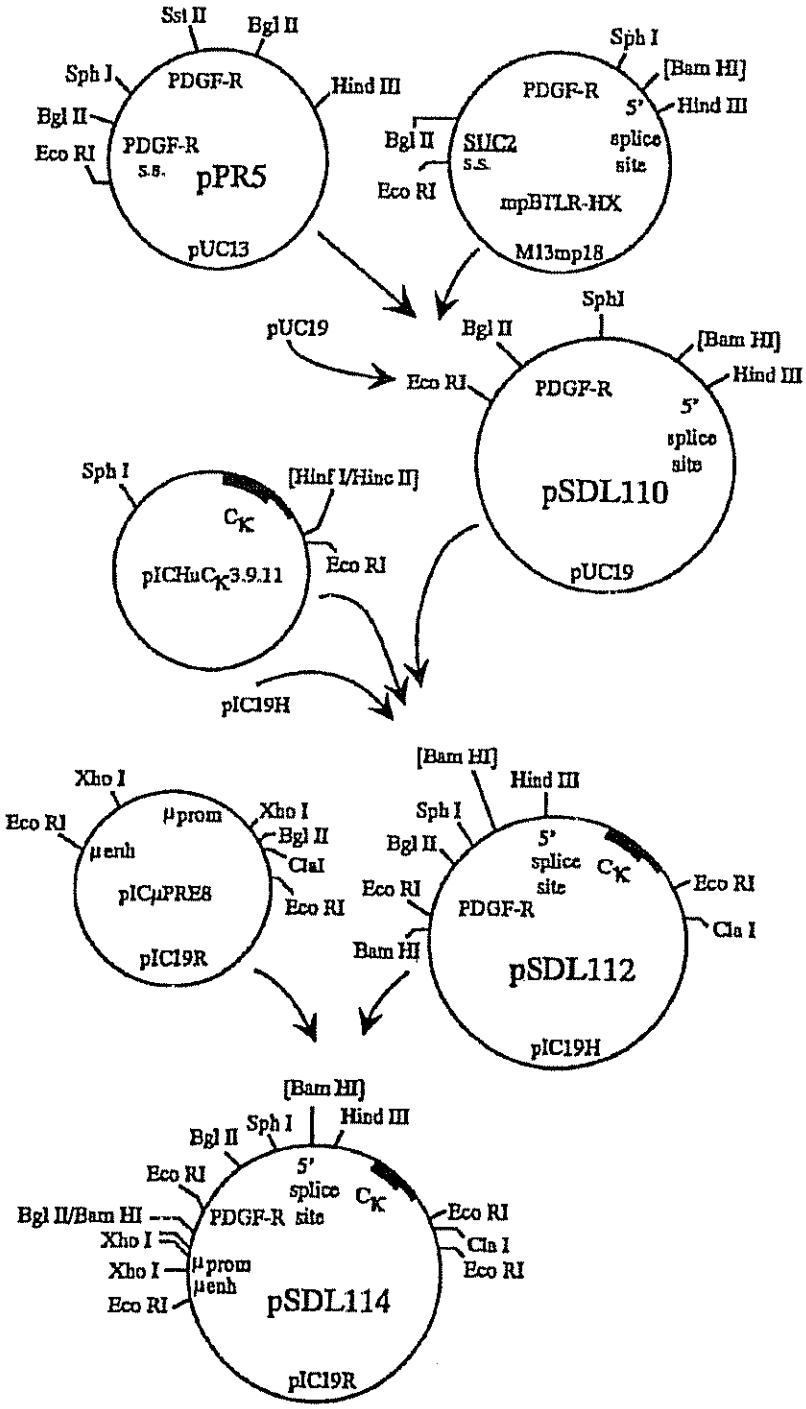

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Figure 8

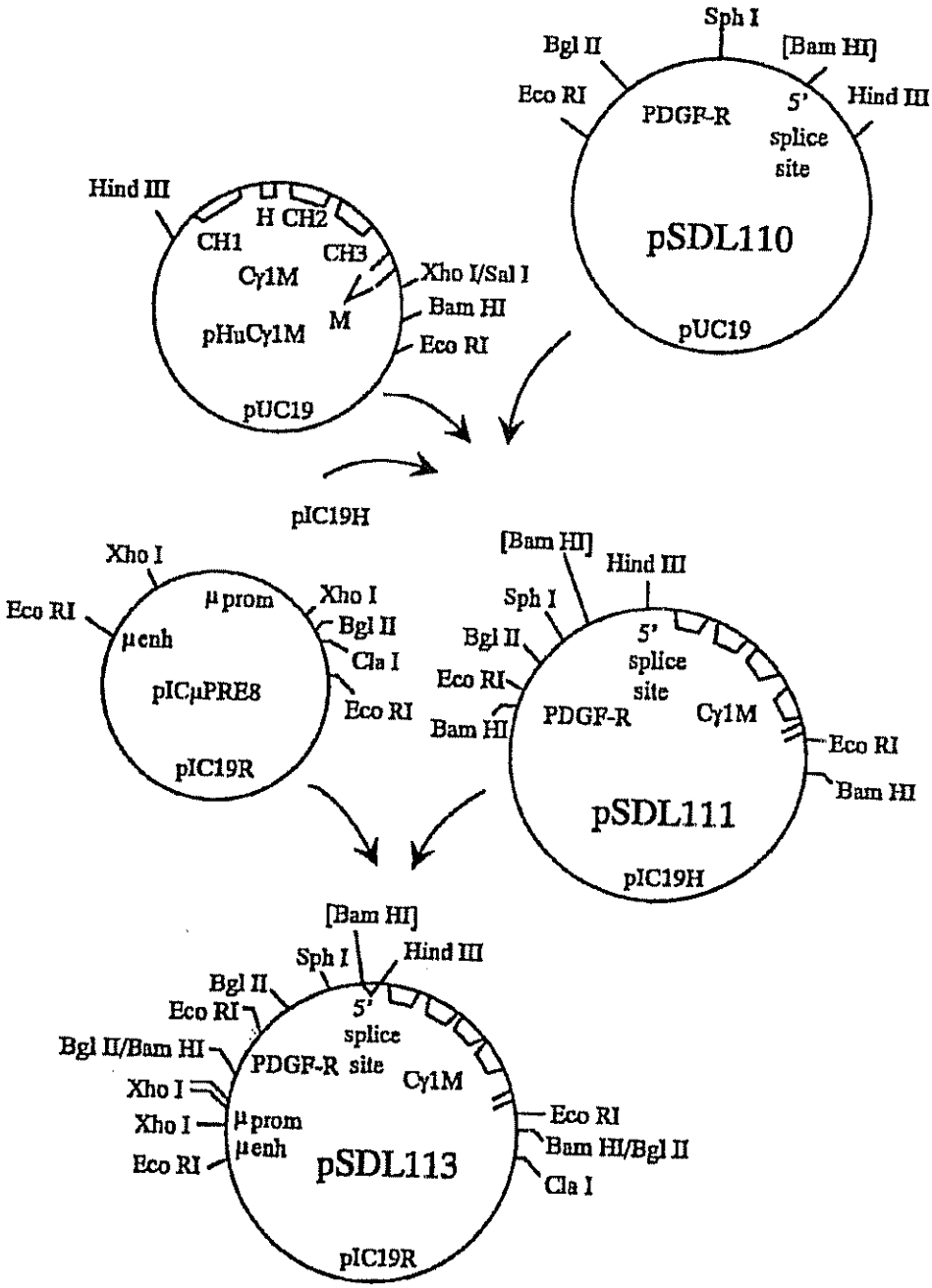

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Cla I
Eco RI
pICμPRE8
pIC19R
[Bam HI]
Hind III
Sph I
Bgl II
Eco RI
Bam HI
5'
splice
site
PDGF-R
Cγ1M
Eco RI
Bam HI
pSDL111
pIC19H
[Bam HI]
Sph I
Hind III
Bgl II
Eco RI
5'
splice
Bgl II/Bam HI
PDGF-R site
Cγ1M
Xho I
Xho I
μprom
μenh
Eco RI
Eco RI
Bam HI/Bgl II
Cla I
pSDL113
pIC19R

Figure 9

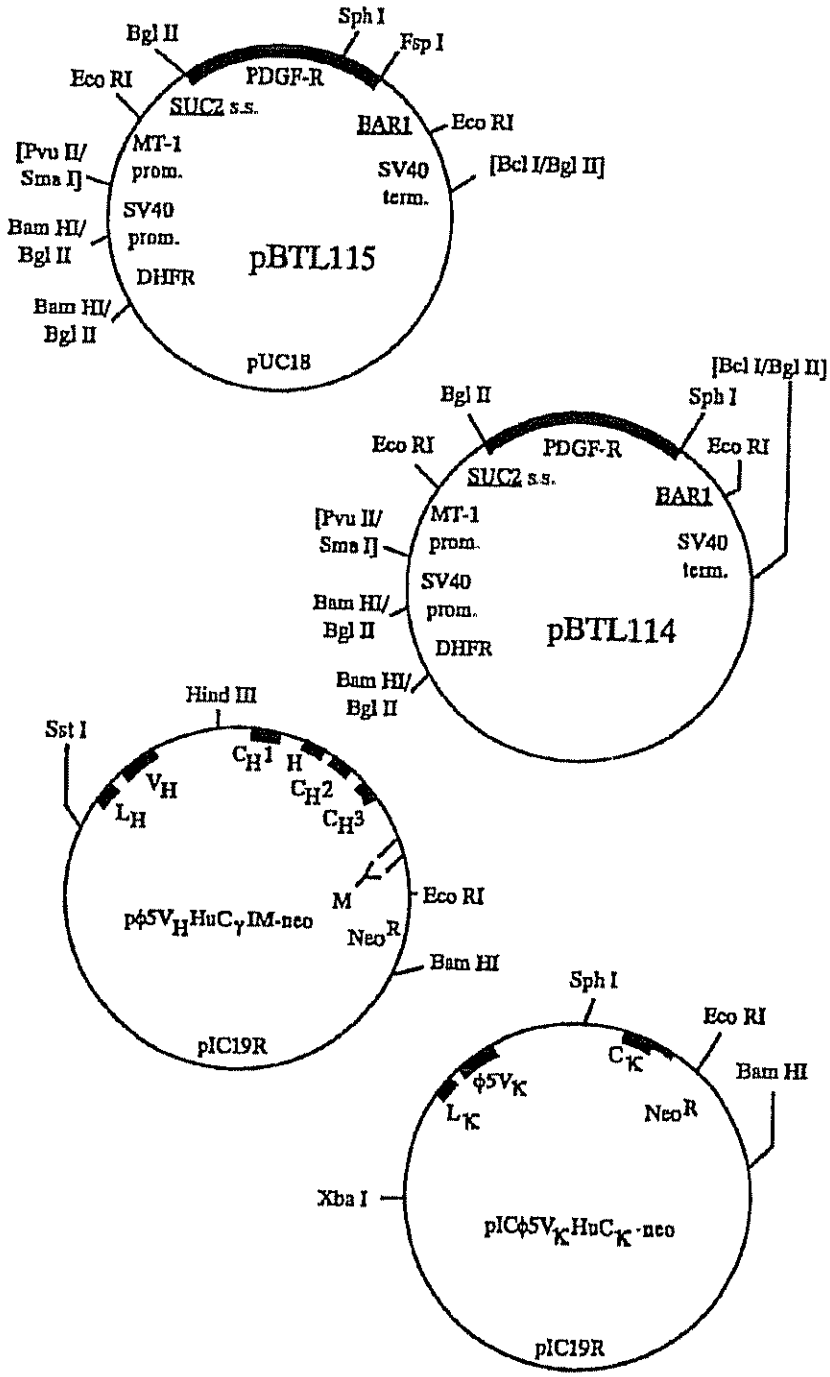

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Fsp I
Eco RI
PDGF-R
SUC2 s.s.
BAR1
Eco RI
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Sma I]
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prom.
SV40
term.
[Bcl I/Bgl II]
SV40
prom.
Bam HI/
Bgl II
pBTL115
DHFR
Bam HI/
Bgl II
pUC18
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Bgl II
Sph I
Eco RI
PDGF-R
Eco RI
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BAR1
[Pvu II/
Sma I]
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prom.
SV40
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SV40
prom.
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Bgl II
pBTL114
DHFR
Bam HI/
Bgl II
Hind III
Sst I
C_H1
H
V_H
C_H2
L_H
C_H3
M
Eco RI
$p\phi5V_HHuC_\gamma IM$-neo
Neo^R
Bam HI
pIC19R
Sph I
Eco RI
C_K
$\phi5V_K$
Bam HI
L_K
Neo^R
Xba I
$pIC\phi5V_KHuC_K$-neo
pIC19R

Figure 10

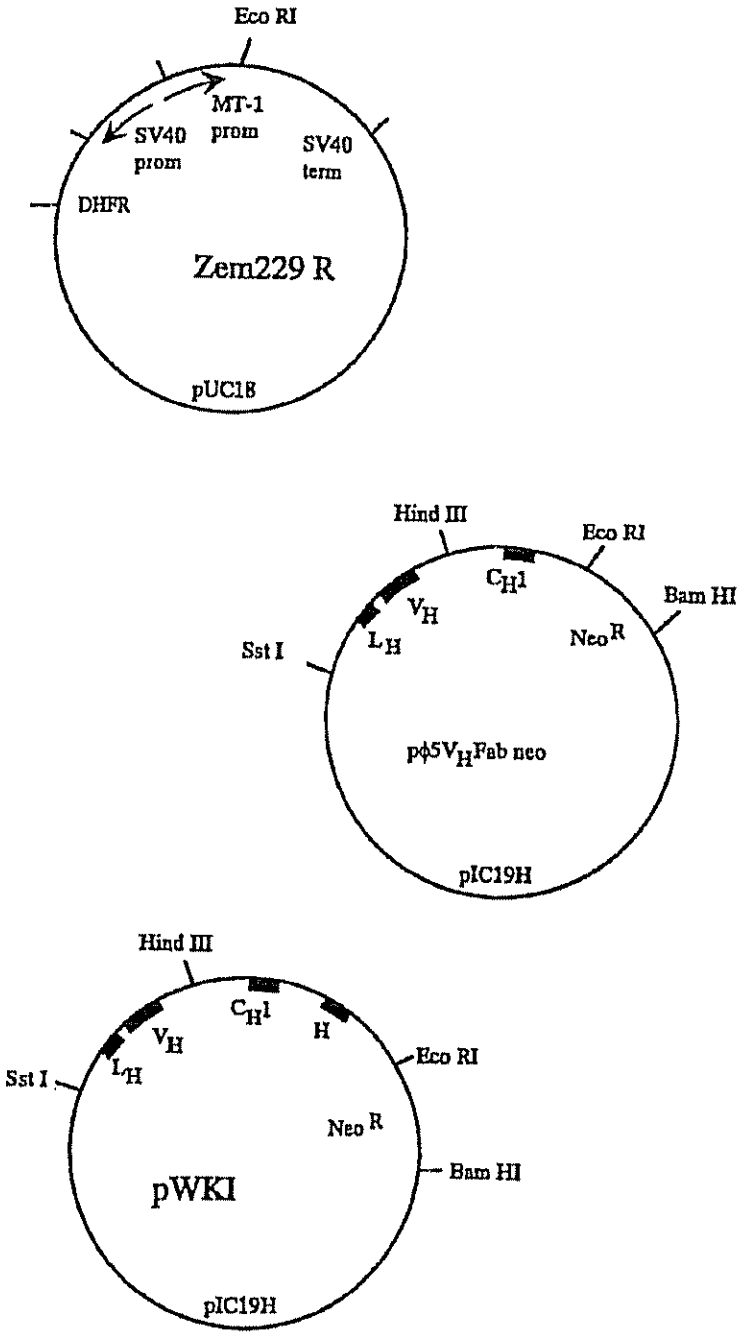

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MT-1 prom
SV40 prom
SV40 term
DHFR
Zem229 R
pUC18
Hind III
Eco RI
C_H1
V_H
L_H
Bam HI
Sst I
Neo^R
$p\phi5V_HFab$ neo
pIC19H
Hind III
C_H1
H
V_H
L_H
Eco RI
Sst I
Neo^R
Bam HI
pWKI
pIC19H

Figure 11A

```
  1 GCCCTGGGGACGGACCGTGGGCGGCGCGCAGCGGCGGGACGCGTTTTGGGGACGTGGTGGCCAGCGCCT
 70 TCCTGCAGACCCACAGGGAAGTACTCCCTTTGACCTCCGGGGAGCTGCGACCAGGTTATACGTTGCTGG

139 TGGAAAAGTGACAATTCTAGGAAAAGAGCTAAAAGCCGGATCGGTGACCGAAAGTTTCCCAGAGCTATG
                                                                        M
                                                                        1

208 GGGACTTCCCATCCGGCGTTCCTGGTCTTAGGCTGTCTTCTCACAGGGCTGAGCCTAATCCTCTGCCAG
    G T S H P A F L V L G C L L T G L S L I L C Q

277 CTTTCATTACCCTCTATCCTTCCAAATGAAAATGAAAAGGTTGTGCAGCTGAATTCATCCTTTTCTCTG
    L S L P S I L P N E N E K V V Q L N S S F S L

346 AGATGCTTTGGGGAGAGTGAAGTGAGCTGGCAGTACCCCATGTCTGAAGAAGAGAGCTCCGATGTGGAA
    R C F G E S E V S W Q Y P M S E E E S S D V E

415 ATCAGAAATGAAGAAAACAACAGCGGCCTTTTTGTGACGGTCTTGGAAGTGAGCAGTGCCTCGGCGGCC
    I R N E E N N S G L F V T V L E V S S A S A A

484 CACACAGGGTTGTACACTTGCTATTACAACCACACTCAGACAGAAGAGAATGAGCTTGAAGGCAGGCAC
    H T G L Y T C Y Y N H T Q T E E N E L E G R H

553 ATTTACATCTATGTGCCAGACCCAGATGTAGCCTTTGTACCTCTAGGAATGACGGATTATTTAGTCATC
    I Y I Y V P D P D V A F V P L G M T D Y L V I

622 GTGGAGGATGATGATTCTGCCATTATACCTTGTCGCACAACTGATCCCGAGACTCCTGTAACCTTACAC
    V E D D D S A I I P C R T T D P E T P V T L H

691 AACAGTGAGGGGGTGGTACCTGCCTCCTACGACAGCAGACAGGGCTTTAATGGGACCTTCACTGTAGGG
    N S E G V V P A S Y D S R Q G F N G T F T V G

760 CCCTATATCTGTGAGGCCACCGTCAAAGGAAAGAAGTTCCAGACCATCCCATTTAATGTTTATGCTTTA
    P Y I C E A T V K G K K F Q T I P F N V Y A L

829 AAAGCAACATCAGAGCTGGATCTAGAAATGGAAGCTCTTAAAACCGTGTATAAGTCAGGGGAAACGATT
    K A T S E L D L E M E A L K T V Y K S G E T I

898 GTGGTCACCTGTGCTGTTTTTAACAATGAGGTGGTTGACCTTCAATGGACTTACCCTGGAGAAGTGAAA
    V V T C A V F N N E V V D L Q W T Y P G E V K
```

Figure 11B

```
967  GGCAAAGGCATCACAATACTGGAAGAAATCAAAGTCCCATCCATCAAATTGGTGTACACTTTGACGGTC
     G  K  G  I  T  I  L  E  E  I  K  V  P  S  I  K  L  V  Y  T  L  T  V

1036 CCCGAGGCCACGGTGAAAGACAGTGGAGATTACGAATGTGCTGCCCGCCAGGCTACCAGGGAGGTCAAA
     P  E  A  T  V  K  D  S  G  D  Y  E  C  A  A  R  Q  A  T  R  E  V  K

1105 GAAATGAAGAAAGTCACTATTTCTGTCCATGAGAAAGGTTTCATTGAAATCAAACCCACCTTCAGCCAG
     E  M  K  K  V  T  I  S  V  H  E  K  G  F  I  E  I  K  P  T  F  S  Q

1174 TTGGAAGCTGTCAACCTGCATGAAGTCAAACATTTTGTTGTAGAGGTGCGGGCCTACCCACCTCCCAGG
     L  E  A  V  N  L  H  E  V  K  H  F  V  V  E  V  R  A  Y  P  P  P  R

1243 ATATCCTGGCTGAAAAACAATCTGACTCTGATTGAAAATCTCACTGAGATCACCACTGATGTGGAAAAG
     I  S  W  L  K  N  N  L  T  L  I  E  N  L  T  E  I  T  T  D  V  E  K

1312 ATTCAGGAAATAAGGTATCGAAGCAAATTAAAGCTGATCCGTGCTAAGGAAGAAGACAGTGGCCATTAT
     I  Q  E  I  R  Y  R  S  K  L  K  L  I  R  A  K  E  E  D  S  G  H  Y

1381 ACTATTGTAGCTCAAAATGAAGATGCTGTGAAGAGCTATACTTTTGAACTGTTAACTCAAGTTCCTTCA
     T  I  V  A  Q  N  E  D  A  V  K  S  Y  T  F  E  L  L  T  Q  V  P  S

1450 TCCATTCTGGACTTGGTCGATGATCACCATGGCTCAACTGGGGGACAGACGGTGAGGTGCACAGCTGAA
     S  I  L  D  L  V  D  D  H  H  G  S  T  G  G  Q  T  V  R  C  T  A  E

1519 GGCACGCCGCTTCCTGATATTGAGTGGATGATATGCAAAGATATTAAGAAATGTAATAATGAAACTTCC
     G  T  P  L  P  D  I  E  W  M  I  C  K  D  I  K  K  C  N  N  E  T  S

1588 TGGACTATTTTGGCCAACAATGTCTCAAACATCATCACGGAGATCCACTCCCGAGACAGGAGTACCGTG
     W  T  I  L  A  N  N  V  S  N  I  I  T  E  I  H  S  R  D  R  S  T  V

1657 GAGGGCCGTGTGACTTTCGCCAAAGTGGAGGAGACCATCGCCGTGCGATGCCTGGCTAAGAATCTCCTT
     E  G  R  V  T  F  A  K  V  E  E  T  I  A  V  R  C  L  A  K  N  L  L

1726 GGAGCTGAGAACCGAGAGCTGAAGCTGGTGGCTCCCACCCTGCGTTCTGAACTCACGGTGGCTGCTGCA
     G  A  E  N  R  E  L  K  L  V  A  P  T  L  R  S  E  L  T  V  A  A  A

1795 GTCCTGGTGCTGTTGGTGATTGTGATCATCTCACTTATTGTCCTGGTTGTCATTTGGAAACAGAAACCG
     V  L  V  L  L  V  I  V  I  I  S  L  I  V  L  V  V  I  W  K  Q  K  P
```

Figure 11C

```
1864  AGGTATGAAATTCGCTGGAGGGTCATTGAATCAATCAGCCCGGATGGACATGAATATATTTATGTGGAC
        R  Y  E  I  R  W  R  V  I  E  S  I  S  P  D  G  H  E  Y  I  Y  V  D

1933  CCGATGCAGCTGCCTTATGACTCAAGATGGGAGTTTCCAAGAGATGGACTAGTGCTTGGTCGGGTCTTG
        P  M  Q  L  P  Y  D  S  R  W  E  F  P  R  D  G  L  V  L  G  R  V  L

2002  GGGTCTGGAGCGTTTGGGAAGGTGGTTGAAGGAACAGCCTATGGATTAAGCCGGTCCCAACCTGTCATG
        G  S  G  A  F  G  K  V  V  E  G  T  A  Y  G  L  S  R  S  Q  P  V  M

2071  AAAGTTGCAGTGAAGATGCTAAAACCCACGGCCAGATCCAGTGAAAAACAAGCTCTCATGTCTGAACTG
        K  V  A  V  K  M  L  K  P  T  A  R  S  S  E  K  Q  A  L  M  S  E  L

2140  AAGATAATGACTCACCTGGGGCCACATTTGAACATTGTAAACTTGCTGGGAGCCTGCACCAAGTCAGGC
        K  I  M  T  H  L  G  P  H  L  N  I  V  N  L  L  G  A  C  T  K  S  G

2209  CCCATTTACATCATCACAGAGTATTGCTTCTATGGAGATTTGGTCAACTATTTGCATAAGAATAGGGAT
        P  I  Y  I  I  T  E  Y  C  F  Y  G  D  L  V  N  Y  L  H  K  N  R  D

2278  AGCTTCCTGAGCCACCACCCAGAGAAGCCAAAGAAAGAGCTGGATATCTTTGGATTGAACCCTGCTGAT
        S  F  L  S  H  H  P  E  K  P  K  K  E  L  D  I  F  G  L  N  P  A  D

2347  GAAAGCACACGGAGCTATGTTATTTTATCTTTTGAAAACAATGGTGACTACATGGACATGAAGCAGGCT
        E  S  T  R  S  Y  V  I  L  S  F  E  N  N  G  D  Y  M  D  M  K  Q  A

2416  GATACTACACAGTATGTCCCCATGCTAGAAAGGAAAGAGGTTTCTAAATATTCCGACATCCAGAGATCA
        D  T  T  Q  Y  V  P  M  L  E  R  K  E  V  S  K  Y  S  D  I  Q  R  S

2485  CTCTATGATCGTCCAGCCTCATATAAGAAGAAATCTATGTTAGACTCAGAAGTCAAAAACCTCCTTTCA
        L  Y  D  R  P  A  S  Y  K  K  K  S  M  L  D  S  E  V  K  N  L  L  S

2554  GATGATAACTCAGAAGGCCTTACTTTATTGGATTTGTTGAGCTTCACCTATCAAGTTGCCCGAGGAATG
        D  D  N  S  E  G  L  T  L  L  D  L  L  S  F  T  Y  Q  V  A  R  G  M

2623  GAGTTTTTGGCTTCAAAAAATTGTGTCCACCGTGATCTGGCTGCTCGCAACGTCCTCCTGGCACAAGGA
        E  F  L  A  S  K  N  C  V  H  R  D  L  A  A  R  N  V  L  L  A  Q  G

2692  AAAATTGTGAAGATCTGTGACTTTGGCCTGGCCAGAGACATCATGCATGATTCGAACTATGTGTCGAAA
        K  I  V  K  I  C  D  F  G  L  A  R  D  I  M  H  D  S  N  Y  V  S  K

2761  GGCAGTACCTTTCTGCCCGTGAAGTGGATGGCTCCTGAGAGCATCTTTGACAACCTCTACACCACACTG
        G  S  T  F  L  P  V  K  W  M  A  P  E  S  I  F  D  N  L  Y  T  T  L
```

Figure 11D

```
2830  AGTGATGTCTGGTCTTATGGCATTCTGCTCTGGGAGATCTTTTCCCTTGGTGGCACCCCTTACCCCGGC
      S  D  V  W  S  Y  G  I  L  L  W  E  I  F  S  L  G  G  T  P  Y  P  G

2899  ATGATGGTGGATTCTACTTTCTACAATAAGATCAAGAGTGGGTACCGGATGGCCAAGCCTGACCACGCT
      M  M  V  D  S  T  F  Y  N  K  I  K  S  G  Y  R  M  A  K  P  D  H  A

2968  ACCAGTGAAGTCTACGAGATCATGGTGAAATGCTGGAACAGTGAGCCGGAGAAGAGACCCTCCTTTTAC
      T  S  E  V  Y  E  I  M  V  K  C  W  N  S  E  P  E  K  R  P  S  F  Y

3037  CACCTGAGTGAGATTGTGGAGAATCTGCTGCCTGGACAATATAAAAAGAGTTATGAAAAAATTCACCTG
      H  L  S  E  I  V  E  N  L  L  P  G  Q  Y  K  K  S  Y  E  K  I  H  L

3106  GACTTCCTGAAGAGTGACCATCCTGCTGTGGCACGCATGCGTGTGGACTCAGACAATGCATACATTGGT
      D  F  L  K  S  D  H  P  A  V  A  R  M  R  V  D  S  D  N  A  Y  I  G

3175  GTCACCTACAAAAACGAGGAAGACAAGCTGAAGGACTGGGAGGGTGGTCTGGATGAGCAGAGACTGAGC
      V  T  Y  K  N  E  E  D  K  L  K  D  W  E  G  G  L  D  E  Q  R  L  S

3244  GCTGACAGTGGCTACATCATTCCTCTGCCTGACATTGACCCTGTCCCTGAGGAGGAGGACCTGGGCAAG
      A  D  S  G  Y  I  I  P  L  P  D  I  D  P  V  P  E  E  E  D  L  G  K

3313  AGGAACAGACACAGCTCGCAGACCTCTGAAGAGAGTGCCATTGAGACGGGTTCCAGCAGTTCCACCTTC
      R  N  R  H  S  S  Q  T  S  E  E  S  A  I  E  T  G  S  S  S  S  T  F

3382  ATCAAGAGAGAGGACGAGACCATTGAAGACATCGACATGATGGACGACATCGGCATAGACTCTTCAGAC
      I  K  R  E  D  E  T  I  E  D  I  D  M  M  D  D  I  G  I  D  S  S  D

3451  CTGGTGGAAGACAGCTTCCTGTAACTGGCGGATTCGAGGGGTTCCTTCCACTTCTGGGGCCACCTCTGG
      L  V  E  D  S  F  L
                        1089

3520  ATCCCGTTCAGAAAACCACTTTATTGCAATGCGGAGGTTGAGAGGAGGACTTGGTTGATGTTTAAAGAG
3589  AAGTTCCCAGCCAAGGGCCTCGGGGAGCGTTCTAAATATGAATGAATGGGATATTTTGAAATGAACTTT
3658  GTCAGTGTTGCCTCTTGCAATGCCTCAGTAGCATCTCAGTGGTGTGTGAAGTTTGGAGATAGATGGATA
3727  AGGGAATAATAGGCCACAGAAGGTGAACTTTGTGCTTCAAGGACATTGGTGAGAGTCCAACAGACACAA
3796  TTTATACTGCGACAGAACTTCAGCATTGTAATTATGTAAATAACTCTAACCAAGGCTGTGTTTAGATTG
3865  TATTAACTATCTTCTTTGGACTTCTGAAGAGACCACTCAATCCATCCTGTACTTCCCTCTTGAAACCTG
3934  ATGTAGCTGCTGTTGAACTTTTTAAAGAAGTGCATGAAAAACCATTTTTGAACCTTAAAAGGTACTGGT
4003  ACTATAGCATTTTGCTATCTTTTTTAGTGTTAAAGAGATAAAGAATAATAAG
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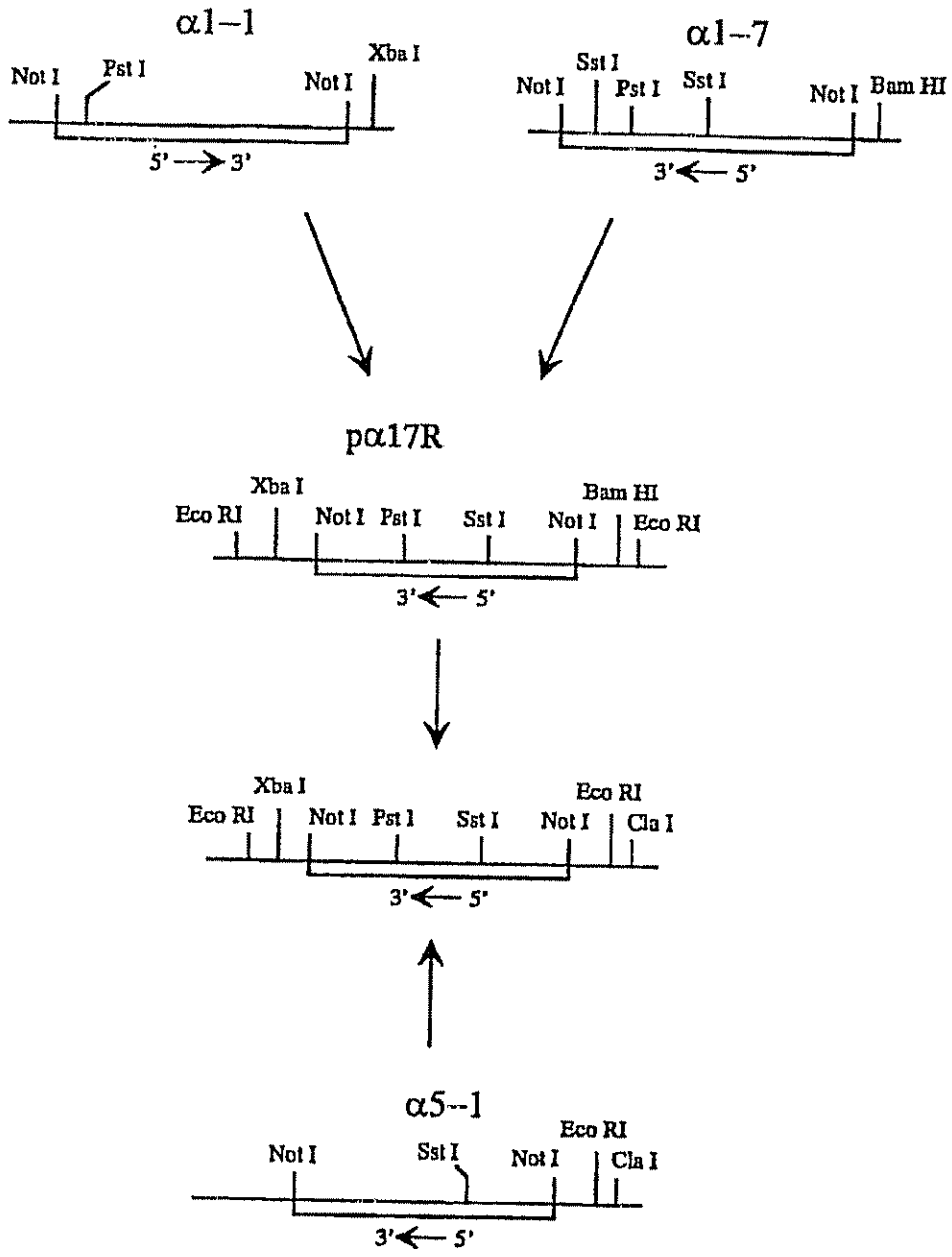

α1–1
Not I
Pst I
Not I
Xba I
5' → 3'
α1–7
Sst I
Not I
Pst I
Sst I
Not I
Bam HI
3' ← 5'
pα17R
Xba I
Eco RI
Not I
Pst I
Sst I
Not I
Bam HI
Eco RI
3' ← 5'
Xba I
Eco RI
Not I
Pst I
Sst I
Not I
Eco RI
Cla I
3' ← 5'
α5–1
Not I
Sst I
Not I
Eco RI
Cla I
3' ← 5'

Figure 12

BIOLOGICALLY ACTIVE DIMERIZED AND MULTIMERIZED POLYPEPTIDE FUSIONS

CROSS REFERENCE TO RELATED APPLICATION

This application is a divisional of U.S. application Ser. No. 8/477,329, filed Jun. 7, 1995, now U.S. Pat. No. 5,750,375 which is a continuation of U.S. application Ser. No. 180,195, filed Jan. 11, 1994,now U.S. Pat. No. 5,567,584, which is a continuation of U.S. application Ser. No. 07/634,510 filed Dec. 27, 1990, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 7/347,291, filed May 2, 1989, now U.S. Pat. No. 5,155,027 which is a continuation-in-part of U.S. application Ser. No/7/146,877, filed Jan. 22, 1988, now abandoned.

TECHNICAL FIELD Technical Field

The present invention is generally directed toward the expression of proteins, and more specifically, toward the expression of growth factor receptor analogs and biologically active dimerized polypeptide fusions.

BACKGROUND OF THE INVENTION

In higher eucaryotic cells, the interaction between receptors and ligands (e.g., hormones) is of central importance in the transmission of and response to a variety of extracellular signals. It is generally accepted that hormones and growth factors elicit their biological functions by binding to specific recognition sites (receptors) in the plasma membranes of their target cells. Upon ligand binding, a receptor undergoes a conformational change, triggering secondary cellular responses that result in the activation or inhibition of intracellular processes. The stimulation or blockade of such an interaction by pharmacological means has important therapeutic implications for a wide variety of illnesses.

Ligands fall into two classes: those that have stimulatory activity, termed agonists; and those that block the effects elicited by the original ligands, termed antagonists. The discovery of agonists that differ in structure and composition from the original ligand may be medically useful. In particular, agonists that are smaller than the original ligand may be especially useful. The bioavailability of these smaller agonists may be greater than that of the original ligand. This may be of particular importance for topical applications and for instances when diffusion of the agonist to its target sites is inhibited by poor circulation. Agonists may also have slightly different spectra of biological activity and/or different potencies, allowing them to be used in very specific situations. Agonists that are smaller and chemically simpler than the native ligand may be produced in greater quantity and at lower cost. The identification of antagonists which specifically block, for example, growth factor receptors has important pharmaceutical applications. Antagonists that block receptors against the action of endogenous, native ligand may be used as therapeutic agents for conditions including atherosclerosis, autocrine tumors, fibroplasia and keloid formation.

The discovery of new ligands that may be used in pharmaceutical applications has centered around designing compounds by chemical modification, complete synthesis, and screening potential ligands by complex and costly screening procedures. The process of designing a new ligand usually begins with the alteration of the structure of the original effector molecule. If the original effector molecule is known to be chemically simple, for example, a catecholamine or

prostaglandin, the task is relatively straightforward. However, if the ligand is structurally complex, for example, a peptide hormone or a growth factor, finding a molecule which is functionally equivalent to the original ligand becomes extremely difficult.

Currently, potential ligands are screened using radioligand binding methods (Lefkowitz et al., *Biochem, Biophys. Res. Comm.* 60: 703–709, 1974; Aurbach et al., *Science* 186: 1223–1225, 1974; Atlas et al., *Proc. Natl. Acad. Sci. USA* 71: 4246–4248, 1974). Potential ligands can be directly assayed by binding the radiolabeled compounds to responsive cells, to the membrane fractions of disrupted cells, or to solubilized receptors. Alternatively, potential ligands may be screened by their ability to compete with a known labeled ligand for cell surface receptors.

The success of these procedures depends on the availability of reproducibly high quality preparations of membrane fractions or receptor molecules, as well as the isolation of responsive cell lines. The preparation of membrane fractions and soluble receptor molecules involves extensive manipulations and complex purification steps. The isolation of membrane fractions requires gentle manipulation of the preparation, a procedure which does not lend itself to commercial production. It is very difficult to maintain high biological activity and biochemical purity of receptors when they are purified by classical protein chemistry methods. Receptors, being integral membrane proteins, require cumbersome purification procedures, which include the use of detergents and other solvents that interfere with their biological activity. The use of these membrane preparations in ligand binding assays typically results in low reproducibility due to the variability of the membrane preparations.

As noted above, ligand binding assays require the isolation of responsive cell lines. Often, only a limited subset of cells is responsive to a particular agent, and such cells may be responsive only under certain conditions. In addition, these cells may be difficult to grow in culture or may possess a low number of receptors. Currently available cell types responsive to platelet-derived growth factor (PDGF), for example, contain only a low number (up to 4×10^4; see Bowen-Pope and Ross, *J. Biol. Chem.* 257: 5161–5171, 1982) of receptors per cell, thus requiring large numbers of cells to assay PDGF analogs or antagonists.

Presently, only a few naturally-occurring secreted receptors, for example, the interleukin-2 receptor (IL-2-R) have been identified. Rubin et al. (*J. Immun.* 135: 3172–3177, 1985) have reported the release of large quantities of IL-2-R into the culture medium of activated T-cell lines. Bailon et al. (*Bio/Technology* 5: 1195–1198, 1987) have reported the use of a matrix-bound interleukin-2 receptor to purify recombinant interleukin-2.

Three other receptors have been secreted from mammalian cells. The insulin receptor (Ellis et al., *J. Cell Biol.* 150: 14a, 1987), the HIV-1 envelope glyco-protein cellular receptor CD4 (Smith et al., *Science* 238: 1704–1707, 1987), the murine IL-7 receptor (*Cell* 60: 941–951, 1990) and the epidermal growth factor (EGF) receptor (Livneh et al., *J. Biol. Chem.* 261: 12490–12497, 1986) have been secreted from mammalian cells using truncated cDNAs that encode portions of the extracellular domains.

Naturally-occurring, secreted receptors have not been widely identified, and there have been only a limited number of reports of secreted recombinant receptors. Secreted receptors may be used in a variety of assays, which include assays to determine the presence of ligand in biological fluids and assays to screen for potential agonists and antagonists.

Current methods for ligand screening and ligand/receptor binding assays have been limited to those using preparations of whole cells or cell membranes for as a source for receptor molecules. The low reproducibility and high cost of producing such preparations does not lend itself to commercial production. There is therefore a need in the art for a method of producing secreted receptors. There is a further need in the art for an assay system that permits high volume screening of compounds that may act on higher eucaryotic cells via specific surface receptors. This assay system should be rapid, inexpensive and adaptable to high volume screening. The present invention discloses such a method and assay system, and further provides other related advantages.

DISCLOSURE OF INVENTION

Briefly stated, the present invention discloses methods for producing secreted receptor analogs, including receptor analogs and secreted platelet-derived growth factor receptor (PDGF-R) analogs. In addition, the present invention discloses methods for producing secreted biologically active dimerized polypeptide fusions.

Within one aspect of the invention a method for producing a secreted PDGF-R analog is disclosed, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding at least a portion of the ligand-binding domain of a PDGF-R, the portion including a ligand-binding domain; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF-R analog encoded by said DNA sequence; and (c) isolating the PDGF-R analog from the host cell.

Within one embodiment of the present invention, a PDGF-R analog comprising the amino acid sequence of FIGS. 1A–1B (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another embodiment, a PDGF-R analog comprising the amino acid sequence of FIGS. 1A–1B (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprising the amino acid sequence of FIGS. 11A–11B (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

Yet another aspect of the present invention discloses a method for producing a secreted, biologically active dimerized polypeptide fusion. The method generally comprises a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said DNA sequence; and (c) isolating the biologically active dimerized polypeptide fusion from the host cell.

Within one embodiment, the dimerizing protein is yeast invertase. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin light chain. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin heavy chain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the first DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (c)

isolating the biologically active dimerized polypeptide fusion from the host cell. In one embodiment, the DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a first polypeptide chain of a non-immunoglobulin polypeptide dimer requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of the non-immunoglobulin polypeptide dimer joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences wherein said dimerized polypeptide fusion exhibits biological activity characteristic of said non-immunoglobulin polypeptide dimer; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

Within one embodiment of the present invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIGS. 1A–1B (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another embodiment, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIGS. 1A–1B (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within another embodiment of the invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIGS. 11A–11B (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIGS. 1A–1B (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 dimerized to the amino acid sequence of FIGS. 11A–11B (sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and (c) isolating the receptor analog from the host cell.

In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_H4; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_H4; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of the receptor analog; and (c) isolating the receptor analog from the host cell. In one embodiment, the DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream of and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_H4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment, the first DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable

region joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream in proper reading frame by a first DNA sequence encoding a first polypeptide chain of a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_E4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of the ligand-binding domain of said receptor joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

Host cells for use in the present invention include cultured mammalian cells and fungal cells. In a preferred embodiment strains of the yeast *Saccharomyces cerevisiae* are used as host cells. Within another preferred embodiment cultured rodent myeloma cells are used as host cells.

Within one embodiment of the present invention, a receptor analog is a PDGF-R analog comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441. Within another embodiment a PDGF-R analog comprises the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531. Within another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 and the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

PDGF-R analogs produced by the above-disclosed methods may be used, for instance, within a method for determining the presence of human PDGF or an isoform thereof in a biological sample.

A method for determining the presence of human PDGF or an isoform thereof in a biological sample is disclosed and comprises (a) incubating a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of containing PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and (b) detecting the presence of receptor/ligand complexes, and therefrom determining the presence of PDGF or an isoform thereof. Suitable biological samples in this regard include blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media, and chemically or physically separated portions thereof.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A–1D (Sequence ID Numbers 1 and 2) illustrate the nucleotide sequence of a representative PDGF β-receptor cDNA and the derived amino acid sequence of the primary translation product and corresponds to Sequence ID Number 1). Numbers above the lines refer to the nucleotide sequence; numbers below the lines refer to the amino acid sequence.

FIG. 2 illustrates the construction of pBTL10, pBTL11 and pBTL12.

FIG. 3 illustrates the construction of pCBS22.

FIG. 4 illustrates the construction of pBTL13 and pBTL14.

FIG. 5 illustrates the construction of pBTL15.

FIG. 6 illustrates the construction of pBTL22 and pBTL26.

FIG. 7 illustrates the construction of pSDL114. Symbols used are S.S., signal sequence, C_κ, immunoglobulin light chain constant region sequence; μ prom, μ promoter; μ enh, μ enhancer.

FIG. 8 illustrates the construction of pSDLB113. Symbols used are S.S., signal sequence; C_H1 , C_H2, C_H3, immunoglobulin heavy chain constant region domain sequences; H, immunoglobulin heavy chain hinge region sequence; M, immunoglobulin membrane anchor sequences; $c_\gamma1M$, immunoglobulin heavy chain constant region and membrane anchor sequences.

FIG. 9 illustrates the constructions pBTL115, pBTL114, $p\phi5V_HHuC_\gamma1M$-neo, $pIC\phi5V_\kappa HuC_\kappa$-neo. Symbols used are set forth in FIGS. 7 and 8, and also include L_H, mouse immunoglobulin heavy chain signal sequence; V_H, mouse immunoglobulin heavy chain variable region sequence; E, mouse immunoglobulin heavy chain enhancer sequence; L_κ, mouse immunoglobulin light chain signal sequence; $\phi5V_\kappa$, mouse immunoglobulin light chain variable region sequence; Neo^R, *E. coli* neomycin resistance gene.

FIG. 10 illustrates the constructions Zem229R, $p\phi5V_HFab$-neo and pWKI. Symbols used are set forth in FIG. 9.

FIGS. 11A–11D illustrate the sequence of a representative PDGF α-receptor CDNA and the deduced amino acid sequence (using standard one-letter codes) encoded by the CDNA and corresponds to Sequence ID Numbers 35 and 36. Numbers at the ends of the lines refer to nucleotide positions. Numbers below the sequence refer to amino acid positions.

FIG. 12 illustrates the assembly of a cDNA molecule encoding a PDGF α-receptor. Complementary DNA sequences are shown as lines. Only those portions of the vectors adjacent to the cDNA inserts are shown.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA Construct

A DNA molecule, or a clone of such a molecule, either single- or double-stranded that has been modified through

human intervention to contain segments of DNA combined and juxtaposed in a manner that as a whole would not otherwise exist in nature.

DNA constructs contain the information necessary to direct the expression and/or secretion of DNA sequences encoding polypeptides of interest. DNA constructs will generally include promoters, enhancers and transcription terminators. DNA constructs containing the information necessary to direct the secretion of a polypeptide will also contain at least one secretory signal sequence.

Secretory Signal Sequence

A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly sythesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Such secretory peptides contain processing sites that allow cleavage of the signal peptides from the mature proteins as it passes through the secretory pathway. Processing sites may be encoded within the signal peptide or may be added to the signal peptide by, for example, in vitro mutagenesis. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast Barrier protein.

Receptor Analog

A non-immunoglobulin polypeptide comprising a portion of a receptor which is capable of binding ligand and/or is recognized by anti-receptor antibodies. The amino acid sequence of the receptor analog may contain additions, substitutions or deletions as compared to the native receptor sequence. A receptor analog may be, for example, the ligand-binding domain of a receptor joined to another protein. Platelet-derived growth factor receptor (PDGF-R) analogs may, for example, comprise a portion of a PDGF receptor capable of binding anti-PDGF receptor antibodies, PDGF, PDGF isoforms, PDGF analogs, or PDGF antagonists.

Dimerizing Protein

A polypeptide chain having affinity for a second polypeptide chain, such that the two chains associate under physiological conditions to form a dimer. The second polypeptide chain may be the same or a different chain.

Biological Activity

A function or set of activities performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile thereof). biological activities may include the induction of extracellular matrix secretion from responsive cell lines, the induction of hormone secretion, the induction of chemotaxis, the induction of mitogenesis, the induction of differentiation, or the inhibition of cell division of responsive cells. A recombinant protein or peptide is considered to be biologically active if it exhibits one or more biological activities of its native counterpart.

Ligand

A molecule capable of being bound by the ligand-binding domain of a receptor or by a receptor analog. The molecule may be chemically synthesized or may occur in nature. Ligands may be grouped into agonists and antagonists. Agonists are those molecules whose binding to a receptor induces the response pathway within a cell. Antagonists are those molecules whose binding to a receptor blocks the response pathway within a cell.

Joined

Two or more DNA coding sequences are said to be joined when, as a result of in-frame fusions between the DNA coding sequences or as a result of the removal of intervening sequences by normal cellular processing, the DNA coding sequences are translated into a polypeptide fusion.

As noted above, the present invention provides methods for producing biologically active dimerized polypeptide fusions and secreted receptor analogs, which include, for example, PDGF receptor analogs. Secreted receptor analogs may be used to screen for new compounds that act as agonists or antagonists when interacting with cells containing membrane-bound receptors. In addition, the methods of the present invention provide dimerized non-immunoglobulin polypeptide fusions of therapeutic value that are biologically active only as diners. Moreover, the present invention provides methods of producing polypeptide dimers that are biologically active only as non-covalently associated diners. Secreted, biologically active dimers that may be produced using the resent invention include nerve growth factor, colony stimulating factor-1, factor XIII, and transforming growth factor β.

As used herein, the ligand-binding domain of a receptor is that portion of the receptor that is involved with binding the natural ligand. While not wishing to be bound by theory, the binding of a natural ligand to a receptor is believed to induce a conformational change which elicits a response to the change within the response pathway of the cell. For membrane-bound receptors, the ligand-binding domain is generally believed to comprise the extracellular domain for the receptor. The structure of receptors may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mt. View, Calif.) or may be predicted according to the methods described, for example, by Kyte and Doolittle, *J. Mol. Biol.* 157:105–132, 1982). The ligand-binding domain of the PDGF β-receptor, for example, has been predicted to include amino acids 29–531 of the published sequence (Gronwald et al., ibid.). The ligand-binding domain of the PDGF α-receptor has been predicted to include amino acids 25–500 of the published α-receptor sequence (Matsui et al., ibid.). As used herein, the ligand-binding domain of the PDGF β-receptor includes amino acids 29–441 of the sequence of FIGS. 1A–1D (Sequence ID Number 1) and C-terminal extensions up to and including amino acid 531 (FIG. 1B). The ligand-binding domain of the PDGF α-receptor is understood to include amino acids 24–524 of FIGS. 11A & 11B (Sequence ID Numbers 35 and 36).

Receptor analogs that may be used in the present invention include the ligand-binding domains of the epidermal growth factor receptor (EGF-R) and the insulin receptor. As used herein, a ligand-binding domain is that portion of the receptor that is involved in binding ligand and is generally a portion or essentially all of the extracellular domain that extends from the plasma membrane into the extracellular space. The ligand-binding domain of the EGF-R, for example, resides in the extracellular domain. EGF-R diners have been found to exhibit higher ligand-binding affinity

than EGF-R monomers (Honi-Schnetzler and Pilch, *Proc. Natl. Acad. Sci. USA* §A:7832–7836, 1987). The insulin receptor (Ullrich et al., *Nature* 313:756–761, 1985) requires dimerization for biological activity.

Another example of a receptor that may be secreted from a host cell is a platelet-derived growth factor receptor (PDGF-R). Two classes of PDGF-Rs, which recognized different isoforms of PDGF, have been identified. (PDGF is a disulfide-bonded, two-chain molecule, which is made up of an A chain and a B chain. These chains may be combined as AB heterodimers, AA homodimers or BB homodimers. These dimeric molecules are referred to herein as "isoforms".) The β-receptor (PDGFβ-R), which recognizes only the BB isoform of PDGF (PDGF-BB), has been described (Claesson-Welsh et al., *Mol. Cell, Biol.* 8:3476–3486, 1988; Gronwald et al., *Proc. Natl. Acad. Sci. USA* 85:3435–3439, 1988). The α-receptor (PDGFα-R), which recognizes all three PDGF isoforms (PDGF-AA, PDGF-AB and PDGF-BB), has been described by Matsui et al. (*Science* 243:800–804, 1989) and Kelly and Murray (pending commonly assigned U.S. patent application Ser. No. 07/355,018, which is incorporated herein by reference). The primary translation products of these receptors indicate that each includes an extracellular domain implicated in the ligand-binding process, a transmembrane domain, and a cytoplasmic domain containing a tyrosine kinase activity.

The present invention provides a standardized assay system, not previously available in the art, for determining the presence of PDGF, PDGF isoforms, PDGF agonists or PDGF antagonists using a secreted PDGF receptor analogs. Such an assay system will typically involve combining the secreted PDGF receptor analog with a biological sample under physiological conditions which permit the formation of receptor-ligand complexes, followed by detecting the presence of the receptor-ligand complexes. The term physiological conditions is meant to include those conditions found within the host organism and include, for example, the conditions of osmolarity, salinity and pH. Detection may be achieved through the use of a label attached to the PDGF receptor analog or through the use of a labeled antibody which is reactive with the receptor analog or the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Receptor-ligand complexes may also be detected visually, i.e., in immunoprecipitation assays which do not require the use of a label. This assay system provides secreted PDGF receptor analogs that may be utilized in a variety of screening assays for, for example, screening for analogs of PDGF. The present invention also provides a methods for measuring the level of PDGF and PDGF isoforms in biological fluids.

As noted above, the present invention provides methods for producing dimerized polypeptide fusions that require dimerization for biological activity or enhancement of biological activity. Polypeptides requiring dimerization for biological activity include, in addition to certain receptors, nerve growth factor, colony-stimulating factor-1 (CSF-1), transforming growth factor β (TGF-β) , PDGF, and factor XIII. Nerve growth factor is a non-covalently linked diner (Harper et al., *J. Biol. Chem.* 257: 8541–8548, 1982). CSF-1, which specifically stimulates the proliferation and differentiation of cells of monomuclear phagocytic lineage, is a disulfide-bonded homodimer (Rettemmier et al., *Mol. Cell. Biol.* 7: 2378–2387, 1987). TGF-β is biologically active as a disulfide-bonded dimer (Assoian et al., *J. Biol. Chem.* 258: 7155–7160, 1983). Factor XIII is a plasma protein that exists as a two chain homodimer n its activated form (Ichinose et al., *Biochem.* 25: 6900– 6906, 1986). PDGF, as noted above, is a disulfide-bonded, two chain molecule (Murray et al., U.S. Pat. No. 4,766,073).

The present invention provides methods by which receptor analogs, including receptor analogs and PDGF-R analogs, requiring dimerization for activity may be secreted from host cells. The methods described herein are particularly advantageous in that they allow the production of large quantities of purified receptors. The receptors may be used in assays for the screening of potential ligands, in assays for binding studies, as imaging agents, and as agonists and antagonists within therapeutic agents.

A DNA sequence encoding a human PDGF receptor may be isolated as a cDNA using techniques known in the art (see, for example, Okayama and Berg, *Mol. Cell. Biol.* 2:161–170, 1982; *Mol. Cell. Biol.* 3: 280–289, 1983) from a library of human genomic or cDNA sequences. Such libraries may be prepared by standard procedures, such as those disclosed by Gubler and Hoffman (*Gene* 25: 263–269, 1983). It is preferred that the molecule is a CDNA molecule because CDNA lack introns and are therefore more suited to manipulation and expression in transfected or transformed cells. Sources of mRNA for use in the preparation of a CDNA library include the MG-63 human osteosarcoma cell line (available from ATCC under accession number CRL 1427), diploid human dermal fibroblasts and human embryo fibroblast and brain cells (Matsui et al., ibid.). A CDNA encoding a PDGFβ-R has been cloned from a diploid human dermal fibroblast cDNA library using oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Gronwald et al., ibid.). A PDGFα-R cDNA has been isolated by Matsui et al. (ibid.) from human embryo fibroblast and brain cells. Alternatively, a cDNA encoding a PDGFα-R may be isolated from a library prepared from MG-63 human osteosarcoma cells using a cDNA probe containing sequences encoding the transmembrane and cytoplasmic domains of the PDGFβ-R (described by Kelly and Murray, ibid.). Partial CDNA clones (fragments) can be extended by re-screening of the library with the cloned CDNA fragment until the full sequence is obtained. In one embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 1A & 1B (Sequence ID Number 1) from amino acid 29 through amino acid 441. In another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 1A & 1B (Sequence ID Number 1) from amino acid 29 through amino acid 531. In yet another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 11A & 11B (Sequence ID Numbers 35 and 36) from amino acid 24 through amino acid 524. One skilled in the art may envision the use of a smaller DNA sequence encoding the ligand-binding domain of a PDGF receptor containing at least 400 amino acids of the extracellular domain.

DNA sequences encoding EGF-R (Ullrich et al., *Nature* 3: 418–425, 1984), the insulin receptor (Ullrich et al., *Nature* 313: 756–761, 1985), nerve growth factor (Ullrich et al. *Nature* 303: 821–825, 1983), colony stimulating factor-1 (Rettenmier et al., ibid.), transforming growth factor β (Derynck et al., *Nature* 3: 701–705, 1985), PDGF (Murray et al., ibid.), and factor XIII (Ichinose et al., ibid.) may also be used within the present invention.

To direct polypeptides requiring dimerization for biological activity or receptor analogs into the secretory pathway of the host cell, at least one secretory signal sequence is used in conjunction with the DNA sequence of interest. Preferred secretory signals include the alpha factor signal sequence (pre-pro sequence) (Kurjan and Herkowitz, *Cell* 30: 933–943, 1982; Kurjan et al., U.S. Pat. No. 4,546,082;

Brake, EP 116,201, 1983), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Pat. No. 4,613,572; MacKay, WO 87/002670), immunoglobulin V_H signal sequences (Loh et al., *Cell* 33: 85–93, 1983; Watson *Nuc. Acids. Res* 12: 5145–5164, 1984) and immunoglobulin V_κ signal sequences (Watson, ibid.). Particularly preferred signal sequences are the SUC2 signal sequence (Carlson et al., *Mol. Cell. Biol.* 3: 439–447, 1983) and PDGF receptor signal sequences. Alternatively, secretory signal sequences may be synthesized according to the rules established, for example, by von Heinje (*Eur. J. Biochem.* 133: 17–21, 1983; *J. Mol. Biol.* 184: 99–105, 1985; *Nuc. Acids. Res.* 14: 4683–3690, 1986).

Secretory signal sequences may be used singly or may be combined. For example, a first secretory signal sequence may be used singly or combined with a sequence encoding the third domain of Barrier (described in co-pending commonly assigned U.S. patent application Ser. No. 07/104,316, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the DNA sequence and in proper reading frame with both the secretory signal sequence and the DNA sequence of interest.

In one embodiment of the present invention, a sequence encoding a dimerizing protein is joined to a sequence encoding a polypeptide chain of a polypeptide dimer or a receptor analog, and this fused sequence is joined in proper reading frame to a secretory signal sequence. As shown herein, the present invention utilizes such an arrangement to drive the association of the polypeptide or receptor analog to form a biologically active molecule upon secretion. Suitable dimerizing proteins include the *S. cerevisiae* repressible acid phosphatase (Mizunaga et al., *J. Biochem.* (Tokyo) 103: 321–326, 1988), the *S. cerevisiae* type 1 killer preprotoxin (Sturley et al., *EMBO J.* 5: 3381–3390, 1986), the *S. calsbergensis* alpha galactosidase melibiase (Sumner-Smith et al., *Gene* 36: 333–340, 1985), the *S. cerevisiae* invertase (Carlson et al., *Mol. Cell. Biol.* 3: 39–447, 1983), the *Neurospora crassa* ornithine decarboxylase (Digangi et al., *J. Biol. Chem.* 6: 7889–7893, 1987), immunoglobulin heavy chain hinge regions (Takahashi et al., *Cell* 29: 671–679, 1982), and other dimerizing immunoglobulin sequences. In a preferred embodiment, *S. cerevisiae* invertase is used to drive the association of polypeptides into dimers. Portions of dimerizing proteins, such as those mentioned above, may be used as dimerizing proteins where those portions are capable of associating as a dimer in a covalent or noncovlent manner. Such portions may be determined by, for example, altering a sequence encoding a dimerizing protein through In vitro mutagenesis to delete portions of the coding sequence. These deletion mutants may be expressed in the appropriate host to determine which portions retain the capability of associating as dimers. Portions of immunoglobulin gene sequences may be used to drive the association of non-immunoglobulin polypeptides. These portions correspond to discrete domains of immunoglobulins. Immunoglobulins comprise variable and constant regions, which in turn comprise discrete domains that show similarity in their three-dimensional conformations. These discrete domains correspond to immunoglobulin heavy chain constant region domain exons, immunoglobulin heavy chain variable region domain exons, immunoglobulin light chain varable region domain exons and immunoglobulin light chain constant region domain exons in immunoglobulin genes (Hood et al., in *Immunology*, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, Calif.; Honjo et al., *Cell* 18: 559–568, 1979; Takahashi et al., *Cell* 29: 671–679, 1982; and Honjo, *Ann. Rev. Immun.* 1:499–528, 1983)). Particularly preferred portions of immunoglobulin heavy chains include Fab and Fab' fragments. (An Fab fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain variable region domain and a heavy chain constant region omain. An Fab' fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain variable region domain, a heavy chain constant region domain and a heavy chain hinge region domain.)

It is preferred to use an immunoglobulin light chain constant region in association with at least one immunoglobulin heavy chain constant region domain. In a another embodiment, an immunoglobulin light chain constant region is associated with at least one immunoglobulin heavy chain constant region domain joined to an immunoglobulin hinge region. In one set of embodiments, an immunoglobulin light chain constant region joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with at least one heavy chain constant region. In a preferred set of embodiments a variable region is joined upstream of and in proper reading frame with at least one immunoglobulin heavy chain constant region. In another set of embodiments, an immunoglobulin heavy chain is joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with an immunoglobulin light chain constant region. In yet another set of embodiments, a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog is joined to at least one immunoglobulin heavy chain constant region which is joined to an immunoglobulin hinge region and is associated with an immunoglobulin light chain constant region. In a preferred set of embodiments an Immunoglobulin variable region is joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

Immunoglobulin heavy chain constant region domains include C_H1, C_H2, C_H3, and C_HA of any class of immunoglobulin heavy chain including γ, α, ε, μ, and δ classes (Honjo, ibid., 1983) A particularly preferred immunoglobulin heavy chain constant region domain is human C_H1. Immunoglobulin variable regions include V_H, V_κ or V_λ.

DNA sequences encoding immunoglobulins may be cloned from a variety of genomic or CDNA libraries known in the art. The techniques for isolating such DNA sequences using probe-based methods are conventional techniques and are well known to those skilled in the art. Probes for isolating such DNA sequences may be based on published DNA sequences (see, for example, Hieter et al., *Cell* 2: 197–207, 1980). Alternatively, the polymerase chain reaction (PCR) method disclosed by Mullis et al. (U.S. Pat. No. 4,683,195) and Mullis (U.S. Pat. No. 4,683,202), incorporated herein by reference may be used. The choice of library and selection of probes for the isolation of such DNA sequences is within the level of ordinary skill in the art.

Host cells for use in practicing the present invention include eukaryotic cells capable of being transformed or transfected with exogenous DNA and grown in culture, such as cultured mammalian and fungal cells. Fungal cells, including species of yeast (e.g., Saccharomyces spp., Schizosaccharomyces spp.), or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred.

Expression units for use in the present invention will generally comprise the following elements, operably linked in a 5' to 3' orientation: a transcriptional promoter, a secre-

tory signal sequence a DNA sequence encoding nonimmunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein and a transcriptional terminator. The selection of suitable promoters, signal sequences and terminators will be determined by the selected host cell and will be evident to one skilled in the art and are discussed more specifically below.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., *Proc. Natl. Acad. Sci. USA* 7: 1035–1039, 1978), YEp13 (Broach et al., *Gene* 8: 121–133, 1979), pJDB249 and pJDB219 (Beggs, *Nature* 275:104–108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al. ibid.), URA3 (Botstein et al., *Gene p:* 17, 1979), HIS3 (Struhl et al., ibid.) or POTI (Kawasaki and Bell, EP 171,142). Other suitable selectable markers include the CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255: 12073–12080, 1980; Alber and Kawasaki, *J. Mol. A Genet.* 1: 419–434, 1982; Kawasaki, U.S. Pat. No. 4,599, 311) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol.* 101: 192–201, 1983). In this regard, particularly preferred promoters are the TPI1 promoter (Kawasaki, U.S. Pat. No. 4,599,311, 1986) and the ADH2–4^{c} promoter (Russell et al., *Nature* 304: 652–654, 1983 and Irani and Kilgore, described in pending, commonly assigned U.S. patent application Ser. No. 07/183,130, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi Aspercillus (McKnight and Upshall, described in commonly assigned U.S. Pat. No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the ADH3 promoter (McKnight et al., *EMBO J.* 4: 2093–2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., ibid.). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75: 1929–1933, 1978), Yelton et al., (*Proc. Natl Acad. Sci. USA* 81: 1740–1747, 1984), and Russell (*Nature* 301: 167–169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In a preferred embodiment, a *Saccharomyces cerevisiae* host cell that contains a genetic deficiency in a gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, the *S. cerevisiae* host cell contains a genetic deficiency in the MNN9 gene (described in pending, commonly assigned U.S. patent application Ser. Nos. 116,095 and 189,547 which are incorporated by reference herein in their entirety). Most preferably, the *S. cerevisiae* host cell contains a disruption of the MNN9 gene. *S. cerevisiae* host cells having such defects may be prepared using standard techniques of mutation and selection. Ballou et al. (*J. Biol. Chem.* 255: 5986–5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized *S. cerevisiae* cells were screened using fluoresceinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties. To optimize production of the heterologous proteins, it is preferred that the host strain carries a mutation, such as the S. cerevisiae pep4 mutation (Jones, *Genetics* 85: 23–33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cell lines are rodent myeloma cell lines, which include p3X63Ag8 (ATCC TIB 9), OF (ATCC CRL 1646), NS-1 (ATCC TIB 18) and 210-RCY-Ag1 (Galfre et al., *Nature* 277: 131, 1979). A particularly preferred rodent myeloma cell line is SP2/0-Ag14 (ATCC CRL 1581). In addition, a number of other cell lines may be used within the present invention, including COS-1 (ATCC CRL 1650), BHK, p363.Ag.8.653 (ATCC CRL 1580) Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CC 29.1), 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36: 59–72, 1977) and DUKX cells (Urlaub and Chasin, *Proc. Patl. Acad. Sci USA* 77: 4216–4220, 1980) A preferred BHK cell line is the tk^{-}ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci USA* 79: 1106–1110, 1982). A preferred BHK cell line is the tk-ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79: 1106–1110, 1982). A tk^{-} BHK cell line is available from the American Type Culture Collection, Rockville, Md., under accession number CRL 1632. A particularly preferred tk^{-} BHK cell line is BHK 570 which is available from the American Type Culture Collection under accession number 10314.

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. referred promoters include viral promoters and cellular promoters. Preferred viral promoters include the major late promoter from adenovirus 2 (Kaufman and Sharp, *Mol. Cell. Biol.* 2: 1304–13199, 1982) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol,* 1: 854–864, 1981). Preferred cellular promoters include the mouse metallothionein 1 promoter (Palmiter et al., *Science* 222: 809–814, 1983) and a mouse V_{k} promoter (Grant et al., *Nuc. Acids Res.* 11: 5496, 1987). A particularly preferred promoter is a mouse V_{H} promoter (Loh et al., ibid.). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman

and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 2: 3719–3730, 1981). A particularly preferred polyadenylation signal is the V_H gene terminator (Loh et al., ibid.). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, *Cell* 33: 717–728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell* 14: 725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7: 603, 1981; Graham and Van der Eb, *Virology* 52: 456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., *EMBO J.* 1: 841–845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene. A particularly preferred amplifiable marker is the DHFR' CDNA (Simonsen and Levinson, *Proc. Natl. Adac. Sci. USA* 80: 2495–2499, 1983). Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*; Butterworth Publishers, Stoneham, Mass.) and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Pat. No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1–2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Host cells containing DNA constructs of the resent invention are grown in an appropriate growth medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which are complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Yeast cells, for example, are preferably grown in a chemically defined medium, comprising a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 6.5. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, Mo.). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 0.1 M and 1.5 M., preferably at 0.5 M or 1.0 M. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The culture medium from appropriately grown transformed or transfected host cells is separated from the cell material, and the presence of dimerized polypeptide fusions or secreted receptor analogs is demonstrated. A preferred method of detecting receptor analogs, for example, is by the binding of the receptors or portions of receptors to a receptor-specific antibody. An anti-receptor antibody may be a monoclonal or polyclonal antibody raised against the receptor in question, for example, an anti-PDGF receptor monoclonal antibody may be used to assay for the presence of PDGF receptor analogs. Another antibody, which may be used for detecting substance P-tagged peptides and proteins, is a commercially available rat anti-substance P monoclonal antibody which may be utilized to visualize peptides or proteins that are fused to the C-terminal amino acids of substance P. Ligand binding assays may also be used to detect the presence of receptor analogs. In the case of PDGF receptor analogs, it is preferable to use fetal foreskin fibroblasts, which express PDGF receptors, to compete against the PDGF receptor analogs of the present invention for labeled PDGF and PDGF isoforms.

Assays for detection of secreted, biologically active peptide dimers and receptor analogs may include Western transfer, protein blot or colony filter. A Western transfer filter may be prepared using the method described by Towbin et al. (*Proc. Natl. Acad. Sci. USA* 76: 4350–4354, 1979). Briefly, samples are electrophoresed in a sodium dodecylsulfate polyacrylamide gel. The proteins in the gel are electrophoretically transferred to nitrocellulose paper. Protein blot filters may be prepared by filtering supernatant samples or concentrates through nitrocellulose filters using, for example, a Minifold (Schleicher & Schuell, Keene, N.H.). Colony filters may be prepared by growing colonies on a nitrocellulose filter that has been laid across an appropriate growth medium. In this method, a solid medium is preferred. The cells are allowed to grow on the filters for at least 12 hours. The cells are removed from the filters by washing with an appropriate buffer that does not remove the proteins bound to the filters. A preferred buffer comprises 25 mM Tris-base, 19 mM glycine, pH 8.3, 20% methanol.

The dimerized polypeptide fusions and receptor analogs present on the Western transfer, protein blot or colony filters may be visualized by specific antibody binding using methods known in the art. For example, Towbin et al. (ibid.) describe the visualization of proteins immobilized on nitrocellulose filters with a specific antibody followed by a labeled second antibody, directed against the first antibody. Kits and reagents required for visualization are commercially available, for example, from Vector Laboratories, (Burlingame, Calif.), and Sigma Chemical Company (St. Louis, Mo.).

Secreted, biologically active dimerized polypeptide fusions and receptor analogs may be isolated from the medium of host cells grown under conditions that allow the secretion of the biologically active dimerized polypeptide fusions and receptor analogs. The cell material is removed from the culture medium, and the biologically active dimerized polypeptide fusions and receptor analogs are isolated using isolation techniques known in the art. Suitable isolation techniques include precipitation and fractionation by a variety of chromatographic methods, including gel filtration, ion exchange chromatography and immunoaffinity chromatography. A particularly preferred purification method is immunoaffinity chromatography using an antibody directed against the receptor analog or dimerized polypeptide fusion. The antibody is preferably immobilized or attached to a solid support or substrate. A particularly preferred substrate is CNBr-activated Sepharose (Pharmacia LKB Technologies, Inc., Piscataway, N.J.). By this method, the medium is combined with the antibody/substrate under conditions that will allow binding to occur. The complex may be washed to remove unbound material, and the receptor analog or peptide dimer is released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods of elution include changes in pH, wherein the immobilized antibody has a high affinity for the ligand at a first pH and a reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs of the present invention can be used within a variety of assays for detecting the presence of and/or screening for native PDGF, PDGF isoforms or PDGF-like molecules. These assays will typically involve combining PDGF receptor analogs, which may be bound to a solid substrate such as polymeric microtiter plate wells, with a biological sample under conditions that permit the formation of receptor/ligand complexes. Screening assays for the detection of PDGF, PDGF isoforms or PDGF-like molecules will typically involve combining soluble PDGF receptor analogs with a biological sample and incubating the mixture with a PDGF isoform or mixture of PDGF isoforms bound to a solid substrate such as polymeric microtiter plates, under conditions that permit the formation of receptor/ligand complexes. Detection may be achieved through the use of a label attached to the receptor or through the use of a labeled antibody which is reactive with the receptor. Alternatively, the labeled antibody may be reactive with the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Complexes may also be detected visually, i.e., in immunoprecipitation assays, which do not require the use of a label.

Secreted PDGF receptor analogs of the present invention may also be labeled with a radioisotope or other imaging agent and used for in vivo diagnostic purposes. Preferred radioisotope imaging agents include iodine-125 and technetium-99, with technetium-99 being particularly preferred. Methods for producing protein-isotope conjugates are well known in the art, and are described by, for example, Eckelman et al. (U.S. Pat. No. 4,652,440), Parker et al. (WO 87/05030) and Wilber et al. (EP 203,764). Alternatively, the secreted receptor analogs may be bound to spin label enhancers and used for magnetic resonance (MR) imaging. Suitable spin label enhancers include stable, sterically hindered, free radical compounds such as nitroxides. Methods for labeling ligands for MR imaging are disclosed by, for example, Coffman et al. (U.S. Pat. No. 4,656,026). For administration, the labeled receptor analogs are combined with a pharmaceutically acceptable carrier or diluent, suchEs sterile saline or sterile water. Administration is preferably by bolus injection, preferably intravenously. These imaging agents are particularly useful in identifying the locations of atherosclerotic plaques, PDGF-producing tumors, and receptor-bound PDGF.

The secreted PDGF receptor analogs of the present invention may also be utilized within diagnostic kits. Briefly, the subject receptor analogs are preferably provided in a lyophilized form or immobilized onto the walls of a suitable container, either alone or in conjunction with antibodies capable of binding to native PDGF or selected PDGF isoform(s) or specific ligands. The antibodies, which may be conjugated to a label or unconjugated, are generally included in the kits with suitable buffers, such as phosphate, stabilizers, inert proteins or the like. Generally, these materials are present in less than about 5% weight based upon the amount of active receptor analog, and are usually present in an amount of at least about 0.001% weight. It may also be desirable to include an inert excipient to dilute the active ingredients. Such an excipient may be present from about 1% to 99% weight of the total composition. In addition, the kits will typically include other standard reagents, instructions and, depending upon the nature of the label involved, reactants that are required to produce a detectable product. Where an antibody capable of binding to the receptor or receptor/ligand complex is employed, this antibody will usually be provided in a separate vial. The antibody is typically conjugated to a label and formulated in an analogous manner with the formulations briefly described above. The diagnostic kits, including the containers, may be produced and packaged using conventional kit manufacturing procedures.

As noted above, the secreted PDGF receptor analogs of the present invention may be utilized within methods for purifying PDGF from a variety of samples. Within a preferred method, the secreted PDGF receptor analogs are immobilized or attached to a substrate or support material, such as polymeric tubes, beads, polysaccharide particulates, polysaccharide-containing materials, polyacrylamide or other water insoluble polymeric materials. Methods for immobilization are well known in the art (Mosbach et al., U.S. Pat. No. 4,415,665; Clarke et al., *Meth. Enzymology* : 436–442, 1979). A common method of immobilization is CNBr activation. Activated substrates are commercially available from a number of suppliers, including Pharmacia (Piscataway, N.J.), Pierce chemical Co. (Rockford, Ill.) and Bio-Rad Laboratories (Richmond, Calif.). A preferred substrate is CNBr-activated Sepharose (Pharmacia, Piscataway, N.J.). Generally, the substrate/receptor analog complex will be in the form of a column. The sample is then combined with the immobilized receptor analog under conditions that allow binding to occur. The substrate with immobilized receptor analog is first equilibrated with a buffer solution of a composition in which the receptor analog has been previously found to bind its ligand. The sample, in the solution used for equilibration, is then applied to the substrate/receptor analog complex. Where the complex is in the form of a column, it is preferred that the sample be passed over the column two or more times to permit full binding of ligand to receptor analog. The complex is then washed with the same solution to elute unbound material. In addition, a second wash solution may be used to minimize nonspecific binding. The bound material may then be released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods include changes in pH, wherein the immobilized receptor has a high affinity for

PDGF at a first pH and reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs fused to dimerizing proteins of the present invention may be used in pharmaceutical compositions for topical or intravenous application. The secreted PDGF receptor analogs of the present invention may be useful in the treatment of atherosclerosis by, for example, binding endogenous PDGF to prevent smooth muscle cell proliferation. The PDGF receptor analogs fused to dimerizing proteins are used in combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include saline and sterile water. Pharmaceutical compositions may also contain stabilizers and adjuvants. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Enzymes, including restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase, were obtained from New England Biolabs (Beverly, Mass.), GIBCO-BRL (Gaithersburg, Md.) and Boerhinger-Mannheim Biochemicals (Indianapolis, Ind.) and were used as directed by the manufacturer or as described in Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, NY, 1982) and Sambrook et al. (*Molecular Cloning: A Laboratory Manual*/Second Edition, Cold Spring Harbor Laboratory, NY, 1989).

Example 1

Cloning PDGF Receptor cDNAs

A. Cloning the PDGF β-Receptor

A cDNA encoding the PDGF β-receptor was cloned as follows. Complementary DNA (cDNA) libraries were prepared from poly(A) RNA from diploid human dermal fibroblast cells, prepared by explant from a normal adult, essentially as described by Hagen et al. (*Proc. Natl. Acad. Sci. USA* 83: 2412–2416, 1986). Briefly, the poly(A) RNA was primed with oligo d(T) and cloned into λgt11 using Eco RI linkers. The random primed library was screened for the presence of human PDGF receptor cDNA's using three oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Yarden et al., *Nature* 323: 226–232, 1986). Approximately one million phage from the random primed human fibroblast cell library were screened using oligonucleotides ZC904, ZC905 and ZC906 (Table 1; Sequence ID Numbers 5, 6 and 7, respectively). Eight positive clones were identified and plaque purified. Two clones, designated RP41 and RP51, were selected for further analysis by restriction enzyme mapping and DNA sequence analysis. RP51 was found to contain 356 bp of 5'-noncoding sequence, the ATG translation initiation codon and 738 bp of the amino terminal coding sequence. RP41 was found to overlap clone RP51 and contained 2649 bp encoding amino acids 43–925 of the β-receptor protein.

TABLE 1

Oligonucleotide Sequences

ZC871 (Sequence ID Number 3)
5' GTC TCT TCC TCA GGT AAA TGA GTG CCA GGG CCG GCA AGC CCC CGC TCC 3'

ZC872 (Sequence ID Number 4)
5' CCG GGG AGC GGG GGC TTG CCG GCC CTG GCA CTC ATT TAC CTG AGG AAG AGA CAC CT 3'

ZC904 (Sequence ID Number 5)
5' CAT GGG CAC GTA ATC TAT AGA TTC ATC CTT GCT CAT ATC CAT GTA 3'

ZC905 (Sequence ID Number 6)
5' TCT TGC CAG GGC ACC TGG GAC ATC TGT TCC CAC ATC ACC GG 3'

ZC906 (Sequence ID Number 7)
5' AAG CTG TCC TCT GCT TCA GCC AGA GGT CCT GGG CAG CC 3'

ZC1380 (Sequence ID Number 8)
5' CAT GGT GGA ATT CCT GCT GAT 3'

ZC1447 (Sequence ID Number 9)
5' TG GTT GTG CAG AGC TCA GGA AGA GAT CGA 3'

ZC1453 (Sequence ID Number 10)
5' AAT TCA TTA TGT TGT TGC AAG CCT TCT TGT TCC TGC TAG CTG GTT TCG CTG TTA A 3'

ZC1454 (Sequence ID Number 11)
5' GAT CTT AAC AGC GAA ACC AGC TAG CAG GAA CAA GAA GGC TTG CAA CAA CAT AAT G 3'

ZC1478 (Sequence ID Number 12)
5' ATC GCG AGC ATG CAG ATC TGA 3'

ZC1479 (Sequence ID Number 13)
5' AGC TTC AGA TCT GCA TGC TCC CGA T 3'

ZC1776 (Sequence ID Number 14)
5' AGC TGA GCG CAA ATG TTG TGT CGA GTG CCC ACC GTG CCC AGC TTA GAA TTC T 3'

ZC1777 (Sequence ID Number 15)
5' CTA GAG AAT TCT AAG CTG GGC ACG GTG GGC ACT CGA CAC AAC ATT TGC GCT C 3'

ZC1845 (Sequence ID Number 16)
5' GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG CCC TTT AAG CA 3'

ZC1847 (Sequence ID Number 17)
5' AGC TTG CTT AAA GGG CAA GGA GTG TGG CAC CAC GAT GAC CTC CTG CGT GTC CTG GCC CAC AGC GTT GCG CAG CGT GCA GCG CAC CGA CAG TGG CC 3'

ZC1886 (Sequence ID Number 18)
5' CCA GTG CCA AGC TTG TCT AGA CTT ACC TTT AAA GGG CAA GGA G 3'

ZC1892 (Sequence ID Number 19)
5' AGC TTG AGC GT 3'

ZC1893 (Sequence ID Number 20)
5' CTA CAC GCT CA 3'

ZC1894 (Sequence ID Number 21)
5' AGC TTC CAG TTC TTC GGC CTC ATG TCA GTT CTT CGG CCT CAT GTG AT 3'

ZC1895 (Sequence ID Number 22)
5' CTA GAT CAC ATG AGG CCG AAG AAC TGA CAT GAG GCC GAA GAA CTG GA 3'

ZC2181 (Sequence ID Number 23)

TABLE 1-continued

Oligonucleotide Sequences

5' AAT TCG GAT CCA CCA TGG GCA CCA GCC ACC CGG CGT TCC TGG TGT TAG GCT GCC TGC TGA CCG GCC 3'

ZC2182 (Sequence ID Number 24)
5' TGA GCC TGA TCC TGT GCC AAC TGA GCC TGC CAT CGA TCC TGC CAA ACG AGA ACG AGA AGG TTG TGC AGC TA 3'

ZC2183 (Sequence ID Number 25)
5' AAT TTA GCT GCA CAA CCT TCT CGT TCT CGT TTG GCA GGA TCG ATG GCA GGC TCA GTT GGC ACA GGA TCA 3'

ZC2184 (Sequence ID Number 26)
5' GGC TCA GGC CGG TCA GCA GGC AGC CTA ACA CCA GGA ACG CCG GGT GGC TGG TGC CCA TGG TGG ATC CG 3'

ZC2311 (Sequence ID Number 27)
5' TGA TCA CCA TGG CTC AAC TG 3'

ZC2351 (Sequence ID Number 28)
5' CGA ATT CCA C 3'

ZC2352 (Sequence ID Number 29)
5' CAT GGT GGA ATT CGA GCT 3'

ZC2392 (Sequence ID Number 30)
5' ACG TAA GCT TGT CTA GAC TTA CCT TCA GAA CGC AGG GTG GG 3'

The 3'-end of the CDNA was not isolated in the first cloning and was subsequently isolated by screening $6x10^5$ phage of the oligo d(T)-primed CDNA library with a 630 bp Sst I-Eco RI fragment derived from the 3'-end of clone RP41. One isolate, designated TO91, was further analyzed by restriction enzyme mapping and DNA sequencing. This clone was found to comprise the 3'-end of the receptor coding region and 1986 bp of 3' untranslated sequence.

Clones RP51, RP41 and TO91 were ligated together to construct a full-length CDNA encoding the entire PDGF β-receptor. RP41 was digested with Acc I and Bam HI to isolate the 2.12 kb fragment. RP51 was digested with Eco RI and Acc I to isolate the 982 bp fragment. The 2.12 kb RP41 fragment and the 982 bp RP51 fragment were joined in a three-part ligation with pUC13, which had been linearized by digestion with Eco RI and Bam HI. The resultant plasmid was designated 51/41. Plasmid 51/41 was digested with Eco RI and Bam HI to isolate the 3 kb fragment comprising the partial PDGF receptor cDNA. OT91 was digested with Bam HI and Xba I to isolate the 1.4 kb fragment containing the 3' portion of the PDGF receptor cDNA. The Eco RI-Bam HI 51/41 fragment, the Bam HI-Xba I TO91 fragment and the Eco RI-Xba I digested pUC13 were joined in a three-part ligation. The resultant plasmid was designated pR-RX1 (FIG. 2).

B. Cloning the PDGF-α Receptor

A cDNA encoding to PDGF α-receptor was cloned as follows. RNA was prepared by the method of Chirgwin et al. (*Biochemistry* 18: 5294, 1979) and twice purified on oligo d(T) cellulose to yield poly(A)+RNA. Complementary DNA was prepared in Igtlo phage using a kit purchased from Invitrogen (San Diego, CA). The resulting λ phage DNA was packaged with a coat particle mixture from Stratagene Cloning Systems (La Jolla, Calif.), infected into *E. coli* strain C600 Hfl⁻ and titered.

Approximately $1.4x10^6$ phage recombinants were plated to produce plaques for screening. Nitrocellulose filter lifts were prepared according to standard methods and were hybridized to a radiolabeled PDGF β-receptor DNA fragment (Gronwald et al., ibid.) comprising the 1.9 kb Fsp I-Hind III fragment that encodes the transmembrane and cytoplasmic domains of the PDGF β-receptor cDNA. Hybridization was performed for 36 hours at 42° C. in a mixture containing 40% formamide, 5×SSCP (SSC containing 25 mM phosphate buffer, pH 6.5), 200 μg/ml denatured salmon sperm DNA, 3×Denhardt's, and 10% dextran sulfate. Following hybridization, the filters were washed extensively at room temperature in 2×SSC, then for 15 minutes at 47–48° C. Following an exposure to X-ray film, the filters were treated to increasingly stringent wash conditions followed by film recording until a final wash with 0.1×SSC at 65° C. was reached. Film analysis showed that a "family" of plaques that hybridized at lower wash stringency but not at the highest stringency. This "family" was selected for further analysis.

Two λ phage clones from the "family" obtained from the initial screening were subcloned into the Not I site of the pUClype plasmid vector pBluescript SK⁺ (obtained from Stratagene Cloning Systems, La Jolla, Calif.) and were analyzed by restriction mapping and sequence analysis. Restriction enzyme analysis of a phage clone, designated α1-1, revealed a restriction fragment pattern dissimilar from that of the PDGF β-receptor CDNA with the exception of a common Bgl II-Bgl II band of approximately 160 bp. The PDGF β-receptor cDNA contains two similarly spaced Bgl II sites within the region coding for the second tyrosine kinase domain.

Restriction analysis of a second plasmid subclone (designated α1-7) revealed an overlap of the 5' approximately 1.2 kb of clone α1-1, and an additional approximately 2.2 kb of sequence extending in the 5' direction. Sequence analysis revealed that the 3' end of this clone encodes the second tyrosine kinase domain, which contains regions of near sequence identity to the corresponding regions in the PDGF β-receptor. The 5' end of clone α1-7 contained non-receptor sequences. Two additional β-receptor clones were obtained by probing with α1-1 sequences. Clone α1-1 was digested with Not I and Spe I, and a 230 bp fragment was recovered. Clone α1-1 was also digested with Bam HI and Not I, and a 550 bp fragment was recovered. A clone that hybridized to the 230 bp probe was designated α5-1. This clone contained the 5'-most coding sequence for the PDGF α-receptor. Another clone, designated α6-3, hybridized to the 550 bp probe and was found to contain 3' coding and non-coding sequences, including the poly(A) tail.

Clone α1-1 was radiolabeled (^{32}p) and used to probe a northern blot (Thomas, *Methods Enzymol*, 100: 225–265, 1983) of the MG-63 poly(A)+RNA used to prepare the cDNA library. A single band of approximately 6.6 kb was observed. RNA prepared from receptor-positive cell lines including the human fibroblast SK4, WI-38 and 7573 cell lines; the mouse fibroblast line DI 3T3; the U2-OS human osteosarcoma cell line and baboon aortic smooth muscle cells, and RNA prepared from receptor-negative lines including A431 (an epithelial cell line) and VA 13 (SV40-transformed WI-38 cells) were probed by northern format with the α1-1 cDNA. In all cases, the amount of the 6.6 kb band detected in these RNA correlated well with the relative levels of α-receptor detected on the respective cell surfaces. The 6.6 kb RNA was not detected in RNA prepared from any tested cell line of hematopoietic origin, in agreement with a lack of PDGF α-receptor protein detected on these cell types.

Clones α1-1 and and α1-7 were joined at a unique Pst I site in the region encoding the transmembrane portion of the receptor. Clone α1-1 was digested with Xba I and Pst I and

the receptor sequence fragment was recovered. Clone α1-7 was digested with Pst I and Bam HI and the receptor fragment was recovered. The two fragments were ligated with Xba I+Bam HI-digested pIC19R (Marsh et al. *Gene* 32: 481–486, 1984) to construct plasmid pα17R (FIG. 12)

The remainder of the 5'-most α-receptor sequence was obtained from clone α5-1 as an Sst I-Cla I fragment. This fragment was joined to the Eco RI-Sst I receptor fragment of pα17R and cloned into Eco RI+Cla I-digested pBluescript SK+plasmid to construct plasmid pα17B (FIG. 12). FIGS. 11A–11D (Sequence ID Numbers 35 and 36) shows the nucleotide sequence and deduced amino acid sequence of the cDNA present in pα17B.

Example 2

Construction of a SUC2 Signal Sequence-PDGF β-Receptor Fusion

To direct the PDGF β-receptor into the yeast secretory pathway, the PDGF β-receptor cDNA was joined to a sequence encoding the *Saccharomyces cerevisiae* SUC2 signal sequence. Oligonucleotides ZC1453 and ZC1454 (Sequence ID Numbers 10 and 11; Table 1) were designed to form an adapter encoding the SUC2 secretory signal flanked by a 5' Eco RI adhesive end and a 3' Bgl II adhesive end. ZC1453 and ZC1454 were annealed under conditions described by Maniatis et al. (ibid.). Plasmid pR-RX1 was digested with Bgl II and Sst II to isolate the 1.7 kb fragment comprising the PDGF β-receptor coding sequence from amino acids 28 to 596. Plasmid pR-RX1 was also cut with Sst II and Hind III to isolate the 1.7 kb fragment comprising the coding sequence from amino acids 597 through the translation termination codon and 124 bp of 3' untranslated DNA. The two 1.7 kb pR-RX1 fragments and the ZC1453/ZC1454 adapter were joined with pUC19, which had been linearized by digestion with Eco RI and Hind III. The resultant plasmid, comprising the SUC2 signal sequence fused in-frame with the PDGF β-receptor cDNA, was designated pBTL10 (FIG. 2).

Example 3

Construction of pCBS22

The BAR1 gene product, Barrier, is an exported protein that has been shown to have three domains. When used in conjunction with a first signal sequence, the third domain of Barrier protein has been shown to aid in the secretion of proteins into the medium (MacKay et al., U.S. patent application Ser. No. 104,316).

The portion of the BAR1 gene encoding the third domain of Barrier was joined to a sequence encoding the C-terminal portion of substance P (subP; Munro and Pelham, *EMBO J.* 3: 3087–3093, 1984). The presence of the substance P amino acids on the terminus of the fusion protein allowed the protein to be detected using commercially available anti-substance P antibodies. Plasmid pZV9 (deposited as a transformant in *E. coli* strain RR1, ATCC accession no. 53283), comprising the entire BAR2 coding region and its associated flanking regions, was cut with Sal I and Bam HI to isolate the 1.3 kb BAR1 fragment. This fragment was subcloned into pUC13, which had been cut with Sal I and Bam HI, to generate the plasmid designated pZV17. Plasmid pZV17 was digested with Eco RI to remove the 3'-most 0.5 kb of the BAR1 coding region. The vector-BAR1 fragment was religated to create the plasmid designated pJH66 (FIG. 3). Plasmid pJH66 was linearized with Eco RI and blunt-ended with DNA polymerase I (Klenow fragment). Kinased Bam HI linkers (5'CCG GAT CCG G 3') were added and excess linkers were removed by digestion with Bam HI before religation. The resultant plasmid was designated pSW8 (FIG. 3).

Plasmid pSW81, comprising the TPI1 promoter, the BAR1 coding region fused to the coding region of the C-terminal portion of substance P (Munro and Pelham, *EMBO J.* 3: 3087–3093, 1984) and the TPI1 terminator, was derived from pSW8. Plasmid pSW8 was cut with Sal I and Bam HI to isolate the 824 bp fragment encoding amino acids 252 through 526 of BAR1. Plasmid pPM2, containing the synthetic oligonucleotide sequence encoding the dimer form of the C-terminal portion of substance P (subP) in M13mp8, was obtained from Hugh Pelham (MRC Laboratory of Molecular Biology, Cambridge, England). Plasmid pPM2 was linearized by digestion with Bam HI and Sal I and ligated with the 824 bp BAR1 fragment from pSW8. The resultant plasmid, pSW14, was digested with Sal I and Sma I to isolate the 871 bp BAR1-substance P fragment. Plasmid pSW16, comprising a fragment of BAR1 encoding amino acids 1 through 250, was cut with Xba I and Sal I to isolate the 767 bp BAR1 fragment. This fragment was ligated with the 871 bp BAR1-substance P fragment in a three-part ligation with pUC18 cut with Xba I and Sma I. The resultant plasmid, designated pSW15, was digested with Xba I and Sma I to isolate the 1.64 kb BAR1-substance P fragment. The ADH1 promoter was obtained from pRL029. Plasmid pRL029, comprising the ADH1 promoter and the BAR1 5' region encoding amino acids 1 to 33 in pUC18, -was digested with Sph I and Xba I to isolate the 0.42 kb ADH1 promoter fragment. The TPI1 terminator (Alber and Kawasaki, ibid.) was provided as a linearized fragment containing the TPI1 terminator and pUC18 with a Klenow-filled Xba I end and an Sph I end. This fragment was ligated with the 0.42 kb ADH1 promoter fragment and the 1.64 kb BAR1-substance P fragment in a three-part ligation to produce plasmid pSW22.

The ADH1 promoter and the coding region of BAR1, from the translation initiation ATG through the Eco RV site present in pSW22, were removed by digestion with Hind III and Eco RV. The 3.9 kb vector fragment, comprising the 401 bp between the Eco RV and the Eco RI sites of the BAR1 gene fused to subp and the TPI1 terminator, was isolated by gel electrophoresis. Oligonucleotide ZC1478 (Sequence ID Number 12; Table 1) was kinased and annealed with oligonucleotide ZC1479 (Sequence ID Number 13; Table 1) using conditions described by Maniatis et al. (ibid.). The annealed oligonucleotides formed an adapter comprising a Hind III adhesive end and a polylinker encoding Bgl II, Sph I, Nru I and Eco RV restriction sites. The ZC1479/ZC1478 adapter was ligated with the gel-purified pSW22 fragment. The resultant plasmid was designated pCBS22 (FIG. 3).

Example 4

Construction of pBTL13

In order to enhance the secretion of the β-receptor and to facilitate the identification of the secreted protein, a sequence encoding the third domain of BAR1 fused to the C-terminal amino acids of substance P was fused in frame with the 5' 1240 bp of the PDGF β-receptor. Plasmid pBTL10 (Example 2) was digested with Sph I and Sst I to isolate the 4 kb fragment comprising the SUC2 signal sequence, a portion of the PDGF β-receptor CDNA and the pUC19 vector sequences. Plasmid pCBS22 was digested with Sph I and Sst I to isolate the 1.2 kb fragment compris-

ing the BAR1-subp fusion and the TPI1 terminator. These two fragments were ligated, and the resultant plasmid was designated pBTL13 (FIG. 4)

Example 5

Construction of an Expression Vector Encoding the Entire PDGF β-Receptor

The entire PDGF β-receptor was directed into the secretory pathway by fusing a SUC2 signal sequence to the 5' end of the PDGF β-receptor coding sequence. This fusion was placed behind the TPIL promoter and inserted into the vector YEp13 for expression in yeast.

The TPI1 promoter was obtained from plasmid pTPIC10 (Alber and Kawasaki, *J. Mol. Apnl. Genet.* 1: 410–434, 1982), and plasmid PFATPOT (Kawasaki and Bell, EP 171,142; ATCC 20699). Plasmid pTPIC10 was cut at the unique Kpn I site, the TPI1 coding region was removed with Bal-31 exonuclease, and an Eco RI linker (sequence: GGA ATT CC) was added to the 3' end of the promoter. Digestion with Bgl II and Eco RI yielded a TPI1 promoter fragment having Bgl II and Eco RI sticky ends. This fragment was then joined to plasmid YRp7' (Stinchcomb et al., *Nature* 282: 39–43, 1979) that had been cut with Bgl II and Eco RI (partial). The resulting plasmid, TE32, was cleaved with Eco RI (partial) and Bam HI to remove a portion of the tetracycline resistance gene. The linearized plasmid was then recircularized by the addition of an Eco RI-Bam HI linker to produce plasmid TEA32. Plasmid TEA32 was digested with Bgl II and Eco RI, and the 900 bp partial TPI1 promoter fragment was gel-purified. Plasmid pIC19H (Marsh et al., *Gene* 32:481–486, 1984) was cut with Bgl II and Eco RI and the vector fragment was gel purified. The TPI1 promoter fragment was then ligated to the linearized pIC19H and the mixture was used to transform *E. coli* RR1. Plasmid DNA was prepared and screened for the presence of a ~900 bp Bgl II-Eco RI fragment. A correct plasmid was selected and designated PICTPIP.

The TPI1 promoter was then subcloned to place convenient restriction sites at its ends. Plasmid pIC7 (Marsh et al., ibid.) was digested with Eco RI, the fragment ends were blunted with DNA polymerase I (Klenow fragment), and the linear DNA was recircularized using T4 DNA ligase. The resulting plasmid was used to transform *E. coli* RR1. Plasmid DNA was prepared from the transformants and was screened for the loss of the Eco RI site. A plasmid having the correct restriction pattern was designated pIC7RI*. Plasmid pIC7RI* was digested with Hind III and Nar I, and the 2500 bp fragment was gel-purified. The partial TPI1 promoter fragment (ca. 900 bp) was removed from pICTPIP using Nar I and Sph I and was gel-purified. The remainder of the TPI1 promoter was obtained from plasmid pFATPOT by digesting the plasmid with Sph I and Hind III, and a 1750 bp fragment, which included a portion of the TPI1 promoter fragment from PICTPIP, and the fragment from pFATPOT were then combined in a triple ligation to produce pMVR1 (FIG. 2).

The TPI1 promoter was then joined to the SUC2-PDGF β-receptor fusion. Plasmid PBTL10 (Example 2) was digested with Eco RI and Hind III to isolate the 3.4 kb fragment comprising the SUC2 signal sequence and the entire PDGF β-receptor coding region. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment and the fragment derived from pBTL10 were joined with YEp13, which had been linearized by digestion with Bam HI and Hind III, in a three-part ligation. The resultant plasmid was designated pBTL12 (FIG. 2).

Example 6

Construction of an Expression Vector Encoding the 5' Extracellular Portion of the PDGF β-Receptor

The extracellular portion of the PDGF β-receptor was directed into the secretory pathway by fusing the coding sequence to the SUC2 signal sequence. This fusion was placed in an expression vector behind the TPI1 promoter. Plasmid pBTL10 (Example 2) was digested with Eco RI and Sph I to isolate the approximately 1.3 kb fragment comprising the SUC2 signal sequence and the PDGF β-receptor extracellular domain coding sequence. Plasmid pMVR1 (Example 5) was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment was joined with the fragment derived from pBTL10 and YEp13, which had been linearized by digestion with Bam HI and Sph I, in a three-part ligation. The resultant plasmid was designated pBTL11 (FIG. 2).

Example 7

Construction of Yeast Expression Vectors pBTL14 and pBTL15, and The Expression of PDGF β-Receptor-BAR1-subP Fusions

A. Construction of pBTL14

The SUC2-PDGFβ-R fusion was joined with the third domain of BAR1 to enhance the secretion of the receptor, and the expression unit was cloned into a derivative of YEp13 termed pJH50. YEp13 was modified to destroy the Sal I site near the LEU2 gene. This was achieved by partially digesting YEp13 with Sal I followed by a complete digestion with Xho I. The 2.0 kb Xho I-Sal I fragment comprising the LEU2 gene and the 8.0 kb linear YEp13 vector fragment were isolated and ligated together. The ligation mixture was transformed into *E. coli* strain RR1. DNA was prepared from the transformants and was analyzed by digestion with Sal I and Xho I. A clone was isolated which showed a single Sal I site and an inactive Xho I site indicating that the LEU2 fragment had inserted in the opposite orientation relative to the parent plasmid YEp13. The plasmid was designated pJH50.

Referring to FIG 4, plasmid pBTL12 (Example 5) was digested with Sal I and Pst I to isolate the 2.15 kb fragment comprising 270 bp of YEp13 vector sequence, the TPI1 promoter, the SUC2 signal sequence, and 927 bp of PDGF β-receptor cDNA. Plasmid pBTL13 (Example 4) was digested with Pst I and Hind III to isolate the 1.48 kb fragment comprising 313 bp of PDGF β-receptor CDNA, the BAR1-subP fusion and the TPI1 terminator. The fragments derived from pBTL12 and pBTL13 were joined with pJH50, which had been linearized by digestion with Hind III and Sal I, in a three-part ligation. The resultant plasmid was designated pBTL14.

B. Construction of pBTL15

Referring to FIG. 5, a yeast expression vector was constructed comprising the TPI1 promoter, the SUC2 signal sequence, 1.45 kb of PDGF β-receptor CDNA sequence fused to the BAR1-subP fusion and the TPI1 terminator. Plasmid pBTL12 (Example 5) was digested with Sal I and Fsp I to isolate the 2.7 kb fragment comprising the TPI1 promoter, the SUC2 signal sequence, the PDGFβ-R coding sequence, and 270 bp of YEp13 vector sequence. Plasmid pBTL13 (Example 4) was digested with Nru I and Hind III to isolate the 1.4 kb fragment comprising the BAR1-subP fusion, the TPI1 terminator and 209 bp of 3' PDGF β-receptor cDNA sequence. The fragments derived from

pBTL12 and pBTL13 were joined in a three-part ligation with pH50, which had been linearized by digestion with Hind III and Sal I. The resultant plasmid was designated pBTL15.

C. Expression of PDGFβ-R-subP Fusions from pBTL14 and pBTL15

Yeast expression vectors pBTL14 and pBTL15 were transformed into Saccharomyces cerevisiae strains ZY100 (MATa leu2-3,112 ade2-101 suc2-A9 ca12 pep4::TPI1prom-CAT) and ZY400 (MATa leu2-3.112 ade2-101 suc2-A9 gal2 pep4::TPI1irom-CAT Δmnn9::URA3). Transformations were carried out using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on –LEUDS (Table 2).

TABLE 2

Media Recipes

-LeuThrTrp Amino Acid Mixture

4 g adenine
3 g L-arginine
5 g L-aspartic acid
2 g L-histidine free base
6 g L-isoleucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
4 g uracil
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

–LEUDS

20 g glucose
6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, Mich.)
0.6 g –LeuThrTrp Amino Acid Mixture
182.2 g sorbitol
18 g Agar

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan. Pour plates and allow to solidify.

–LEUDS+sodium succinate, pH 6.5

20 g Yeast Nitrogen Base without amino acids
0.6 g –LeuTrpThr Amino Acid Mixture
182.2 g sorbitol
11.8 g succinic acid

Mix all ingredients in distilled water to a final volume of 1 liter. Adjust the pH of the solution to pH 6.5. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

Fermentation Medium

7 g/l yeast nitrogen base without amino acids or ammonium sulfate (DIFCO Laboratories)
0.6 g/l ammonium sulfate
0.5 M sorbitol
0.39 g/l adenine sulfate
0.01% polypropylene glycol

Mix all ingredients in distilled water. Autoclave 15 minutes. Add 80 ml 50% glucose for each liter of medium.

Super Synthetic –LEUD, pH 6.5 [liquid or solid medium]

6.7 g Yeast Nitrogen Base without amino acids or ammonium sulfate (DIFCO)
6 g ammonium sulfate
160 g adenine
0.6 g –LeuThrTrp Amino Acid Mixture
20 g glucose
11.8g succinic acid

Mix all ingredients and add distilled water to a final volume of 800 ml. Adjust the pH of the solution to pH 6.4. Autoclave 15 minutes. For solid medium, add 18 g agar before autoclaving, autoclave and pour plates.

Super Synthetic –LEUDS, pH 6,4 (liquid or solid medium)

Use the same recipe as Super Synthetic –LEUD, pH 6.4, but add 182.2 g sorbitol before autoclaving.

YEPD

20 g glucose
20 g Bacto Peptone (DIFCO Laboratories)
10 g Bacto Yeast Extract (DIFCO Labloratories)
18 g agar
4 ml adenine 1%
8 ml 1% L-leucine

Mix all ingredients in distilled water, and bring to a final volume of 1 liter. Autoclave 25 minutes and pour plates.

The transformants were assayed for binding to an anti-PDGF β-receptor monoclonal antibody (PR7212) or an anti-substance P antibody by protein blot assay. ZY100 [pBTL14] and ZY100[pBTL15] transformants were grown overnight at 30° C. in 5 ml Super Synthetic -LEUD, pH 6.4 (Table 2). ZY400[pBTL14] and ZY400[pBTL15] transformants were grown overnight at 30° C. in 5 ml Super Synthetic-LEUDS, pH 6.4 (Table 2). The cultures were pelleted by centrifugation and the supernatants were assayed for the presence of secreted PDGF β-receptor anlogs by protein blot assay using methods described in Example 18. Results of assays using PR7212 are shown in Table 3.

TABLE 3

Results of a protein blot probed with PR7212

Transformant:	
ZY100[pBTL14]	+
ZY400[pBTL14]	++
ZY100[pBTL15]	+
ZY400[pBTL15]	+

Example 8

Construction of a SUC2-PDGFβ-R Fusion Comprising the Complete PDGFβ-R Extracellular Domain

A. Construction of pBTL22

The PDGFβ-R coding sequence present in pBTL10 was modified to delete the coding region 3' to the extracellular PDGFβ-R domain. As shown in FIG. 6, plasmid pBTL10 was digested with Sph I and Bam HI and with Sph I and Sst II to isolate the 4.77 kb fragment and the 466 bp fragment, respectively. The 466 bp fragment was then digested with Sau 3A to isolate the 0.17 kb fragment. The 0.17 kb fragment and the 4.77 kb were joined by ligation. The resultant plasmid was designated pBTL21.

Plasmid pBTL21, containing a Bam HI site that was regenerated by the ligation of the Bam HI and Sau 3A sites, was digested with Hind III and Bam HI to isolate the 4.2 kb fragment. Synthetic oligonucleotides ZC1846 (Sequence ID Number 16; Table 1) and ZC1847 (Sequence ID Number 17; Table 1) were designed to form an adapter encoding the

PDGFβ-R from the Sau 3A site after bp 1856 (FIG. 1B; (Sequence ID Number 1)) to the end of the extracellular domain at 1958 bp (FIG. 1B; Sequence ID Number 1), having a 5' Bam HI adhesive end that destroys the Bam HI site and a 3' Hind III adhesive end. oligonucleotides ZC1846 and ZC1847 were annealed under conditions described by Maniatis et. al. (ibid.). The 4.2 pBTL21 fragment and the ZC1846/ZC1847 adapter were joined by ligation. The resultant plasmid, designated pBTL22, comprises the SUC2 signal sequence fused in proper reading frame to the extracellular domain of PDGFβ-R in the vector pUC19 (FIG. 6).

B. Construction of pBTL28

An in-frame translation stop codon was inserted immediately after the coding region of the PDGFβ-R in pBTL22 using oligonucleotides ZC1892 (Sequence ID Number 19; Table 1) and ZC1893 (Sequence ID Number 20; Table 1). These oligonucleotides were designed to form an adapter encoding a stop codon in-frame with the PDGFβ-R coding sequence from pBTL22 flanked by a 5' Hind III adhesive end and a 3' Xba I adhesive end. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1892 and ZCIB93 were annealed to form a Hind III-Xba I adapter. The 1.6 kb SUC2-PDGFβ-R fragment, the 3.86 kb pMVR1 fragment and the ZC1892/ZC1893 adapter were joined in a three-part ligation. The resultant plasmid was designated pBTL27.

The expression unit present in pBTL27 was inserted into the yeast expression vector pJH50 by first digesting pJH50 with Bam HI and Sal I to isolate the 10.3 kb vector fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI and with Xho I and Eco RI to isolate the 0.9 kb TPI1 promoter fragment and the 1.65 kb fragment, respectively. The 10.3 kb pJH50 vector fragment, the 0.9 kb TPI1 promoter fragment and 1.65 kb fragment were joined in a three-part ligation. The resultant plasmid was designated pBTL28.

C. Construction of Plasmid pBTL30

The PDGFβ-R coding sequence present in plasmid pBTL22 was modified to encode the twelve C-terminal amino acids of substance P and an in-frame stop codon. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pIC7RI* and the TPI1 terminator. Synthetic oligonucleotides ZC1894 (Sequence ID Number 21; Table 1) and ZC1895 (Sequence ID Number 22; Table 1) were annealed to form an adapter containing the codons for the twelve C-terminal amino acids of substance P followed by an in-frame stop codon and flanked on the 5' end with a Hind III adhesive end and on the 3' end with an Xba I adhesive end. The ZC1894/ZC1895 adapter, the 1.6 kb SUC2-PDGFβ-R fragment and the pMVR1 fragment were joined in a three-part ligation. The resultant plasmid, designated pBTL29, was digested with Eco RI and Xho I to isolate the 1.69 kb SUC2-PDGFβ-R-subP-TPI1 terminator fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. Plasmid pJH50 was digested with Bam HI and Sal I to isolate the 10.3 kb vector fragment. The 1.69 kb pBTL29 fragment, the 0.9 kb TPI1 promoter fragment and the 10.3 kb vector fragment were joined in a three-part ligation. The resulting plasmid was designated pBTL30.

Example 9

Construction and Expression of a SUC2-PDGFβ-R-IgG Hinge

Expression Vector

An expression unit comprising the TPI1 promoter, the SUC2 signal sequence, the PDGFβ-R extracellular domain, an immunoglobulin hinge region and the TPI1 terminator was constructed. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.56 kb fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.7 kb fragment, comprising the TPI1 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1776 (Sequence ID Number 14; Table 1) and ZC1777 (Sequence ID Number 15; Table 1) were designed to form, when annealed, an adapter encoding an immunoglobulin hinge region with a 5' Hind III adhesive end and a 3'Xba I adhesive end. Oligonucleotides ZC1776 and ZC1777 were annealed under conditions described by Maniatis et al. (ibid.). The 1.56 kb pBTL22 fragment, the 3.7 kb fragment and the ZC1776/ZC1777 adapter were joined in a three-part ligation, resulting in plasmid pBTL24.

The expression unit of pBTL24, comprising the TPI1 promoter, SUC2 signal sequence, PDGFβ-R extracellular domain sequence, hinge region sequence, and TPI1 terminator, was inserted into pJH50. Plasmid pBTL24 was digested with Xho I and Hind III to isolate the 2.4 kb expression unit. Plasmid pJH50 was digested with Hind III and Sal I to isolate the 9.95 kb fragment. The 2.4 kb pBTL24 fragment and 9.95 kb pJH50 vector fragment were joined by ligation. The resultant plasmid was designated pBTL25.

Plasmid pBTL25 was transformed into *Saccharomyces cerevisiae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). The transformants were tested for their ability to bind the anti-PDGFβ-R monoclonal antibody PR7212 using the colony assay method described in Example 18. Plasmid pBTL25 transformants were patched onto nitrocellulose filters that had been wetted and supported by YEPD solid medium. Antibody PR7212 was found to bind to the PDGFβ-R-IgG hinge fusion secreted by ZY400[pBTL25] transformants.

Example 10

Construction and Expression of a SUC2 Signal Sequence-PDGFβ-R Extracellular Domain-SUC2 Fusion

As shown in FIG. 6, an expression unit comprising the TPI1 promoter, SUC2 signal sequence, PDGFβ-R extracellular domain sequence, and SUC2 coding sequence was constructed as follows. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R fragment. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The SUC2 coding region was obtained from pJH40. Plasmid pJH40 was constructed by inserting the 2.0 kb Hind III-Hind III SUC2 fragment from pRB58 (Carlson et al., *Cell* 28:145--154, 1982) into the Hind III site of pUC19 followed by the destruction of the Hind III site 3' to the coding region. Plasmid pJH40 was digested with Hind III and Sal I to isolate the 2.0 kb SUC2 coding sequence. Plasmid pJH50 was digested with Sal I and Bam HI to isolate the 10.3 kb vector fragment. The 0.9 kb Bgl II-Eco RI TPI1 promoter fragment, the 1.6 kb Eco RI-Hind III SUC2-PDGFβ-R, the 2.0 kb Hind III-Sal I SUC2 fragment and the 10.3 kb Bam HI-Sal I vector fragment were joined in a four-part ligation. The resultant plasmid was designated pBTL26 (FIG. 6).

Plasmid pBTL26 was transformed into *Saccharomyces cerevisae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). ZY400 transformants (ZY400[pBTL26]) were assayed by protein blot (Example 18), colony blot (Example 18) and competition assay.

Protein blot assays were carried out on ZY400[pBTL26] and ZY400[pJH50] (control) transformants that had been grown in flasks. Two hundred-fifty microliters of a 5 ml overnight cultures of ZY400[pBTL26] and ZY400 [pJH50] in −LEUDS+sodium succinate, pH 6.5 (Table 2) were inoculated into 50 ml of −LEUDS+sodium succinate, pH 6.5. The cultures were incubated for 35 hours in an airbath shaker at 30° C. The culture supernatants were harvested by centrifugation. The culture supernatants were assayed as described in Example 18 and were found to bind PR7212 antibody.

Colony assays were carried out on ZY400[pBTL26] transformants. ZY400[pBTL26] transformants were patched onto wetted nitrocellulose filters that were supported on a YEPD plate. The colony assay carried out as described in Example 8.A. showed that ZY400[pBTL26] antibodies bound PR7212 antibodies.

Competition binding assays were carried out on ZY400 [pBTL26] and ZY400[pJH50] transformants. The transformants were grown in two liters of fermentation medium (Table 2) in a New Brunswick Bioflo2 fermentor (New Brunswick, Philadelphia, Pa.) with continuous pH control at pH 6.4. The cultures were adjusted to pH 7.5 immediately prior to harvesting. Culture supernatants were concentrated in an Amicon concentrator (Amicon, San Francisco, Calif.) using an Amicon 10^4 mw spiral filter cartridge. The concentrated supernatants were further concentrated using Amicon Centriprep 10's. Fifteen milliliters of the concentrated supernatant samples were added to the Centripreps, and the Centripreps were spun in a Beckman GRP centrifuge (Beckman Instruments Inc., Carlsbad, Calif.) at setting 5 for a total of 60 minutes. The concentrates were removed from the Centripreps and were assayed in the competition assay.

The competition binding assay measured the amount of ^{125}I-PDGF left to bind to fetal foreskin fibroblast cells after preincubation with the concentrate containing the PDGFβ-R-SUC2 fusion protein. PDGF-AA and PDGF-AB were iodinated using the Iodopead method (Pierce Chemical). PDGF-BB_{sy} was iodinated and purified as described in Example 18.F. The concentrate was serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-AA, PDGF-BB_{sy}, or PDGF-AB, and the mixtures were incubated for two hours at room temperature. Three hundred micrograms of unlabeled PDGF-BB was added to each sample mixture. The sample mixtures were added to 24-well plates containing confluent fetal foreskin fibroblast cells (AG1523, available from the Human Genetic Mutant *Cell Repository, Camden, N.J.). The cells were incubated with the mixture for four hours at* 4° C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held at 4° C. (PBS; Sigma, St. Louis, Mo.). Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the PDGFβ-R-SUC2 fusion protein was able to competitively bind all three isoforms of PDGF.

The PDGFβ-R produced from ZY400 [pBTL26] transformants was tested for cross reactivity to fibroblast growth factor (FGF) and transforming growth factorβ (TGF-β) using the competition assay essentially described above. Supernatant concentrates from ZY400[pBTL26] and ZY400 [JH50] (control) transformants were serially diluted in binding medium (Table 4). The dilutions were mixed with 7.9 ng of iodinated FGF or 14 ng of iodinated TGF-β, and the mixtures were incubated for two hours at room temperature. Fourteen micrograms of unlabeled FGF was added to each mixture containing labeled FGF, and 7 μg of unlabeled TGF-β was added to each mixture containing labeled TGF-β. The sample mixtures were added to 24-well plates containing confluent human dermal fibroblast cells. (Human dermal fibroblast cells express both FGF receptors and TGFβ receptors.) The cells were incubated with the mixtures for four hours at 4° C. Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated FGF or TGF-β bound to the cells was determined. The results of these assays showed that the PDGFβ-R-SUC2 fusion protein did not cross react with FGF or TGF-β.

TABLE 4

Reagent Recipes

Binding Medium

500 ml	Ham's F-12 medium
12 ml	1M HEPES, pH 7.4
5 ml	100× PSN (Penicillin/Streptomycin/Neomycin, Gibco)
1 g	rabbit serum albumin

Western Transfer Buffer

25 mM	Tris, pH 8.3
19 mM	glycine, pH 8.3
20%	methanol

Western Buffer A

50 ml	1 M Tris, pH 7.4
20 ml	0.25 mM EDTA, pH 7.0
5 ml	10% NP-40
37.5 ml	4 M NaCl
2.5 g	gelatin

The Tris, EDTA, NP-40 and NaCl were diluted to a final volume of one liter with distilled water. The gelatin was added to 300 ml of this solution and the solution was heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the remainder of the first solution and stirred at 4° C. until cool. The buffer was stored at 4° C.

Western Buffer B

50 ml	1 M Tris, pH 7.4
20 ml	0.25 M EDTA, pH 7.0
5 ml	10% NP-40
58.4 g	NaCl
2.5 g	gelatin
4 g	N-lauroyl sarcosine

The Tris, EDTA, NP-40, and the NaCl were mixed and diluted to a final volume of one liter. The gelatin was added to 300 ml of this solution and heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the original solution and the N-lauroyl sarcosine was added. The final mixture was stirred at 4° C. until the solids were completely dissolved. This buffer was stored at 4° C.

2x Loading Buffer

36 ml	0.5 M Tris-HCl, pH 6.8
16 ml	glycerol
16 ml	20% SDS
4 ml	0.5% Bromphenol Blue in 0.5 M Tris-HCl, pH 6.8

Mix all ingredients. Immediately before use, add 100 μl β-mercaptoethanol to each 900 μl dye mix

Example 11

Construction and Expression of PDGF Receptor Analogs From BHK cells

A. Construction of pBTL114 and pBTL115

The portions of the PDGF β-receptor extracellular domain present in pBTL14 and pBTL15 were placed in a mammalian expression vector. Plasmids pBTL14 and pBTL15 were digested with Eco RI to isolate the 1695 bp and 1905 bp SUC2 signal-PDGFβ-R-BAR1 fragments. The 1695 bp fragment and the 1905 bp fragment were each ligated to Zem229R that had been linearized by digestion with Eco RI.

The vector Zem229R was constructed as shown in FIG. 10 from Zem229. Plasmid Zem229 is a pUC18-based expression vector containing a unique Bam HI site for insertion of cloned DNA between the mouse metallothionein-1 promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 transcription terminator. Zem229 was modified to delete the Eco RI sites flanking the Bam HI cloning site and to replace the Bamr HI site with a single Eco RI cloning site. The plasmid was partially digested with Eco RI, treated with DNA polymerase I (Klenow fragment) and dNTPs, and religated. Digestion of the plasmid with Bam HI followed by ligation of the linearized plasmid with a Bam HI-Eco I adapter resulted in a unique Eco RI cloning site. The resultant plasmid was designated Zem229R.

The ligation mixtures were transformed into *E. coli* strain RR1. Plasmid DNA was prepared and the plasmids were subjected to restriction enzyme analysis. A plasmid having the 1695 bp pBTL14 fragment inserted into Zem229R in the correct orientation was designated pBTL114 (FIG 9). A plasmid having the 1905 bp pBTL15 fragment inserted into Zem229R in the correct orientation was designated pBTL115 (FIG. 9).

B. Expression of Secreted PDGF β-Receptor Analogs in tk⁻ts13 BHK Cells

Plasmids pBTL114 and pBTL115 were each transfected into tk⁻ts13 cells using calcium phosphate precipitation (essentially as described by Graham and van der Eb, *J. Gen. Virol.* 36: 59–72, 1977). The transfected cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1xPSN antibiotic mix (Gibco 600–5640), 2.0 mM L-glutamine. The cells were selected in 250 nM methotrexate (MTX) for 14 days, and the resulting colonies were screened by the immunofilter assay (McCracken and Brown, *Biotechniques*, 82–87, March/April 1984). Plates were rinsed with PBS or No Serum medium (DMEM plus 1xPSN antibiotic mix). Teflon® mesh (Spectrum Medical Industries, Los Angeles, Calif.) was then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After six hours incubation at 37° C., filters were removed and placed in Wester buffer A (Table 4) overnight at room temperature. The filters were developed using the antibody PR7212 and the procedure described in Example 8. The filters showed that conditioned media from pBTL114-transfected and pBTL115-transfected BHK cells bound the PR7212 antibody indicating the presence of biologically active secreted PDGFβ-R.

Example 12

Expression of PDGF β-Receptor Analogs in Cultured Mouse Myeloma Cells

A. Construction of pICpPRE8

The immunoglobulin μ heavy chain promoter and enhancer were subcloned into pIC19H to provide a unique Hind III site 3' to the promoter. Plasmid $p\mu$ (Grosschedl and Baltimore, *Cell* 41: 885–897, 1985) was digested with Sal I and Eco RI to isolate the 3.1 kb fragment comprising the μ promoter. Plasmid pIC19H was linearized by digestion with Eco RI and Xho I. The p promoter fragment and the linearized pIC19H vector fragment were joined by ligation. The resultant plasmid, designated pICμ3, was digested with Ava II to isolate the 700 bp μ promoter fragment. The 700 bp fragment was blunt-ended by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. Plasmid pIC19H was linearized by digestion with Xho I, and the adhesive ends were filled in by treatment with DNA polymerase I (Klenow fragement) and deoxynucleotide triphosphates. The blunt-ended Ava II fragment was ligated with the blunt-ended, linearized pIC19H, and the ligation mixture was transformed into *E. coli* JM83. Plasmid DNA was prepared from the transformants and was analyzed by restriction digest. A plasmid with a Bgl II site 5' to the promoter was designated pICμPR1(–). Plasmid pICμPR1 (–) was digested with Hind III and Bgl II to isolate the 700 bp μ promoter fragment. Plasmid pIC19R was linearized by digestion with Hind III and Bam HI. The 700 bp promoter fragment was joined with the linearized pIC19R by ligation. The resultant plasmid, designated pICμPR7, comprised the μ promoter with an unique Sma I site 5' to the promoter and a unique Hind III site 3' to the promoter.

The immunoglobulin heavy chain μ enhancer (Gillies et al., *Cell* 33: 717–728, 1983) was inserted into the unique Sma I site to generate plasmid pICμPRE8. Plasmid pJ4 (obtained from F. Blattner, Univ. Wisconsin, Madison, Wis.), comprising the 1.5 kb Hind III-Eco RI A enhancer fragment in the vector pAT153 (Amersham, Arlington Heights, Ill.), was digested with Hind III and Eco RI to isolate the 1.5 kb enhancer fragment. The adhesive ends of the enhancer fragment were filled in by treatment with T4 DNA polymerase and deoxynucleotide triphosphates. The blunt-ended fragment and pICμPR7, which had been linearized by digestion with Sma I, were joined by ligation. The ligation mixture was transformed into *E. coli* RR1. Plasmid DNA was prepared from the transformants, and the plasmids were analyzed by restriction digests. A plasmid comprising the μ enhancer and the A promoter was designated pICμPRE8 (FIG. 7).

B. Construction of pSDL114

The DNA sequence encoding the extracellular domain of the PDGF β-receptor was joined with the DNA sequence encoding the human immunoglobulin light chain constant region. The PDGF β-receptor extracellular domain was obtained from mpBTL22, which comprised the Eco RI-Hind III fragment from pBTL22 (Example 8.A.) cloned into Eco RI-Hind III cut M13mp18. Single stranded DNA was prepared from a mpBTL22 phage clone, and the DNA was subjected to in vitro mutagenesis using the oligonucleotide ZC1886 (Table 1) and the method described by Kunkel (*Proc. Natl. Acad. Sci. USA* 82: 488–492, 1985). A phage clone comprising the mutagenized PDGFβ-R with a donor splice site (5' splice site) at the 3' end of the PDGFβ-R extracellular domain was designated pBTLR-HX (FIG. 7).

The native PDGFβ-R signal sequence was obtained from pPR5. Plasmid PPR5, comprising 738 bp of 5' coding sequence with an Eco RI site immediately 5' to the translation initiation codon, was constructed by in vitro mutagenesis of the PDGFβ-R CDNA fragment from RP51 (Example 1). Replicative form DNA of RP51 was digested with Eco RI to isolate the 1.09 kb PDGFβ-R fragment. The PDGFβ-R fragment was cloned into the Eco RI site of M13mp18. Single stranded template DNA was prepared from a phage clone containing the PDGFβ-R fragment in the proper orientation. The template DNA was subjected to in vitro mutagenesis using oligonucleotide ZC1380 (Sequence ID Number 8; Table 1) and the method described by Zoller and

Smith (*Meth. Enzymol.* 100: 468–500, 1983). The mutagenesis resulted in the placement of an Eco RI site immediately 5' to the translation initiation codon. Mutagenized phage clones were analyzed by dideoxy sequence analysis. A phage clone containing the ZC1380 mutation was selected, and replicative form (RF) DNA was prepared from the phage clone. The RF DNA was digested with Eco RI and Acc I to isolate the 0.63 kb fragment. Plasmid pR-RXI (Example 1) was digested with Acc I and Eco RI to isolate the 3.7 kb fragment. The 0.63 kb fragment and the 3.7 kb fragment were joined by ligation resulting in plasmid pPR5 (FIG. 7).

As shown in FIG. 7, the PDGFβ-R signal peptide and part of the extracellular domain were obtained from plasmid pPR5 as a 1.4 kb Eco RI-Sph I fragment. Replicative form DNA from phage clone pBTLR-HX was digested with Sph I and Hind III to isolate the approximately 0.25 kb PDGFβ-R fragment. Plasmid pUC19 was linearized by digestion with Eco RI and Hind III. The 1.4 kb Eco RI-Sph I PDGFβ-R fragment, the 0.25 kb Sph I-Hind III fragment from pBTLR-HX and the Eco RI-Hind III cut pUC19 were joined in a three-part ligation. The resultant plasmid, pSDL110, was digested with Eco RI and Hind III to isolate the 1.65 kb PDGFβ-R fragment.

Plasmid pICHuCr3.9.11 was used as the source of the human immunoglobulin light chain gene (FIG. 7). The human immunoglobulin light chain gene was isolated from a human genomic library using an oligonucleotide probe (5' TGT GAC ACT CTC CTG GGA GTT A 3'; Sequence ID Number 32), which was based on a published human kappa C gene sequence (Hieter et al., *Cell* 22: 197–207, 1980). The human light chain (kappa) constant region was subdloned as a 1.1 kb Sph I-Hinf I genomic fragment of the human kappa gene, which has been treated with DNA polymerase DNA I (Klenow Fragment) to fill in the Hinf I adhesive end, into Sph I-Hinc II cut pUC19. The 1.1 kb human kappa constant region was susbsequently isolated as a 1.1 kb Sph I-Bam HI fragment that was subdloned into Sph I-Bgl II cut pIC19R (Marsh et al., ibid.). The resultant plasmid was designated pICHuCX3.9.11. Plasmid pICHuC_{κ} 3.9.11 was digested with Hind III and Eco RI to isolate the 1.1 kb kappa constant region gene. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGFβ-R fragment, the 1.1 kb human kappa constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL112, was digested with Bam HI and Cla I to isolate the 2.75 kb fragment. Plasmid ppPRE8 was linearized with Bgl II and Cla I. The 2.75 kb fragment and the linearized ppPRE8 were joined by ligation. The resultant plasmid was designated pSDL114 (FIG. 7).

Plasmid pSDL114 was linearized by digestion with Cla I and was cotransfected with Pvu I-digested p416 into SP2/0-Ag14 (ATCC CRL 1581) by electroporation using the method essentially described by Neumann et al. (*EMBO J.* 1: 841–845, 1982). (Plasmid p416 comprises the Adenovirus 5 ori, SV40 enhancer, Adenovirus 2 major late promoter, Adenovirus 2 tripartite leader, 5' and 3' splice sites, the DHFR^r CDNA, the SV40 polyadenylation signal and 1 ML-1 (Lusky and Botchan, *Nature* 2: 79–81, 1981) vector sequences.) Transfectants were selected in growth medium containing methotrexate.

Media from drug resistant clones were tested for the presence of secreted PDGF β-receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml polyclonal goat anti-human kappa chain (Cappel Laboratories, Melvern, Pa.) diluted in phosphate buffered saline (PBS; Sigma) overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the well were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 -in PBS. One hundred microliters of peroxidase-conjugated goat anti-human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well and the wells were incubated for one hour at 4° C. One hundred microliters of chromophore (100 u1 ABTS (2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt; Sigma)+1 μl 30% H_2O_2+12.5 ml citrate/phosphate buffer (9.04 g/l citric acid, 10.16 g/l Na_2HPO_4)) was added to each well, and the wells were incubated to thirty minutes at room temperature. The samples were measured at 405 nm. The results of the assay showed that the PDGFβ-R analog secreted by the transfectants contained an immunoglobulin light chain.

Spent media from drug resistant clones was also tested for the presence of secreted PDGF β-receptor analogs by immunoprecipitation. Approximately one million drug resistant transfectants were metabolically labeled by growth in DMEM medium lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μCi ^{35}S-cysteine. Media was harvested from the labeled cells and 250 μl of the spent media was assayed by immunoprecipitation with the anti-PDGF β-receptor antibody PR7212 to detect the presence of metabolically labeled PDGF β-receptor analogs. PR7212, diluted in PBS, was added to the media to a final concentration of 2.5 μg per 250 μl spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to the PR7212/media mixtures. The immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at −70° C. The results of the immunoprecipitation showed that the PDGF β-receptor analog secreted by the transfectants was bound by the anti-PDGF β-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF β-receptor analog secreted by the transfectants contained both the PDGF β-receptor ligand-binding domain and the human light chain constant region.

C. Cotransfection of pSDL114 with an Immunoglobulin Heavy Chain

Plasmid pSDL114 was cotransfected with pϕ5$V_H$huC$_\gamma$1M-neo, which encodes a neomycin resistance gene expression unit and a complete mouse/human chimeric immunoglobulin heavy chain gene expression unit.

Plasmid pϕ5$V_H$huC$_\gamma$1M-neo was constructed as follows. The mouse immunoglobulin heavy chain gene was isolated from a lambda genomic DNA library constructed from the murine hybridoma cell line NR-ML-05 (Serafini et al., *Eur. J. Nucl. Med.* 14: 232, 1988) using an oligonucleotide probe designed to span the $V_H/D/J_H$ junction (5'GCA TAG TAG TTA CCA TAT CCT CTT GCA CAG 3'; Sequence ID Number 33). The human immunoglobulin gamma-1 C gene was isolated from a human genomic library using a cloned human gamma-4 constant region gene (Ellison et al., *DNA* 1: 11–18, 1981). The mouse immunoglobulin variable region was isolated as a 5.3 kb Sst I-Hind III fragment from the original phage clone and the human gamma-1 C gene was obtained from the original phage clone as a 6.0 kb Hind III-Xho I fragment. The chimeric gamma-1 C gene was created by joining the V_H and C_H fragments via the common Hind III site and incorporating them with the *E. coli* neomycin resistance gene expression unit into pIC19H to yield pϕ5$V_H$huC$_\gamma$1M-neo.

Plasmid pSDL114 was linearized by digestion with Cla I and was co-transfected into SP2/0-Ag14 cells with Asp 718 linearized $p\phi5V_H huC_\gamma 1M$-neo. The transfectants were selected in growth medium containing methotrexate and neomycin. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay.

The competition binding assay measured the amount of ^{125}-PDGF left to bind to human dermal fibroblast cells after preincubation with the spent media from pSDL114-$p\phi5V_H huC_\gamma 1M$-neo transfected cells. The media were serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-BB or iodinated PDGF-AA, and the mixtures were incubated for two hours at room temperature. Three hundred micrograms of unlabeled PDGF-BB or unlabeled PDGF-AA was added to one tube from each series. The sample mixtures were added to 24 well plates containing confluent human dermal fibroblast cells. The cells were incubated with the mixture for four hours at 4° C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held a 4° C. (PBS; Sigma, St. Louis, Mo.). Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the protein produced from pSDL114-$p\phi5V_H huC_\gamma 1M$- neo transfected cells was able to competetively bind PDGF-BB but did not bind PDGF-AA.

The PDGF β-receptor analog produced from a pSDL114-$p\phi5V_H huC_\gamma 1M$-neo transfectant was assayed to determine if the receptor analog was able to bind PDGF-BB with high affinity. Eight and one half milliliters of spent media containing the PDGFβ-R analogs from a pSDL114-$p\phi5V_H huC_\gamma 1M$-neo transfectant was added to 425 μl of Sepharose Cl-4B-Protein A beads (Sigma, St. Louis, Mo.), and the mixture was incubated for 10 minutes at 4° C. The beads were pelleted by centrifugation and washed with binding medium (Table 4). Following the wash the beads were resuspended in 8.5 ml of binding media, and 0.25 ml aliquots were dispensed to 1.5 ml tubes. Binding reactions were prepared by adding iodinated PDGF-BB_{Jyr} (Example 18.F) diluted in DMEM+10% fetal calf serum to the identical aliquots of receptor-bound beads to final PDGF-BB_{Jyr} concentrations of between 4.12 pM and 264 pM. Nonspecific binding was determined by adding a 100 fold excess of unlabeled BB to an identical set of binding reactions. Mixtures were incubated overnight at 4° C.

The beads were pelleted by centrifugation, and unbound PDGF-BB was removed with three washes in PBS. The beads were resuspended in 100 μl of PBS and were counted. Results of the assay showed that the PDGFβ-R analog was able to bind PDGF-BB with high affinity.

D. Construction of pSDL113.

As shown in FIG. 8, the DNA sequence encoding the extracellular domain of the PDGF β-receptor was joined with the DNA sequence encoding a human immunoglobulin heavy chain constant region joined to a hinge sequence. Plasmid pSDL110 was digested with Eco RI and Hind III to isolate the 1.65 kb PDGFβ-R fragment. Plasmid $pICHu_\gamma$-1M was used as the source of the heavy chain constant region and hinge region. Plasmid $pICHu_\gamma$-1M comprises the approximately 6 kb Hind III-Xho I fragment of a human gamma-1 C gene subcloned into pUC19 that had been linearized by digestion with Hind III and Sal I. Plasmid $pICHu_\gamma$-1M was digested with Hind III and Eco RI to isolate the 6 kb fragment encoding the human heavy chain constant region. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGFβ-R fragment, the 6 kb heavy chain constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL111, was digested with Bam HI to isolate the 7.7 kb fragment. Plasmid pμPRE8 was linearized with Bgl II and was treated with calf intestinal phosphatase to prevent self-ligation. The 7.7 kb fragment and the linearized pμPRE8 were joined by ligation. A plasmid containing the insert in the proper orientation was designated pSDL113 (FIG. 8).

Plasmid pSDL113 is linearized by digestion with Cla I and is cotransfected with Pvu I-digested p416 into SP2/0-Ag14 by electroporation. Transfectants are selected in growth medium containing methotrexate.

Media from drug resistant clones are tested for the presence of secreted PDGFβ-R analogs by immunoprecipitation using the method described in Example 12.B.

E. Cotransfection of pSDL113 with an Immunoglobulin Light Chain Gene

Plasmid pSDL113 is linearized by digestion with Cla I and was cotransfected with $pIC\phi5V_\kappa HuC_\kappa$-Neo, which encodes a neomycin resistance gene and a mouse/human chimeric immunoglobulin light chain gene. The mouse immunoglobulin light chain gene was isolated from a lambda genomic DNA library constructed from the murine hybridoma cell line NR-ML-05 (Woodhouse et al, ibid.) using an oligonucleotide probe designed to span the V_κ/J_κ junction (5' ACC GAA CGT GAG AGG AGT GCT ATA A 3'; Sequence ID Number 34). The human immunoglobulin light chain constant region gene was isolated as described in Example 12.B. The mouse NR-ML-05 immunoglobulin light chain variable region gene was subcloned from the original mouse genomic phage clone into pIC19R as a 3 kb Xba I-Hinc II fragment. The human kappa C gene was subcloned from the original human genomic phage clone into pUC19 as a 2.0 kb Hind III-Eco RI fragment. The chimeric kappa gene was created by joining the NR-ML-05 light chain variable region gene and human light chain constant region gene via the common Sph I site and incorporating them with the *E. coli* neomycin resistance gene into pIC19H to yield $pIC\phi5V_\kappa HuC_\kappa$-Neo (FIG. 9).

The linearized pSDL113 and $pIC\phi5V_\kappa HuC_\kappa$-Neo are transfected into SP2/0-Ag14 cells by electroporation. The transfectants are selected in growth medium containing methotrexate and neomycin.

F. Cotransfection of pSDL113 and pSDL114

A clone of SP2/0-Ag14 stably transfected with pSDL114 and p416 was co-transfected with Cla I-digested pSDL113 and Bam HI-digested pICneo by electroporation. (Plasmid pICneo comprises the SV40 promoter operatively linked to the *E. coli* neomycin resistance gene and pIC19H vector sequences.) Transfected cells were selected in growth medium containing methotrexate and G418. Media from drug-resistanct clones were tested for their ability to bind PDGF-BB or PDGF-AA in a competition binding assay as described in Example 12.C. The results of the assay showed that the transfectants secreted a PDGF β-receptor analog which was capable of competitively binding PDGF-BB but did not detectably bind to PDGF-AA.

G. Cotransfection of pSDL114 with Fab

A clone of SP2/0-AG14 stably transfected with pSDL114 and p416 was transfected with the Fab region of the human gamma-4 gene (γ_4) in plasmid $p\phi5V_H$Fab-neo.

Plasmid $p\phi5V_H$Fab-neo was constructed by first digesting plasmid p24BRH (Ellison et al., *DNA* 1: 11, 1988) was digested with Xma I and Eco RI to isolate the 0.2 kb

fragment comprising the immunoglobulin 3' untranslated region. Synthetic oligonucleotides ZC871 (Sequence ID Number 3; Table 1) and ZC872 (Sequence ID Number 4; Table 1) were kinased and annealed using essentially the methods described by Maniatis et al. (ibid.). The annealed oligonucleotides ZC871/ZC872 formed an Sst I-Xma I adapter. The ZC871/ZC872 adapter, the 0.2 kb p24BRH fragment and Sst I-Eco RI linearized pUC19 were joined in a three-part ligation to form plasmid $p\gamma_4 3'$. Plasmid $p\gamma_4 3'$ was linearized by digestion with Bam HI and Hind III. Plasmid p24BRH was cut with Hind III and Bgl II to isolate the 0.85 kb fragment comprising the $C_H 1$ region. The $p\gamma_4 3'$ fragment and the Hind III-Bgl II p24BRH fragment were joined by ligation to form plasmid $p\gamma_4$Fab. Plasmid $p\gamma_4$Fab was digested with Hind III and Eco RI to isolate the 1.2 kb fragment comprising γ_4Fab. Plasmid pICneo, comprising the SV40 promoter operatively linked to the *E. coli* neomycin resistance gene and pIC19H vector sequences, was linearized by digestion with Sst I and Eco RI. Plasmid $p\phi 5V_H$, comprising the mouse immunoglobulin heavy chain gene variable region and pUC18 vector sequences, was digested with Sst I and Hind III to isolate the 5.3 kb V_H fragment. The linearized pICneo was joined with the 5.3 kb Sst I-Hind III fragment and the 1.2 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated $p\phi 5V_H$Fab-neo (FIG. 10).

A pSDL114/p416-transfected SP2/0-AG14 clone was transfected with Sca I-linearized $p\phi 5V_H$Fab-neo. Transfected cells were selected in growth medium containing methotrexate and G418. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay as described in Example 12.C. The results of the assay showed that the PDGF β-receptor analog secreted from the transfectants was capable of competitively binding PDGF-BB.

H. Cotransfection of pSDL114 with Fab'

A stably transfected SP2/0-AG14 isolate containing pSDL114 and p416 was transfected with plasmid pWKI, which contained the Fab' portion of an immunoglobulin heavy chain gene. Plasmid pWKI was constructed as follows.

The immunoglobulin gamma-1 Fab' sequence, comprising the $C_H 1$ and hinge regions sequences, was derived from the gamma-1 gene clone described in Example 12.C. The gamma-1 gene clone was digested with Hind III and Eco RI to isolate the 3.0 kb fragment, which was subcloned into Hind III-Eco RI linearized M13mp19. Single-stranded template DNA from the resultant phage was subjected to site-directed mutagenesis using oligonucleotide ZC1447 (Sequence ID Number 9; Table 1) and essentially the method of Zoller and Smith (ibid.). A phage clone was identified having a ZC1447 induced deletion resulting in the fusion of the hinge region to a DNA sequence encoding the amino acids Ala-Leu-His-Asn-His-Tyr-Thr-Gln-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly-Lys (Sequence ID Number 31) followed in-frame by a stop codon. Replicative form DNA from a positive phage clone was digested with Hind III and Eco RI to isolate the 1.9 kb fragment comprising the $C_H 1$ and hinge regions. Plasmid $p\phi 5V_H$ was digested with Sst I and Hind III to isolate the 5.3 kb fragment comprising the mouse immunoglobulin heavy chain gene variable region. Plasmid pICneo was linearized by digestion with Sst I and Eco RI. The linearized pICneo was joined with the 5.3 kb Hind III-Sst I fragment and the 1.9 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated pWKI (FIG. 10).

An SP2/0-AG14 clone stably transfected with pSDL114 and p416 was transfected with Asp 718-linearized pWKI. Transfected cells were selected by growth in medium containing methotrexate and G418. Media samples from transfected cells were assayed using the competition assay described in Example 12.C. Results from the assays showed that the transfected cells produced a PDGF β-receptor analog capable of competitively binding PDGF-BB.

Example 13

Purification and Characterization of PDGF β-Receptor Analogs from Mammalian cells Co-transfected With pSDL113 and pSDL114

A. Purification of PDGF β-Receptor Analogs

The PDGF β-receptor analog was purified from conditioned culture media from a clone of transfected cells grown in a hollow fiber system. The media was passed over a protein-A sepharose column, and the column was washed sequentially with phosphate buffered saline, pH 7.2 (PBS; Sigma, St. Louis, Mo.) and 0.1 M citrate, pH 5.0. The PDGF β-receptor analog was eluted from the protein-A column with 0.1 M citrate pH 2.5 and immediately neutralized by the addition of Tris-base, pH 7.4. The eluate fractions containing PDGF β-receptor analog, as determined by silver stain, were pooled and chromatographed over an S-200 column (Pharmacia LKB Technologies, Inc., Piscataway, N.J.) equilibrated with PBS. The peak fractions from the S-200 column were pooled and concentrated on a centriprep-10 concentrator (Amicon). Glycerol (10% final volume) was added to the preparation and the sample frozen at −80° C. PDGF β-receptor analogs purified from pSDL114+pSDL113 co-transfected cells were termed "tetrameric PDGF α-receptors".

B. Measurement of The Relative Binding Affinity of Tetrameric PDGF β-Receptor Analog by Soluble Receptor Assay

Purified tetrameric PDGF β-receptor analog was compared to detergent solubilized extracts of human dermal fibroblasts for ^{125}I-labeled PDGF-BB binding activity in a soluble receptor assay essentially as described by Hart et al. (*J. Biol. Chem.* 262: 10780–10785, 1987). Human dermal fibroblast cells were extracted at 20×10^6 cell equivalents per ml in TNEN extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 10% glycerol). Two hundred and fifty thousand PDGF β-receptor-subunits per cell was used to calculate the tetrameric PDGF β-receptor analog number per volume of extract. This value has been previously published by Seifert et al. (*J. Biol Chem.* 264: 8771–8778, 1989). The PDGF β-receptor analog number was determined from the protein concentration of the PDGF β-receptor analog assuming an average molecular weight of 140 kDa for each immunoglobulin-PDGF β-receptor monomer, and four monomers per tetramer. Thus, each tetrameric molecule contains four receptor molecules.

Increasing amounts of either detergent solubilized extracts of human dermal fibroblast cells or purified PDGF β-receptor analog were incubated with 1 ng of ^{125}I-labeled PDGF-BB for one hour at 37° C. The sample was then diluted with 1 ml binding media and was added to monolayers of human dermal fibroblast cells grown in 24-well culture dishes. The samples were incubated for two hours at 4° C. The wells were washed to remove unbound, ^{125}I-labeled PDGF-BB. On half of a milliliter of extraction buffer (PBS+1% Nonidet P-40) was added to each well followed by a 5 minute incubation. The extraction mixtures were harvested and counted in a gamma counter.

The results showed that the PDGF β-receptor analog had the same relative binding affinity as solubilized PDGF

β-receptor-subunit from mammalian cells in a solution phase binding assay.

C. Determination of the Binding Affinity of the PDGF β-Receptor Analog in a Solid Phase Format

The apparent dissociation constant K_D(app) of the PDGF β-receptor analog was determined essentially as described by Bowen-Pope and Ross (*Methods in Enzymology* 109: 69–100, 1985), using the concentration of ^{125}I-labeled PDGF-BB giving half-maximal specific ^{125}I-labeled PDGF-BB binding. Saturation binding assays to determine the concentration of ^{125}I-labeled PDGF-BB that gave half-maximal binding to immobilized PDGF β-receptor analog were conducted as follows.

Affinity purified goat anti-human IgG, H- and L-chain (Commercially available from Cappel Labs) was diluted into 0.1 M Na_2HCO_3, pH 9.6 to a concentration of 2 μg/ml. One hundred microliters of the antibody solution was coated onto each well of 96-well microtiter plates for 18 hours at 4° C. The wells were washed once with ELISA C buffer (PBS+ 0.05% Tween-20) followed by an incubation with 175 μl/well of ELISA B buffer (PBS+1% BSA+0.05% Tween-20) to block the wells. The wells were washed once with ELISA B buffer. One hundred microliters of 12.1 ng/ml or 24.3 ng/ml of tetrameric PDGF β-receptor analog protein diluted in ELISA B was added to each well and the plates were incubated for 2 hours at 37° C. Unbound protein was removed from the wells by two washes with ELISA C. ^{125}I-labeled PDGF-BB_{2y} (Example 18.F.) was serially diluted into binding media (25 mM HEPES, pH 7.2, 0.25% rabbit serum albumin diluted in HAMs P-12 medium (GIBCO-BRL)), and 100 μl of the dilutions were added to the wells. The plates were incubated for two hours at room temperature. The unbound ^{125}I-labeled PDGF-BB was removed, and the wells were washed three times with binding media. Following the last wash, 100 μl of 0.1 M citrate, pH 2.5 was added to each well. After five minutes, the citrate buffer was removed, transferred to a tube and counted in a gamma counter. The counts reflect counts of ^{125}I-labeled PDGF-BB_{2y} bound by the receptor analog. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-BB_{2y} was determined by a parallel assay wherein separate wells coated only with goat anti-human IgG were incubated with the ^{125}I-labeled PDGF-BB concentrations. Nonspecific binding was determined to be 2.8% of the total input counts per well and averaged 6% of the total counts bound.

Saturation binding assay on 12.1 and 24.3 ng/ml of tetrameric PDGF β-receptor analog gave half-maximal binding at 0.8 and 0.82 ng/ml ^{125}I-labeled PDGF-BB_{2y}, respectively. By Scatchard analysis (Scatchard, *Ann. NY Acad. Sci* 51: 660–667, 1949) these values were shown to correspond to a K_D(app) of 2.7×10^{-11} which agree with the published values for PDGF receptors on mammalian cells.

Example 14

Solid Phase Ligand Binding Assay Using the PDGF β-Receptor Analog

A. Solid Phase Radioreceptor Competition Binding Assay

In a solid phase radioreceptor competition binding assay (RRA), the wells of 96-well microtiter plates were coated with 100 μl of 2 μg/ml affinity purified goat anti-human IgG (Cappel Labs) diluted in 0.1 M Na_2HCO_3, pH 9.6. After an eighteen hour incubation at 4° C., the wells were washed once with ELISA C. The wells were blocked by incubation for 2 hours at 37° C. with 175 μl/well ELISA B. The wells were washed once with ELISA B then incubated for 2 hours at 37° C. with 50 ng/ml tetrameric PDGF β-receptor analog diluted in ELISA B. The unbound receptor was removed, and the test wells were incubated with increasing concentrations of serially diluted, unlabeled PDGF-BB (diluted in binding media. Following a two hour incubation at room temperature, the wells were washed three times with binding media. One hundred microliters of 5 ng/ml ^{125}I-labeled PDGF-BB_{2y} (Example 18.F.) was added to each well, and the plates were incubated for an additional two hours at room temperature. The wells were washed three times with binding media followed by a 5 minute incubation with 100 μl/well of 0.1 M citrate, pH 2.5. The samples were harvested and counted in a gamma counter.

Radioreceptor assay (RRA) competition binding curves were generated for PDGF β-receptor analog protein plated at 48.6 ng/ml. The sensitivity of the assays is 1 ng/ml of PDGF-BB, with 8 ng/ml giving 50% inhibition in ^{125}I-PDGF-BB binding, and a working range between 1 and 32 ng/ml of PDGF-BB. The values were similar to those obtained using monolayers of SK-5 cells in an RRA.

B. Use of Tetrameric PDGF β-Receptor Analogs As Antagonists for PDGF-Stimulated Mitogenesis.

A tetrameric PDGF β-receptor analog, purified as described in Example 13, was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF β-receptor analog were mixed with 5 ng of PDGF. The mixtures were then added to cultures of mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of ^{3}H-thymidine, was determined essentially as described (Raines and Ross, *Methods in Enzymology* 109: 749–773, 1985, which is incorporated by reference herein). The tetrameric PDGF β-receptor analog demonstrated a dose response inhibition of PDGF-BB-stimulated ^{3}H-thymidine incorporation, while having essentially no effect on PDGF-AA- and PDGF-AB-stimulated ^{3}H-thymidine incorporation.

C. Binding of Tetrameric PDGF β-receptor Analog to Immobilized PDGF.

A tetrameric PDGF β-receptor analog, purified as described in Example 13, was analyzed for its ability to bind to immobilized PDGF. PDGF-BB (100 ng/ml) was coated onto wells a 96-well microtiter plate, and the plates were incubated 18 hours at 4° C. followed by one wash with ELISA C buffer. The wells were incubated for 2 hours 37° C. with ELISA B buffer to block the wells. Increasing concentrations of ^{125}I-labeled tetrameric PDGF β-receptor analog, diluted in binding media, was added to the wells for two hours at room temperature. The wells were washed four times with ELISA C buffer to remove unbound receptor analog. One hundred microliters of 1 M H_2SO_4 was added to each well and the plates were incubated for five minutes at room temperature. The solution was then harvested and transferred to tubes to be counted in a gamma counter. Nonspecific binding was determined to be less than 10% of the total counts bound.

A receptor competition binding assay was developed using this assay format. The assay was carried out as described above, and simultaneous to the addition of the ^{125}I-labeled tetrameric PDGF β-receptor analog, increasing amounts of PDGF-AA, AB or BB were added to the PDGF-BB coated wells. Under these conditions, only PDGF-BB was found to significantly block the binding of the labeled PDGF β-receptor analog to the immobilized PDGF-BB.

Example 15

Construction and Expression of PDGFα-R Analogs in Cultured Mouse Myeloma Cells

A. Construction of an optimized PDGFα-R cDNA

The PDGF α-receptor coding region was optimized for expression in mammalian cells as follows. The 5' end of the cDNA was modified to include an optimized Kozak consensus translation initiation sequence (Kozak, *Nuc. Acids Res.* 12: 857–872, 1984) and Eco RI and Bam HI sites just 5' of the initiation methionine codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 (Sequence ID Numbers 23, 24, 25 and 26, respectively; Table 1) were designed to form, when annealed, an adapter having an Eco RI adhesive end, a Bam HI restriction site, a sequence encoding a Kozak consensus sequence 5' to the initation methionine codon, a mammalian codon optimized sequence encoding amino acids 1–42 of FIGS. 11A–11D, and an Eco RI adhesive end that destroys the Eco RI site within the PDGFα-R coding sequence. The adapter also introduced a diagnostic Cla I site 3' to the initiation methionine codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 were kinased, annealed and ligated. Plasmid pα17B was linearized by partial digestion with Eco RI. The linearized pα17B was ligated with the ZC2181/ZC2182/ZC2183/ZC2184 oligonucleotide adapter, and the ligation mixture was transformed into *E. coli*. Plasmid DNA prepared from the transformants was analyzed by restriction analysis and a positive clone having the oligonucleotide adapter in the correct orientation was digested with Eco RI and Pst I to isolate the 1.6 kb fragment. This fragment was subcloned into Eco RI+Pst I-linearized M13mp19. The resultant phage clone was designated 792-8. Single-stranded 792-8 DNA was sequenced to confirm the orientation of the adapter.

A fragment encoding the ligand-binding domain of the PDGF α-receptor (PDGFα-R) was then generated as follows. Restriction sites and a splice donor sequence were introduced at the 3' end of the PDGFα-R extracellular domain by PCR amplification of the 792-8 DNA and oligonucleotides ZC2311 and ZC2392 (Sequence ID Numbers 27 and 30, Table 1). Oligonucleotide ZC2311 is a sense primer encoding nucleotides 1470 to 1489 of FIG. 11B. Oligonucleotide ZC2392 is an antisense primer that encodes nucleotides 1759 to 1776 of FIG. 11B followed by a splice donor and Xba I and Hind III restriction sites. The 792-8 DNA was amplified using manufacturer recommended (Perkin Elmer Cetus, Norwalk, Conn.) conditions and the GeneAmp™ DNA amplification reagent kit (Perkin Elmer Cetus), and blunt-ended 329 bp fragment was isolated. The blunt-end fragment was digested with Nco I and Hind III and ligated with Sma I-digested pUC18. A plasmid having an insert with the Nco I site distal to the Hind III site present in the pUC18 polylinker was designated pUC18 Sma-PCR Nco HIII #13. The Hind III site present in the insert was not regenerated upon ligation with the linearized pUC18. Plasmid pUC18 Sma-PCR Nco HIII #13 was digested with Nco I and Hind III to isolate the 355 bp PDGFα-R containing fragment encoding PDGFαR. oligonucleotides ZC2351 and ZC2352 (Table 1; Sequence ID Numbers 28 and 29) were kinased and annealed to form an Sst I-Nco I adapter encoding an internal Eco RI site and a Kozak consensus translation initiation site. The 355 bp Nco I-Hind III fragment, the ZC2351/ZC2352 adapter and a 1273 bp Nco I fragment comprising the extracellular domain of of PDGF α-R derived from 792-8 were ligated with Hind III+SstI-digested pUC18, and tranformed into *E. coli*. Plasmid DNA was isolated from the transformants and analyzed by restriction analysis. None of the isolates contained the 1273 bp Nco I fragment. A plasmid containing the Nco I-Hind III fragment and the ZC2351/ZC2352 adapter was designated pUC18 Hin Sst Δ Nco #46. Plasmid pUC18 Hin Sst Δ Nco #46 was linearized by digestion and joined by ligation with the 1273 bp Nco I fragment comprisising the extracellular domain of the PDGFα-R from clone α18 R-19. The ligations were transformed into *E. coli*, and plasmid DNA was isolated from the transformants. Analysis of the plasmid DNA showed that only clones with the Nco I fragment in the wrong orientation were isolated. A clone having the Nco I fragment in the wrong orientation was digested with Nco I, religated and transformed into *E. coli*. Plasmid DNA was isolated from the transformants and was analyzed by restriction analysis. A plasmid having the Nco I insert in the correct orientation was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb fragment comprising the extracellular domain of the PDGFα-R preceded by a consensus initiation sequence (Kozak, ibid.) and followed by a splice donor site.

B. Construction of pPAB7

The DNA sequence encoding the extracellular domain of the PDGFα-R was joined to the immunoglobulin μ enhancer-promoter and to a DNA sequence encoding an immunoglobulin light chain constant region. The immunoglobulin μ enhancer-promoter was obtained from plasmid pJH1 which was derived from plasmid PIC$_\mu$PRE8 (Example 12.A.) by digestion with Eco RI and Sst I to isolate the 2.2 kb fragment comprising the immunoglobulin enhancer and heavy chain variable region promoter. The 2.2 kb Sst I-Eco RI fragment was ligated with Sst I+Eco RI-linearized pUC19. The resulting plasmid, designated pJH1, contained the immunoglobulin enhancer and heavy chain variable region promoter immediately 5' to the pUC19 linker sequences. Plasmid pJH1 was linearized by digestion with Sst I and Hind III and joined with the 1.6 kb partial Sst I-Hind III fragment containing the PDGFα-R extracellular domain sequences. The resulting plasmid having the immunoglobulin μ enhancer-promoter joined to the PDGFα-R extracellular domain was designated pPAB6. Plasmid pSDL112 was digested with Hind III to isolate the 1.2 kb fragment encoding the immunoglobulin light chain constant region (Cκ). The 1.2 kb Hind III fragment was ligated with Hind III-linearized pPAB6. A plasmid having the C_κ sequence in the correct orientation was desginated pPAB7.

C. Construction of pPAB9

The partial Sst I-Hind III fragment encoding the extracellular domain of the PDGFα-R was joined to the immunoglobulin heavy chain constant region. For convenience, the internal Xba I site in plasmid pJH1 was removed by digestion with Xba I, blunt-ending with T4 DNA polymerase, and religation. A plasmid which did not contain the internal Xba I site, but retained the Xba I site in the polylinker was designated 11.28.3.6. Plasmid 11.28.3.6 was linearized by digestion with Sst I and Xba I. Plasmid pPAB6 was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb Sst I-Hind III fragment containing the PDGFα-R extracellular domain. Plasmid pφ5V$_H$huC$_\gamma$1M-neo (Example 12.C.) was digested wtih Hind III and Xba I to isolate the 6.0 kb fragment encoding the immunoglobulin heavy chain constant region (huC$_\gamma$1M). The Sst I-Hind III-linearized 11.28.3.6, the 1.6 kb Sst I-Hind III PDGFα-R fragment and the 6.0 kb Hind III-Xba I huC$_\gamma$1M fragment were ligated to form plasmid pPAB9.

D. Expression of pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Pvu I-linearized pPAB9 were cotransfected with Pvu I-linearized p416 into SP2/0-Ag14 cells by electroporation. Transfected cells were initially selected in growth medium containing 50 nM methotrexate and were subsequently amplified in a growth medium containing 100 μM methotrexate. Media from drug

resistant clones were tested for the presence of secreted PDGF α-receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml monoclonal antibody 292.1.8 which is specific for the PDGF α-receptor diluted in phosphate buffered saline (PBS; Sigma] overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the plates were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human IgG heavy chain antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well, and the plates were incubated for one hour at 4° C. One hundred microliters of chromophore (100 μl ABTS [2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid] diammonium salt; Sigma]+1 μl 30% H_2O_2+12.5 ml citrate/phosphate buffer [9.04 g/l citric acid, 10.16 g/l Na_2HPO_4]) was added to each well, and the wells were incubated for 30 minutes at room temperature. The samples were measured at 405 nm. The results of the assay showed that the PDGF α-receptor analogs secreted by the transfectants contained an immunoglobulin heavy chain.

Analysis of spent media from transfected cells by Northern analysis, Western analysis and by radioimmunoprecipitation showed that the transfectants did not express a PDGF α-receptor analog from the pPAB7 construction. Transfectants were subsequently treated as containing only pPAB9.

Drug resistant clones were also tested for the presence of secreted PDGF α-receptor analogs by immunoprecipitation. For each clone, approximately one million drug resistant transfectants were grown in DMEM lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μCi ^{35}S-cysteine. The spent media was harvested from the labeled cells and 250 μl of medium from each clone was assayed for binding to the anti-PDGF α-receptor antibody 292.18. Monoclonal antibody 292.18 diluted in PBS was added to each sample to a final concentration of 2.5 μg per 250 μl spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample, and the immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at −70° C. The results of the immunoprecipitation showed that the PDGF α-receptor analog secreted by the transfectants was bound by the anti-PDGF α-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF α-receptor analog secreted by the transfectants contained both the PDGF α-receptor ligand-binding domain and the human heavy chain.

Spent medium from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay esssentially as described in Example 12.C. The results of the assay showed that the transfectants secreted a PDGF α-receptor analog capable of binding PDGF-AA. A clone containing the pPAB9 was desginated 3.17.1.57.

E. Co-expression of pPAB7 and pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Bam HI-linearized pICneo were cotransfected into clone 3.17.1.57, and transfected cells were selected in the presence of neomycin. Media from drug resistant cells were assayed for the presence of immunoglobulin heavy chain, immunoglobulin light chain and the PDGF α-receptor ligand-binding domain by ELISA essentially as described above. Briefly, ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml goat anti-human IgG Fc antibody (Sigma) or 100 μl of 1 μg/ml 292.18 overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well of each plate, and the plates were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human IgG antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well of the plate coated with the anti-Fc antibody, and 100 μl of peroxidase-conjugated goat anti human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well of the plate coated with 292.18. The plates were incubated for one hour at 4° C. One hundred microliters of chromophore (100 μl ABTS (2,2'-Azinobis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt; Sigma]+1 μl 30% H_2O_2+12.5 ml citrate/phosphate buffer [9.04 g/l citric acid, 10.16 g/l Na_2HPO_4]) was added to each well of each plate, and the plates were incubated to 30 minutes at room temperature. The samples were measured at 405 nm, the wavelength giving maximal absorbance of the chromogenic substrate, to identify clones having absorbances higher than background indicating the presence of immunoglobulin heavy chain. Clones that gave positive results in both ELISA assays (showing that the clones produced proteins containing heavy chain regions, light chain constant regions and the PDGF α-receptor ligand-binding region) were selected for further characterization.

Drug resistant clones that were positive for both ELISA assays were subsequently tested for the presence of secreted PDGF α-receptor analogs by immunoprecipitation. For each positive clone, approximately one million drug resistant transfectants were grown in DMEM lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μCI ^{35}S-cysteine. The spent media was harvested from the labeled cells and 250 μl of medium from each clone was assayed for binding to monoclonal antibody 292.18. Monoclonal antibody 292.18 diluted in PBS was added to each sample to a final concentration of 2.5 μg. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample and the immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at −70° C. The results of the immunoprecipitation showed that the PDGF α-receptor analog secreted by the transfectants was bound by the anti-PDGF α-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF α-receptor analog secreted by the transfectants contained the PDGF α-receptor ligand-binding domain, the human heavy chain and the human light chain constant region. A clone that secreted a PDGF α-receptor analog that was positive for both the above-described ELISA assays and the immunoprecipitation assay was designated 5.6.2.1.

Example 16

Purification and characterization of PDGF α-Receptor Analogs

A. Purification of PDGF α-Receptor Analogs From Clone 3.17.1.57

The PDGF α-Receptor analog was purified from the conditioned culture media of clone 3.17.1.57 by cycling cell-conditioned medium over an immunoaffinity column composed of monoclonal antibody 292.18 bound to a CNBr-

activated Sepharose 4B resin, which is specific for the PDGF α-receptor. The column was washed with PBA, then eluted with 0.1 M citrate, pH 3.0. The peak column fractions containing the α-receptor were pooled, neutralized to pH 7.2 by the addition of 2 M Tris, pH 7.4, then passed over a protein-A Sepharose column. This column was washed sequentially with PBS, then with 0.1 M citrate, pH 5.0. The PDGF α-receptor analog was then eluted with 0.1 M citrate, pH 3.0. The peak eluate fractions were pooled, and glycerol was added to a final concentration of 1nt. The sample was concentrated on a centriprep 10 concentrator (Amicon). The PDGF α-receptor analog purified from clone 3 17 1.57 was termed a "dimeric PDGF α-receptor analog".

B. Purification of PDGF α-Receptor Analogs From Clone 5.6.2.1

The PDGF α-receptor analog was purified from the conditioned culture media of clone 5.6.2.1 by cycling cell-conditioned medium over the immunoaffinity column described above. The column was washed with PBS then eluted with 0.1 M citrate, pH 3.0. The peak column fractions containing the α-receptor were pooled, neutralized to pH 7.2 by the addition of 2 M Tris (what pH 7.4), then passed over a protein-A sepharose column. This column was washed sequentially with PBS then with 0.1 M citrate, pH 5.0. The PDGF α-receptor analog was then eluted with 0.1 M citrate, pH 3.0. The peak eluate fractions were pooled and glycerol was added to a final concentration of 10%. The sample was concentrated on a centriprep 10 concentrator. The PDGF α-receptor analogs purified from clone 5.6.2.1 was termed a "tetrameric PDGF α-receptor analog".

Example 17

A. Use of the PDGF α-receptor Analogs in Ligand Binding Studies

Purified tetrameric PDGF α-receptor analog and purified dimeric PDGF α-receptor analog were compared to monolayers of a control cell line of canine kidney epithelial cells, which do not naturally express the PDGF α-receptor, transfected with the human PDGF α-receptor cDNA for ligand binding activity. The dissociation constant (Kd) of the receptor preparations was determined by saturation binding and subsequent Scatchard analysis.

Ligand binding of the purified PDGF α-receptor analogs was determined using a solid phase binding assay. Affinity-purified goat anti-human IgG was diluted to a concentration of 2 μg/ml in 0.1 M Na_2HCO_3, pH 9.6 and 100 μl/well of the solution was used to coat 96-well microtiter plates for 18 hours at 4° C. Excess antibody was removed from the wells with one wash with ELISA C buffer (PBS, 0.05% Tween-20). The plates were incubated with 175 μl/well of ELISA B buffer (PBS, 1% BSA, 0.05% Tween-20) to block the wells, followed by two washes with ELISA C buffer. One hundred microliters of 50 ng/ml PDGF α-receptor analog (dimeric or tetrameric) diluted in ELISA buffer B was added to each well and the plates were incubated over night at 4° C. Unbound protein was removed from the wells with two washes with ELISA buffer B. ^{125}I-labeled PDGF-AA was serially diluted in binding media (Hams F-12, 25 mM HEPES pH 7.2, 0.25% rabbit serum albumin), and 100 μl of each dilution was added to the wells. The samples were incubated for two hours at room temperature. Unbound ^{125}I-labeled PDGF-AA was removed with three washes with binding media. One hundred microliters of 0.1 M citrate, pH 2.5 was added to each well, and the plates were incubated for five minutes. After the incubation, the citrate buffer was removed and transferred to a tube for counting in a gamma counter. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-AA was determined by a parallel assay wherein separate wells coated only with goat anti-human IgG were incubated with the ^{125}I-labeled PDGF-AA samples.

A saturation binding assay was performed on alpha T-7 cells transfected with the PDGF α-receptor. The cells were grown to confluency in 24-well culture plates. The cells were washed one time with binding media. Iodinated PDGF-AA was serially diluted in binding media. One milliliter of each dilution was added to the wells, and the plates were incubated for 3 hours at 4° C. Unbound ^{125}I-labeled PDGF-AA was removed and the cells were washed three times with binding media. PBS containing 1% Triton X-100 was added to the cells for 5 minutes. The extracts were harvested and counted in a gamma counter. Nonspecific binding was determined at a single concentration of ^{125}-labeled PDGF-AA using a 500-fold excess PDGF-BB.

The dissociation constants determined by Scatchard analysis (ibid.) of the saturation binding assays for the tetrameric PDGF α-receptor analog, dimeric PDGF α-receptor analog and the control cells (Table 5).

Table 5

Dissociation Constants for the Tetrameric PDGF α-Receptor, the Dimeric PDGF α-receptor and control cells Transfected with the PDGF α-receptor

TABLE 5

Dissociation Constants for the Tetrameric PDGF α-Receptor, the Dimeric PDGF α-receptor and control cells Transfected with the PDGF α-receptor	
Receptor	kD
Tetrameric PDGF α-receptor analog	1.6×10^{-12}
Dimeric PDGF α-receptor analog	8.51×10^{-13}
Control cells [PDGF α-receptor]	3.7×10^{-13}

A solid-phase competition binding assay was established using the tetrameric PDGF α-receptor analog. Ninety six-well microtiter plates were coated with goat anti-human IgG (2 μg/ml), the wells blocked with ELISA B buffer, 50 ng/ml of purified tetrameric PDGF α-receptor analog diluted in binding media was added, and the plates were incubated two hours at room temperature. Unbound receptor was removed and the wells were washed with binding media. The plates were incubated for two hours at room temperature with increasing concentrations of either PDGF-AA or PDGF-BB diluted in binding media. The wells were washed, then incubated for two hours at room temperature with 3 ng/ml ^{125}I-labeled PDGF-AA diluted in binding media. Unbound labeled PDGF-AA was removed, the wells were subsequently washed with binding media, and the bound, labeled PDGF-AA was harvested by the addition of 0.1 M citrate, pH 2.5, as described for the saturation binding studies. PDGF-AB, PDGF-AA and PDGF-BB were found to compete for receptor binding with ^{125}I-PDGF-AA.

B. Use of Tetrameric PDGF α-Receptor Analogs As Antagonists for PDGF-Stimulated Mitogenesis

A dimeric PDGF α-receptor analog, purified as described in Example 16.B., was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF α-receptor analog were mixed with PDGF-AA, -AB or -BB ranging 0.6 to 5 ng. The mixtures were then added to cultures of confluent mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of ^{3}H-thymidine, was determined essentially as

described (Raines and Ross, *Methods in Enzymology* 109: 749–773, 1985, which is incorporated by reference herein). The dimeric PDGF α-receptor analog demonstrated a dose response inhibition of PDGF-stimulated ^{3}H-thymidine incorporation for all three isoforms of PDGF.

C. Inverse Ligand-Receptor Radioreceptor Assay

An inverse ligand-receptor radioreceptor assay was designed to screen for the presence of PDGF-BB, PDGF-BB binding proteins, PDGF-BB related molecules, and PDGF-β receptor antagonists in test samples. PDGF-BB (100 ng/ml) was coated onto the walls of 96-well microtiter plates, and the plates were incubated at 4° C. for 16 hours. The wells were washed once with ELISA C buffer and then incubated with ELISA B buffer to block the nonspecific binding sites. To the wells were added 50 μl of either PDGF standard or a test sample and 50 μl of ^{125}I-labeled tetrameric PDGF β-receptor analog. The samples were incubated for one hour at room temperature. The wells were washed once with ELISA C buffer, and 0.1 M citrate, pH 2.5 containing 1% NP-40 was added to each well to disrupt the ligand-receptor analog bond and elute the bound receptor analog. The acid wash was collected and counted in a gamma counter. The presence of PDGF or a molecule which mimics PDGF or otherwise interferes with the binding of the well-bound PDGF-BB with its receptor will cause a decrease in the binding of the radiolabeled tetrameric PDGF β-receptor. Using this assay, PDGF-BB was found to inhibit receptor binding while PDGF-AA and PDGF-AB caused no significant decrease in receptor binding.

Example 18

Assay Methods

A. Preparation of Nitrocellulose Filters for Colony Assay

Colonies of transformants were tested for secretion of PDGF β-receptor analogs by first growing the cells on nitrocellulose filters that had been laid on top of solid growth medium. Nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) were placed on top of solid growth medium and were allowed to be completely wetted. Test colonies were patched onto the wetted filters and were grown at 30° C. for approximately 40 hours. The filters were then removed from the solid medium, and the cells were removed by four successive rinses with Western Transfer Buffer (Table 4). The nitrocellulose filters were soaked in Western Buffer A (Table 4) for one hour at room temperature on a shaking platform with two changes of buffer. Secreted PDGFβ-R analogs were visualized on the filters described below.

B. Preparation of Protein Blot Filters

A nitrocellulose filter was soaked in Western Buffer A (Table 4) without the gelatin and placed in a Minifold (Schleicher & Schuell, Keene, N.H.). Five milliliters of culture supernatant was added without dilution to the Minifold wells, and the liquid was allowed to pass through the nitrocellulose filter by gravity. The nitrocellulose filter was removed from the minifold and was soaked in Western Buffer A (Table 3) for one hour on a shaking platform at room temperature. The buffer was changed three times during the hour incubation.

C. Preparation of Western Blot Filters

The transformants were analyzed by Western blot, essentially as described by Towbin et al. (*Proc. Natl. Acad. Sci. USA* 76: 4350–4354, 1979) and Gordon et al. (U.S. Pat. No. 4,452,901). Culture supernatants from appropriately grown transformants were diluted with three volumes of 95% ethanol. The ethanol mixtures were incubated overnight at −70° C. The precipitates were spun out of solution by centrifugation in an SS-24 rotor at 18,000 rpm for 20 minutes. The supernatants were discarded and the precipitate pellets were resuspended in 200 μl of dH_2O. Two hundred microliters of 2× loading buffer (Table 4) was added to each sample, and the samples were incubated in a boiling water bath for 5 minutes.

The samples were electrophoresed in a 15% sodium dodecylsulfate polyacrylamide gel under non-reducing conditions. The proteins were electrophoretically transferred to nitrocellulose paper using conditions described by Towbin et al. (ibid.). The nitrocellulose filters were then incubated in Western Buffer A (Table 4) for 75 minutes at room temperature on a platform rocker.

D. Processing the Filters for Visualization with Antibody

Filters prepared as described above were screened for proteins recognized by the binding of a PDGF β-receptor specific monoclonal antibody, designated PR7212. The filters were removed from the Western Buffer A (Table 4) and placed in sealed plastic bags containing a 10 ml solution comprising 10 μg/ml PR7212 monoclonal antibody diluted in Western Buffer A. The filters were incubated on a rocking platform overnight at 4° C. or for one hour at room temperature. Excess antibody was removed with three 15-minute washes with Western Buffer A on a shaking platform at room temperature.

Ten microliters biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, Calif.) in 20 ml Western Buffer A was added to the filters. The filters were re-incubated for one hour at room temperature on a platform shaker, and unbound conjugated antibody was removed with three fifteen-minute washes with Western Buffer A.

The filters were pre-incubated for one hour at room temperature with a solution comprising 50 μl Vectastain Reagent A (Vector Laboratories) in 10 ml of Western Buffer A that had been allowed to incubate at room temperature for 30 minutes before use. The filters were washed with one quick wash with distilled water followed by three 15-minute washes with Western Buffer B (Table 4) at room temperature. The Western Buffer B washes were followed by one wash with distilled water.

During the preceding wash step, the substrate reagent was prepared. Sixty mg of horseradish peroxidase reagent (Bio-Rad, Richmond, Calif.) was dissolved in 20 ml HPLC grade methanol. Ninety milliliters of distilled water was added to the dissolved peroxidase followed by 2.5 ml 2 M Tris, pH 7.4 and 3.8 ml 4 M NaCl. One hundred microliters of 30% H_2O_2 was added just before use. The washed filters were incubated with 75 ml of substrate and incubated at room temperature for 10 minutes with vigorous shaking. After the 10 minute incubation, the buffer was changed, and the filters were incubated for an additional 10 minutes. The filters were then washed in distilled water for one hour at room temperature. Positives were scored as those samples which exhibited coloration.

E. Processing the Filters For Visualization with an Anti-Substance P Antibody

Filters prepared as described above were probed with an anti-substance P antibody. The filters were removed from the Western Buffer A and rinsed with Western transfer buffer, followed by a 5-minute wash in phosphate buffered saline (PBS, Sigma, St. Louis, Mo.). The filters were incubated with a 10 ml solution containing 0.5 M 1-ethyl-3-3-dimethylamino propyl carbodiimide (Sigma) in 1.0 M NH_4Cl for 40 minutes at room temperature. After incubation, the filters were washed three times, for 5 minutes per wash, in PBS. The filters were blocked with 2% powdered milk diluted in PBS.

The filters were then incubated with a rat anti-substance P monoclonal antibody (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Ten microliters of the antibody was diluted in 10 ml of antibody solution (PBS containing 20% fetal calf serum and 0.5% Tween-20). The filters were incubated at room temperature for 1 hour. Unbound antibody was removed with four 5-minute washes with PBS.

The filters were then incubated with a biotin-conjugated rabbit anti-rat peroxidase antibody (Cappel Laboratories, Malvern, Pa.). The conjugated antibody was diluted 1:1000 in 10 ml of antibody solution for 2 hours at room temperature. Excess conjugated antibody was removed with four 5-minute washes with PBS.

The filters were pre-incubated for 30 minutes at room temperature with a solution containing 50 μl Vectastain Reagent A (Vector Laboratories) and 50 μl Vectastain Reagent B (Vector Laboratories) in 10 ml of antibody solution that had been allowed to incubate for 30 minutes before use. Excess Vectastain reagents were removed by four 5-minute washes with PBS.

During the preceding wash step, the substrate reagent was prepared. Sixty milligrams of horseradish peroxidase reagent (Bio-Rad Laboratories, Richmond, Calif.) was dissolved in 25 ml of HPLC grade methanol. Approximately 100 ml of PBS and 200 μl H_2O_2 were added just before use. The filters were incubated with the substrate reagent for 10 to 20 minutes. The substrate was removed by a vigorous washing distilled water.

F. Iodination of PDGF-BB

A PDGF-BB mutant molecule having a tyrosine replacing the phenylalanine at position 23 (PDGF-BB_{Tyr}) was iodinated and subsequently purified, using a purification method which produces 125I-labeled PDGF-BB with a higher specific activity than primary-labeled material and which was found to substantially decrease the nonspecific binding component. The PDGF-BB_{Tyr} was labeled using the Iodobead method (Pierce Chemical). The labeled protein was gel filtered over a C-25 desalting column (Pharmacia LKB Technologies) equilibrated with 10 mM acetic acid, 0.25% gelatin and 100 mM NaCl. The peak fractions were pooled and pH adjusted to 7.2 by the addition of Tris-base. The labeled mixture was chromatographed over an affinity column composed of PDGF β-receptor analog protein coupled to CnBr-activated Sepharose (Pharmacia LKB Technologies, Inc.). The column was washed with phosphate buffered saline and eluted with 0.1 M citrate, pH 2.5 containing 0.25% gelatin. The peak eluate fractions were pooled and assayed by ELISA to determine the PDGF-BB concentration.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 36

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4465 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Skin
(G) CELL TYPE: fibroblasts

(vii) IMMEDIATE SOURCE:
(B) CLONE: pR-rX1

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 354..3671
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

CCCTCAGCCC TGCTGCCCAG CACGAGCCTG TGCTCGCCCT GCCCAACGCA GACAGCCAGA 60

CCCAGGGCGG CCCCTCTGGC GGCTCTGCTC CTCCCGAAGG ATGCTTGGGG AGTGAGGCGA 120

AGCTGGGCGC TCCTCTCCCC TACAGCAGCC CCCTTCCTCC ATCCCTCTGT TCTCCTGAGC 180

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CTTCAGGAGC CTGCACCAGT CCTGCCTGTC CTTCTACTCA GCTGTTACCC ACTCTGGGAC    240

CAGCAGTCTT TCTGNTAACT GGGAGAGGGC AGTAAGGAGG ACTTCCTGGA GGGGGTGACT    300

GTCCAGAGCC TGGAACTGTG CCCACACCAG AAGCCATCAG CAGCAAGGAC ACC ATG       356
                                                            Met
                                                             1

CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC GAG CTG CTG       404
Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu Leu
                5                  10                  15

TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT CAG GGC CTG      452
Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly Leu
        20                  25                  30

GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC      500
Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser Thr
    35                  40                  45

TTC GTT CTG ACC TGC TCG GCT TCA GCT CCG GTG GTG TGG GAA CGG ATG      548
Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg Met
 50                  55                  60                  65

TCC CAG GAG CCC CCA CAG GAA ATG GCC AAG GCC CAG GAT GGC ACC TTC      596
Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr Phe
                70                  75                  80

TCC AGC GTG CTC ACA CTG ACC AAC CTC ACT GGG CTA GAC ACG GGA GAA      644
Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly Glu
            85                  90                  95

TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA CTG GAG ACC GAT GAG CGG      692
Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu Arg
        100                 105                 110

AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG GGC TTC CTC CCT      740
Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu Pro
    115                 120                 125

AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA ACT GAG ATC      788
Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu Ile
130                 135                 140                 145

ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG ACA CTG CAC      836
Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu His
                150                 155                 160

GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT CAC CAA CGT      884
Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln Arg
            165                 170                 175

GGC TTT TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC AAA ACC ACC      932
Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr Thr
        180                 185                 190

ATT GGG GAC AGG GAG GTG GAT TCT GAT GCC TAC TAT GTC TAC AGA CTC      980
Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg Leu
    195                 200                 205

CAG GTG TCA TCC ATC AAC GTC TCT GTG AAC GCA GTG CAG ACT GTG GTC     1028
Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val Val
210                 215                 220                 225

CGC CAG GGT GAG AAC ATC ACC CTC ATG TGC ATT GTG ATC GGG AAT GAG     1076
Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn Glu
                230                 235                 240

GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC AAA GAA AGT GGG CGG CTG     1124
Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg Leu
            245                 250                 255

GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT TAC CAC ATC CGC     1172
Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile Arg
        260                 265                 270

TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG GGG ACC TAC     1220
Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr Tyr
    275                 280                 285
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ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT GAA AAG GCC    1260
Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys Ala
290                 295                 300                 305

ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC CTG GGA GAG    1316
Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly Glu
                310                 315                 320

GTG GGC ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG ACA CTG CAG    1364
Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu Gln
            325                 330                 335

GTA GTG TTC GAG GCC TAC CCA CCG CCC ACT GTC CTG TGG TTC AAA GAC    1412
Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys Asp
        340                 345                 350

AAC CGC ACC CTG GGC GAC TCC AGC GCT GGC GAA ATC GCC CTG TCC ACG    1460
Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser Thr
    355                 360                 365

CGC AAC GTG TCG GAG ACC CGG TAT GTG TCA GAG CTG ACA CTG GTT CGC    1508
Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val Arg
370                 375                 380                 385

GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC ATG CGG GCC TTC CAT GAG    1556
Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His Glu
                390                 395                 400

GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC AAT GTC CCT GTC    1604
Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro Val
            405                 410                 415

CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG GAA CAG ACA    1652
Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln Thr
        420                 425                 430

GTC CGC TGT CGT GGC CGG GGC ATG CCC CAG CCG AAC ATC ATC TGG TCT    1700
Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp Ser
    435                 440                 445

GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG CCC ACG CTG    1748
Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr Leu
450                 455                 460                 465

CTG GGG AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT AAC GTG ACG    1796
Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val Thr
                470                 475                 480

TAC TGG GAG GAG GAG CAG GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG    1844
Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg Leu
            485                 490                 495

CAG CAC GTG GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT    1892
Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn Ala
        500                 505                 510

GTG GGC CAG GAC ACC CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG CCC    1940
Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu Pro
    515                 520                 525

TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG GCC CTG GTG GTG CTC ACC    1988
Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu Thr
530                 535                 540                 545

ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG AAG AAG CCA CGT    2036
Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro Arg
                550                 555                 560

TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT GAC GGC CAT    2084
Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly His
            565                 570                 575

GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC TCC ACG TGG    2132
Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr Trp
        580                 585                 590

GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC GGC TCT GGG    2180
Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser Gly
    595                 600                 605
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GCC TTT GGG CAG GTG GTG GAG GCC ACG GCT CAT GGC CTG AGC CAT TCT     2228
Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His Ser
610                 615                 620                 625

CAG GCC ACG ATG AAA GTG GCC GTC AAG ATG CTT AAA TCC ACA GCC CGC     2276
Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala Arg
                630                 635                 640

AGC AGT GAG AAG CAA GCC CTT ATG TCG GAG CTG AAG ATC ATG AGT CAC     2324
Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser His
            645                 650                 655

CTT GGG CCC CAC CTG AAC GTG GTC AAC CTG TTG GGG GCC TGC ACC AAA     2372
Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys
        660                 665                 670

GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC TGC CGC TAC GGA GAC CTG     2420
Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp Leu
    675                 680                 685

GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG CAG CAC CAC TCC     2468
Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His Ser
690                 695                 700                 705

GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC AGC AAT GCT CTG CCC     2516
Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu Pro
                710                 715                 720

GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG GAG AGC GAC     2564
Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser Asp
            725                 730                 735

GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC TAT GTG CCC     2612
Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val Pro
        740                 745                 750

ATG CTG GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC GAG TCC TCC     2660
Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser Ser
    755                 760                 765

AAC TAC ATG GCC CCT TAC GAT AAC TAC GTT CCC TCT GCC CCT GAG AGG     2708
Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu Arg
770                 775                 780                 785

ACC TGC CGA GCA ACT TTG ATC AAC GAG TCT CCA GTG CTA AGC TAC ATG     2756
Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr Met
                790                 795                 800

GAC CTC GTG GGC TTC AGC TAC CAG GTG GCC AAT GGC ATG GAG TTT CTG     2804
Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe Leu
            805                 810                 815

GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG GCG GCT AGG AAC GTG CTC     2852
Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
        820                 825                 830

ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT GGC CTG GCT CGA     2900
Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala Arg
    835                 840                 845

GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC ACC TTT TTG     2948
Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe Leu
850                 855                 860                 865

CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC CTC TAC ACC     2996
Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr Thr
                870                 875                 880

ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG GAG ATC TTC     3044
Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Phe
            885                 890                 895

ACC TTG GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC GAG CAG TTC     3092
Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln Phe
        900                 905                 910

TAC AAT GCC ATC AAA CGG GGT TAC CGC ATG GCC CAG CCT GCC CAT GCC     3140
Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His Ala
    915                 920                 925
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TCC GAC GAG ATC TAT GAG ATC ATG CAG AAG TGC TGG GAA GAG AAG TTT     3188
Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys Phe
930                 935                 940                 945

GAG ATT CGG CCC CCC TTC TCC CAG CTG GTG CTG CTT CTC GAG AGA CTG     3236
Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Glu Arg Leu
                950                 955                 960

TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG CAG GTG GAT GAG GAG TTT     3284
Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu Phe
            965                 970                 975

CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG GCC CGC TTG CCT     3332
Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu Pro
        980                 985                 990

GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC GTC CTC TAT     3380
Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu Tyr
    995                 1000                1005

ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC ATC CCC CTG     3428
Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro Leu
1010                1015                1020                1025

CCT GAC CCC AAA CCC GAG GTT GCT GAC GAG GGC CCA CTG GAG GGT TCC     3476
Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly Ser
                1030                1035                1040

CCC AGC CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC TCC TCA ACC     3524
Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser Thr
            1045                1050                1055

ATC TCC TGT GAC AGC CCC CTG GAG CCC CAG GAC GAA CCA GAG CCA GAG     3572
Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro Glu
        1060                1065                1070

CCC CAG CTT GAG CTC CAG GTG GAG CCG GAG CCA GAG CTG GAA CAG TTG     3620
Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln Leu
    1075                1080                1085

CCG GAT TCG GGG TGC CCT GCG CCT CGG GCG GAA GCA GAG GAT AGC TTC     3668
Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser Phe
1090                1095                1100                1105

CTG TAGGGGGCTG GCCCCTACCC TGCCCTGCCT GAAGCTCCCC CCCTGCCAGC          3721
Leu

ACCCAGCATC TCCTGGCCTG GCCTGACCGG GCTTCCTGTC AGCCAGGCTG CCCTTATCAG   3781

CTGTCCCCTT CTGGAAGCTT TCTGCTCCTG AGGTGTTGTG CCCCAAACCC TGGGGCTGGC   3841

TTAGGAGGCA AGAAAACTGC AGGGGCCCTG ACCAGCCCTC TGCCTCCAGG GAGGCCAACT   3901

GACTCTGAGC CAGGGTTCCC CCAGGGAACT CAGTTTTCCC AEATGTAAGA TGGGAAAGTT   3961

AGGCTTGATG ACCCAGAATC TAGGATTCTC TCCCTGGCTG ACACGGTGCC GAGACCGAAT   4021

CCCTCCCTGG GAAGATTCTT GGAGTTACTG AGGTGGTAAA TTAACATTTT TTCTGTTCAG   4081

CCAGCTACCC CTCAAGGAAT CATAGCTCTC TCCTCGGACT TTTTATCCAC CCAGGAGCTA   4141

GGGAAGAGAC CCTAGCCTCC CTGGCTGCTG GCTGAGCTAG GGCCTAGCTT GAGCAGTGTT   4201

GCCTCATCCA GAAGAAAGCC AGTCTCCTCC CTATGATGCC AGTCCCTGCC TTCCCTGGCC   4261

CGAGCTGGTC TGGGGCCATT AGGCAGCCTA ATTAATGCTG GAGGCTGAGC CAAGTACAGG   4321

ACACCCCCAG CCTGCAGCCC TTGCCCAGGG CACTTGGAGC ACACGCAGCC ATAGCAAGTG   4381

CCTGTGTCCC TGTCCTTCAG GCCCATCAGT CCTGGGGCTT TTTCTTTATC ACCCTCAGTC   4441

TTAATCCATC CACCAGAGTC TAGA                                          4465
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1106 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu
  1               5                  10                  15

Leu Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly
             20                  25                  30

Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser
         35                  40                  45

Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg
     50                  55                  60

Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr
 65                  70                  75                  80

Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly
                 85                  90                  95

Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu
            100                 105                 110

Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu
        115                 120                 125

Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu
    130                 135                 140

Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu
145                 150                 155                 160

His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln
                165                 170                 175

Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr
            180                 185                 190

Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg
        195                 200                 205

Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val
    210                 215                 220

Val Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn
225                 230                 235                 240

Glu Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg
                245                 250                 255

Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile
            260                 265                 270

Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr
        275                 280                 285

Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys
    290                 295                 300

Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly
305                 310                 315                 320

Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu
                325                 330                 335

Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys
            340                 345                 350

Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser
        355                 360                 365

Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val
    370                 375                 380

Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His
385                 390                 395                 400
```

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Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro
405 410 415

Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln
420 425 430

Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp
435 440 445

Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr
450 455 460

Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val
465 470 475 480

Thr Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg
485 490 495

Leu Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn
500 505 510

Ala Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu
515 520 525

Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu
530 535 540

Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro
545 550 555 560

Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly
565 570 575

His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr
580 585 590

Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser
595 600 605

Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His
610 615 620

Ser Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala
625 630 635 640

Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser
645 650 655

His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr
660 665 670

Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp
675 680 685

Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His
690 695 700

Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu
705 710 715 720

Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser
725 730 735

Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val
740 745 750

Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser
755 760 765

Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu
770 775 780

Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr
785 790 795 800

Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe
805 810 815

Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val
820 825 830

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```
Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala
        835                 840                 845

Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe
    850                 855                 860

Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr
865                 870                 875                 880

Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile
                885                 890                 895

Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln
            900                 905                 910

Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His
        915                 920                 925

Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys
    930                 935                 940

Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg
945                 950                 955                 960

Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu
                965                 970                 975

Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu
            980                 985                 990

Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu
        995                 1000                1005

Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro
    1010                1015                1020

Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly
1025                1030                1035                1040

Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser
                1045                1050                1055

Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro
            1060                1065                1070

Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln
        1075                1080                1085

Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser
    1090                1095                1100

Phe Leu
1105
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC871

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATTATACGCT CTCTTCCTCA GGTAAATGAG TGCCAGGGCC GGCAAGCCCC CGCTCCA        57
```

(2) INFORMATION FOR SEQ ID NO:4:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 56 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC872

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGGGGAGCG GGGGCTTGCC GGCCCTGGCA CTCATTTACC TGAGGAAGAG AGAGCT 56

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC904

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGGGCACG TAATCTATAG ATTCATCCTT GCTCATATCC ATGTA 45

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC906

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCCT GGGCAGCC 38

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC905

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCCT GGGCAGCC 38

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATGGTGGAA TTCCTGCTGA T 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1447

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGTTGTGCA GAGCTGAGGA AGAGATGGA 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1453

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTCATTAT GTTGTTGCAA GCCTTCTTGT TCCTGCTAGC TGGTTTCGCT GTTAA 55

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1454

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCTTAACA GCGAAACCAG CTAGCAGGAA CAAGAAGGCT TGCAACAACA TAATG 55

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1478

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATCGCGAGCA TGCAGATCTG A 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1479

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTCAGAT CTGCATGCTG CCGAT 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1776

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTGAGCGC AAATGTTGTG TCGAGTGCCC ACCGTCCCCA GCTTAGAATT CT 52

-continued

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1777

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTAGAGAATT CTAAGCTGGG CACGGTGGGC ACTCGACACA ACATTTGCGC TC 52

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1646

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCGGCCAC TGTCGGTGCG CTGCACGCTG CGCAACGCTG TGGGCCAGGA CACGCAGGAG 60

GTCATCGTGG TGCCACACTC CTTGCCCTTT AAGCA 95

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1647

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTTGCTTA AAGGGCAAGG AGTGTGGCAC CACGATGACC TCCTGCGTGT CCTGGCCCAC 60

AGCGTTGCGC AGCGTGCAGC GCACCGACAG TGCCC 95

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

-continued

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1886

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

CCAGTGCCAA GCTTGTCTAG ACTTACCTTT AAAGGGCAAG GAG 43

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1992

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCTTGAGCG T 11

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1993

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTAGACGCTC A 11

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1894

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTTCCAGT TCTTCGGCCT CATGTCAGTT CTTCGGCCTC ATGTGAT 47

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC1895

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGATCACA TGRGGCCGAA GAACTGACAT GAGGCCGAAG AACTGGA 47

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2181

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATTCGGATC CACCATGGGC ACCAGCCACC CGGCGTTCCT GGTGTTAGGC YGCCTGCTGA 60

CCGGCC 66

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2182

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGAGCCTGAT CCTGTGCCAA CTGAGCCTGC CATCGATCCT GCCAAACGAG AACGAGAAGG 60

TTGTGCAGCT A 71

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2183

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AATTTAGCTG CACAACCTTC TCGTTCTCGT TTGGCAGGAT CGATGGCAGG CTCAGTTGGC 60

ACAGGATCA 69

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2184

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGCTCAGGCC GGTCAGCAGG CAGCCTAACA CCAGGAACGC CGGGTGGCTG GTGCCCATGG 60

TGGATCCG 68

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2311

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGATCACCAT GGCTCAACTG 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2351

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATTCCAC 10

-continued

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2352

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATTATACGCA TGGTGGAATT CGAGCT 26

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: EC2392

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ACGTAAGCTT GTCTAGACTT ACCTTCAAAA CGCAGGGTGG G 41

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(vii) IMMEDIATE SOURCE:
(B) CLONE: pWX1

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Leu His Asn His Tyr Thr Glu Lys Ser Leu Ser Leu Ser Pro Gly
1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGTGACACTC TCCTGGGAGT TA 22

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCATAGTAGT TACCATATCC TCTTGCACAG 30

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ACCGAACGTG AGAGGAGTGC TATAA 25

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4054 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:
(B) CLONE: p-alpha-17B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 285..3471
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GCCCTCGGGA CCGACCGTGG GCGGCGCGCA GCGGCGGGAC GCGTTTTCGG GACGTCGTGC 60

CCAGCGCCTT CCTGCAGACC CACAGGGAAG TACTCCCTTT GACCTCCGGG GAGCTGCGAC 120

CAGGTTATAC GTTGCTGGTG GAAAAGTGAC AATTCTAGGA AAAGAGCTAA AAGCCCGATC 180

GGTGAACGAA AGTTTCCCAG AGCT ATG GGG ACT TCC CAT CCG GCG TTC CTG 231
Met Gly Thr Ser His Pro Ala Phe Leu
1 5

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GTC TTA GGC TGT CTT CTC ACA GGG CTG AGC CTA ATC CTC TGC CAG CTT      279
Val Leu Gly Cys Leu Leu Thr Gly Leu Ser Leu Ile Leu Cys Gln Leu
 10                  15                  20                  25

TCA TTA CCC TCT ATC CTT CCA AAT GAA AAT GAA AAG GTT GTG CAG CTG      327
Ser Leu Pro Ser Ile Leu Pro Asn Glu Asn Glu Lys Val Val Gln Leu
             30                  35                  40

AAT TCA TCC TTT TCT CTG AGA TGC TTT GGG GAG AGT GAA GTG AGC TGG      375
Asn Ser Ser Phe Ser Leu Arg Cys Phe Gly Glu Ser Glu Val Ser Trp
         45                  50                  55

CAG TAC CCC ATG TCT GAA GAA GAG AGC TCC GAT GTG GAA ATC AGA AAT      423
Gln Tyr Pro Met Ser Glu Glu Glu Ser Ser Asp Val Glu Ile Arg Asn
     60                  65                  70

GAA GAA AAC AAC AGC GGC CTT TTT GTG ACG GTC TTG GAA GTG AGC AGT      471
Glu Glu Asn Asn Ser Gly Leu Phe Val Thr Val Leu Glu Val Ser Ser
 75                  80                  85

GCC TCG GCG GCC CAC ACA GGG TTG TAC ACT TGC TAT TAC AAC CAC ACT      519
Ala Ser Ala Ala His Thr Gly Leu Tyr Thr Cys Tyr Tyr Asn His Thr
 90                  95                 100                 105

CAG ACA GAA GAG AAT GAG CTT GAA GGC AGG CAC ATT TAC ATC TAT GTG      567
Gln Thr Glu Glu Asn Glu Leu Glu Gly Arg His Ile Tyr Ile Tyr Val
                110                 115                 120

CCA GAC CCA GAT GTA GCC TTT GTA CCT CTA GGA ATG ACG GAT TAT TTA      615
Pro Asp Pro Asp Val Ala Phe Val Pro Leu Gly Met Thr Asp Tyr Leu
            125                 130                 135

GTC ATC GTG GAG GAT GAT GAT TCT GCC ATT ATA CCT TGT CGC ACA ACT      663
Val Ile Val Glu Asp Asp Asp Ser Ala Ile Ile Pro Cys Arg Thr Thr
        140                 145                 150

GAT CCC GAG ACT CCT GTA ACC TTA CAC AAC AGT GAG GGG GTG GTA CCT      711
Asp Pro Glu Thr Pro Val Thr Leu His Asn Ser Glu Gly Val Val Pro
    155                 160                 165

GCC TCC TAC GAC AGC AGA CAG GGC TTT AAT GGG ACC TTC ACT GTA GGG      759
Ala Ser Tyr Asp Ser Arg Gln Gly Phe Asn Gly Thr Phe Thr Val Gly
170                 175                 180                 185

CCC TAT ATC TGT GAG GCC ACC GTC AAA GGA AAG AAG TTC CAG ACC ATC      807
Pro Tyr Ile Cys Glu Ala Thr Val Lys Gly Lys Lys Phe Gln Thr Ile
                190                 195                 200

CCA TTT AAT GTT TAT GCT TTA AAA GCA ACA TCA GAG CTG GAT CTA GAA      855
Pro Phe Asn Val Tyr Ala Leu Lys Ala Thr Ser Glu Leu Asp Leu Glu
            205                 210                 215

ATG GAA GCT CTT AAA ACC GTG TAT AAG TCA GGG GAA ACG ATT GTG GTC      903
Met Glu Ala Leu Lys Thr Val Tyr Lys Ser Gly Glu Thr Ile Val Val
        220                 225                 230

ACC TGT GCT GTT TTT AAC AAT GAG GTG GTT GAC CTT CAA TGG ACT TAC      951
Thr Cys Ala Val Phe Asn Asn Glu Val Val Asp Leu Gln Trp Thr Tyr
    235                 240                 245

CCT GGA GAA GTG AAA GGC AAA GGC ATC ACA ATA CTG GAA GAA ATC AAA      999
Pro Gly Glu Val Lys Gly Lys Gly Ile Thr Ile Leu Glu Glu Ile Lys
250                 255                 260                 265

GTC CCA TCC ATC AAA TTG GTG TAC ACT TTG ACG GTC CCC GAG GCC ACG     1047
Val Pro Ser Ile Lys Leu Val Tyr Thr Leu Thr Val Pro Glu Ala Thr
                270                 275                 280

GTG AAA GAC AGT GGA GAT TAC GAA TGT GCT GCC CGC CAG GCT ACC AGG     1095
Val Lys Asp Ser Gly Asp Tyr Glu Cys Ala Ala Arg Gln Ala Thr Arg
            285                 290                 295

GAG GTC AAA GAA ATG AAG AAA GTC ACT ATT TCT GTC CAT GAG AAA GGT     1143
Glu Val Lys Glu Met Lys Lys Val Thr Ile Ser Val His Glu Lys Gly
        300                 305                 310

TTC ATT GAA ATC AAA CCC ACC TTC AGC CAG TTG GAA GCT GTC AAC CTG     1191
Phe Ile Glu Ile Lys Pro Thr Phe Ser Gln Leu Glu Ala Val Asn Leu
    315                 320                 325
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CAT GAA GTC AAA CAT TTT GTT GTA GAG GTG CGG GCC TAC CCA CCT CCC     1239
His Glu Val Lys His Phe Val Val Glu Val Arg Ala Tyr Pro Pro Pro
330                 335                 340                 345

AGG ATA TCC TGG CTG AAA AAC AAT CTG ACT CTG ATT GAA AAT CTC ACT     1287
Arg Ile Ser Trp Leu Lys Asn Asn Leu Thr Leu Ile Glu Asn Leu Thr
                350                 355                 360

GAG ATC ACC ACT GAT GTG GAA AAG ATT CAG GAA ATA AGG TAT CGA AGC     1335
Glu Ile Thr Thr Asp Val Glu Lys Ile Gln Glu Ile Arg Tyr Arg Ser
            365                 370                 375

AAA TTA AAG CTG ATC CGT GCT AAG GAA GAA GAC AGT GGC CAT TAT ACT     1383
Lys Leu Lys Leu Ile Arg Ala Lys Glu Glu Asp Ser Gly His Tyr Thr
        380                 385                 390

ATT GTA GCT CAA AAT GAA GAT GCT GTG AAG AGC TAT ACT TTT GAA CTG     1431
Ile Val Ala Gln Asn Glu Asp Ala Val Lys Ser Tyr Thr Phe Glu Leu
    395                 400                 405

TTA ACT CAA GTT CCT TCA TCC ATT CTG GAC TTG GTC GAT GAT CAC CAT     1479
Leu Thr Gln Val Pro Ser Ser Ile Leu Asp Leu Val Asp Asp His His
410                 415                 420                 425

GGC TCA ACT GGG GGA CAG ACG GTG AGG TGC ACA GCT GAA GGC ACG CCG     1527
Gly Ser Thr Gly Gly Gln Thr Val Arg Cys Thr Ala Glu Gly Thr Pro
                430                 435                 440

CTT CCT GAT ATT GAG TGG ATG ATA TGC AAA GAT ATT AAG AAA TGT AAT     1575
Leu Pro Asp Ile Glu Trp Met Ile Cys Lys Asp Ile Lys Lys Cys Asn
            445                 450                 455

AAT GAA ACT TCC TGG ACT ATT TTG GCC AAC AAT GTC TCA AAC ATC ATC     1623
Asn Glu Thr Ser Trp Thr Ile Leu Ala Asn Asn Val Ser Asn Ile Ile
        460                 465                 470

ACG GAG ATC CAC TCC CGA GAC AGG AGT ACC GTG GAG GGC CGT GTG ACT     1671
Thr Glu Ile His Ser Arg Asp Arg Ser Thr Val Glu Gly Arg Val Thr
    475                 480                 485

TTC GCC AAA GTG GAG GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT     1719
Phe Ala Lys Val Glu Glu Thr Ile Ala Val Arg Cys Leu Ala Lys Asn
490                 495                 500                 505

CTC CTT GGA GCT GAG AAC CGA GAG CTG AAG CTG GTG GCT CCC ACC CTG     1767
Leu Leu Gly Ala Glu Asn Arg Glu Leu Lys Leu Val Ala Pro Thr Leu
                510                 515                 520

CGT TCT GAA CTC ACG GTG GCT GCT GCA GTC CTG GTG CTG TTG GTG ATT     1815
Arg Ser Glu Leu Thr Val Ala Ala Ala Val Leu Val Leu Leu Val Ile
            525                 530                 535

GTG ATC ATC TCA CTT ATT GTC CTG GTT GTC ATT TGG AAA CAG AAA CCG     1863
Val Ile Ile Ser Leu Ile Val Leu Val Val Ile Trp Lys Gln Lys Pro
        540                 545                 550

AGG TAT GAA ATT CGC TGG AGG GTC ATT GAA TCA ATC AGC CCG GAT GGA     1911
Arg Tyr Glu Ile Arg Trp Arg Val Ile Glu Ser Ile Ser Pro Asp Gly
    555                 560                 565

CAT GAA TAT ATT TAT GTG GAC CCG ATG CAG CTG CCT TAT GAC TCA AGA     1959
His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Arg
570                 575                 580                 585

TGG GAG TTT CCA AGA GAT GGA CTA GTG CTT GGT CGG GTC TTG GGG TCT     2007
Trp Glu Phe Pro Arg Asp Gly Leu Val Leu Gly Arg Val Leu Gly Ser
                590                 595                 600

GGA GCG TTT GGG AAG GTC GTT GAA GGA ACA GCC TAT GGA TTA AGC CGG     2055
Gly Ala Phe Gly Lys Val Val Glu Gly Thr Ala Tyr Gly Leu Ser Arg
            605                 610                 615

TCC CAA CCT GTC ATG AAA GTT GCA GTG AAG ATG CTA AAA CCC ACG GCC     2103
Ser Gln Pro Val Met Lys Val Ala Val Lys Met Leu Lys Pro Thr Ala
        620                 625                 630

AGA TCC AGT GAA AAA CAA GCT CTC ATG TCT GAA CTG AAG ATA ATG ACT     2151
Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Thr
    635                 640                 645
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CAC CTG GGG CCA CAT TTG AAC ATT GTA AAC TTG CTG GGA GCC TGC ACC     2199
His Leu Gly Pro His Leu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr
650                 655                 660                 665

AAG TCA GGC CCC ATT TAC ATC ATC ACA GAG TAT TGC TTC TAT GGA GAT     2247
Lys Ser Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Phe Tyr Gly Asp
                670                 675                 680

TTG GTC AAC TAT TTG CAT AAG AAT AGG GAT AGC TTC CTG AGC CAC CAC     2295
Leu Val Asn Tyr Leu His Lys Asn Arg Asp Ser Phe Leu Ser His His
            685                 690                 695

CCA GAG AAG CCA AAG AAA GAG CTG GAT ATC TTT GGA TTG AAC CCT GCT     2343
Pro Glu Lys Pro Lys Lys Glu Leu Asp Ile Phe Gly Leu Asn Pro Ala
        700                 705                 710

GAT GAA AGC ACA CGG AGC TAT GTT ATT TTA TCT TTT GAA AAC AAT GGT     2391
Asp Glu Ser Thr Arg Ser Tyr Val Ile Leu Ser Phe Glu Asn Asn Gly
    715                 720                 725

GAC TAC ATG GAC ATG AAG CAG GCT GAT ACT ACA CAG TAT GTC CCC ATG     2439
Asp Tyr Met Asp Met Lys Gln Ala Asp Thr Thr Gln Tyr Val Pro Met
730                 735                 740                 745

CTA GAA AGG AAA GAG GTT TCT AAA TAT TCC GAC ATC CAG AGA TCA CTC     2487
Leu Glu Arg Lys Glu Val Ser Lys Tyr Ser Asp Ile Gln Arg Ser Leu
                750                 755                 760

TAT GAT CGT CCA GCC TCA TAT AAG AAG AAA TCT ATG TTA GAC TCA GAA     2535
Tyr Asp Arg Pro Ala Ser Tyr Lys Lys Lys Ser Met Leu Asp Ser Glu
            765                 770                 775

GTC AAA AAC CTC CTT TCA GAT GAT AAC TCA GAA GGC CTT ACT TTA TTG     2583
Val Lys Asn Leu Leu Ser Asp Asp Asn Ser Glu Gly Leu Thr Leu Leu
        780                 785                 790

GAT TTG TTG AGC TTC ACC TAT CAA GTT GCC CGA GGA ATG GAG TTT TTG     2631
Asp Leu Leu Ser Phe Thr Tyr Gln Val Ala Arg Gly Met Glu Phe Leu
    795                 800                 805

GCT TCA AAA AAT TGT GTC CAC CGT GAT CTG GCT GCT CGC AAC GTC CTC     2679
Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
810                 815                 820                 825

CTG GCA CAA GGA AAA ATT GTG AAG ATC TGT GAC TTT GGC CTG GCC AGA     2727
Leu Ala Gln Gly Lys Ile Val Lys Ile Cys Asp Phe Gly Leu Ala Arg
                830                 835                 840

GAC ATC ATG CAT GAT TCG AAC TAT GTG TCG AAA GGC AGT ACC TTT CTG     2775
Asp Ile Met His Asp Ser Asn Tyr Val Ser Lys Gly Ser Thr Phe Leu
            845                 850                 855

CCC GTG AAG TGG ATG GCT CCT GAG AGC ATC TTT GAC AAC CTC TAC ACC     2823
Pro Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Asn Leu Tyr Thr
        860                 865                 870

ACA CTG AGT GAT GTC TGG TCT TAT GGC ATT CTG CTC TGG GAG ATC TTT     2871
Thr Leu Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe
    875                 880                 885

TCC CTT GGT GGC ACC CCT TAC CCC GGC ATG ATG GTG GAT TCT ACT TTC     2919
Ser Leu Gly Gly Thr Pro Tyr Pro Gly Met Met Val Asp Ser Thr Phe
890                 895                 900                 905

TAC AAT AAG ATC AAG AGT GGG TAC CGG ATG GCC AAG CCT GAC CAC GCT     2967
Tyr Asn Lys Ile Lys Ser Gly Tyr Arg Met Ala Lys Pro Asp His Ala
                910                 915                 920

ACC AGT GAA GTC TAC GAG ATC ATG GTG AAA TGC TGG AAC AGT GAG CCG     3015
Thr Ser Glu Val Tyr Glu Ile Met Val Lys Cys Trp Asn Ser Glu Pro
            925                 930                 935

GAG AAG AGA CCC TCC TTT TAC CAC CTG AGT GAG ATT GTG GAG AAT CTG     3063
Glu Lys Arg Pro Ser Phe Tyr His Leu Ser Glu Ile Val Glu Asn Leu
        940                 945                 950

CTG CCT GGA CAA TAT AAA AAG AGT TAT GAA AAA ATT CAC CTG GAC TTC     3111
Leu Pro Gly Gln Tyr Lys Lys Ser Tyr Glu Lys Ile His Leu Asp Phe
    955                 960                 965
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CTG AAG AGT GAC CAT CCT GCT GTG GCA CGC ATG CGT GTG GAC TCA GAC     3159
Leu Lys Ser Asp His Pro Ala Val Ala Arg Met Arg Val Asp Ser Asp
970                 975                 980                 985

AAT GCA TAC ATT GGT GTC ACC TAC AAA AAC GAG GAA GAC AAG CTG AAG     3207
Asn Ala Tyr Ile Gly Val Thr Tyr Lys Asn Glu Glu Asp Lys Leu Lys
                990                 995                1000

GAC TGG GAG GGT GGT CTG GAT GAG CAG AGA CTG AGC GCT GAC AGT GGC     3255
Asp Trp Glu Gly Gly Leu Asp Glu Gln Arg Leu Ser Ala Asp Ser Gly
            1005                1010                1015

TAC ATC ATT CCT CTG CCT GAC ATT GAC CCT GTC CCT GAG GAG GAG GAC     3303
Tyr Ile Ile Pro Leu Pro Asp Ile Asp Pro Val Pro Glu Glu Glu Asp
        1020                1025                1030

CTG GGC AAG AGG AAC AGA CAC AGC TCG CAG ACC TCT GAA GAG AGT GCC     3351
Leu Gly Lys Arg Asn Arg His Ser Ser Gln Thr Ser Glu Glu Ser Ala
    1035                1040                1045

ATT GAG ACG GGT TCC AGC AGT TCC ACC TTC ATC AAG AGA GAG GAC GAG     3399
Ile Glu Thr Gly Ser Ser Ser Ser Thr Phe Ile Lys Arg Glu Asp Glu
1050                1055                1060                1065

ACC ATT GAA GAC ATC GAC ATG ATG GAC GAC ATC GGC ATA GAC TCT TCA     3447
Thr Ile Glu Asp Ile Asp Met Met Asp Asp Ile Gly Ile Asp Ser Ser
                1070                1075                1080

GAC CTG GTG GAA GAC AGC TTC CTG TAACTGGCGG ATTCGAGGGG TTCCTTCCAC    3501
Asp Leu Val Glu Asp Ser Phe Leu
            1085

TTCTGGGGCC ACCTCTGGAT CCCGTTCAGA AAACCACTTT ATTGCAATGC GGAGGTTGAG   3561

AGGAGGACTT GGTTGATGTT TAAAGAGAAG TTCCCAGCCA AGGGCCTCGG GGAGCGTTCT   3621

AAATATGAAT GAATGGGATA TTTTGAAATG AACTTTGTCA GTGTTGCCTC TTGCAATGCC   3681

TCAGTAGCAT CTCAGTGGTG TGTGAAGTTT GGAGATAGAT GGATAAGGGA ATAATAGGCC   3741

ACAGAAGGTG AACTTTGTGC TTCAAGGACA TTGGTGAGAG TCCAACAGAC ACAATTTATA   3801

CTGCGACAGA ACTTCAGCAT TGTAATTATG TAAATAACTC TAACCAAGGC TGTGTTTAGA   3861

TTGTATTAAC TATCTTCTTT GGACTTCTGA AGAGACCACT CAATCCATCC TGTACTTCCC   3921

TCTTGAAACC TGATGTAGCT GCTGTTGAAC TTTTTAAAGA AGTGCATGAA AAACCATTTT   3981

TGAACCTTAA AAGGTACTGG TACTATAGCA TTTTGCTATC TTTTTTAGTG TTAAAGAGAT   4041

AAAGAATAAT AAG                                                      4054
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(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1089 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu Leu Thr
 1               5                  10                  15

Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile Leu Pro
            20                  25                  30

Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser Leu Arg
        35                  40                  45

Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser Glu Glu
    50                  55                  60

Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser Gly Leu
65                  70                  75                  80

Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His Thr Gly
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                85                  90                  95

Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn Glu Leu
            100                 105                 110

Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val Ala Phe
        115                 120                 125

Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp Asp Asp
    130                 135                 140

Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro Val Thr
145                 150                 155                 160

Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser Arg Gln
                165                 170                 175

Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu Ala Thr
            180                 185                 190

Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr Ala Leu
        195                 200                 205

Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys Thr Val
    210                 215                 220

Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe Asn Asn
225                 230                 235                 240

Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys Gly Lys
                245                 250                 255

Gly Ile Thr Ile Leu Glu Glu Ile Lys Val Pro Ser Ile Lys Leu Val
            260                 265                 270

Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly Asp Tyr
        275                 280                 285

Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met Lys Lys
    290                 295                 300

Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys Pro Thr
305                 310                 315                 320

Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His Phe Val
                325                 330                 335

Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu Lys Asn
            340                 345                 350

Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp Val Glu
        355                 360                 365

Lys Ile Gln Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile Arg Ala
    370                 375                 380

Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn Glu Asp
385                 390                 395                 400

Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro Ser Ser
                405                 410                 415

Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly Gln Thr
            420                 425                 430

Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu Trp Met
        435                 440                 445

Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp Thr Ile
    450                 455                 460

Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser Arg Asp
465                 470                 475                 480

Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu Glu Thr
                485                 490                 495

Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu Asn Arg
            500                 505                 510
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Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr Val Ala
515 520 525

Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu Ile Val
530 535 540

Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg Trp Arg
545 550 555 560

Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr Val Asp
565 570 575

Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg Asp Gly
580 585 590

Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys Val Val
595 600 605

Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met Lys Val
610 615 620

Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys Gln Ala
625 630 635 640

Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His Leu Asn
645 650 655

Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile Tyr Ile
660 665 670

Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu His Lys
675 680 685

Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys Lys Glu
690 695 700

Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg Ser Tyr
705 710 715 720

Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met Lys Gln
725 730 735

Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu Val Ser
740 745 750

Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala Ser Tyr
755 760 765

Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu Ser Asp
770 775 780

Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe Thr Tyr
785 790 795 800

Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys Val His
805 810 815

Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys Ile Val
820 825 830

Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp Ser Asn
835 840 845

Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met Ala Pro
850 855 860

Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser
865 870 875 880

Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr Pro Tyr
885 890 895

Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys Ser Gly
900 905 910

Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr Glu Ile
915 920 925

Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser Phe Tyr
930 935 940

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His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr Lys Lys
945                 950                 955                 960

Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His Pro Ala
                965                 970                 975

Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly Val Thr
            980                 985                 990

Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly Leu Asp
        995                 1000                1005

Glu Gln Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu Pro Asp
    1010                1015                1020

Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn Arg His
1025                1030                1035                1040

Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser Ser Ser
                1045                1050                1055

Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile Asp Met
            1060                1065                1070

Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp Ser Phe
        1075                1080                1085

Leu
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We claim:

1. A biologically active, dimerized polypeptide fusion, comprising:

first and second polypeptide chains, wherein each of said polypeptide chains comprises a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein heterologous to said non-immunoglobulin polypeptide.

2. The biologically active, dimerized polypeptide fusion of claim 1 which is a homodimer.

3. The biologically active, dimerized polypeptide fusion of claim 1 wherein the dimerizing protein of one of said polypeptide chains comprises an immunoglobulin heavy chain constant region.

4. The biologically active, dimerized polypeptide fusion of claim 3 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin hinge region.

5. The biologically active, dimerized polypeptide fusion of claim 3 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin variable region.

6. The biologically active, dimerized polypeptide fusion of claim 5 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

7. The biologically active, dimerized polypeptide fusion of claim 1 wherein the dimerizing protein one of said polypeptide chains comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 of a γ, α, ϵ, μ, or δ class immunoglobulin heavy chain.

8. The biologically active, dimerized polypeptide fusion of claim 1 wherein the dimerizing protein of one of said polypeptide chains comprises an immunoglobulin light chain constant region.

9. A biologically active, multimerized polypeptide fusion, comprising:

a non-immunoglobulin polypeptide requiring multimerization for biological activity joined to an immunoglobulin light chain constant region; and

an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4.

10. The biologically active, multimerized polypeptide fusion of claim 9 which is a tetramer comprising four polypeptide fusions each having a non-immunoglobulin polypeptide joined to a multimerizing protein.

11. The biologically active, multimerized polypeptide fusion of claim 9 including a multimerizing protein which comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 of a γ, α, ϵ, μ, or δ class immunoglobulin heavy chain.

12. A biologically active, multimerized polypeptide fusion comprising: a non-immunoglobulin polypeptide requiring multimerization for biological activity joined to an immunoglobulin heavy chain constant region; and

an immunoglobulin heavy chain.

13. The biologically active, multimerized polypeptide fusion of claim 12 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin hinge region.

14. The biologically active, multimerized polypeptide fusion of claim 12 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin variable region.

15. The biologically active, multimerized polypeptide fusion of claim 14 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

16. A biologically active, heteromultimeric polypeptide fusion, comprising:

a first polypeptide fusion comprising a first non-immunoglobulin polypeptide joined to a first multimerizing protein heterologous to said first non-immunoglobulin polypeptide and a second polypeptide fusion comprising a second non-immunoglobulin polypeptide joined to a second multimerizing protein heterologous to said second non-immunoglobulin polypeptide, wherein said first multimerizing protein and said second multimerizing protein are capable of associating to form a multimer.

17. The biologically active, heteromultimeric polypeptide fusion of claim 16 wherein the first and second non-

immunoglobulin polypeptides each comprise an amino acid sequence selected from the group consisting of (A) the amino acid sequence Sequence ID NO:2, and (B) the amino acid sequence of Sequence ID NO:36.

18. The biologically active, heteromultimeric polypeptide fusion of claim 16 wherein the first multimerizing protein is different from the second multimerizing protein.

19. The biologically active, heteromultimeric polypeptide fusion of claim 18 wherein the first and second non-immunoglobulin polypeptides are the same.

20. The biologically active, heteromultimeric polypeptide fusion of claim 16 wherein the first and second multimerizing proteins each comprise an immunoglobulin heavy chain constant region or an immunoglobulin light chain constant region.

21. The biologically active, heteromultimeric polypeptide fusion of claim 16 which comprises a first polypeptide fusion having a first non-immunoglobulin polypeptide joined to a first immunoglobulin constant region and a second polypeptide fusion having a second non-immunoglobulin polypeptide fused to a second immunoglobulin constant region different from the first immunoglobulin constant region.

22. The biologically active, heteromultimeric polypeptide fusion of claim 21 wherein the first multimerizing protein comprises an immunoglobulin heavy chain constant region and the second multimerizing protein comprises an immunoglobulin light chain constant region.

23. The biologically active, heteromultimeric polypeptide fusion of claim 20 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region joined to an immunoglobulin hinge region.

24. The biologically active, heteromultimeric polypeptide fusion of claim 20 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region joined to an immunoglobulin variable region.

25. The biologically active, heteromultimeric polypeptide fusion of claim 24 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

26. The biologically active, heteromultimeric polypeptide fusion of claim 16 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

27. The biologically active, heteromultimeric polypeptide fusion of claim 16 wherein said multimerized polypeptide fusion comprises a first polypeptide fusion comprising a first receptor or receptor domain requiring multimerization for activity joined to a first immunoglobulin constant region and a second polypeptide fusion comprising a second receptor or receptor domain requiring multimerization for activity joined to a second immunoglobulin constant region.

28. The biologically active, heteromultimeric polypeptide fusion of claim 27 wherein one of said receptor domains comprises a ligand binding domain.

29. The biologically active, heteromultimeric polypeptide fusion of claim 16 which is a heterotetramer comprising four polypeptide fusions each having a non-immunoglobulin polypeptide joined to a multimerizing protein.

* * * * *

EXHIBIT 2

US005843725C1

(12) EX PARTE REEXAMINATION CERTIFICATE (5480th)
United States Patent
Sledziewski et al.

(10) Number: US 5,843,725 C1
(45) Certificate Issued: Aug. 29, 2006

(54) METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

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Certificate of Correction Issued Jan. 1, 2002

Related U.S. Application Data

(60) Division of application No. 08/180,195, filed on Jan. 11, 1994, now Pat. No. 5,567,584, which is a continuation of application No. 07/634,510, filed on Dec. 27, 1990, now abandoned, which is a continuation-in-part of application No. 07/347,291, filed on May 2, 1989, now Pat. No. 5,155,027, which is a continuation-in-part of application No. 07/146,877, filed on Jan. 22, 1988, now abandoned.

(30) Foreign Application Priority Data

Jan. 18, 1989 (EP) 8910087

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C12N 15/10 (2006.01)
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(52) U.S. Cl. 435/69.7; 435/325; 435/320.1; 435/172.3; 530/387.1; 530/387.3; 530/333; 530/350; 530/399

(58) Field of Classification Search 530/350, 530/387.3, 388.24, 399, 402, 387.1, 333; 435/69.7, 320.1, 325, 172.3
See application file for complete search history.

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(57) ABSTRACT

Methods for producing secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof.

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EX PARTE REEXAMINATION CERTIFICATE ISSUED UNDER 35 U.S.C. 307

THE PATENT IS HEREBY AMENDED AS INDICATED BELOW.

Matter enclosed in heavy brackets [ ] appeared in the patent, but has been deleted and is no longer a part of the patent; matter printed in italics indicates additions made to the patent.

AS A RESULT OF REEXAMINATION, IT HAS BEEN DETERMINED THAT:

The patentability of claims 9–13, 15–20 and 28–49 is confirmed.

Claims 1, 14 and 21 are determined to be patentable as amended.

Claims 2–8 and 22–27, dependent on an amended claim, are determined to be patentable.

New claims 50 and 51 are added and determined to be patentable.

1. A method for producing a secreted active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory-signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein heterologous to said non-immunoglobulin polypeptide;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said DNA sequence; and

isolating said dimerized polypeptide fusion from said host cell,

wherein the dimerizing protein is selected from the group consisting of yeast invertase; at least a portion of an immunoglobulin light chain comprising at least an immunoglobulin light chain constant region domain; and at least a portion of an immunoglobulin heavy chain comprising at least an immunoglobulin heavy chain constant region domain.

14. The method of claim 9 wherein said non-immunoglobulin polypeptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531, a polypeptide comprising the amino acid sequence of [FOGS] *FIGS.* 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of FIGS. 11A–11 D (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524.

21. A method for producing a secreted receptor analog, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to a dimerizing protein heterologous to said ligand-binding domain, and optionally an immunoglobulin hinge or variable region *joined* to said dimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and

isolating said receptor analog from said host cell,

wherein the dimerizing protein is selected from the group consisting of yeast invertase; at least a portion of an immunoglobulin light chain comprising at least an immunoglobulin light chain constant region domain; and at least a portion of an immunoglobulin heavy chain comprising at least an immunoglobulin heavy chain constant region domain.

50. The method of claim 1 wherein the dimerizing protein is at least a portion of an immunoglobulin heavy chain comprising at least an immunoglobulin heavy chain constant region domain joined to an immunoglobulin heavy chain hinge region.

51. The method of claim 21 wherein the dimerizing protein is at least a portion of an immunoglobulin heavy chain comprising at least an immunoglobulin heavy chain constant region domain, and an immunoglobulin heavy chain hinge region is joined to the dimerizing protein.

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US005843725A

United States Patent [19]

Sledziewski et al.

[11] Patent Number: 5,843,725

[45] Date of Patent: *Dec. 1, 1998

[54] METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

[75] Inventors: Andrzej Z. Sledziewski; Lillian Anne Bell; Wayne R. Kindsvogel, all of Seattle, Wash.

[73] Assignee: ZymoGenetics, Inc., Seattle, Wash.

[*] Notice: The term of this patent shall not extend beyond the expiration date of Pat. Nos. 5,155,027 and 5,750,375.

[21] Appl. No.: 475,458

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Related U.S. Application Data

[62] Division of Ser. No. 180,195, Jan. 11, 1994, Pat. No. 5,567,584, which is a continuation of Ser. No. 634,510, Dec. 27, 1990, abandoned, which is a continuation-in-part of Ser. No. 347,291, May 2, 1989, Pat. No. 5,155,027, which is a continuation-in-part of Ser. No. 146,877, Jan. 22, 1988, abandoned.

[30] Foreign Application Priority Data

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[52] U.S. Cl. 435/69.7; 435/325; 435/320.1; 435/172.3; 530/387.1; 530/387.3; 530/333; 530/350; 530/399

[58] Field of Search 435/69.7, 320.1, 435/172.3, 325; 530/387.3, 399, 333, 350, 387.1

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Attorney, Agent, or Firm—Townsend and Townsend and Crew

[57] ABSTRACT

Methods for producing secreted receptor analogs and biologically active peptide dimers are disclosed. The methods for producing secreted receptor analogs and biologically active peptide dimers utilize a DNA sequence encoding a receptor analog or a peptide requiring dimerization for biological activity joined to a dimerizing protein. The receptor analog includes a ligand-binding domain. Polypeptides comprising essentially the extracellular domain of a human PDGF receptor fused to dimerizing proteins, the portion being capable of binding human PDGF or an isoform thereof, are also disclosed. The polypeptides may be used within methods for determining the presence of and for purifying human PDGF or isoforms thereof.

49 Claims, 18 Drawing Sheets



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   1 GGCCCCTCAGCCCTGCTGCCCAGCACGAGCCTGTGCTCCCCTGCCCAACGCAGACAGCCAGACCCAGG   69

  70 GCGGCCCCTCTGGCGGCTCTGCTCCTCCCGAAGGATGCTTGGGGAGTGAGGCGAAGCTGGGCGCTCCTC  138

 139 TCCCCTACAGCAGCCCCCTTCCTCCATCCCTCTGTTCTCCTGAGCCTTCAGGAGCCTGCACCAGTCCTG  207

 208 CCTGTCCTTCTACTCAGCTGTTACCCACTCTGGGACCAGCAGTCTTTCTGATAACTGGGAGAGGGCAGT  276

 277 AAGGAGGACTTCCTGGAGGGGGTGACTGTCCAGAGCCTGGAACTGTGCCCACACCAGAAGCCATCAGCA  345

 346 GCAAGGACACCATGCGGCTTCCGGGTGCGATGCCAGCTCTGGCCCTCAAAGGCGAGCTGCTGTTGCTGT  414
                 M  R  L  P  G  A  M  P  A  L  A  L  K  G  E  L  L  L  L  S   20

 415 CTCTCCTGTTACTTCTGGAACCACAGATCTCTCAGGGCCTGGTCGTCACACCCCCGGGGCCAGAGCTTG  483
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 484 TCCTCAATGTCTCCAGCACCTTCGTTCTGACCTGCTCGGGTTCAGCTCCGGTGGTGTGGGAACGGATGT  552
       L  N  V  S  S  T  F  V  L  T  C  S  G  S  A  P  V  V  W  E  R  M  S   66

 553 CCCAGGAGCCCCCACAGGAAATGGCCAAGGCCCAGGATGGCACCTTCTCCAGCGTGCTCACACTGACCA  621
       Q  E  P  P  Q  E  M  A  K  A  Q  D  G  T  F  S  S  V  L  T  L  T  N   89

 622 ACCTCACTGGGCTAGACACGGGAGAATACTTTTGCACCCACAATGACTCCCGTGGACTGGAGACCGATG  690
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 691 AGCGGAAACGGCTCTACATCTTTGTGCCAGATCCCACCGTGGGCTTCCTCCCTAATGATGCCGAGGAAC  759
       R  K  R  L  Y  I  F  V  P  D  P  T  V  G  F  L  P  N  D  A  E  E  L  135

 760 TATTCATCTTTCTCACGGAAATAACTGAGATCACCATTCCATGCCGAGTAACAGACCCACAGCTGGTGG  828
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 829 TGACACTGCACGAGAAGAAAGGGGACGTTGCACTGCCTGTCCCCTATGATCACCAACGTGGCTTTTCTG  897
       T  L  H  E  K  K  G  D  V  A  L  P  V  P  Y  D  H  Q  R  G  F  S  G  181

 898 GTATCTTTGAGGACAGAAGCTACATCTGCAAAACCACCATTGGGGACAGGGAGGTGGATTCTGATGCCT  966
       I  F  E  D  R  S  Y  I  C  K  T  T  I  G  D  R  E  V  D  S  D  A  Y  204

 967 ACTATGTCTACAGACTCCAGGTGTCATCCATCAACGTCTCTGTGAACGCAGTGCAGACTGTGGTCCGCC 1035
       Y  V  Y  R  L  Q  V  S  S  I  N  V  S  V  N  A  V  Q  T  V  V  R  Q  227

1036 AGGGTGAGAACATCACCCTCATGTGCATTGTGATCGGGAATGAGGTGGTCAACTTCGAGTGGACATACC 1104
       G  E  N  I  T  L  M  C  I  V  I  G  N  E  V  V  N  F  E  W  T  Y  P  250

1105 CCCGCAAAGAAAGTGGGCGGCTGGTGGAGCCGGTGACTGACTTCCTCTTGGATATGCCTTACCACATCC 1173
       R  K  E  S  G  R  L  V  E  P  V  T  D  F  L  L  D  M  P  Y  H  I  R  273

1174 GCTCCATCCTGCACATCCCCAGTGCCGAGTTAGAAGACTCGGGGACCTACACCTGCAATGTGACGGAGA 1242
       S  I  L  H  I  P  S  A  E  L  E  D  S  G  T  Y  T  C  N  V  T  E  S  296

1243 GTGTGAATGACCATCAGGATGAAAAGGCCATCAACATCACCGTGGTTGAGAGCGGCTACGTGCGGCTCC 1311
       V  N  D  H  Q  D  E  K  A  I  N  I  T  V  V  E  S  G  Y  V  R  L  L  319
```

Fig. 1A

```
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1381 CCTACCCACCGCCCACTGTCCTGTGGTTCAAAGACAACCGCACCCTGGGCGACTCCAGCGCTGGCGAAA 1449
       Y  P  P  P  T  V  L  W  F  K  D  N  R  T  L  G  D  S  S  A  G  E  I  365

1450 TCGCCCTGTCCACGCGCAACGTGTCGGAGACCCGGTATGTGTCAGAGCTGACACTGGTTCGCGTGAAGG 1518
       A  L  S  T  R  N  V  S  E  T  R  Y  V  S  E  L  T  L  V  R  V  K  V  388

1519 TGGCAGAGGCTGGCCACTACACCATGCGGGCCTTCCATGAGGATGCTGAGGTCCAGCTCTCCTTCCAGC 1587
       A  E  A  G  H  Y  T  M  R  A  F  H  E  D  A  E  V  Q  L  S  F  Q  L  411

1588 TACAGATCAATGTCCCTGTCCGAGTGCTGGAGCTAAGTGAGAGCCACCCTGACAGTGGGGAACAGACAG 1656
       Q  I  N  V  P  V  R  V  L  E  L  S  E  S  H  P  D  S  G  E  Q  T  V  434

1657 TCCGCTGTCGTGGCCGGGGCATGCCCCAGCCGAACATCATCTGGTCTGCCTGCAGAGACCTCAAAAGGT 1725
       R  C  R  G  R  G  H  P  Q  P  N  I  I  W  S  A  C  R  D  L  K  R  C  457

1726 GTCCACGTGAGCTGCCGCCCACGCTGCTGGGGAACAGTTCCGAAGAGGAGAGCCAGCTGGAGACTAACG 1794
       P  R  E  L  P  P  T  L  L  G  N  S  S  E  E  E  S  Q  L  E  T  N  V  480

1795 TGACGTACTGGGAGGAGGAGCAGGAGTTTGAGGTGCTGAGCACACTGCGTCTGCAGCACGTGGATCGGC 1863
       T  Y  W  E  E  E  Q  E  F  E  V  V  S  T  L  R  L  Q  H  V  D  R  P  503

1864 CACTGTCGGTGCGCTGCACGCTGCGCAACGCTGTGGGCCAGGACACGCAGGAGGTCATCGTGGTGCCAC 1932
       L  S  V  R  C  T  L  R  N  A  V  G  Q  D  T  Q  E  V  I  V  V  P  H  526

1933 ACTCCTTGCCCTTTAAGGTGGTGGTGATCTCAGCCATCCTGGCCCTGGTGGTGCTCACCATCATCTCCC 2001
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2002 TTATCATCCTCATCATGCTTTGGCAGAAGAAGCCACGTTACGAGATCCGATGGAAGGTGATTGAGTCTG 2070
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2071 TGAGCTCTGACGGCCATGAGTACATCTACGTGGACCCCATGCAGCTGCCCTATGACTCCACGTGGGAGC 2139
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2140 TGCCGCGGGACCAGCTTGTGCTGGGACGCACCCTCGGCTCTGGGGCCTTTGGGCAGGTGGTGGAGGCCA 2208
       P  R  D  Q  L  V  L  G  R  T  L  G  S  G  A  F  G  Q  V  V  E  A  T  618

2209 CGGCTCATGGCCTGAGCCATTCTCAGGCCACGATGAAAGTGGCCGTCAAGATGCTTAAATCCACAGCCC 2277
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2278 GCAGCAGTGAGAAGCAAGCCCTTATGTCGGAGCTGAAGATCATGAGTCACCTTGGGCCCCACCTGAACG 2346
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2347 TGGTCAACCTGTTGGGGGCCTGCACCAAAGGAGGACCCATCTATATCATCACTGAGTACTGCCGCTACG 2415
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2416 GAGACCTGGTGGACTACCTGCACCGCAACAAACACACCTTCCTGCAGCACCACTCCGACAAGCGCCGCC 2484
       D  L  V  D  Y  L  H  R  N  K  H  T  F  L  Q  H  H  S  D  K  R  R  P  710

2485 CGCCCAGCGCGGAGCTCTACAGCAATGCTCTGCCCGTTGGGCTCCCCTTGCCCAGCCATGTGTCCTTGA 2553
       P  S  A  E  L  Y  S  N  A  L  P  V  G  L  P  L  P  S  H  V  S  L  T  733
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Fig. 1B

```
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2623 ACATGAAAGGAGACGTCAAATATGCAGACATCGAGTCCTCCAACTACATGGCCCCTTACGATAACTACG 2691
       M  K  G  D  V  K  Y  A  D  I  E  S  S  N  Y  M  A  P  Y  D  N  Y  V  779

2692 TTCCCTCTGCCCCTGAGAGGACCTGCCGAGCAACTTTGATCAACGAGTCTCCAGTGCTAACCTACATGG 2760
       P  S  A  P  E  R  T  C  R  A  T  L  I  N  E  S  P  V  L  S  Y  M  D  802

2761 ACCTCGTGGGCTTCAGCTACCAGGTGGCCAATGGCATGGAGTTTCTGGCCTCCAAGAACTGCGTCCACA 2829
       L  V  G  F  S  Y  Q  V  A  N  G  M  E  F  L  A  S  K  N  C  V  H  R  825

2830 GAGACCTGGCGGCTAGGAACGTGCTCATCTGTGAAGGCAAGCTGGTCAAGATCTGTGACTTTGGCCTGG 2898
       D  L  A  A  R  N  V  L  I  C  E  G  K  L  V  K  I  C  D  F  G  L  A  848

2899 CTCGAGACATCATGCGGGACTCGAATTACATCTCCAAAGGCAGCACCTTTTTGCCTTTAAAGTGGATGG 2967
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2968 CTCCGGAGAGCATCTTCAACAGCCTCTACACCACCCTGAGCGACGTGTGGTCCTTCGGGATCCTGCTCT 3036
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3106 TCAAACGGGGTTACCGCATGGCCCAGCCTGCCCATGCCTCCGACGAGATCTATGAGATCATGCAGAAGT 3174
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3175 GCTGGGAAGAGAAGTTTGAGATTCGGCCCCCCTTCTCCCAGCTGGTCCTGCTTCTCGAGAGACTGTTGG 3243
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3244 GCGAAGGTTACAAAAAGAAGTACCAGCAGGTGGATGAGGAGTTTCTGAGGAGTGACCACCCAGCCATCC 3312
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3382 ATACTGCCGTGCAGCCCAATGAGGGTGACAACGACTATATCATCCCCCTGCCTGACCCCAAACCCGAGG 3450
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3589 TCCAGGTGGAGCCGGAGCCAGAGCTGGAACAGTTGCCGGATTCGGGGTGCCCTGCGCCTCGGGCGGAAG 3657
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3658 CAGAGGATAGCTTCCTGTAGGGGGCTGGCCCCTACCCTGCCCTGCCTGAAGCTCCCCCCCTGCCAGCAC 3726
       E  D  S  F  L  .                                                    1106

3727 CCAGCATCTCCTGGCCTGGCCTGACCGGGCTTCCTGTCAGCCAGGCTGCCCTTATCAGCTGTCCCCTTC 3795
```

Fig. 1C

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3796 TGGAAGCTTTCTGCTCCTGACGTGTTGTGCCCCAAACCCTGGGGCTGGCTTAGGAGGCAAGAAAACTGC 3864
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3934 TCAGTTTTCCCATATGTAAGATGGGAAAGTTAGGCTTGATGACCCAGAATCTAGGATTCTCTCCCTGGC 4002
4003 TGACACGGTGGGGAGACCGAATCCCTCCCTGGGAAGATTCTTGGAGTTACTGAGGTGGTAAATTAACAT 4071
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5314 GGGTTGGGAAGGGGGTGCAGGAAGCTCAACCCCTCTGGGCACCAACCCTGCATTGCAGGTTGGCACCTT 5382
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5452 ATCACCTAGGTTTACAAATATTTTTAGGACTCACGTTAACTCACATTTATACAGCAGAAATGCTATTTT 5520
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Fig. 1D

Figure 2

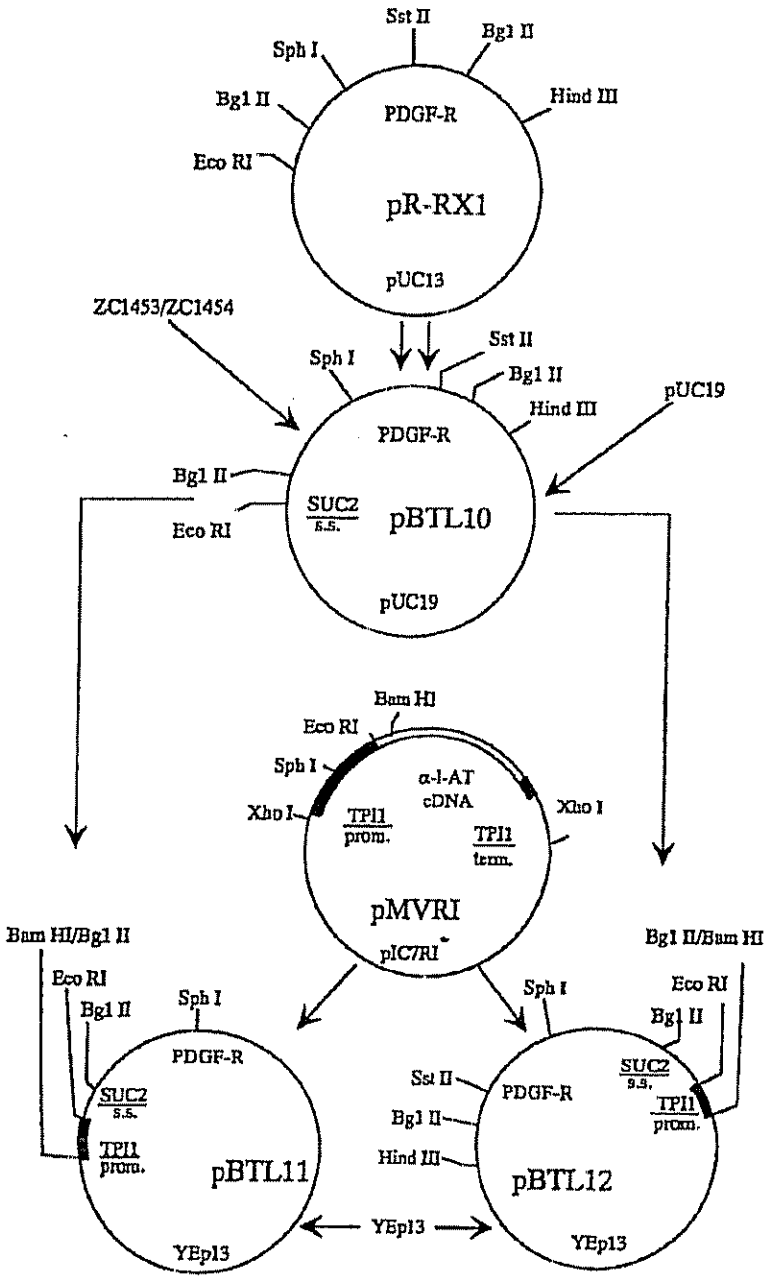

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Sph I
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cDNA
Xho I
Xho I
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prom.
TPI1
term.
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prom.
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YEp13

Figure 3

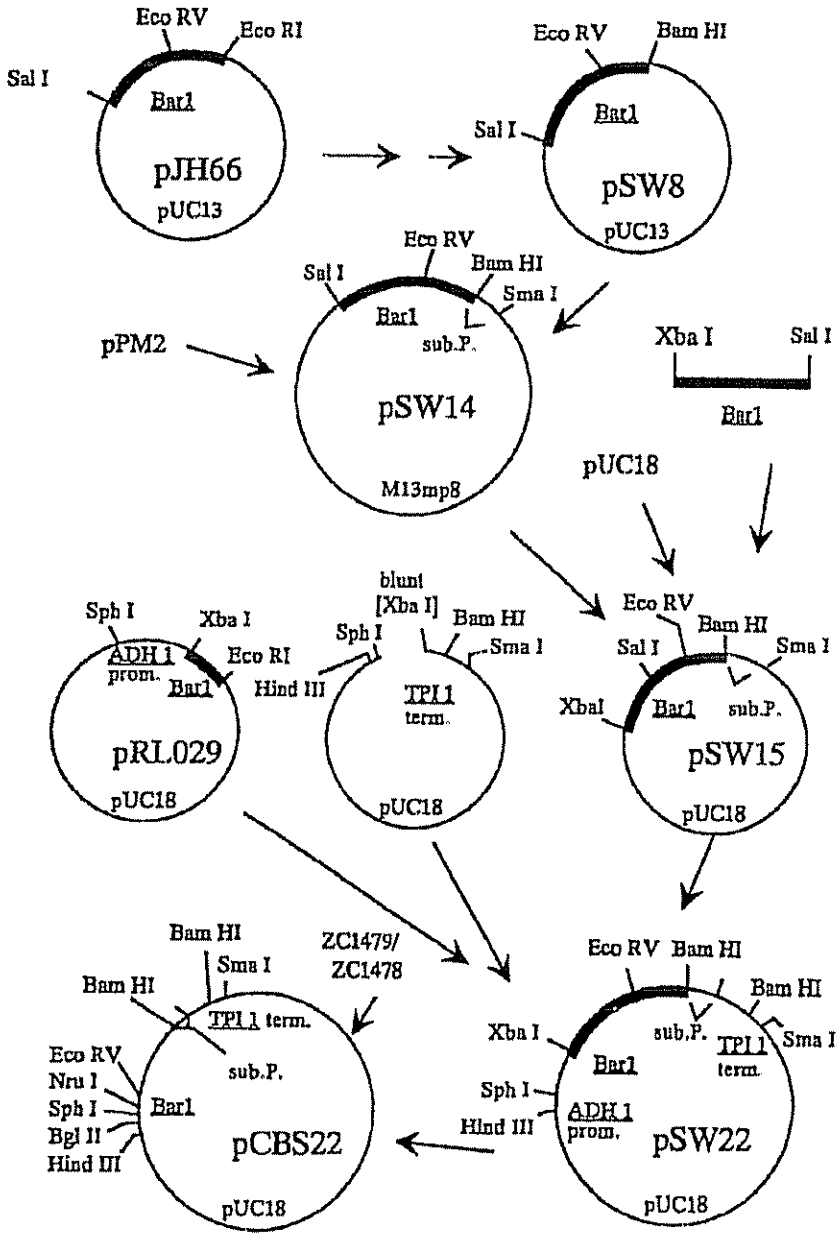

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Sma I
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pPM2
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prom.
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Bam HI
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prom.
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Figure 4

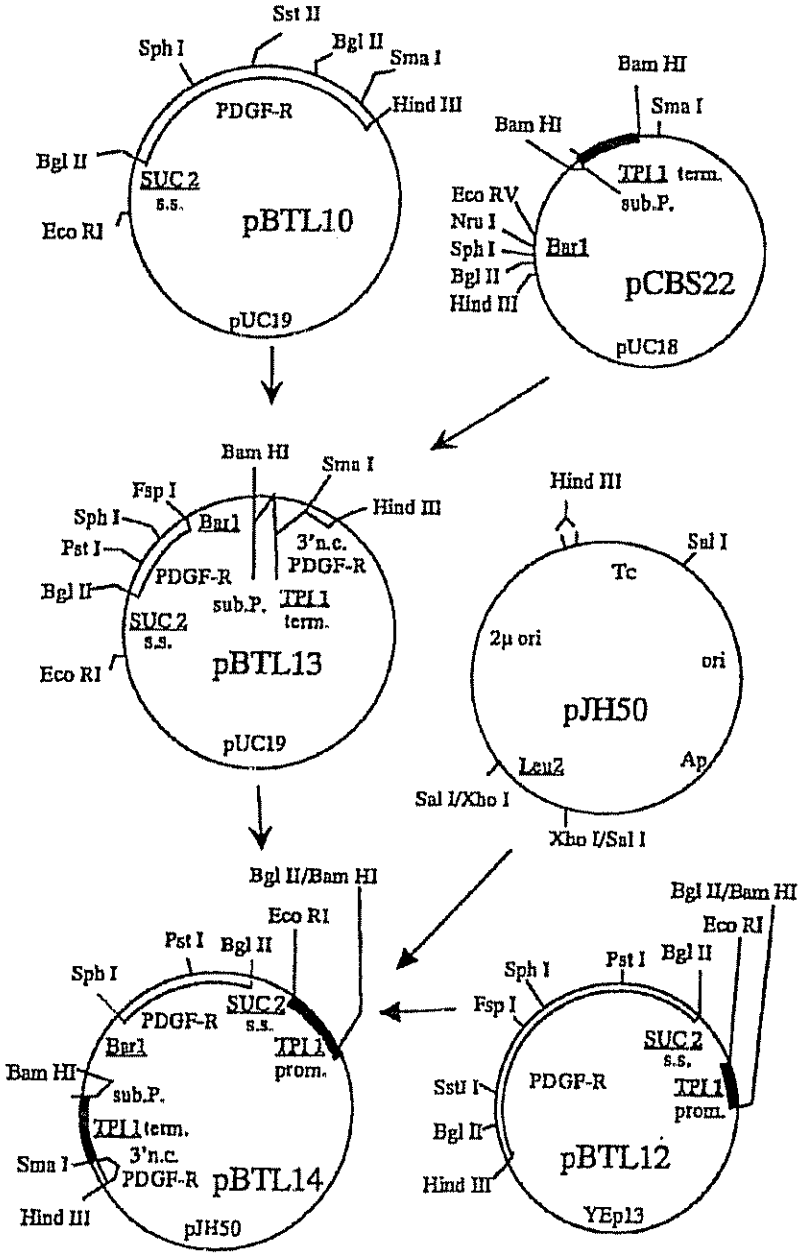

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Bar1
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term.
SUC2
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Tc
2μ ori
ori
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Leu2
Ap
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Xho I/Sal I
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Bgl II
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prom.
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Bgl II
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Figure 5

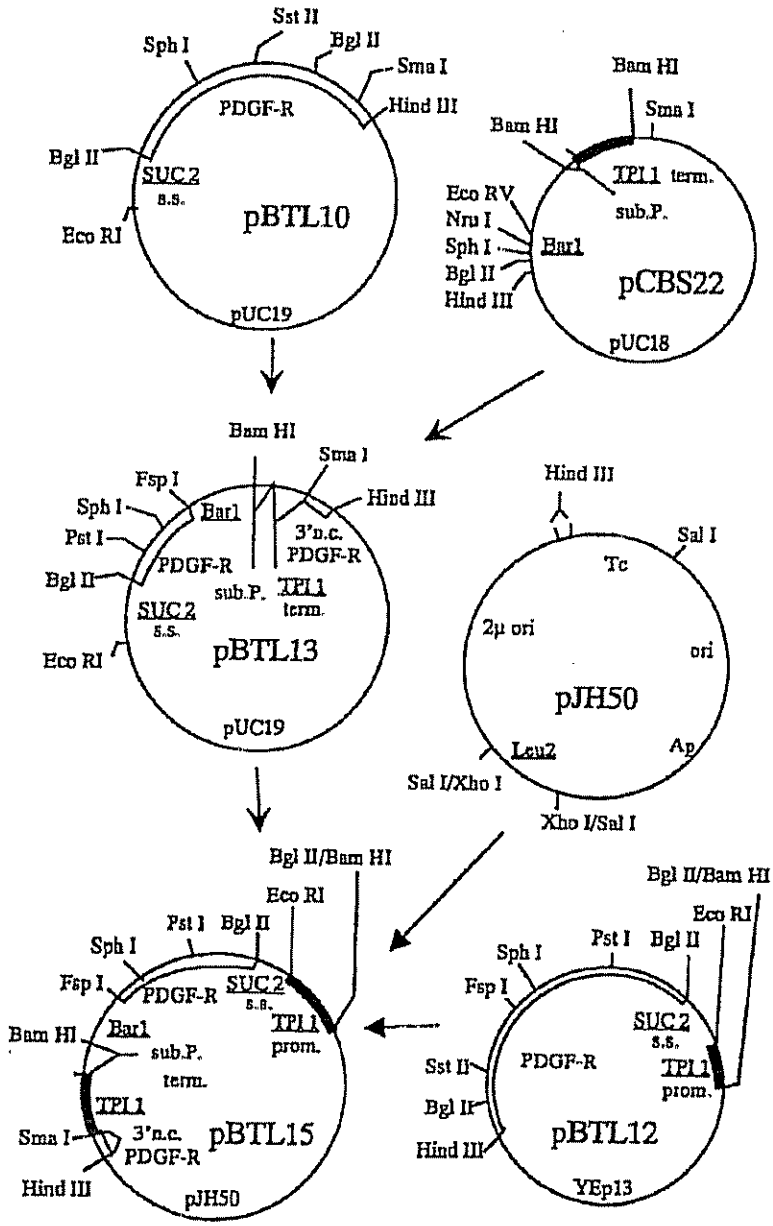

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Fsp I
Sph I
Bar1
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Leu2
Ap
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Xho I/Sal I
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Bar1
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Figure 6

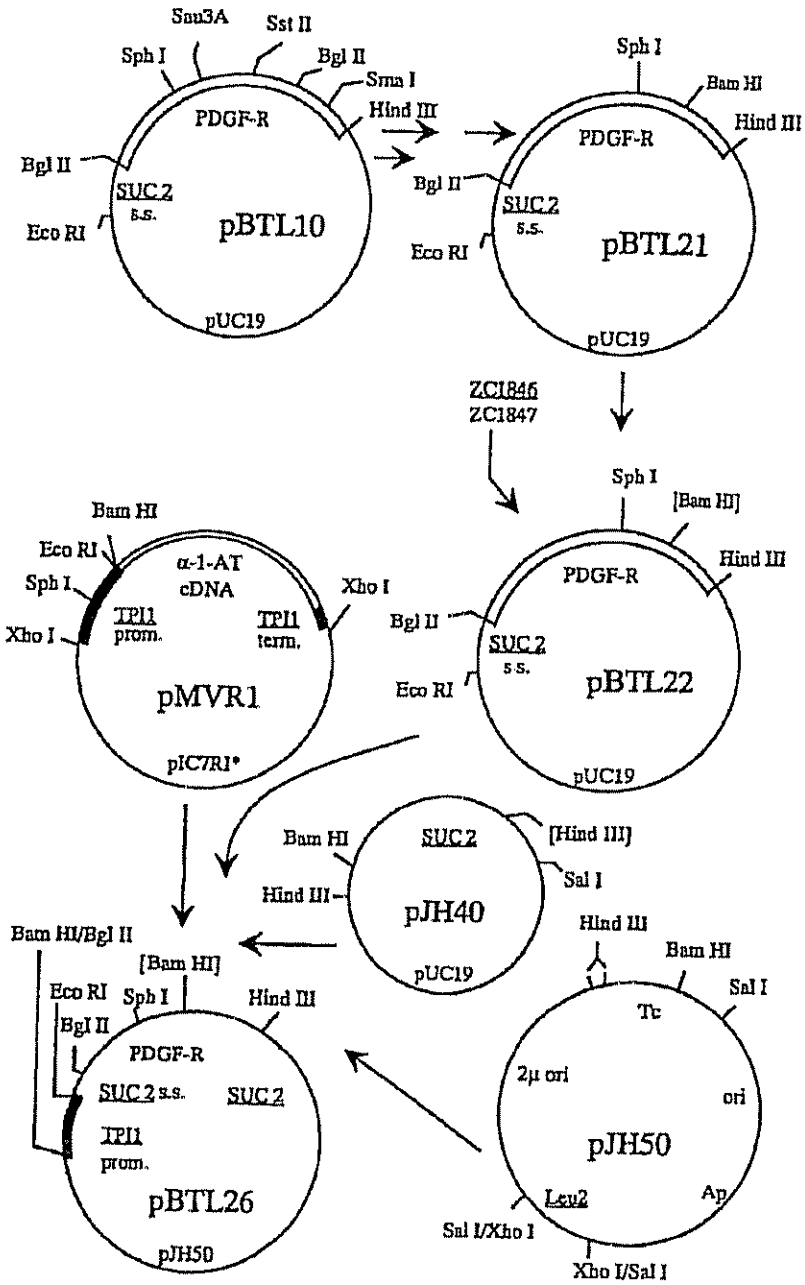

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Ap
Sal I/Xho I
Xho I/Sal I

Figure 7

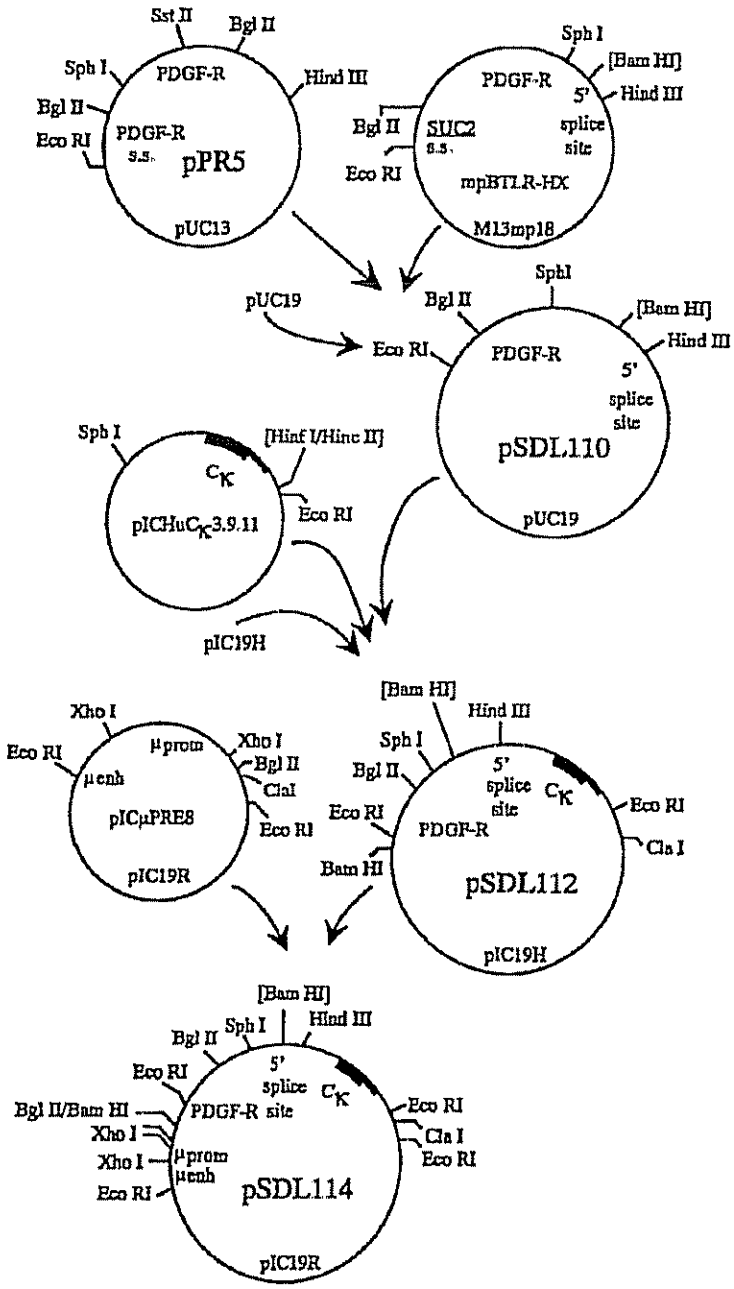

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Figure 8

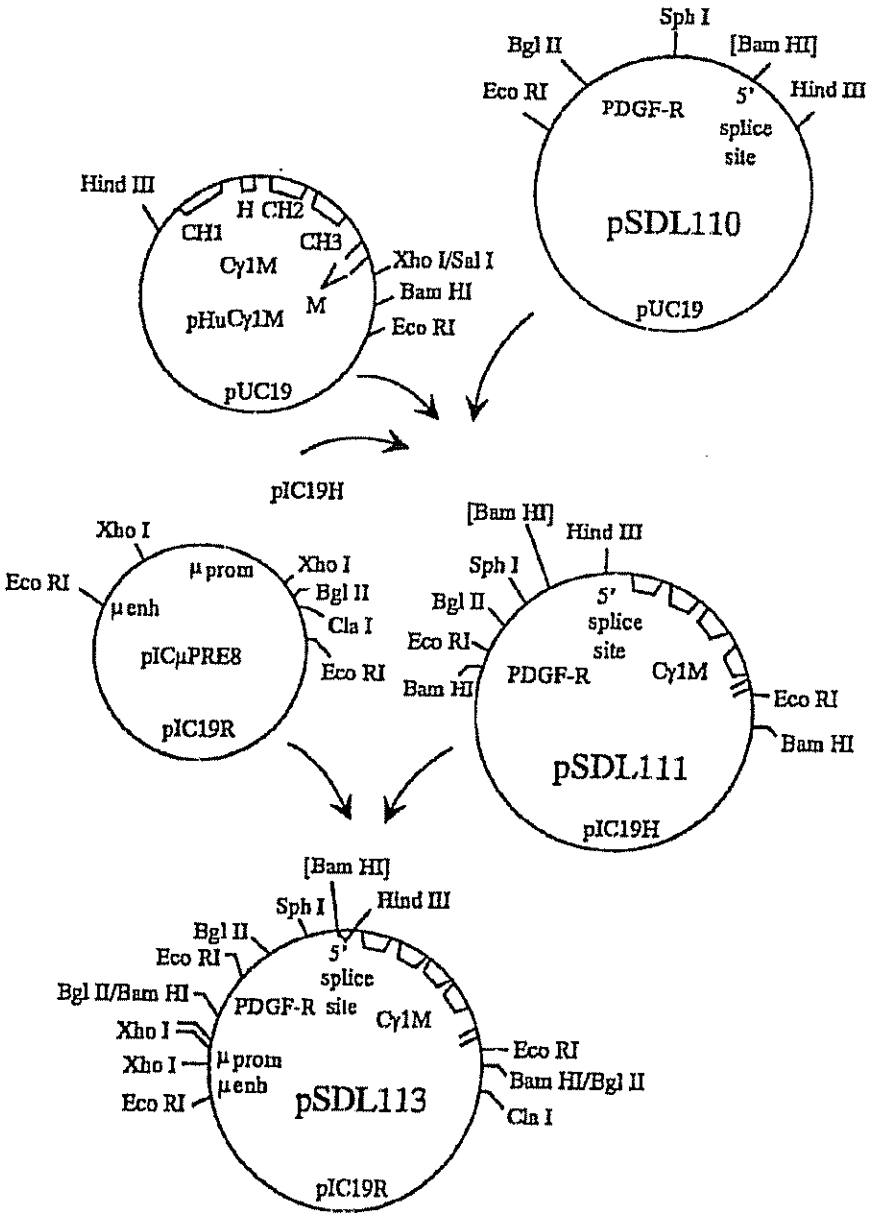

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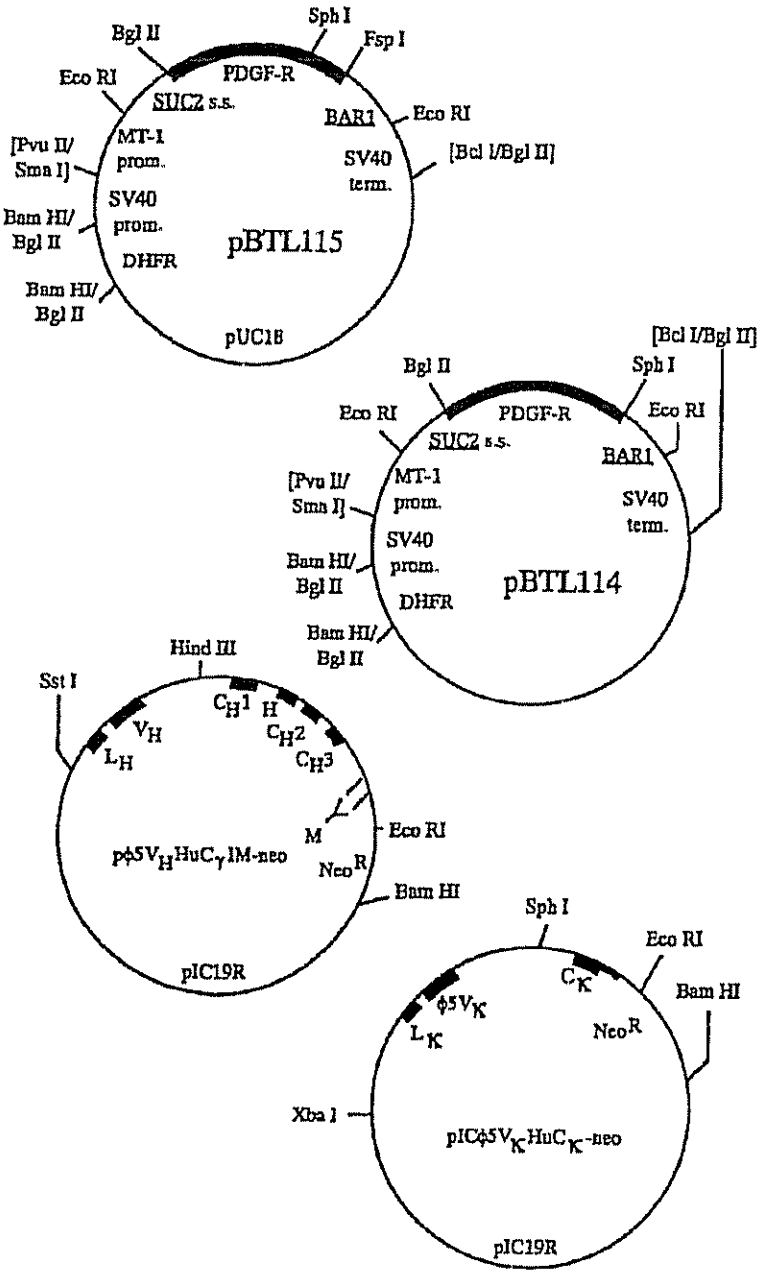

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Sma I]
MT-1
prom.
SV40
term.
[Bcl I/Bgl II]
SV40
prom.
Bam HI/
Bgl II
DHFR
pBTL115
Bam HI/
Bgl II
pUC18
[Bcl I/Bgl II]
Bgl II
Sph I
Eco RI
PDGF-R
Eco RI
SUC2 s.s.
BAR1
[Pvu II/
Sma I]
MT-1
prom.
SV40
term.
SV40
prom.
Bam HI/
Bgl II
pBTL114
DHFR
Bam HI/
Bgl II
Hind III
Sst I
C_H1
H
V_H
C_H2
L_H
C_H3
M
Eco RI
$p\phi5V_HHuC_\gamma1M$-neo
Neo^R
Bam HI
pIC19R
Sph I
Eco RI
C_K
$\phi5V_K$
Bam HI
L_K
Neo^R
Xba I
$pIC\phi5V_KHuC_K$-neo
pIC19R

Figure 10

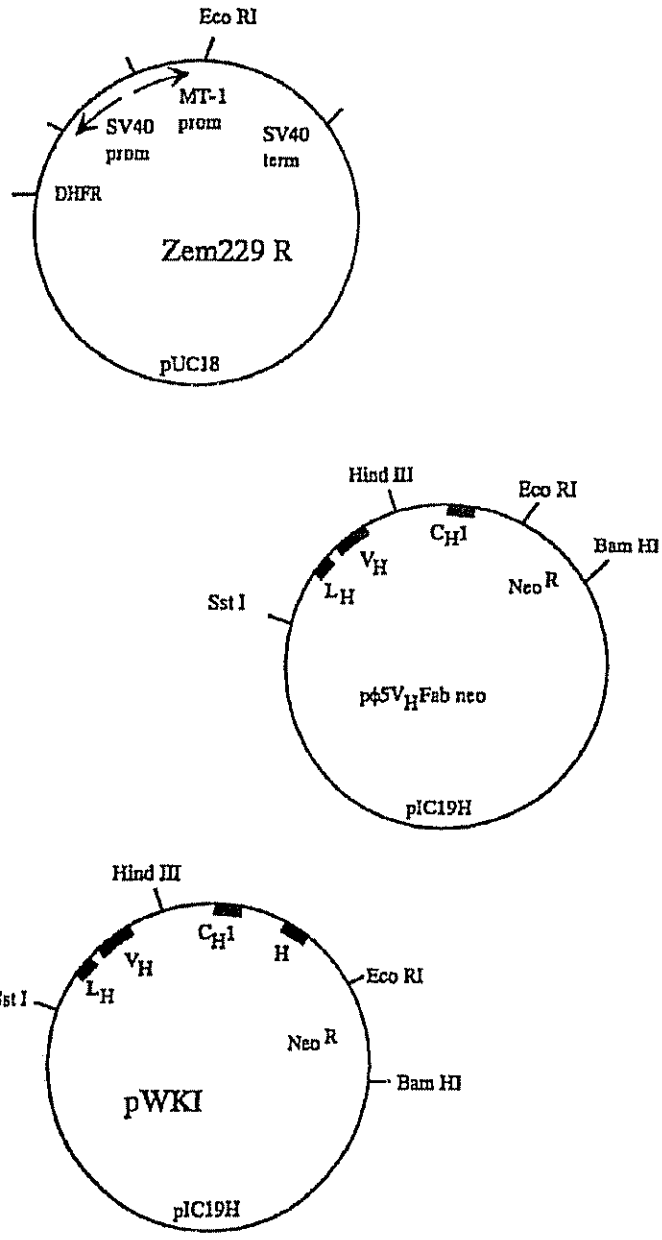

Eco RI
MT-1 prom
SV40 prom
SV40 term
DHFR
Zem229 R
pUC18
Hind III
Eco RI
C_H1
V_H
L_H
Bam HI
Sst I
Neo^R
$p\phi5V_HFab$ neo
pIC19H
Hind III
C_H1
H
V_H
L_H
Eco RI
Sst I
Neo^R
Bam HI
pWKI
pIC19H

Figure 11A

```
  1 GCCCTGGGGACGGACCGTGGGCGGCGCGCAGCGGCGGGACGCGTTTTGGGGACGTGGTGGCCAGCGCCT
 70 TCCTGCAGACCCACAGGGAAGTACTCCCTTTGACCTCCGGGGAGCTGCGACCAGGTTATACGTTGCTGG

139 TGGAAAAGTGACAATTCTAGGAAAAGAGCTAAAAGCCGGATCGGTGACCGAAAGTTTCCCAGAGCTATG
                                                                    M
                                                                    1

208 GGGACTTCCCATCCGGCGTTCCTGGTCTTAGGCTGTCTTCTCACAGGGCTGAGCCTAATCCTCTGCCAG
    G T S H P A F L V L G C L L T G L S L I L C Q

277 CTTTCATTACCCTCTATCCTTCCAAATGAAAATGAAAAGGTTGTGCAGCTGAATTCATCCTTTTCTCTG
    L S L P S I L P N E N E K V V Q L N S S F S L

346 AGATGCTTTGGGGAGAGTGAAGTGAGCTGGCAGTACCCCATGTCTGAAGAAGAGAGCTCCGATGTGGAA
    R C F G E S E V S W Q Y P M S E E E S S D V E

415 ATCAGAAATGAAGAAAACAACAGCGGCCTTTTTGTGACGGTCTTGGAAGTGAGCAGTGCCTCGGCGGCC
    I R N E E N N S G L F V T V L E V S S A S A A

484 CACACAGGGTTGTACACTTGCTATTACAACCACACTCAGACAGAAGAGAATGAGCTTGAAGGCAGGCAC
    H T G L Y T C Y Y N H T Q T E E N E L E G R H

553 ATTTACATCTATGTGCCAGACCCAGATGTAGCCTTTGTACCTCTAGGAATGACGGATTATTTAGTCATC
    I Y I Y V P D P D V A F V P L G M T D Y L V I

622 GTGGAGGATGATGATTCTGCCATTATACCTTGTCGCACAACTGATCCCGAGACTCCTGTAACCTTACAC
    V E D D D S A I I P C R T T D P E T P V T L H

691 AACAGTGAGGGGGTGGTACCTGCCTCCTACGACAGCAGACAGGGCTTTAATGGGACCTTCACTGTAGGG
    N S E G V V P A S Y D S R Q G F N G T F T V G

760 CCCTATATCTGTGAGGCCACCGTCAAAGGAAAGAAGTTCCAGACCATCCCATTTAATGTTTATGCTTTA
    P Y I C E A T V K G K K F Q T I P F N V Y A L

829 AAAGCAACATCAGAGCTGGATCTAGAAATGGAAGCTCTTAAAACCGTGTATAAGTCAGGGGAAACGATT
    K A T S E L D L E M E A L K T V Y K S G E T I

898 GTGGTCACCTGTGCTGTTTTTAACAATGAGGTGGTTGACCTTCAATGGACTTACCCTGGAGAAGTGAAA
    V V T C A V F N N E V V D L Q W T Y P G E V K
```

Figure 11B

```
967  GGCAAAGGCATCACAATACTGGAAGAAATCAAAGTCCCATCCATCAAATTGGTGTACACTTTGACGGTC
     G  K  G  I  T  I  L  E  E  I  K  V  P  S  I  K  L  V  Y  T  L  T  V

1036 CCCGAGGCCACGGTGAAAGACAGTGGAGATTACGAATGTGCTGCCCGCCAGGCTACCAGGGAGGTCAAA
     P  E  A  T  V  K  D  S  G  D  Y  E  C  A  A  R  Q  A  T  R  E  V  K

1105 GAAATGAAGAAAGTCACTATTTCTGTCCATGAGAAAGGTTTCATTGAAATCAAACCCACCTTCAGCCAG
     E  M  K  K  V  T  I  S  V  H  E  K  G  F  I  E  I  K  P  T  F  S  Q

1174 TTGGAAGCTGTCAACCTGCATGAAGTCAAACATTTTGTTGTAGAGGTGCGGGCCTACCCACCTCCCAGG
     L  E  A  V  N  L  H  E  V  K  H  F  V  V  E  V  R  A  Y  P  P  P  R

1243 ATATCCTGGCTGAAAAACAATCTGACTCTGATTGAAAATCTCACTGAGATCACCACTGATGTGGAAAAG
     I  S  W  L  K  N  N  L  T  L  I  E  N  L  T  E  I  T  T  D  V  E  K

1312 ATTCAGGAAATAAGGTATCGAAGCAAATTAAAGCTGATCCGTGCTAAGGAAGAAGACAGTGGCCATTAT
     I  Q  E  I  R  Y  R  S  K  L  K  L  I  R  A  K  E  E  D  S  G  H  Y

1381 ACTATTGTAGCTCAAAATGAAGATGCTGTGAAGAGCTATACTTTTGAACTGTTAACTCAAGTTCCTTCA
     T  I  V  A  Q  N  E  D  A  V  K  S  Y  T  F  E  L  L  T  Q  V  P  S

1450 TCCATTCTGGACTTGGTCGATGATCACCATGGCTCAACTGGGGGACAGACGGTGAGGTGCACAGCTGAA
     S  I  L  D  L  V  D  D  H  H  G  S  T  G  G  Q  T  V  R  C  T  A  E

1519 GGCACGCCGCTTCCTGATATTGAGTGGATGATATGCAAAGATATTAAGAAATGTAATAATGAAACTTCC
     G  T  P  L  P  D  I  E  W  M  I  C  K  D  I  K  K  C  N  N  E  T  S

1588 TGGACTATTTTGGCCAACAATGTCTCAAACATCATCACGGAGATCCACTCCCGAGACAGGAGTACCGTG
     W  T  I  L  A  N  N  V  S  N  I  I  T  E  I  H  S  R  D  R  S  T  V

1657 GAGGGCCGTGTGACTTTCGCCAAAGTGGAGGAGACCATCGCCGTGCGATGCCTGGCTAAGAATCTCCTT
     E  G  R  V  T  F  A  K  V  E  E  T  I  A  V  R  C  L  A  K  N  L  L

1726 GGAGCTGAGAACCGAGAGCTGAAGCTGGTGGCTCCCACCCTGCGTTCTGAACTCACGGTGGCTGCTGCA
     G  A  E  N  R  E  L  K  L  V  A  P  T  L  R  S  E  L  T  V  A  A  A

1795 GTCCTGGTGCTGTTGGTGATTGTGATCATCTCACTTATTGTCCTGGTTGTCATTTGGAAACAGAAACCG
     V  L  V  L  L  V  I  V  I  I  S  L  I  V  L  V  V  I  W  K  Q  K  P
```

Figure 11C

```
1864  AGGTATGAAATTCGCTGGAGGGTCATTGAATCAATCAGCCCGGATGGACATGAATATATTTATGTGGAC
        R  Y  E  I  R  W  R  V  I  E  S  I  S  P  D  G  H  E  Y  I  Y  V  D

1933  CCGATGCAGCTGCCTTATGACTCAAGATGGGAGTTTCCAAGAGATGGACTAGTGCTTGGTCGGGTCTTG
        P  M  Q  L  P  Y  D  S  R  W  E  F  P  R  D  G  L  V  L  G  R  V  L

2002  GGGTCTGGAGCGTTTGGGAAGGTGGTTGAAGGAACAGCCTATGGATTAAGCCGGTCCCAACCTGTCATG
        G  S  G  A  F  G  K  V  V  E  G  T  A  Y  G  L  S  R  S  Q  P  V  M

2071  AAAGTTGCAGTGAAGATGCTAAAACCCACGGCCAGATCCAGTGAAAAACAAGCTCTCATGTCTGAACTG
        K  V  A  V  K  M  L  K  P  T  A  R  S  S  E  K  Q  A  L  M  S  E  L

2140  AAGATAATGACTCACCTGGGGCCACATTTGAACATTGTAAACTTGCTGGGAGCCTGCACCAAGTCAGGC
        K  I  M  T  H  L  G  P  H  L  N  I  V  N  L  L  G  A  C  T  K  S  G

2209  CCCATTTACATCATCACAGAGTATTGCTTCTATGGAGATTTGGTCAACTATTTGCATAAGAATAGGGAT
        P  I  Y  I  I  T  E  Y  C  F  Y  G  D  L  V  N  Y  L  H  K  N  R  D

2278  AGCTTCCTGAGCCACCACCCAGAGAAGCCAAAGAAAGAGCTGGATATCTTTGGATTGAACCCTGCTGAT
        S  F  L  S  H  H  P  E  K  P  K  K  E  L  D  I  F  G  L  N  P  A  D

2347  GAAAGCACACGGAGCTATGTTATTTTATCTTTTGAAAACAATGGTGACTACATGGACATGAAGCAGGCT
        E  S  T  R  S  Y  V  I  L  S  F  E  N  N  G  D  Y  M  D  M  K  Q  A

2416  GATACTACACAGTATGTCCCCATGCTAGAAAGGAAAGAGGTTTCTAAATATTCCGACATCCAGAGATCA
        D  T  T  Q  Y  V  P  M  L  E  R  K  E  V  S  K  Y  S  D  I  Q  R  S

2485  CTCTATGATCGTCCAGCCTCATATAAGAAGAAATCTATGTTAGACTCAGAAGTCAAAAACCTCCTTTCA
        L  Y  D  R  P  A  S  Y  K  K  K  S  M  L  D  S  E  V  K  N  L  L  S

2554  GATGATAACTCAGAAGGCCTTACTTTATTGGATTTGTTGAGCTTCACCTATCAAGTTGCCCGAGGAATG
        D  D  N  S  E  G  L  T  L  L  D  L  L  S  F  T  Y  Q  V  A  R  G  M

2623  GAGTTTTTGGCTTCAAAAAATTGTGTCCACCGTGATCTGGCTGCTCGCAACGTCCTCCTGGCACAAGGA
        E  F  L  A  S  K  N  C  V  H  R  D  L  A  A  R  N  V  L  L  A  Q  G

2692  AAAATTGTGAAGATCTGTGACTTTGGCCTGGCCAGAGACATCATGCATGATTCGAACTATGTGTCGAAA
        K  I  V  K  I  C  D  F  G  L  A  R  D  I  M  H  D  S  N  Y  V  S  K

2761  GGCAGTACCTTTCTGCCCGTGAAGTGGATGGCTCCTGAGAGCATCTTTGACAACCTCTACACCACACTG
        G  S  T  F  L  P  V  K  W  M  A  P  E  S  I  F  D  N  L  Y  T  T  L
```

Figure 11D

```
2830  AGTGATGTCTGGTCTTATGGCATTCTGCTCTGGGAGATCTTTTCCCTTGGTGGCACCCCTTACCCCGGC
      S  D  V  W  S  Y  G  I  L  L  W  E  I  F  S  L  G  G  T  P  Y  P  G

2899  ATGATGGTGGATTCTACTTTCTACAATAAGATCAAGAGTGGGTACCGGATGGCCAAGCCTGACCACGCT
      M  M  V  D  S  T  F  Y  N  K  I  K  S  G  Y  R  M  A  K  P  D  H  A

2968  ACCAGTGAAGTCTACGAGATCATGGTGAAATGCTGGAACAGTGAGCCGGAGAAGAGACCCTCCTTTTAC
      T  S  E  V  Y  E  I  M  V  K  C  W  N  S  E  P  E  K  R  P  S  F  Y

3037  CACCTGAGTGAGATTGTGGAGAATCTGCTGCCTGGACAATATAAAAAGAGTTATGAAAAAATTCACCTG
      H  L  S  E  I  V  E  N  L  L  P  G  Q  Y  K  K  S  Y  E  K  I  H  L

3106  GACTTCCTGAAGAGTGACCATCCTGCTGTGGCACGCATGCGTGTGGACTCAGACAATGCATACATTGGT
      D  F  L  K  S  D  H  P  A  V  A  R  M  R  V  D  S  D  N  A  Y  I  G

3175  GTCACCTACAAAAACGAGGAAGACAAGCTGAAGGACTGGGAGGGTGGTCTGGATGAGCAGAGACTGAGC
      V  T  Y  K  N  E  E  D  K  L  K  D  W  E  G  G  L  D  E  Q  R  L  S

3244  GCTGACAGTGGCTACATCATTCCTCTGCCTGACATTGACCCTGTCCCTGAGGAGGAGGACCTGGGCAAG
      A  D  S  G  Y  I  I  P  L  P  D  I  D  P  V  P  E  E  E  D  L  G  K

3313  AGGAACAGACACAGCTCGCAGACCTCTGAAGAGAGTGCCATTGAGACGGGTTCCAGCAGTTCCACCTTC
      R  N  R  H  S  S  Q  T  S  E  E  S  A  I  E  T  G  S  S  S  S  T  F

3382  ATCAAGAGAGAGGACGAGACCATTGAAGACATCGACATGATGGACGACATCGGCATAGACTCTTCAGAC
      I  K  R  E  D  E  T  I  E  D  I  D  M  M  D  D  I  G  I  D  S  S  D

3451  CTGGTGGAAGACAGCTTCCTGTAACTGGCGGATTCGAGGGGTTCCTTCCACTTCTGGGGCCACCTCTGG
      L  V  E  D  S  F  L
                     1089

3520  ATCCCGTTCAGAAAACCACTTTATTGCAATGCGGAGGTTGAGAGGAGGACTTGGTTGATGTTTAAAGAG
3589  AAGTTCCCAGCCAAGGGCCTCGGGGAGCGTTCTAAATATGAATGAATGGGATATTTTGAAATGAACTTT
3658  GTCAGTGTTGCCTCTTGCAATGCCTCAGTAGCATCTCAGTGGTGTGTGAAGTTTGGAGATAGATGGATA
3727  AGGGAATAATAGGCCACAGAAGGTGAACTTTGTGCTTCAAGGACATTGGTGAGAGTCCAACAGACACAA
3796  TTTATACTGCGACAGAACTTCAGCATTGTAATTATGTAAATAACTCTAACCAAGGCTGTGTTTAGATTG
3865  TATTAACTATCTTCTTTGGACTTCTGAAGAGACCACTCAATCCATCCTGTACTTCCCTCTTGAAACCTG
3934  ATGTAGCTGCTGTTGAACTTTTTAAAGAAGTGCATGAAAAACCATTTTTGAACCTTAAAAGGTACTGGT
4003  ACTATAGCATTTTGCTATCTTTTTTAGTGTTAAAGAGATAAAGAATAATAAG
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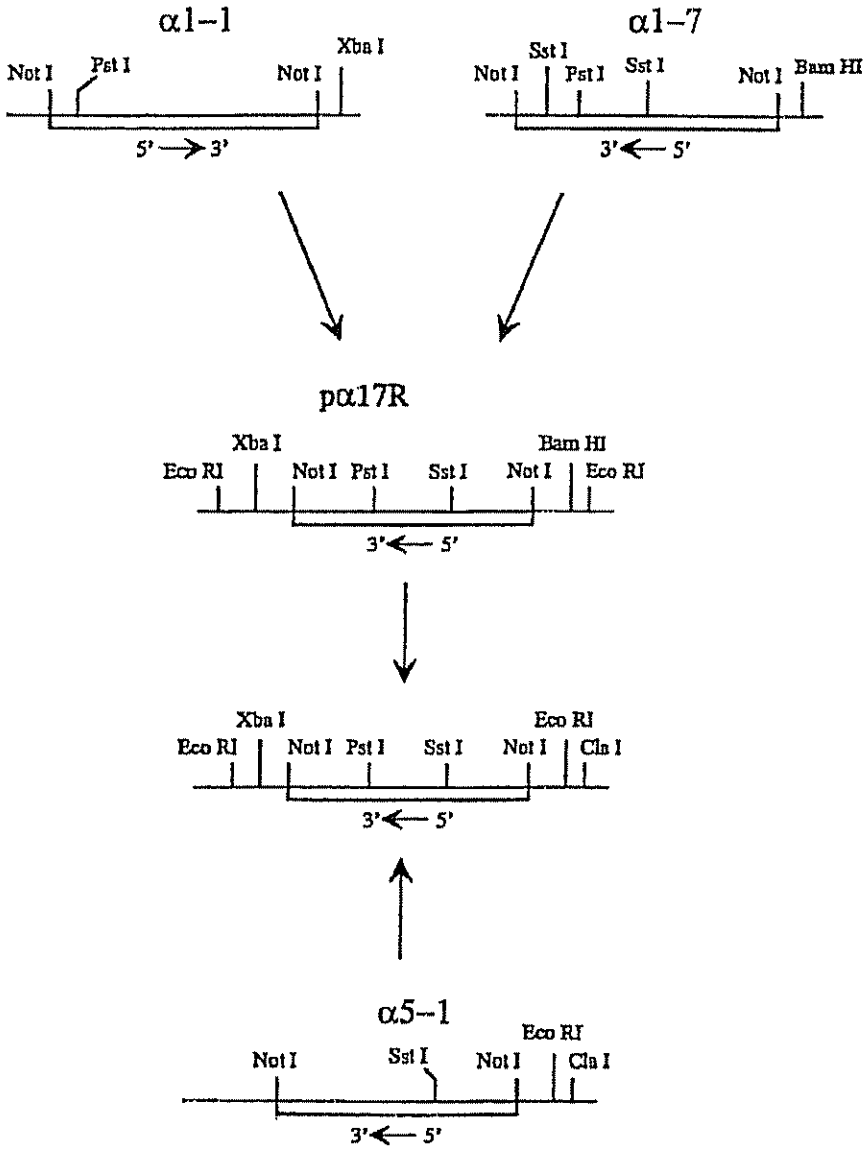

α1–1
Not I
Pst I
Not I
Xba I
5' → 3'
α1–7
Not I
Sst I
Pst I
Sst I
Not I
Bam HI
3' ← 5'
pα17R
Xba I
Eco RI
Not I
Pst I
Sst I
Not I
Bam HI
Eco RI
3' ← 5'
Xba I
Eco RI
Not I
Pst I
Sst I
Not I
Eco RI
Cla I
3' ← 5'
α5–1
Not I
Sst I
Not I
Eco RI
Cla I
3' ← 5'

Figure 12

METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE DIMERIZED POLYPEPTIDE FUSIONS

This application is a divisional of U.S. application Ser. No. 08/180,195, filed Jan. 11, 1994, now U.S. Pat. No. 5,567,584, which is a continuation of U.S. application Ser. No. 07/634,510 filed Dec. 27, 1990, now abandoned, which is a continuation-in-part of U.S. application Ser. No. 07/347,291, filed May 2, 1989, now U.S. Pat. No. 5,155,027, which is a continuation-in-part of U.S. application Ser. No. 07/146,877, filed Jan. 22, 1988, now abandoned.

TECHNICAL FIELD

The present invention is generally directed toward the expression of proteins, and more specifically, toward the expression of growth factor receptor analogs and biologically active dimerized polypeptide fusions.

BACKGROUND OF THE INVENTION

In higher eucaryotic cells, the interaction between receptors and ligands (e.g., hormones) is of central importance in the transmission of and response to a variety of extracellular signals. It is generally accepted that hormones and growth factors elicit their biological functions by binding to specific recognition sites (receptors) in the plasma membranes of their target cells. Upon ligand binding, a receptor undergoes a conformational change, triggering secondary cellular responses that result in the activation or inhibition of intracellular processes. The stimulation or blockade of such an interaction by pharmacological means has important therapeutic implications for a wide variety of illnesses.

Ligands fall into two classes: those that have stimulatory activity, termed agonists; and those that block the effects elicited by the original ligands, termed antagonists. The discovery of agonists that differ in structure and composition from the original ligand may be medically useful. In particular, agonists that are smaller than the original ligand may be especially useful. The bioavailability of these smaller agonists may be greater than that of the original ligand. This may be of particular importance for topical applications and for instances when diffusion of the agonist to its target sites is inhibited by poor circulation. Agonists may also have slightly different spectra of biological activity and/or different potencies, allowing them to be used in very specific situations. Agonists that are smaller and chemically simpler than the native ligand may be produced in greater quantity and at lower cost. The identification of antagonists which specifically block, for example, growth factor receptors has important pharmaceutical applications. Antagonists that block receptors against the action of endogenous, native ligand may be used as therapeutic agents for conditions including atherosclerosis, autocrine tumors, fibroplasia and keloid formation.

The discovery of new ligands that may be used in pharmaceutical applications has centered around designing compounds by chemical modification, complete synthesis, and screening potential ligands by complex and costly screening procedures. The process of designing a new ligand usually begins with the alteration of the structure of the original effector molecule. If the original effector molecule is known to be chemically simple, for example, a catecholamine or prostaglandin, the task is relatively straightforward. However, if the ligand is structurally complex, for example, a peptide hormone or a growth factor, finding a molecule which is functionally equivalent to the original ligand becomes extremely difficult.

Currently, potential ligands are screened using radioligand binding methods (Lefkowitz et al., *Biochem. Biophys. Res. Comm.* 60: 703–709, 1974; Aurbach et al., Science 186: 1223–1225, 1974; Atlas et al., *Proc. Natl. Acad. Sci. USA* 71: 4246–4248, 1974). Potential ligands can be directly assayed by binding the radiolabeled compounds to responsive cells, to the membrane fractions of disrupted cells, or to solubilized receptors. Alternatively, potential ligands may be screened by their ability to compete with a known labeled ligand for cell surface receptors.

The success of these procedures depends on the availability of reproducibly high quality preparations of membrane fractions or receptor molecules, as well as the isolation of responsive cell lines. The preparation of membrane fractions and soluble receptor molecules involves extensive manipulations and complex purification steps. The isolation of membrane fractions requires gentle manipulation of the preparation, a procedure which does not lend itself to commercial production. It is very difficult to maintain high biological activity and biochemical purity of receptors when they are purified by classical protein chemistry methods. Receptors, being integral membrane proteins, require cumbersome purification procedures, which include the use of detergents and other solvents that interfere with their biological activity. The use of these membrane preparations in ligand binding assays typically results in low reproducibility due to the variability of the membrane preparations.

As noted above, ligand binding assays require the isolation of responsive cell lines. Often, only a limited subset of cells is responsive to a particular agent, and such cells may be responsive only under certain conditions. In addition, these cells may be difficult to grow in culture or may possess a low number of receptors. Currently available cell types responsive to platelet-derived growth factor (PDGF), for example, contain only a low number (up to 4×10^4; see Bowen-Pope and Ross, *J. Biol. Chem.* 257: 5161–5171, 1982) of receptors per cell, thus requiring large numbers of cells to assay PDGF analogs or antagonists.

Presently, only a few naturally-occurring secreted receptors, for example, the interleukin-2 receptor (IL-2-R) have been identified. Rubin et al. (*J. Immun.* 135: 3172–3177, 1985) have reported the release of large quantities of IL-2-R into the culture medium of activated T-cell lines. Bailon et al. (*BioTechnology* 5: 1195–1198, 1987) have reported the use of a matrix-bound interleukin-2 receptor to purify recombinant interleukin-2.

Three other receptors have been secreted from mammalian cells. The insulin receptor (Ellis et al., *J. Cell Biol.* 150: 14a, 1987), the HIV-1 envelope glyco-protein cellular receptor CD4 (Smith et al., Science 238: 1704–1707, 1987), the murine IL-7 receptor (Cell 60: 941–951, 1990) and the epidermal growth factor (EGF) receptor (Livneh et al., *J. Biol. Chem.* 261: 12490–12497, 1986) have been secreted from mammalian cells using truncated cDNAs that encode portions of the extracellular domains.

Naturally-occurring, secreted receptors have not been widely identified, and there have been only a limited number of reports of secreted recombinant receptors. Secreted receptors may be used in a variety of assays, which include assays to determine the presence of ligand in biological fluids and assays to screen for potential agonists and antagonists. Current methods for ligand screening and ligand/receptor binding assays have been limited to those using preparations of whole cells or cell membranes for as a source for receptor

molecules. The low reproducibility and high cost of producing such preparations does not lend itself to commercial production. There is therefore a need in the art for a method of producing secreted receptors. There is a further need in the art for an assay system that permits high volume screening of compounds that may act on higher eucaryotic cells via specific surface receptors. This assay system should be rapid, inexpensive and adaptable to high volume screening. The present invention discloses such a method and assay system, and further provides other related advantages.

DISCLOSURE OF INVENTION

Briefly stated, the present invention discloses methods for producing secreted receptor analogs, including receptor analogs and secreted platelet-derived growth factor receptor (PDGF-R) analogs. In addition, the present invention discloses methods for producing secreted biologically active dimerized polypeptide fusions.

Within one aspect of the invention a method for producing a secreted PDGF-R analog is disclosed, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding at least a portion of the ligand-binding domain of a PDGF-R, the portion including a ligand-binding domain; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF-R analog encoded by said DNA sequence; and (c) isolating the PDGF-R analog from the host cell.

Within one embodiment of the present invention, a PDGF-R analog comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another embodiment, a PDGF-R analog comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprising the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

Yet another aspect of the present invention discloses a method for producing a secreted, biologically active dimerized polypeptide fusion. The method generally comprises a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encodes by said DNA sequence; and (c) isolating the biologically active dimerized polypeptide fusion from the host cell.

Within one embodiment, the dimerizing protein is yeast invertase. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin light chain. Within another embodiment, the dimerizing protein is at least a portion of an immunoglobulin heavy chain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment, the first DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences; and (c) isolating the biologically active dimerized polypeptide fusion from the host cell. In one embodiment, the DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted, biologically active dimerized polypeptide fusion, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a first polypeptide chain of a non-immunoglobulin polypeptide dimer requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_HA; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of the non-immunoglobulin polypeptide dimer joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said first and second DNA sequences wherein said dimerized polypeptide fusion exhibits biological activity characteristic of said non-immunoglobulin polypeptide dimer; and (d) isolating the dimerized polypeptide fusion from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

Within one embodiment of the present invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, is secreted. Within another embodiment, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 is secreted. Within another embodiment of the invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a biologically active dimerized polypeptide fusion comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 dimerized to the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to a dimerizing protein; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and (c) isolating the receptor analog from the host cell.

In yet another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin light chain constant region; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain, selected from the group consisting of C_H1, C_H2, C_H3, and C_HA; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment, the second DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin varisble region joined upstream of and in proper reading frame with the immunoglobulin heavy chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_HA; (b) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of the receptor analog; and (c) isolating the receptor analog from the host cell. In one embodiment, the DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream of and in proper reading frame with a first DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_HA; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment, the first DNA sequence further encodes an immunoglobulin heavy chain hinge region wherein the hinge region is joined to the heavy chain constant region domain. In a preferred embodiment, the second DNA sequence further encodes an immunoglobulin variable region joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

In another aspect of the invention, a method is disclosed for producing a secreted receptor analog, comprising (a) introducing into a eukaryotic host cell a first DNA construct

comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream in proper reading frame by a first DNA sequence encoding a first polypeptide chain of a ligand-binding domain of a receptor requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain, selected from the group C_H1, C_H2, C_H3, and C_M4; (b) introducing into the host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of the ligand-binding domain of said receptor joined to an immunoglobulin light chain constant region domain; (c) growing the host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said first and second DNA sequences; and (d) isolating the receptor analog from the host cell. In one embodiment the first DNA sequence further encodes an immunoglobulin heavy chain hinge region domain wherein the hinge region is joined to the immunoglobulin heavy chain constant region domain.

Host cells for use in the present invention include cultured mammalian cells and fungal cells. In a preferred embodiment strains of the yeast *Saccharomyces cerevisiae* are used as host cells. Within another preferred embodiment cultured rodent myeloma cells are used as host cells.

Within one embodiment of the present invention, a receptor analog is a PDGF-R analog comprising the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441. Within another embodiment a PDGF-R analog comprises the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531. Within another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted. Within yet another embodiment of the invention, a PDGF-R analog comprises the amino acid sequence of FIG. 1 (Sequence ID Numbers 1 and 2) from isoleucine, number 29 to lysine, number 531 and the amino acid sequence of FIG. 11 (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524 is secreted.

PDGF-R analogs produced by the above-disclosed methods may be used, for instance, within a method for determining the presence of human PDGF or an isoform thereof in a biological sample.

A method for determining the presence of human PDGF or an isoform thereof in a biological sample is disclosed and comprises (a) incubating a polypeptide comprising a PDGF receptor analog fused to a dimerizing protein with a biological sample suspected of containing PDGF or an isoform thereof under conditions that allow the formation of receptor/ligand complexes; and (b) detecting the presence of receptor/ligand complexes, and therefrom determining the presence of PDGF or an isoform thereof. Suitable biological samples in this regard include blood, urine, plasma, serum, platelet and other cell lysates, platelet releasates, cell suspensions, cell-conditioned culture media, and chemically or physically separated portions thereof.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1A–D (Sequence ID Numbers 1 and 2) illustrate the nucleotide sequence of a representative PDGF β-receptor cDNA and the derived amino acid sequence of the primary translation product and corresponds to Sequence ID Number 1). Numbers above the lines refer to the nucleotide sequence; numbers below the lines refer to the amino acid sequence.

FIG. 2 illustrates the construction of pBTL10, pBTL11 and pBTL12.

FIG. 3 illustrates the construction of pCBS22.

FIG. 4 illustrates the construction of pBTL13 and pBTL14.

FIG. 5 illustrates the construction of pBTL15.

FIG. 6 illustrates the construction of pBTL22 and pBTL26.

FIG. 7 illustrates the construction of pSDL114. Symbols used are S.S., signal sequence, C_λ, immunoglobulin light chain constant region sequence; μprom, μ promoter, μ enh; μ enhancer.

FIG. 8 illustrates the construction of pSDLB113. Symbols used are S.S., signal sequence; C_H1, C_H2, C_H3, immunoglobulin heavy chain constant region domain sequences; H, immunoglobulin heavy chain hinge region sequence; M, immunoglobulin membrane anchor sequences; Cγ1M, immunoglobulin heavy chain constant region and membrane anchor sequences.

FIG. 9 illustrates the constructions pBTL115, pBTL114, pϕ5V_HHuC$_\gamma$1M-neo, plCϕ5V_κHuC$_\kappa$-neo. Symbols used are set forth in FIGS. 7 and 8, and also include L_H, mouse immunoglobulin heavy chain signal sequence; V_H, mouse immunoglobulin heavy chain variable region sequence; E, mouse immunoglobulin heavy chain enhancer sequence; L_κ, mouse immunoglobulin light chain signal sequence; ϕ5V_κ, mouse immunoglobulin light chain variable region sequence; Neo^R, *E. coli* neomycin resistance gene.

FIG. 10 illustrates the constructions Zem229R, pϕ5V_HFab-neo and pWK1. Symbols used are set forth in FIG. 9.

FIGS. 11A–D illustrates the sequence of a representative PDGF α-receptor cDNA and the deduced amino acid sequence (using standard one-letter codes) encoded by the cDNA and corresponds to Sequence ID Numbers 35 and 36. Numbers at the ends of the lines refer to nucleotide positions. Numbers below the sequence refer to amino acid positions.

FIG. 12 illustrates the assembly of a cDNA molecule encoding a PDGF α-receptor. Complementary DNA sequences are shown as lines. Only those portions of the vectors adjacent to the cDNA inserts are shown.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

DNA Construct: A DNA molecule, or a clone of such a molecule, either single- or double-stranded that has been modified through human intervention to contain segments of DNA combined and juxtaposed in a manner that as a whole would not otherwise exist in nature.

DNA constructs contain the information necessary to direct the expression and/or secretion of DNA sequences encoding polypeptides of interest. DNA constructs will generally include promoters, enhancers and transcription terminators. DNA constructs containing the information necessary to direct the secretion of a polypeptide will also contain at least one secretory signal sequence.

Secretory Signal Sequence: A DNA sequence encoding a secretory peptide. A secretory peptide is an amino acid sequence that acts to direct the secretion of a mature polypeptide or protein from a cell. Secretory peptides are characterized by a core of hydrophobic amino acids and are typically (but not exclusively) found at the amino termini of newly sythesized proteins. Very often the secretory peptide is cleaved from the mature protein during secretion. Such secretory peptides contain processing sites that allow cleavage of the signal peptides from the mature proteins as it passes through the secretory pathway. Processing sites may be encoded within the signal peptide or may be added to the signal peptide by, for example, in vitro mutagenesis. Certain secretory peptides may be used in concert to direct the secretion of polypeptides and proteins. One such secretory peptide that may be used in combination with other secretory peptides is the third domain of the yeast Barrier protein.

Receptor Analog: A non-immunoglobulin polypeptide comprising a portion of a receptor which is capable of binding ligand and/or is recognized by anti-receptor antibodies. The amino acid sequence of the receptor analog may contain additions, substitutions or deletions as compared to the native receptor sequence. A receptor analog may be, for example, the ligand-binding domain of a receptor joined to another protein. Platelet-derived growth factor receptor (PDGF-R) analogs may, for example, comprise a portion of a PDGF receptor capable of binding anti-PDGF receptor antibodies, PDGF, PDGF isoforms, PDGF analogs, or PDGF antagonists.

Dimerizing Protein: A polypeptide chain having affinity for a second polypeptide chain, such that the two chains associate under physiological conditions to form a dimer. The second polypeptide chain may be the same or a different chain.

Biological activity: A function or set of activities performed by a molecule in a biological context (i.e., in an organism or an in vitro facsimile thereof). Biological activities may include the induction of extracellular matrix secretion from responsive cell lines, the induction of hormone secretion, the induction of chemotaxis, the induction of mitogenesis, the induction of differentiation, or the inhibition of cell division of responsive cells. A recombinant protein or peptide is considered to be biologically active if it exhibits one or more biological activities of its native counterpart.

Ligand: A molecule capable of being bound by the ligand-binding domain of a receptor or by a receptor analog. The molecule may be chemically synthesized or may occur in nature. Ligands may be grouped into agonists and antagonists. Agonists are those molecules whose binding to a receptor induces the response pathway within a cell. Antagonists are those molecules whose binding to a receptor blocks the response pathway within a cell.

Joined: Two or more DNA coding sequences are said to be joined when, as a result of in-frame fusions between the DNA coding sequences or as a result of the removal of intervening sequences by normal cellular processing, the DNA coding sequences are translated into a polypeptide fusion.

As noted above, the present invention pr vides methods for producing biologically active dimerized polypeptide fusions and secreted receptor analogs, which include, for example, PDGF receptor analogs. Secreted receptor analogs may be used to screen for new compounds that act as agonists or antagonists when interacting with cells containing membrane-bound receptors. In addition, the methods of the present invention provide dimerized non-immunoglobulin polypeptide fusions of therapeutic value that are biologically active only as dimers. Moreover, the present invention provides methods of producing polypeptide dimers that are biologically active only as non-covalently associated dimers. Secreted, biologically active dimers that may be produced using the present invention include nerve growth factor, colony stimulating factor-1, factor XIII, and transforming growth factor β.

As used herein, the ligand-binding domain of a receptor is that portion of the receptor that is involved with binding the natural ligand. While not wishing to be bound by theory, the binding of a natural ligand to a receptor is believed to induce a conformational change which elicits a response to the change within the response pathway of the cell. For membrane-bound receptors, the ligand-binding domain is generally believed to comprise the extracellular domain for the receptor. The structure of receptors may be predicted from the primary translation products using the hydrophobicity plot function of, for example, P/C Gene or Intelligenetics Suite (Intelligenetics, Mt. View, Calif.) or may be predicted according to the methods described, for example, by Kyte and Doolittle, *J. Mol. Biol.* 157:105–132, 1982). The ligand-binding domain of the PDGF p-receptor, for example, has been predicted to include amino acids 29-531 of the published sequence (Gronwald et al., ibid.). The ligand-binding domain of the PDGF α-receptor has been predicted to include amino acids 25–500 of the published α-receptor sequence (Matsui et al., ibid.). As used herein, the ligand-binding domain of the PDGF β-receptor includes amino acids 29–441 of the sequence of FIGS. 1A and 1B (Sequence ID Number 1) and C-terminal extensions up to and including amino acid 531(FIG. 1B). The ligand-binding domain of the PDGF α-receptor is understood to include amino acids 24–524 of FIGS. 11A and 11B (Sequence ID Numbers 35 and 36).

Receptor analogs that may be used in the present invention include the ligand-binding domains of the epidermal growth factor receptor (EGF-R) and the insulin receptor. As used herein, a ligand-binding domain is that portion of the receptor that is involved in binding ligand and is generally a portion or essentially all of the extracellular domain that extends from the plasma membrane into the extracellular space. The ligand-binding domain of the EGF-R, for example, resides in the extracellular domain. EGF-R dimers have been found to exhibit higher ligand-binding affinity than EGF-R monomers (Boni-Schnetzler and Pilch, *Proc. Natl. Acad. Sci. USA* 84:7832–7836, 1987). The insulin receptor (Ullrich et al., *Nature* 313:756–761, 1985) requires dimerization for biological activity.

Another example of a receptor that may be secreted from a host cell is a platelet-derived growth factor receptor (PDGF-R). Two classes of PDGF-Rs, which recognized different isoforms of PDGF, have been identified. (PDGF is a disulfide-bonded, two-chain molecule, which is made up of an A chain and a B chain. These chains may be combined as AB heterodimers, AA homodimers or BB homodimers. These dimeric molecules are referred to herein as "isoforms".) The β-receptor (PDGFβ-R), which recognizes only the BB isoform of PDGF (PDGF-BB), has been described (Claesson-Welsh et al., *Mol. Cell. Biol.* 8:3476–3486, 1988; Gronwald et al., *Proc. Natl. Acad. Sci. USA* 85:3435–3439, 1988). The α-receptor (PDGFα-R), which recognizes all three PDGF isoforms (PDGF-AA, PDGF-AB and PDGF-BB), has been described by Matsui et al. (Science 243:800–804, 1989) and Kelly and Murray (pending commonly assigned U.S. patent application Ser.

No. 07/355,018, which is incorporated herein by reference). The primary translation products of these receptors indicate that each includes an extracellular domain implicated in the ligand-binding process, a transmembrane domain, and a cytoplasmic domain containing a tyrosine kinase activity.

The present invention provides a standardized assay system, not previously available in the art, for determining the presence of PDGF, PDGF isoforms, PDGF agonists or PDGF antagonists using a secreted PDGF receptor analogs. Such an assay system will typically involve combining the secreted PDGF receptor analog with a biological sample under physiological conditions which permit the formation of receptor-ligand complexes, followed by detecting the presence of the receptor-ligand complexes. The term physiological conditions is meant to include those conditions found within the host organism and include, for example, the conditions of osmolarity, salinity and pH. Detection may be achieved through the use of a label attached to the PDGF receptor analog or through the use of a labeled antibody which is reactive with the receptor analog or the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Receptor-ligand complexes may also be detected visually, i.e., in immunoprecipitation assays which do not require the use of a label. This assay system provides secreted PDGF receptor analogs that may be utilized in a variety of screening assays for, for example, screening for analogs of PDGF. The present invention also provides a methods for measuring the level of PDGF and PDGF isoforms in biological fluids.

As noted above, the present invention provides methods for producing dimerized polypeptide fusions that require dimerization for biological activity or enhancement of biological activity. Polypeptides requiring dimerization for biological activity include, in addition to certain receptors, nerve growth factor, colony-stimulating factor-1 (CSF-1), transforming growth factor β (TGF-β), PDGF, and factor XIII. Nerve growth factor is a non-covalently linked dimer (Harper et al., *J. Biol. Chem.* 257: 8541–8548, 1982). CSF-1, which specifically stimulates the proliferation and differentiation of cells of mononuclear phagocytic lineage, is a disulfide-bonded homodimer (Rettenmier et al., *Mol. Cell. Biol.* 7: 2378–2387, 1987). TGF-β is biologically active as a disulfide-bonded dimer (Assoian et al., *J. Biol. Chem.* 258: 7155–7160, 1983). Factor XIII is a plasma protein that exists as a two chain homodimer in its activated form (Ichinose et al., *Biochem.* 25: 6900–6906, 1986). PDGF, as noted above, is a disulfide-bonded, two chain molecule (Murray et al., U.S. Pat. No. 4,766,073).

The present invention provides methods by which receptor analogs, including receptor analogs and PDGF-R analogs, requiring dimerization for activity may be secreted from host cells. The methods described herein are particularly advantageous in that they allow the production of large quantities of purified receptors. The receptors may be used in assays for the screening of potential ligands, in assays for binding studies, as imaging agents, and as agonists and antagonists within therapeutic agents.

A DNA sequence encoding a human PDGF receptor may be isolated as a cDNA using techniques known in the art (see, for example, Okayama and Berg, *Mol. Cell. Biol.* 2: 161–170, 1982; *Mol. Cell. Biol.* 3: 280–289, 1983) from a library of human genomic or cDNA sequences. Such libraries may be prepared by standard procedures, such as those disclosed by Gubler and Hoffman (*Gene* 25: 263–269, 1983). It is preferred that the molecule is a cDNA molecule because cDNA lack introns and are therefore more suited to manipulation and expression in transfected or transformed cells. Sources of mRNA for use in the preparation of a cDNA library include the MG-63 human osteosarcoma cell line (available from ATCC under accession number CRL 1427), diploid human dermal fibroblasts and human embryo fibroblast and brain cells (Matsui et al., ibid.). A cDNA encoding a PDGFβ-R has been cloned from a diploid human dermal fibroblast cDNA library using oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Gronwald et al., ibid.). A PDGFα-R cDNA has been isolated by Matsui et al. (ibid.) from human embryo fibroblast and brain cells. Alternatively, a cDNA encoding a PDGFα-R may be isolated from a library prepared from MG-63 human osteosarcoma cells using a cDNA probe containing sequences encoding the transmembrane and cytoplasmic domains of the PDGFβ-R (described by Kelly and Murray, ibid.). Partial cDNA clones (fragments) can be extended by re-screening of the library with the cloned cDNA fragment until the full sequence is obtained. In one embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 1A and 1B (Sequence ID Number 1) from amino acid 29 through amino acid 441. In another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 1A and 1B (Sequence ID Number 1) from amino acid 29 through amino acid 531. In yet another embodiment, a ligand-binding domain of a PDGF receptor is encoded by the sequence of FIGS. 11A and 11B (Sequence ID Numbers 35 and 36) from amino acid 24 through amino acid 524. One skilled in the art may envision the use of a smaller DNA sequence encoding the ligand-binding domain of a PDGF receptor containing at least 400 amino acids of the extracellular domain.

DNA sequences encoding EGF-R (Ullrich et al., *Nature* 304: 418–425, 1984), the insulin receptor (Ullrich et al., *Nature* 313: 756–761, 1985), nerve growth factor (Ullrich et al. *Nature* 303: 821–825, 1983), colony stimulating factor-1 (Rettenmier et al., ibid.), transforming growth factor β (Derynck et al., *Nature* 316: 701–705, 1985), PDGF (Murray et al., ibid.), and factor XIII (Ichinose et al., ibid.) may also be used within the present invention.

To direct polypeptides requiring dimerization for biological activity or receptor analogs into the secretory pathway of the host cell, at least one secretory signal sequence is used in conjunction with the DNA sequence of interest. Preferred secretory signals include the alpha factor signal sequence (pre-pro sequence) (Kurjan and Herkowitz, *Cell* 30: 933–943, 1982; Kurjan et al., U.S. Pat. No. 4,546,082; Brake, EP 116,201, 1983), the PHO5 signal sequence (Beck et al., WO 86/00637), the BAR1 secretory signal sequence (MacKay et al., U.S. Pat. No. 4,613,572; MacKay, WO 87/002670), immunoglobulin V_H signal sequences (Lob et al., *Cell* 33: 85–93, 1983; Watson *Nuc. Acids. Res.* 12: 5145–5164, 1984) and immunoglobulin V_κ signal sequences (Watson, ibid.). Particularly preferred signal sequences are the SUC2 signal sequence (Carlson et al., *Mol. Cell. Biol.* 3: 439–447, 1983) and PDGF receptor signal sequences. Alternatively, secretory signal sequences may be synthesized according to the rules established, for example, by von Heinje (*Eur. J. Biochem.* 133: 17–21, 1983; *J. Mol. Biol.* 184: 99–105, 1985; *Nuc. Acids. Res.* 14: 4683–3690, 1986).

Secretory signal sequences may be used singly or may be combined. For example, a first secretory signal sequence may be used singly or combined with a sequence encoding the third domain of Barrier (described in co-pending commonly assigned U.S. patent application Ser. No. 07/104,316, which is incorporated by reference herein in its entirety). The third domain of Barrier may be positioned in proper reading frame 3' of the DNA sequence of interest or 5' to the

DNA sequence and in proper reading frame with both the secretory signal sequence and the DNA sequence of interest.

In one embodiment of the present invention, a sequence encoding a dimerizing protein is joined to a sequence encoding a polypeptide chain of a polypeptide dimer or a receptor analog, and this fused sequence is joined in proper reading frame to a secretory signal sequence. As shown herein, the present invention utilizes such an arrangement to drive the association of the polypeptide or receptor analog to form a biologically active molecule upon secretion. Suitable dimerizing proteins include the *S. cerevisiae* repressible acid phosphatase (Mizunaga et al., *J. Biochem.* (Tokyo) 103: 321–326, 1988), the *S. cerevisiae* type 1 killer preprotoxin (Sturley et al., *EMBO J.* 5: 3381–3390, 1986), the *S. calsbergensis* alpha galactosidase melibiase (Sumner-Smith et al., *Gene* 36: 333–340, 1985), the *S. cerevisiae* invertase (Carlson et al., *Mol. Cell. Biol.* 3: 439–447, 1983), the *Neurospora crassa* ornithine decarboxylase (Digangi et al., *J. Biol. Chem.* 262: 7889–7893, 1987), immunoglobulin heavy chain hinge regions (Takahashi et al., *Cell* 29: 671–679, 1982), and other dimerizing immunoglobulin sequences. In a preferred embodiment, *S. cerevisiae* invertase is used to drive the association of polypeptides into dimers. Portions of dimerizing proteins, such as those mentioned above, may be used as dimerizing proteins where those portions are capable of associating as a dimer in a covalent or noncovlent manner. Such portions may be determined by, for example, altering a sequence encoding a dimerizing protein through in vitro mutagenesis to delete portions of the coding sequence. These deletion mutants may be expressed in the appropriate host to determine which portions retain the capability of associating as dimers. Portions of immunoglobulin gene sequences may be used to drive the association of non-immunoglobulin polypeptides. These portions correspond to discrete domains of immunoglobulins. Immunoglobulins comprise variable and constant regions, which in turn comprise discrete domains that show similarity in their three-dimensional conformations. These discrete domains correspond to immunoglobulin heavy chain constant region domain exons, immunoglobulin heavy chain variable region domain exons, immunoglobulin light chain variable region domain exons and immunoglobulin light chain constant region domain exons in immunoglobulin genes (Hood et al., in *Immunology*, The Benjamin/Cummings Publishing Company, Inc., Menlo Park, Calif.; Honjo et al., *Cell* 18: 559–568, 1979; Takahashi et al., *Cell* 29: 671–679, 1982; and Honjo, *Ann. Rev. Immun.* 1:499–528, 1983)). Particularly preferred portions of immunoglobulin heavy chains include Fab and Fab' fragments. (An Fab fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain variable region domain and a heavy chain constant region domain. An Fab' fragment is a portion of an immunoglobulin heavy chain that includes a heavy chain variable region domain, a heavy chain constant region domain and a heavy chain hinge region domain.)

It is preferred to use an immunoglobulin light chain constant region in association with at least one immunoglobulin heavy chain constant region domain. In another embodiment, an immunoglobulin light chain constant region is associated with at least one immunoglobulin heavy chain constant region domain joined to an immunoglobulin hinge region. In one set of embodiments, an immunoglobulin light chain constant region joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with at least one heavy chain constant region. In a preferred set of embodiments a variable region is joined upstream of and in proper reading frame with at least one immunoglobulin heavy chain constant region. In another set of embodiments, an immunoglobulin heavy chain is joined in frame with a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog and is associated with an immunoglobulin light chain constant region. In yet another set of embodiments, a polypeptide chain of a non-immunoglobulin polypeptide dimer or receptor analog is joined to at least one immunoglobulin heavy chain constant region which is joined to an immunoglobulin hinge region and is associated with an immunoglobulin light chain constant region. In a preferred set of embodiments an immunoglobulin variable region is joined upstream of and in proper reading frame with the immunoglobulin light chain constant region.

Immunoglobulin heavy chain constant region domains include C_H1, C_H2, C_H3, and C_H4 of any class of immunoglobulin heavy chain including γ, α, ε, μ, and δ classes (Honjo, ibid., 1983) A particularly preferred immunoglobulin heavy chain constant region domain is human C_H1. Immunoglobulin variable regions include V_H, V_κ, or V_λ. DNA sequences encoding immunoglobulins may be cloned from a variety of genomic or cDNA libraries known in the art. The techniques for isolating such DNA sequences using probe-based methods are conventional techniques and are well known to those skilled in the art. Probes for isolating such DNA sequences may be based on published DNA sequences (see, for example, Hieter et al., *Cell* 22: 197–207, 1980). Alternatively, the polymerase chain reaction (PCR) method disclosed by Mullis et al. (U.S. Pat. No. 4,683,195) and Mullis (U.S. Pat. No. 4,683,202), incorporated herein by reference may be used. The choice of library and selection of probes for the isolation of such DNA sequences is within the level of ordinary skill in the art.

Host cells for use in practicing the present invention include eukaryotic cells capable of being transformed or transfected with exogenous DNA and grown in culture, such as cultured mammalian and fungal cells. Fungal cells, including species of yeast (e.g., Saccharomyces spp., Schizosaccharomyces spp.), or filamentous fungi (e.g., Aspergillus spp., Neurospora spp.) may be used as host cells within the present invention. Strains of the yeast *Saccharomyces cerevisiae* are particularly preferred.

Expression units for use in the present invention will generally comprise the following elements, operably linked in a 5' to 3' orientation: a transcriptional promoter, a secretory signal sequence a DNA sequence encoding non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein and a transcriptional terminator. The selection of suitable promoters, signal sequences and terminators will be determined by the selected host cell and will be evident to one skilled in the art and are discussed more specifically below.

Suitable yeast vectors for use in the present invention include YRp7 (Struhl et al., *Proc. Natl. Acad. Sci. USA* 76: 1035–1039, 1978), YEp13 (Broach et al., *Gene* 8: 121–133, 1979), pJDB249 and pJDB219 (Beggs, *Nature* 275:104–108, 1978) and derivatives thereof. Such vectors will generally include a selectable marker, which may be one of any number of genes that exhibit a dominant phenotype for which a phenotypic assay exists to enable transformants to be selected. Preferred selectable markers are those that complement host cell auxotrophy, provide antibiotic resistance or enable a cell to utilize specific carbon sources, and include LEU2 (Broach et al. ibid.), URA3 (Botstein et al., *Gene* 8: 17, 1979), HIS3 (Struhl et al., ibid.) or POT1 (Kawasaki and Bell, EP 171,142). Other suitable selectable

markers include the CAT gene, which confers chloramphenicol resistance on yeast cells.

Preferred promoters for use in yeast include promoters from yeast glycolytic genes (Hitzeman et al., *J. Biol. Chem.* 255: 12073–12080, 1980; Alber and Kawasaki, *J. Mol. Appl. Genet.* 1: 419–434, 1982; Kawasaki, U.S. Pat. No. 4,599, 311) or alcohol dehydrogenase genes (Young et al., in *Genetic Engineering of Microorganisms for Chemicals*, Hollaender et al., (eds.), p. 355, Plenum, New York, 1982; Ammerer, *Meth. Enzymol.* 101: 192–201, 1983). In this regard, particularly preferred promoters are the TPII promoter (Kawasaki, U.S. Pat. No. 4,599,311, 1986) and the ADH2-4^c promoter (Russell et al., *Nature* 304: 652–654, 1983 and Irani and Kilgore, described in pending, commonly assigned U.S. patent application Ser. No. 07/183,130, which is incorporated herein by reference). The expression units may also include a transcriptional terminator. A preferred transcriptional terminator is the TPI1 terminator (Alber and Kawasaki, ibid.).

In addition to yeast, proteins of the present invention can be expressed in filamentous fungi, for example, strains of the fungi Aspergillus (McKnight and Upshall, described in commonly assigned U.S. Pat. No. 4,935,349, which is incorporated herein by reference). Examples of useful promoters include those derived from *Aspergillus nidulans* glycolytic genes, such as the ADH3 promoter (McKnight et al., *EMBO J.* 4: 2093–2099, 1985) and the tpiA promoter. An example of a suitable terminator is the ADH3 terminator (McKnight et al., ibid.). The expression units utilizing such components are cloned into vectors that are capable of insertion into the chromosomal DNA of Aspergillus.

Techniques for transforming fungi are well known in the literature, and have been described, for instance, by Beggs (ibid.), Hinnen et al. (*Proc. Natl. Acad. Sci. USA* 75: 1929–1933, 1978), Yelton et al., (*Proc. Natl. Acad. Sci. USA* 81: 1740–1747, 1984), and Russell (*Nature* 301: 167–169, 1983). The genotype of the host cell will generally contain a genetic defect that is complemented by the selectable marker present on the expression vector. Choice of a particular host and selectable marker is well within the level of ordinary skill in the art.

In a preferred embodiment, a *Saccharomyces cerevisiae* host cell that contains a genetic deficiency in a gene required for asparagine-linked glycosylation of glycoproteins is used. Preferably, the *S. cerevisiae* host cell contains a genetic deficiency in the MNN9 gene (described in pending, commonly assigned U.S. patent application Ser. Nos. 116,095 and 189,547 which are incorporated by reference herein in their entirety). Most preferably, the *S. cerevisiae* host cell contains a disruption of the MNN9 gene. *S. cerevisiae* host cells having such defects may be prepared using standard techniques of mutation and selection. Ballou et al. (*J. Biol. Chem.* 255: 5986–5991, 1980) have described the isolation of mannoprotein biosynthesis mutants that are defective in genes which affect asparagine-linked glycosylation. Briefly, mutagenized *S. cerevisiae* cells were screened using fluorescinated antibodies directed against the outer mannose chains present on wild-type yeast. Mutant cells that did not bind antibody were further characterized and were found to be defective in the addition of asparagine-linked oligosaccharide moieties. To optimize production of the heterologous proteins, it is preferred that the host strain carries a mutation, such as the *S. cerevisiae* pep4 mutation (Jones, *Genetics* 85: 23–33, 1977), which results in reduced proteolytic activity.

In addition to fungal cells, cultured mammalian cells may be used as host cells within the present invention. Preferred cell lines are rodent myeloma cell lines, which include p3X63Ag8 (ATCC TIB 9), FO (ATCC CRL 1646), NS-1 (ATCC TIB 18) and 210-RCY-Ag1 (Galfre et al., *Nature* 277: 131, 1979). A particularly preferred rodent myeloma cell line is SP2/0-Ag14 (ATCC CRL 1581). In addition, a number of other cell lines may be used within the present invention, including COS-1 (ATCC CRL 1650), BHK, p363.Ag.8.653 (ATCC CRL 1580) Rat Hep I (ATCC CRL 1600), Rat Hep II (ATCC CRL 1548), TCMK (ATCC CCL 139), Human lung (ATCC CCL 75.1), Human hepatoma (ATCC HTB-52), Hep G2 (ATCC HB 8065), Mouse liver (ATCC CC 29.1), 293 (ATCC CRL 1573; Graham et al., *J. Gen. Virol.* 36: 59–72, 1977) and DUKX cells (Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA* 77: 4216–4220, 1980). A preferred BHK cell line is the tk$^-$ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79: 1106–1110, 1982). A preferred BHK cell line is the tk$^-$ts13 BHK cell line (Waechter and Baserga, *Proc. Natl. Acad. Sci. USA* 79: 1106–1110, 1982). A tk$^-$ BHK cell line is available from the American Type Culture Collection, Rockville, Md., under accession number CRL 1632. A particularly preferred tk$^-$ BHK cell line is BHK 570 which is available from the American Type Culture Collection under accession number 10314.

Mammalian expression vectors for use in carrying out the present invention will include a promoter capable of directing the transcription of a cloned gene or cDNA. Preferred promoters include viral promoters and cellular promoters. Preferred viral promoters include the major late promoter from adenovirus 2 (Kaufman and Sharp, *Mol. Cell. Biol.* 2: 1304–13199, 1982) and the SV40 promoter (Subramani et al., *Mol. Cell. Biol.* 1: 854–864, 1981). Preferred cellular promoters include the mouse metallothionein 1 promoter (Palmiter et al., *Science* 222: 809–814, 1983) and a mouse V_κ promoter (Grant et al., *Nuc. Acids Res.* 15: 5496, 1987). A particularly preferred promoter is a mouse V_H promoter (Loh et al., ibid.). Such expression vectors may also contain a set of RNA splice sites located downstream from the promoter and upstream from the DNA sequence encoding the peptide or protein of interest. Preferred RNA splice sites may be obtained from adenovirus and/or immunoglobulin genes. Also contained in the expression vectors is a polyadenylation signal located downstream of the coding sequence of interest. Polyadenylation signals include the early or late polyadenylation signals from SV40 (Kaufman and Sharp, ibid.), the polyadenylation signal from the adenovirus 5 E1B region and the human growth hormone gene terminator (DeNoto et al., *Nuc. Acids Res.* 9: 3719–3730, 1981). A particularly preferred polyadenylation signal is the V_H gene terminator (Loh et al., ibid.). The expression vectors may include a noncoding viral leader sequence, such as the adenovirus 2 tripartite leader, located between the promoter and the RNA splice sites. Preferred vectors may also include enhancer sequences, such as the SV40 enhancer and the mouse μ enhancer (Gillies, *Cell* 33: 717–728, 1983). Expression vectors may also include sequences encoding the adenovirus VA RNAs.

Cloned DNA sequences may be introduced into cultured mammalian cells by, for example, calcium phosphate-mediated transfection (Wigler et al., *Cell* 14: 725, 1978; Corsaro and Pearson, *Somatic Cell Genetics* 7: 603, 1981; Graham and Van der Eb, *Virology* 52: 456, 1973.) Other techniques for introducing cloned DNA sequences into mammalian cells, such as electroporation (Neumann et al., *EMBO J.* 1: 841–845, 1982), may also be used. In order to identify cells that have integrated the cloned DNA, a selectable marker is generally introduced into the cells along with

the gene or cDNA of interest. Preferred selectable markers for use in cultured mammalian cells include genes that confer resistance to drugs, such as neomycin, hygromycin, and methotrexate. The selectable marker may be an amplifiable selectable marker. A preferred amplifiable selectable marker is the DHFR gene.

A particularly preferred amplifiable marker is the DHFR^r cDNA (Simonsen and Levinson, *Proc. Natl. Adac. Sci. USA* 80: 2495–2499, 1983). Selectable markers are reviewed by Thilly (*Mammalian Cell Technology*, Butterworth Publishers, Stoneham, Mass.) and the choice of selectable markers is well within the level of ordinary skill in the art.

Selectable markers may be introduced into the cell on a separate plasmid at the same time as the gene of interest, or they may be introduced on the same plasmid. If on the same plasmid, the selectable marker and the gene of interest may be under the control of different promoters or the same promoter, the latter arrangement producing a dicistronic message. Constructs of this type are known in the art (for example, Levinson and Simonsen, U.S. Pat. No. 4,713,339). It may also be advantageous to add additional DNA, known as "carrier DNA" to the mixture which is introduced into the cells.

Transfected mammalian cells are allowed to grow for a period of time, typically 1–2 days, to begin expressing the DNA sequence(s) of interest. Drug selection is then applied to select for growth of cells that are expressing the selectable marker in a stable fashion. For cells that have been transfected with an amplifiable selectable marker the drug concentration may be increased in a stepwise manner to select for increased copy number of the cloned sequences, thereby increasing expression levels.

Host cells containing DNA constructs of the present invention are grown in an appropriate growth medium. As used herein, the term "appropriate growth medium" means a medium containing nutrients required for the growth of cells. Nutrients required for cell growth may include a carbon source, a nitrogen source, essential amino acids, vitamins, minerals and growth factors. The growth medium will generally select for cells containing the DNA construct by, for example, drug selection or deficiency in an essential nutrient which are complemented by the selectable marker on the DNA construct or co-transfected with the DNA construct. Yeast cells, for example, are preferably grown in a chemically defined medium, comprising a non-amino acid nitrogen source, inorganic salts, vitamins and essential amino acid supplements. The pH of the medium is preferably maintained at a pH greater than 2 and less than 8, preferably at pH 6.5. Methods for maintaining a stable pH include buffering and constant pH control, preferably through the addition of sodium hydroxide. Preferred buffering agents include succinic acid and Bis-Tris (Sigma Chemical Co., St. Louis, Mo.). Yeast cells having a defect in a gene required for asparagine-linked glycosylation are preferably grown in a medium containing an osmotic stabilizer. A preferred osmotic stabilizer is sorbitol supplemented into the medium at a concentration between 25 0.1M and 1.5M., preferably at 0.5M or 1.0M. Cultured mammalian cells are generally grown in commercially available serum-containing or serum-free media. Selection of a medium appropriate for the particular cell line used is within the level of ordinary skill in the art.

The culture medium from appropriately grown transformed or transfected host cells is separated from the cell material, and the presence of dimerized polypeptide fusions or secreted receptor analogs is demonstrated. A preferred method of detecting receptor analogs, for example, is by the binding of the receptors or portions of receptors to a receptor-specific antibody. An anti-receptor antibody may be a monoclonal or polyclonal antibody raised against the receptor in question, for example, an anti-PDGF receptor monoclonal antibody may be used to assay for the presence of PDGF receptor analogs. Another antibody, which may be used for detecting substance P-tagged peptides and proteins, is a commercially available rat anti-substance P monoclonal antibody which may be utilized to visualize peptides or proteins that are fused to the C-terminal amino acids of substance P. Ligand binding assays may also be used to detect the presence of receptor analogs. In the case of PDGF receptor analogs, it is preferable to use fetal foreskin fibroblasts, which express PDGF receptors, to compete against the PDGF receptor analogs of the present invention for labeled PDGF and PDGF isoforms.

Assays for detection of secreted, biologically active peptide dimers and receptor analogs may include Western transfer, protein blot or colony filter. A Western transfer filter may be prepared using the method described by Towbin et al (*Proc. Natl. Acad. Sci. USA* 76: 4350–4354, 1979). Briefly, samples are electrophoresed in a sodium dodecylsulfate polyacrylamide gel. The proteins in the gel are electrophoretically transferred to nitrocellulose paper. Protein blot filters may be prepared by filtering supernatant samples or concentrates through nitrocellulose filters using, for example, a Minifold (Schleicher & Schuell, Keene, N.H.). Colony filters may be prepared by growing colonies on a nitrocellulose filter that has been laid across an appropriate growth medium. In this method, a solid medium is preferred. The cells are allowed to grow on the filters for at least 12 hours. The cells are removed from the filters by washing with an appropriate buffer that does not remove the proteins bound to the filters. A preferred buffer comprises 25 mM Tris-base, 19 mM glycine, pH 8.3, 20% methanol.

The dimerized polypeptide fusions and receptor analogs present on the Western transfer, protein blot or colony filters may be visualized by specific antibody binding using methods known in the art. For example, Towbin et al. (ibid.) describe the visualization of proteins immobilized on nitrocellulose filters with a specific antibody followed by a labeled second antibody, directed against the first antibody. Kits and reagents required for visualization are commercially available, for example, from Vector Laboratories, (Burlingame, Calif.), and Sigma Chemical Company (St. Louis, Mo.).

Secreted, biologically active dimerized polypeptide fusions and receptor analogs may be isolated from the medium of host cells grown under conditions that allow the secretion of the biologically active dimerized polypeptide fusions and receptor analogs. The cell material is removed from the culture medium, and the biologically active dimerized polypeptide fusions and receptor analogs are isolated using isolation techniques known in the art. Suitable isolation techniques include precipitation and fractionation by a variety of chromatographic methods, including gel filtration, ion exchange chromatography and immunoaffinity chromatography. A particularly preferred purification method is immunoaffinity chromatography using an antibody directed against the receptor analog or dimerized polypeptide fusion. The antibody is preferably immobilized or attached to a solid support or substrate. A particularly preferred substrate is CNBr-activated Sepharose (Pharmacia LKB Technologies, Inc., Piscataway, N.J.). By this method, the medium is combined with the antibody/substrate under conditions that will allow binding to occur. The complex

may be washed to remove unbound material, and the receptor analog or peptide dimer is released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods of elution include changes in pH, wherein the immobilized antibody has a high affinity for the ligand at a first pH and a reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs of the present invention can be used within a variety of assays for detecting the presence of and/or screening for native PDGF, PDGF isoforms or PDGF-like molecules. These assays will typically involve combining PDGF receptor analogs, which may be bound to a solid substrate such as polymeric microtiter plate wells, with a biological sample under conditions that permit the formation of receptor/ligand complexes. Screening assays for the detection of PDGF, PDGF isoforms or PDGF-like molecules will typically involve combining soluble PDGF receptor analogs with a biological sample and incubating the mixture with a PDGF isoform or mixture of PDGF isoforms bound to a solid substrate such as polymeric microtiter plates, under conditions that permit the formation of receptor/ligand complexes. Detection may be achieved through the use of a label attached to the receptor or through the use of a labeled antibody which is reactive with the receptor. Alternatively, the labeled antibody may be reactive with the ligand. A wide variety of labels may be utilized, such as radionuclides, fluorophores, enzymes and luminescers. Complexes may also be detected visually, i.e., in immunoprecipitation assays, which do not require the use of a label.

Secreted PDGF receptor analogs of the present invention may also be labeled with a radioisotope or other imaging agent and used for in vivo diagnostic purposes. Preferred radioisotope imaging agents include iodine-125 and technetium-99, with technetium-99 being particularly preferred. Methods for producing protein-isotope conjugates are well known in the art, and are described by, for example, Eckelman et al. (U.S. Pat. No. 4,652,440), Parker et al. (WO 87/05030) and Wilbur et al. (EP 203,764). Alternatively, the secreted receptor analogs may be bound to spin label enhancers and used for magnetic resonance (MR) imaging. Suitable spin label enhancers include stable, sterically hindered, free radical compounds such as nitroxides. Methods for labeling ligands for MR imaging are disclosed by, for example, Coffman et al. (U.S. Pat. No. 4,656,026). For administration, the labeled receptor analogs are combined with a pharmaceutically acceptable carrier or diluent, such as sterile saline or sterile water. Administration is preferably by bolus injection, preferably intravenously. These imaging agents are particularly useful in identifying the locations of atherosclerotic plaques, PDGF-producing tumors, and receptor-bound PDGF.

The secreted PDGF receptor analogs of the present invention may also be utilized within diagnostic kits. Briefly, the subject receptor analogs are preferably provided in a lyophilized form or immobilized onto the walls of a suitable container, either alone or in conjunction with antibodies capable of binding to native PDGF or selected PDGF isoform(s) or specific ligands. The antibodies, which may be conjugated to a label or unconjugated, are generally included in the kits with suitable buffers, such as phosphate, stabilizers, inert proteins or the like. Generally, these materials are present in less than about 5% weight based upon the amount of active receptor analog, and are usually present in an amount of at least about 0.001% weight. It may also be desirable to include an inert excipient to dilute the active ingredients. Such an excipient may be present from about 1% to 99% weight of the total composition. In addition, the kits will typically include other standard reagents, instructions and, depending upon the nature of the label involved, reactants that are required to produce a detectable product. Where an antibody capable of binding to the receptor or receptor/ligand complex is employed, this antibody will usually be provided in a separate vial. The antibody is typically conjugated to a label and formulated in an analogous manner with the formulations briefly described above. The diagnostic kits, including the containers, may be produced and packaged using conventional kit manufacturing procedures.

As noted above, the secreted PDGF receptor analogs of the present invention may be utilized within methods for purifying PDGF from a variety of samples. Within a preferred method, the secreted PDGF receptor analogs are immobilized or attached to a substrate or support material, such as polymeric tubes, beads, polysaccharide particulates, polysaccharide-containing materials, polyacrylamide or other water insoluble polymeric materials. Methods for immobilization are well known in the art (Mosbach et al., U.S. Pat. No. 4,415,665; Clarke et al., *Meth. Enzymology* 68: 436–442, 1979). A common method of immobilization is CNBr activation. Activated substrates are commercially available from a number of suppliers, including Pharmacia (Piscataway, N.J.), Pierce Chemical Co. (Rockford, Ill.) and Bio-Rad Laboratories (Richmond, Calif.). A preferred substrate is CNBr-activated Sepharose (Pharmacia, Piscataway, N.J.). Generally, the substrate/receptor analog complex will be in the form of a column. The sample is then combined with the immobilized receptor analog under conditions that allow binding to occur. The substrate with immobilized receptor analog is first equilibrated with a buffer solution of a composition in which the receptor analog has been previously found to bind its ligand. The sample, in the solution used for equilibration, is then applied to the substrate/receptor analog complex. Where the complex is in the form of a column, it is preferred that the sample be passed over the column two or more times to permit full binding of ligand to receptor analog. The complex is then washed with the same solution to elute unbound material. In addition, a second wash solution may be used to minimize nonspecific binding. The bound material may then be released or eluted through the use of conditions unfavorable to complex formation. Particularly useful methods include changes in pH, wherein the immobilized receptor has a high affinity for PDGF at a first pH and reduced affinity at a second (higher or lower) pH; changes in concentration of certain chaotropic agents; or through the use of detergents.

The secreted PDGF receptor analogs fused to dimerizing proteins of the present invention may be used in pharmaceutical compositions for topical or intravenous application. The secreted PDGF receptor analogs of the present invention may be useful in the treatment of atherosclerosis by, for example, binding endogenous PDGF to prevent smooth muscle cell proliferation. The PDGF receptor analogs fused to dimerizing proteins are used in combination with a physiologically acceptable carrier or diluent. Preferred carriers and diluents include saline and sterile water. Pharmaceutical compositions may also contain stabilizers and adjuvants. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with a sterile aqueous solution prior to administration.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

Enzymes, including restriction enzymes, DNA polymerase I (Klenow fragment), T4 DNA polymerase, T4 DNA ligase and T4 polynucleotide kinase, were obtained from New England Biolabs (Beverly, Mass.), GIBCO-BRL (Gaithersburg, Md.) and Boerhinger-Mannheim Biochemicals (Indianapolis, Ind.) and were used as directed by the manufacturer or as described in Maniatis et al. (*Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y., 1982) and Sambrook et al. (*Molecular Cloning: A Laboratory Manual*/Second Edition, Cold Spring Harbor Laboratory, N.Y., 1989).

EXAMPLE 1

Cloning PDGF Receptor cDNAs

A. Cloning the PDGF β-Receptor

A cDNA encoding the PDGF β-receptor was cloned as follows. Complementary DNA (cDNA) libraries were prepared from poly(A) RNA from diploid human dermal fibroblast cells, prepared by explant from a normal adult, essentially as described by Hagen et al. (*Proc. Natl. Acad. Sci. USA* 83: 2412–2416, 1986). Briefly, the poly(A) RNA was primed with oligo d(T) and cloned into λgt11 using Eco RI linkers. The random primed library was screened for the presence of human PDGF receptor cDNA's using three oligonucleotide probes complementary to sequences of the mouse PDGF receptor (Yarden et al., *Nature* 323: 226–232, 1986). Approximately one million phage from the random primed human fibroblast cell library were screened using oligonucleotides ZC904, ZC905 and ZC906 (Table 1; Sequence ID Numbers 5, 6 and 7, respectively). Eight positive clones were identified and plaque purified. Two clones, designated RP41 and RP51, were selected for further analysis by restriction enzyme mapping and DNA sequence analysis. RP51 was found to contain 356 bp of 5'-noncoding sequence, the ATG translation initiation codon and 738 bp of the amino terminal coding sequence. RP41 was found to overlap clone RP51 and contained 2649 bp encoding amino acids 43–925 of the β-receptor protein.

TABLE 1

Oligonucleotide Sequences

ZC873 (Sequence ID Number 3)

5'CTC TCT TCC TCA GGT AAA TGA GTG CCA GGG CCG GCA AGC CCC CGC TCC 3'

ZC872 (Sequence ID Number 4)

5'CCG GGG AGC GGG GGC TTG CCG GCC CTG GCA CTC ATT TAC CTG AGG AAG AGA GAG CT 3'

ZC904 (Sequence ID Number 5)

5'CAT GGG CAC GTA ATC TAT AGA TTC ATC CTT GCT CAT ATC CAT GTA 3'

ZC905 (Sequence ID Number 6)

5'TCT TGC CAG GGC ACC TGG GAC ATC TGT TCC CAC ATC ACC GG 3'

ZC906 (Sequence ID Number 7)

5'AAG CTG TCC TCT GCT TCA GCC AGA GGT CCT GGG CAG CC 3'

ZC1380 (Sequence ID Number 8)

5'CAT GGT GGA ATT CCT GCT GAT 3'

ZC1447 (Sequence ID Number 9)

5'TG GTT GTG CAG AGC TGA GGA AGA GAT GGA 3'

ZC1453 (Sequence ID Number 10)

5'AAT TCA TTA TGT TGT TGC AAG CCT TCT TGT TCC TGC TAG CTG GTT TCG CTG TTA A 3'

ZC1454 (Sequence ID Number 11)

5'GAT CTT AAC AGC GAA ACC AGC TAG CAG GAA CAA GAA GGC TTG CAA CAA CAT AAT G 3'

ZC1478 (Sequence ID Number 12)

5'ATC GCG AGC ATG CAG ATC TGA 3

ZC1479 (Sequence ID Number 13)

TABLE 1-continued

5'AGC TTC AGA TCT GCA TGC TGC CGA T 3'

ZC1776 (Sequence ID Number 14)

5'AGC TGA GCG CAA ATG TTG TGT CGA GTG CCC ACC GTG CCC AGC TTA GAA TTC T 3'

ZC1777 (Sequence ID Number 15)

5'CTA GAG AAT TCT AAG CTG GGC ACG GTG GGC ACT CGA CAC AAC ATT TGC GCT C 3'

ZC1846 (Sequence ID Number 16)

5'GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT GTG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG CCC TTT AAG CA 3'

ZC1847 (Sequence ID Number 17)

5'AGC TTG CTT AAA GGG CAA GGA GTG TGG CAC CAC GAT GAC CTC CTG CGT GTC CTG GCC CAC AGC GTT GCG CAG CGT GCA GCG CAC CGA CAG TGG CC 3'

ZC1886 (Sequence ID Number 18)

5'CCA GTG CCA AGC TTG TCT AGA CTT ACC TTT AAA GGG CAA GGA G 3'

ZC1892 (Sequence ID Number 19)

5'AGC TTG AGC GT 3'

ZC1893 (Sequence ID Number 20)

5'CTA GAC GCT CA 3'

ZC1894 (Sequence ID Number 21)

5'AGC TTC CAG TTC TTC GGC CTC ATG TCA GTT CTT CGG CCT CAT GTG AT 3'

ZC1895 (Sequence ID Number 22)

5'CTA GAT CAC ATG AGG CCG AAG AAC TGA CAT GAG GCC GAA GAA CTG GA 3'

ZC2181 (Sequence ID Number 23)

5'AAT TCG GAT CCA CCA TGG GCA CCA GCC ACC CGG CGT TCC TGG TGT TAG GCT GCC TGC TGA CCG GCC 3'

ZC2182 (Sequence ID Number 24)

5'TGA GCC TGA TCC TGT GCC AAC TGA GCC TGC CAT CGA TCC TGC CAA ACG AGA ACG AGA AGG TTG TGC AGC TA 3'

ZC2183 (Sequence ID Number 25)

5'AAT TTA GCT GCA CAA CCT TCT CGT TCT CGT TTG GCA GGA TCG ATG GCA GGC TCA GTT GGC ACA GGA TCA 3'

ZC2184 (Sequence ID Number 26)

5'GGC TCA GGC CGG TCA GCA GGC AGC CTA ACA CCA GGA ACG CCG GGT GGC TGG TGC CCA TGG TGG ATC CG 3'

ZC2311 (Sequence ID Number 27)

5' TGA TCA CCA TGG CTC AAC TG 3'

ZC2351 (Sequence ID Number 28)

5'CGA ATT CCA C 3'



TABLE 1-continued

ZC2352 (Sequence ID Number 29)

5'CAT GGT GGA ATT CGA GCT 3'

ZC2392 (Sequence ID Number 30)

5'ACG TAA GCT TGT CTA GAC TTA CCT TCA GAA CGC AGG GTG GG 3'

The 3'-end of the cDNA was not isolated in the first cloning and was subsequently isolated by screening 6×10^5 phage of the oligo d(T)-primed cDNA library with a 630 bp Sst I-Eco RI fragment derived from the 3'-end of clone RP41. One isolate, designated OT91, was further analyzed by restriction enzyme mapping and DNA sequencing. This clone was found to comprise the 3'-end of the receptor coding region and 1986 bp of 3' untranslated sequence.

Clones RP51, RP41 and OT91 were ligated together to construct a full-length cDNA encoding the entire PDGF β-receptor. RP41 was digested with Acc I and Bam HI to isolate the 2.12 kb fragment. RP51 was digested with Eco RI and Acc I to isolate the 982 bp fragment. The 2.12 kb RP41 fragment and the 982 bp RP51 fragment were joined in a three-part ligation with pUC13, which had been linearized by digestion with Eco RI and Bam HI. The resultant plasmid was designated 51/41. Plasmid 51/41 was digested with Eco RI and Bam HI to isolate the 3 kb fragment comprising the partial PDGF receptor cDNA. OT91 was digested with Bam HI and Xba I to isolate the 1.4 kb fragment containing the 3' portion of the PDGF receptor cDNA. The Eco RI-Bam HI 51/41 fragment, the Bam HI-Xba I OT91 fragment and the Eco RI-Xba I digested pUC13 were joined in a three-part ligation. The resultant plasmid was designated pR-RX1 (FIG. 2).

B. Cloning the PDGF-α Receptor

A cDNA encoding to PDGF α-receptor was cloned as follows. RNA was prepared by the method of Chirgwin et al. (*Biochemistry* 18: 5294, 1979) and twice purified on oligo d(T) cellulose to yield poly(A)+RNA. Complementary DNA was prepared in λgt10 phage using a kit purchased from Invitrogen (San Diego, Calif.). The resulting λ phage DNA was packaged with a coat particle mixture from Stratagene Cloning Systems (La Jolla, Calif.), infected into *E. coli* strain C600 Hfl^{-} and titered.

Approximately 1.4×10^6 phage recombinants were plated to produce plaques for screening. Nitrocellulose filter lifts were prepared according to standard methods and were hybridized to a radiolabeled PDGF β-receptor DNA fragment (Gronwald et al., ibid) comprising the 1.9 kb Fsp I-Hind III fragment that encodes the transmembrane and cytoplasmic domains of the PDGF β-receptor cDNA. Hybridization was performed for 36 hours at 42° C. in a mixture containing 40% formamide, 5×SSCP (SSC containing 25 mM phosphate buffer, pH 6.5), 200 μg/ml denatured salmon sperm DNA, 3×Denhardt's, and 10% dextran sulfate. Following hybridization, the filters were washed extensively at room temperature in 2×SSC, then for 15 minutes at 47°–48° C. Following an exposure to X-ray film, the filters were treated to increasingly stringent wash conditions followed by film recording until a final wash with 0.1×SSC at 65° C. was reached. Film analysis showed that a "family" of plaques that hybridized at lower wash stringency but not at the highest stringency. This "family" was selected for further analysis.

Two λ phage clones from the "family" obtained from the initial screening were subcloned into the Not I site of the pUC-type plasmid vector pBluescript SK^{+} (obtained from Stratagene Cloning Systems, La Jolla, Calif.) and were analyzed by restriction mapping and sequence analysis. Restriction enzyme analysis of a phage clone, designated α1-1, revealed a restriction fragment pattern dissimilar from that of the PDGF β-receptor cDNA with the exception of a common Bgl II-Bgl II band of approximately 160 bp. The PDGF β-receptor cDNA contains two similarly spaced Bgl II sites within the region coding for the second tyrosine kinase domain.

Restriction analysis of a second plasmid subclone (designated α1-7) revealed an overlap of the 5' approximately 1.2 kb of clone α1-1, and an additional approximately 2.2 kb of sequence extending in the 5' direction. Sequence analysis revealed that the 3' end of this clone encodes the second tyrosine kinase domain, which contains regions of near sequence identity to the corresponding regions in the PDGF β-receptor. The 5' end of clone α1-7 contained non-receptor sequences. Two additional α-receptor clones were obtained by probing with α1-1 sequences. Clone α1-1 was digested with Not I and Spe I, and a 230 bp fragment was recovered. Clone α1-1 was also digested with Bam HI and Not I, and a 550 bp fragment was recovered. A clone that hybridized to the 230 bp probe was designated α5-1. This clone contained the 5'-most coding sequence for the PDGF α-receptor. Another clone, designated α6-3, hybridized to the 550 bp probe and was found to contain 3' coding and non-coding sequences, including the poly(A) tail.

Clone α1-1 was radiolabeled (^{32}P) and used to probe a northern blot (Thomas, *Methods Enzymol.* 100: 225–265, 1983) of the MG-63 poly(A)+RNA used to prepare the cDNA library. A single band of approximately 6.6 kb was observed. RNA prepared from receptor-positive cell lines including the human fibroblast SK4, WI-38 and 7573 cell lines; the mouse fibroblast line DI 3T3; the U2-OS human osteosarcoma cell line and baboon aortic smooth muscle cells, and RNA prepared from receptor-negative lines including A431 (an epithelial cell line) and VA 13 (SV40-transformed WI-38 cells) were probed by northern format with the α1-1 cDNA. In all cases, the amount of the 6.6 kb band detected in these RNA correlated well with the relative levels of α-receptor detected on the respective cell surfaces. The 6.6 kb RNA was not detected in RNA prepared from any tested cell line of hematopoietic origin, in agreement with a lack of PDGF α-receptor protein detected on these cell types.

Clones α1-1 and α1-7 were joined at a unique Pst I site in the region encoding the transmembrane portion of the receptor. Clone α1-1 was digested with Xba I and Pst I and the receptor sequence fragment was recovered. Clone α1-7 was digested with Pst I and Bam HI and the receptor fragment was recovered. The two fragments were ligated

with Xba I+Bam HI-digested pIC19R (Marsh et al. *Gene* 32: 481–486, 1984) to construct plasmid pα17R (FIG. 12).

The remainder of the 5'-most α-receptor sequence was obtained from clone α5-1 as an Sst I-C1a I fragment. This fragment was joined to the Eco RI-Sst I receptor fragment of pα17R and cloned into Eco RI+C1a I-digested pBluescript SK+plasmid to construct plasmid pα17B (FIG. 12). FIGS. 11A–D (Sequence ID Numbers 35 and 36) shows the nucleotide sequence and deduced amino acid sequence of the cDNA present in pα17B.

EXAMPLE 2

Construction of a SUC2 Signal Sequence-PDGF β-Receptor Fusion

To direct the PDGF β-receptor into the yeast secretory pathway, the PDGF β-receptor cDNA was joined to a sequence encoding the *Saccharomyces cerevisiae* SUC2 signal sequence. Oligonucleotides ZC1453 and ZC1454 (Sequence ID Numbers 10 and 11; Table 1) were designed to form an adapter encoding the SUC2 secretory signal flanked by a 5' Eco RI adhesive end and a 3' Bgl II adhesive end. ZC1453 and ZC1454 were annealed under conditions described by Maniatis et al. (ibid.). Plasmid pR-RX1 was digested with Bgl II and Sst II to isolate the 1.7 kb fragment comprising the PDGF β-receptor coding sequence from amino acids 28 to 596. Plasmid pR-RX1 was also cut with Sst II and Hind III to isolate the 1.7 kb fragment comprising the coding sequence from amino acids 597 through the translation termination codon and 124 bp of 3' untranslated DNA. The two 1.7 kb pR-RX1 fragments and the ZC1453/ZC1454 adapter were joined with pUC19, which had been linearized by digestion with Eco RI and Hind III. The resultant plasmid, comprising the SUC2 signal sequence fused in-frame with the PDGF β-receptor cDNA, was designated pBTL10 (FIG. 2).

EXAMPLE 3

Construction of pCBS22

The BAR1 gene product, Barrier, is an exported protein that has been shown to have three domains. When used in conjunction with a first signal sequence, the third domain of Barrier protein has been shown to aid in the secretion of proteins into the medium (MacKay et al., U.S. patent application Ser. No. 104,316).

The portion of the BAR1 gene encoding the third domain of Barrier was joined to a sequence encoding the C-terminal portion of substance P (subp; Munro and Pelham, *EMBO J.* 3: 3087–3093, 1984). The presence of the substance P amino acids on the terminus of the fusion protein allowed the protein to be detected using commercially available anti-substance P antibodies. Plasmid pZV9 (deposited as a transformant in *E. coli* strain RR1, ATCC accession no. 53283), comprising the entire BAR1 coding region and its associated flanking regions, was cut with Sal I and Bam HI to isolate the 1.3 kb BAR1 fragment. This fragment was subcloned into pUC13, which had been cut with Sal I and Bam HI, to generate the plasmid designated pZV17. Plasmid pZV17 was digested with Eco RI to remove the 3'-most 0.5 kb of the BAR1 coding region. The vector-BAR1 fragment was religated to create the plasmid designated pJH66 (FIG. 3). Plasmid pJH66 was linearized with Eco RI and blunt-ended with DNA polymerase I (Klenow fragment). Kinased Bam HI linkers (5' CCG GAT CCG G 3') were added and excess linkers were removed by digestion with Bam HI before religation. The resultant plasmid was designated pSW8 (FIG. 3).

Plasmid pSW81, comprising the TPI1 promoter, the BAR1 coding region fused to the coding region of the C-terminal portion of substance P (Munro and Pelham, *EMBO J.* 3: 3087–3093, 1984) and the TPI1 terminator, was derived from pSW8. Plasmid pSW8 was cut with Sal I and Bam HI to isolate the 824 bp fragment encoding amino acids 252 through 526 of BAR1. Plasmid pPM2, containing the synthetic oligonucleotide sequence encoding the dimer form of the C-terminal portion of substance P (subP) in M13mp8, was obtained from Hugh Pelham (MRC Laboratory of Molecular Biology, Cambridge, England). Plasmid pPM2 was linearized by digestion with Bam HI and Sal I and ligated with the 824 bp BAR1 fragment from pSW8. The resultant plasmid, pSW14, was digested with Sal I and Sma I to isolate the 871 bp BAR1-substance P fragment. Plasmid pSW16, comprising a fragment of BAR1 encoding amino acids 1 through 250, was cut with Xba I and Sal I to isolate the 767 bp BAR1 fragment. This fragment was ligated with the 871 bp BAR1-substance P fragment in a three-part ligation with pUC18 cut with Xba I and Sma I. The resultant plasmid, designated pSW15, was digested with Xba I and Sma I to isolate the 1.64 kb BAR1-substance P fragment. The ADH1 promoter was obtained from pRL029. Plasmid pRL029, comprising the ADH1 promoter and the BAR1 5' region encoding amino acids 1 to 33 in pUC18, was digested with Sph I and Xba I to isolate the 0.42 kb ADH1 promoter fragment. The TPI1 terminator (Alber and Kawasaki, ibid.) was provided as a linearized fragment containing the TPI1 terminator and pUC18 with a Klenow-filled Xba I end and an Sph I end. This fragment was ligated with the 0.42 kb ADH1 promoter fragment and the 1.64 kb BAR1-substance P fragment in a three-part ligation to produce plasmid pSW22.

The ADH1 promoter and the coding region of BAR1, from the translation initiation ATG through the Eco RV site present in pSW22, were removed by digestion with Hind III and Eco RV. The 3.9 kb vector fragment, comprising the 401 bp between the Eco RV and the Eco RI sites of the BAR1 gene fused to subP and the TPI1 terminator, was isolated by gel electrophoresis. Oligonucleotide ZC1478 (Sequence ID Number 12; Table 1) was kinased and annealed with oligonucleotide ZC1479 (Sequence ID Number 13; Table 1) using conditions described by Maniatis et al. (ibid.). The annealed oligonucleotides formed an adapter comprising a Hind III adhesive end and a polylinker encoding Bgl II, Sph I, Nru I and Eco RV restriction sites. The ZC1479/ZC1478 adapter was ligated with the gel-purified pSW22 fragment. The resultant plasmid was designated pCBS22 (FIG. 3).

EXAMPLE 4

Construction of pBTL13

In order to enhance the secretion of the PDGF β-receptor and to facilitate the identification of the secreted protein, a sequence encoding the third domain of BAR1 fused to the C-terminal amino acids of substance P was fused in frame with the 5' 1240 bp of the PDGF β-receptor. Plasmid pBTL10 (Example 2) was digested with Sph I and Sst I to isolate the 4 kb fragment comprising the SUC2 signal sequence, a portion of the PDGF β-receptor cDNA and the pUC19 vector sequences. Plasmid pCBS22 was digested with Sph I and Sst I to isolate the 1.2 kb fragment comprising the BAR1-subP fusion and the TPI1 terminator. These two fragments were ligated, and the resultant plasmid was designated pBTL13 (FIG. 4).

EXAMPLE 5

Construction of an Expression Vector Encoding the Entire PDGF β-Receptor

The entire PDGF β-receptor was directed into the secretory pathway by fusing a SUC2 signal sequence to the 5' end

of the PDGF β-receptor coding sequence. This fusion was placed behind the TPI1 promoter and inserted into the vector YEp13 for expression in yeast.

The TPI1 promoter was obtained from plasmid pTPIC10 (Alber and Kawasaki, *J. Mol. Appl. Genet.* 1: 410–434, 1982), and plasmid pFATPOT (Kawasaki and Bell, EP 171,142; ATCC 20699). Plasmid pTPIC10 was cut at the unique Kpn I site, the TPI1 coding region was removed with Bal-31 exonuclease, and an Eco RI linker (sequence: GGA ATT CC) was added to the 3' end of the promoter. Digestion with Bgl II and Eco RI yielded a TPI1 promoter fragment having Bgl II and Eco RI sticky ends. This fragment was then joined to plasmid YRp7' (Stinchcomb et al., *Nature* 282: 39–43, 1979) that had been cut with Bgl II and Eco RI (partial). The resulting plasmid, TE32, was cleaved with Eco RI (partial) and Bam HI to remove a portion of the tetracycline resistance gene. The linearized plasmid was then recircularized by the addition of an Eco RI-Bam HI linker to produce plasmid TEA32. Plasmid TEA32 was digested with Bgl II and Eco RI, and the 900 bp partial TPI1 promoter fragment was gel-purified. Plasmid pIC19H (Marsh et al., *Gene* 32:481–486, 1984) was cut with Bgl II and Eco RI and the vector fragment was gel purified. The TPI1 promoter fragment was then ligated to the linearized pIC19H and the mixture was used to transform *E. coli* RR1. Plasmid DNA was prepared and screened for the presence of a ~900 bp Bgl II-Eco RI fragment. A correct plasmid was selected and designated pICTPIP.

The TPI1 promoter was then subcloned to place convenient restriction sites at its ends. Plasmid pIC7 (Marsh et al., ibid.) was digested with Eco RI, the fragment ends were blunted with DNA polymerase I (Klenow fragment), and the linear DNA was recircularized using T4 DNA ligase. The resulting plasmid was used to transform *E. coli* RR1. Plasmid DNA was prepared from the transformants and was screened for the loss of the Eco RI site. A plasmid having the correct restriction pattern was designated pIC7RI*. Plasmid pIC7RI* was digested with Hind III and Nar I, and the 2500 bp fragment was gel-purified. The partial TPI1 promoter fragment (ca. 900 bp) was removed from pICTPIP using Nar I and Sph I and was gel-purified. The remainder of the TPI1 promoter was obtained from plasmid pFATPOT by digesting the plasmid with Sph I and Hind III, and a 1750 bp fragment, which included a portion of the TPI1 promoter fragment from pICTPIP, and the fragment from PFATPOT were then combined in a triple ligation to produce pMVR1 (FIG. 2).

The TPI1 promoter was then joined to the SUC2-PDGF β-receptor fusion. Plasmid pBTL10 (Example 2) was digested with Eco RI and Hind III to isolate the 3.4 kb fragment comprising the SUC2 signal sequence and the entire PDGF β-receptor coding region. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment and the fragment derived from pBTL10 were joined with YEp13, which had been linearized by digestion with Bam HI and Hind III, in a three-part ligation. The resultant plasmid was designated pBTL12 (FIG. 2).

EXAMPLE 6

Construction of an Expression Vector Encoding the 5' Extracellular Portion of the PDGF β-Receptor

The extracellular portion of the PDGF β-receptor was directed into the secretory pathway by fusing the coding sequence to the SUC2 signal sequence. This fusion was placed in an expression vector behind the TPI1 promoter. Plasmid pBTL10 (Example 2) was digested with Eco RI and Sph I to isolate the approximately 1.3 kb fragment comprising the SUC2 signal sequence and the PDGF β-receptor extracellular domain coding sequence. Plasmid pMVR1 (Example 5) was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The TPI1 promoter fragment was joined with the fragment derived from pBTL10 and YEp13, which had been linearized by digestion with Bam HI and Sph I, in a three-part ligation. The resultant plasmid was designated pBTL11 (FIG. 2).

EXAMPLE 7

Construction of Yeast Expression Vectors pBTL14 and pBTL15, and The Expression of PDGF β-Receptor-BAR1-subP Fusions

A. Construction of pBTL14

The SUC2-PDGFβ-R fusion was joined with the third domain of BAR1 to enhance the secretion of the receptor, and the expression unit was cloned into a derivative of YEp13 termed pJH50. YEp13 was modified to destroy the Sal I site near the LEU2 gene. This was achieved by partially digesting YEp13 with Sal I followed by a complete digestion with Xho I. The 2.0 kb Xho I-Sal I fragment comprising the LEU2 gene and the 8.0 kb linear YEp13 vector fragment were isolated and ligated together. The ligation mixture was transformed into *E. coli* strain RR1. DNA was prepared from the transformants and was analyzed by digestion with Sal I and Xho I. A clone was isolated which showed a single Sal I site and an inactive Xho I site indicating that the LEU2 fragment had inserted in the opposite orientation relative to the parent plasmid YEp13. The plasmid was designated pJH50.

Referring to FIG. 4, plasmid pBTL12 (Example 5) was digested with Sal I and Pst I to isolate the 2.15 kb fragment comprising 270 bp of YEp13 vector sequence, the TPI1 promoter, the SUC2 signal sequence, and 927 bp of PDGF β-receptor cDNA. Plasmid pBTL13 (Example 4) was digested with Pst I and Hind III to isolate the 1.48 kb fragment comprising 313 bp of PDGF β-receptor cDNA, the BAR1-subP fusion and the TPI1 terminator. The fragments derived from pBTL12 and pBTL13 were joined with pJH50, which had been linearized by digestion with Hind III and Sal I, in a three-part ligation. The resultant plasmid was designated pBTL14.

B. Construction of pBTL15

Referring to FIG. 5, a yeast expression vector was constructed comprising the TPI1 promoter, the SUC2 signal sequence, 1.45 kb of PDGF β-receptor cDNA sequence fused to the BAR1-subP fusion and the TPI1 terminator. Plasmid pBTL12 (Example 5) was digested with Sal I and Fsp I to isolate the 2.7 kb fragment comprising the TPI1 promoter, the SUC2 signal sequence, the PDGFβ-R coding sequence, and 270 bp of YEp13 vector sequence. Plasmid pBTL13 (Example 4) was digested with Nru I and Hind III to isolate the 1.4 kb fragment comprising the BAR1-subP fusion, the TPI1 terminator and 209 bp of 3' PDGF β-receptor cDNA sequence. The fragments derived from pBTL12 and pBTL13 were joined in a three-part ligation with pJH50, which had been linearized by digestion with Hind III and Sal I. The resultant plasmid was designated pBTL15.

C. Expression of PDGFβ-R-subP fusions from pBTL14 and pBTL15

Yeast expression vectors pBTL14 and pBTL15 were transformed into *Saccharomyces cerevisiae* strains ZY100 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2 pep4::TPI1prom-CAT) and ZY400 (MATa leu2-3,112 ade2-101 suc2-Δ9 gal2

pep4::TPI1prom-CAT Amnn9::URA3). Transformations were carried out using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2).

TABLE 2

Media Recipes

-LeuThrTrp Amino Acid Mixture
4 g adenine
3 g L-arginine
5 g L-aspartic acid
2 g L-histidine free base
6 g L-isoleucine
4 g L-lysine-mono hydrochloride
2 g L-methionine
6 g L-phenylalanine
5 g L-serine
5 g L-tyrosine
4 g uracil
6 g L-valine

Mix all the ingredients and grind with a mortar and pestle until the mixture is finely ground.

-LEUDS
20 g glucose
6.7 g Yeast Nitrogen Base without amino acids (DIFCO Laboratories Detroit, Mich.)
0.6 g -LeuThrTrp Amino Acid Mixture
182.2 g sorbitol
18 g Agar

Mix all the ingredients in distilled water. Add distilled water to a final volume of 1 liter. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan. Pour plates and allow to solidify.

-LEUDS+sodium succinate, pH 6.5
20 g Yeast Nitrogen Base without amino acids
0.6 g -LeuTrpThr Amino Acid Mixture
182.2 g sorbitol
11.8 g succinic acid

Mix all ingredients in distilled water to a final volume of 1 liter. Adjust the pH of the solution to pH 6.5. Autoclave 15 minutes. After autoclaving add 150 mg L-threonine and 40 mg L-tryptophan.

Fermentation Medium
7 g/l yeast nitrogen base without amino acids or ammonium sulfate (DIFCO Laboratories)
0.6 g/l ammonium sulfate
0.5M sorbitol
0.39 g/l adenine sulfate
0.01% polypropylene glycol

Mix all ingredients in distilled water. Autoclave 15 minutes. Add 80 ml 50% glucose for each liter of medium.

Super Synthetic -LEUD. pH 6.5 (liquid or solid medium)
6.7 g Yeast Nitrogen Base without amino acids or ammonium sulfate (DIFCO)
6 g ammonium sulfate
160 g adenine
0.6 g -LeuThrTrp Amino Acid Mixture
20 g glucose
11.8g succinic acid

Mix all ingredients and add distilled water to a final volume of 800 ml. Adjust the pH of the solution to pH 6.4. Autoclave 15 minutes. For solid medium, add 18 g agar before autoclaving, autoclave and pour plates.

Super Synthetic-LEUDS, pH 6.4 (liquid or solid medium)

Use the same recipe as Super Synthetic -LEUD, pH 6.4, but add 182.2 g sorbitol before autoclaving.

YEPD
20 g glucose
20 g Bacto Peptone (DIFCO Laboratories)
10 g Bacto Yeast Extract (DIFCO Laboratories)
18 g agar
4 ml adenine 1%
8 ml 1% L-leucine

Mix all ingredients in distilled water, and bring to a final volume of 1 liter. Autoclave 25 minutes and pour plates.

The transformants were assayed for binding to an anti-PDGF β-receptor monoclonal antibody (PR7212) or an anti-substance P antibody by protein blot assay. ZY100 [pBTL14] and ZY100[pBTL15] transformants were grown overnight at 30° 0C. in 5 ml Super Synthetic -LEUD, pH 6.4 (Table 2). ZY400[pBTL14] and ZY400[pBTL15] transformants were grown overnight at 30° C. in 5 ml Super Synthetic-LEUDS, pH 6.4 (Table 2). The cultures were pelleted by centrifugation and the supernatants were assayed for the presence of secreted PDGF β-receptor anlogs by protein blot assay using methods described in Example 18. Results of assays using PR7212 are shown in Table 3.

TABLE 3

Results of a protein blot probed with PR7212

Transformant	
ZY100[pBTL14]	+
ZY400[pBTL14]	++
ZY100[pBTL15]	+
ZY400[pBTL15]	+

EXAMPLE 8

Construction of a SUC2-PDGFβ-R Fusion Comprising the Complete PDGFβ-R Extracellular Domain

A. Construction of pBTL22

The PDGFβ-R coding sequence present in pBTL10 was modified to delete the coding region 3' to the extracellular PDGFβ-R domain. As shown in FIG. 6, plasmid pBTL10 was digested with Sph I and Bam HI and with Sph I and Sst II to isolate the 4.77 kb fragment and the 466 bp fragment, respectively. The 466 bp fragment was then digested with Sau 3A to isolate the 0.17 kb fragment. The 0.17 kb fragment and the 4.77 kb were joined by ligation. The resultant plasmid was designated pBTL21.

Plasmid pBTL21, containing a Bam HI site that was regenerated by the ligation of the Bam HI and Sau 3A sites, was digested with Hind III and Bam HI to isolate the 4.2 kb fragment. Synthetic oligonucleotides ZC1846 (Sequence ID Number 16; Table 1) and ZC1847 (Sequence ID Number 17; Table 1) were designed to form an adapter encoding the PDGFβ-R from the Sau 3A site after bp 1856 (FIG. 1B; (Sequence ID Number 1)) to the end of the extracellular domain at 1958 bp (FIG. 1B; Sequence ID Number 1), having a 5' Bam HI adhesive end that destroys the Bam HI site and a 3' Hind III adhesive end. Oligonucleotides ZC1846 and ZC1847 were annealed under conditions described by Maniatis et. al. (ibid.). The 4.2 pBTL21 fragment and the ZC1846/ZC1847 adapter were joined by ligation. The resultant plasmid, designated pBTL22, comprises the SUC2 signal sequence fused in proper reading frame to the extracellular domain of PDGFβ-R in the vector pUC19 (FIG. 6).

B. Construction of pBTL28

An in-frame translation stop codon was inserted immediately after the coding region of the PDGFβ-R in pBTL22

using oligonucleotides ZC1892 (Sequence ID Number 19; Table 1) and ZC1893 (Sequence ID Number 20; Table 1). These oligonucleotides were designed to form an adapter encoding a stop codon in-frame with the PDGFβ-R coding sequence from pBTL22 flanked by a 5' Hind III adhesive end and a 3' Xba I adhesive end. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1892 and ZC1893 were annealed to form a Hind III-Xba I adapter. The 1.6 kb SUC2-PDGFβ-R fragment, the 3.86 kb pMVR1 fragment and the ZC1892/ZC1893 adapter were joined in a three-part ligation. The resultant plasmid was designated pBTL27.

The expression unit present in pBTL27 was inserted into the yeast expression vector pJH50 by first digesting pJH50 with Bam HI and Sal I to isolate the 10.3 kb vector fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI and with Xho I and Eco RI to isolate the 0.9 kb TPI1 promoter fragment and the 1.65 kb fragment, respectively. The 10.3 kb pJH50 vector fragment, the 0.9 kb TPI1 promoter fragment and 1.65 kb fragment were joined in a three-part ligation. The resultant plasmid was designated pBTL28.

C. Construction of Plasmid pBTL30

The PDGFβ-R coding sequence present in plasmid pBTL22 was modified to encode the twelve C-terminal amino acids of substance P and an in-frame stop codon. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.68 kb fragment comprising the TPI1 promoter, pIC7RI* and the TPI1 terminator. Synthetic oligonucleotides ZC1894 (Sequence ID Number 21; Table 1) and ZC1895 (Sequence ID Number 22; Table 1) were annealed to form an adapter containing the codons for the twelve C-terminal amino acids of substance P followed by an in-frame stop codon and flanked on the 5' end with a Hind III adhesive end and on the 3' end with an Xba I adhesive end. The ZC1894/ZC1895 adapter, the 1.6 kb SUC2-PDGFβ-R fragment and the pMVR1 fragment were joined in a three-part ligation. The resultant plasmid, designated pBTL29, was digested with Eco RI and Xho I to isolate the 1.69 kb SUC2-PDGFβ-R-subP-TPI1 terminator fragment. Plasmid pBTL27 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. Plasmid pJH50 was digested with Bam HI and Sal I to isolate the 10.3 kb vector fragment. The 1.69 kb pBTL29 fragment, the 0.9 kb TPI1 promoter fragment and the 10.3 kb vector fragment were joined in a three-part ligation. The resulting plasmid was designated pBTL30.

EXAMPLE 9

Construction and Expression of a SUC2-PDGFβ-R-IgG Hinge Expression Vector

An expression unit comprising the TPI1 promoter, the SUC2 signal sequence, the PDGFβ-R extracellular domain, an immunoglobulin hinge region and the TPI1 terminator was constructed. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.56 kb fragment. Plasmid pMVR1 was digested with Eco RI and Xba I to isolate the 3.7 kb fragment, comprising the TPI1 promoter, pIC7RI* vector sequences and the TPI1 terminator. Oligonucleotides ZC1776 (Sequence ID Number 14; Table 1) and ZC1777 (Sequence ID Number 15; Table 1) were designed to form, when annealed, an adapter encoding an immunoglobulin hinge region with a 5' Hind III adhesive end and a 3' Xba I adhesive end. Oligonucleotides ZC1776 and ZC1777 were annealed under conditions described by Maniatis et al. (ibid.). The 1.56 kb pBTL22 fragment, the 3.7 kb fragment and the ZC1776/ZC1777 adapter were joined in a three-part ligation, resulting in plasmid pBTL24.

The expression unit of pBTL24, comprising the TPI1 promoter, SUC2 signal sequence, PDGFβ-R extracellular domain sequence, hinge region sequence, and TPI1 terminator, was inserted into pJH50. Plasmid pBTL24 was digested with Xho I and Hind III to isolate the 2.4 kb expression unit. Plasmid pJH50 was digested with Hind III and Sal I to isolate the 9.95 kb fragment. The 2.4 kb pBTL24 fragment and 9.95 kb pJH50 vector fragment were joined by ligation. The resultant plasmid was designated pBTL25.

Plasmid pBTL25 was transformed into *Saccharomyces cerevisiae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). The transformants were tested for their ability to bind the anti-PDGFβ-R monoclonal antibody PR7212 using the colony assay method described in Example 18. Plasmid pBTL25 transformants were patched onto nitrocellulose filters that had been wetted and supported by YEPD solid medium. Antibody PR7212 was found to bind to the PDGFβ-R-IgG hinge fusion secreted by ZY400[pBTL25] transformants.

EXAMPLE 10

Construction and Expression of a SUC2 signal sequence-PDGFβ-R Extracellular Domain-SUC2 Fusion

As shown in FIG. 6, an expression unit comprising the TPI1 promoter, SUC2 signal sequence, PDGFβ-R extracellular domain sequence, and SUC2 coding sequence was constructed as follows. Plasmid pBTL22 was digested with Eco RI and Hind III to isolate the 1.6 kb SUC2-PDGFβ-R fragment. Plasmid pMVR1 was digested with Bgl II and Eco RI to isolate the 0.9 kb TPI1 promoter fragment. The SUC2 coding region was obtained from pJH40. Plasmid pJH40 was constructed by inserting the 2.0 kb Hind III-Hind III SUC2 fragment from pRB58 (Carlson et al., *Cell* 28:145–154, 1982) into the Hind III site of pUC19 followed by the destruction of the Hind III site 3' to the coding region. Plasmid pJH40 was digested with Hind III and Sal I to isolate the 2.0 kb SUC2 coding sequence. Plasmid pJH50 was digested with Sal I and Bam HI to isolate the 10.3 kb vector fragment. The 0.9 kb Bgl II-Eco RI TPI1 promoter fragment, the 1.6 kb Eco RI-Hind III SUC2-PDGFβ-R, the 2.0 kb Hind III-Sal I SUC2 fragment and the 10.3 kb Bam HI-Sal I vector fragment were joined in a four-part ligation. The resultant plasmid was designated pBTL26 (FIG. 6).

Plasmid pBTL26 was transformed into *Saccharomyces cerevisiae* strain ZY400 using the method essentially described by Beggs (ibid.). Transformants were selected for their ability to grow on -LEUDS (Table 2). ZY400 transformants (ZY400[pBTL26]) were assayed by protein blot (Example 18), colony blot (Example 18) and competition assay.

Protein blot assays were carried out on ZY400[pBTL26] and ZY400[pJH50] (control) transformants that had been grown in flasks. Two hundred-fifty microliters of a 5 ml overnight cultures of ZY400[pBTL26] and ZY400 [pJH50] in -LEUDS+sodium succinate, pH 6.5 (Table 2) were inoculated into 50 ml of -LEUDS+sodium succinate, pH 6.5. The cultures were incubated for 35 hours in an airbath shaker at 30° C. The culture supernatants were harvested by centrifu-

gation. The culture supernatants were assayed as described in Example 18 and were found to bind PR7212 antibody.

Colony assays were carried out on ZY400[pBTL26] transformants. ZY400[pBTL26] transformants were patched onto wetted nitrocellulose filters that were supported on a YEPD plate. The colony assay carried out as described in Example 8.A. showed that ZY400[pBTL26] antibodies bound PR7212 antibodies.

Competition binding assays were carried out on ZY400 [pBTL26] and ZY400[pJH50] transformants. The transformants were grown in two liters of fermentation medium (Table 2) in a New Brunswick Biofio2 fermentor (New Brunswick, Philadelphia, Pa.) with continuous pH control at pH 6.4. The cultures were adjusted to pH 7.5 immediately prior to harvesting. Culture supernatants were concentrated in an Amicon concentrator (Amicon, San Francisco, Calif.) using an Amicon 10^4 mw spiral filter cartridge. The concentrated supernatants were further concentrated using Amicon Centriprep 10's. Fifteen milliliters of the concentrated supernatant samples were added to the Centripreps, and the Centripreps were spun in a Beckman GRP centrifuge (Beckman Instruments Inc., Carlsbad, Calif.) at setting 5 for a total of 60 minutes. The concentrates were removed from the Centripreps and were assayed in the competition assay.

The competition binding assay measured the amount of ^{125}I-PDGF left to bind to fetal foreskin fibroblast cells after preincubation with the concentrate containing the PDGFβ-R-SUC2 fusion protein. PDGF-AA and PDGF-AB were iodinated using the Iodopead method (Pierce Chemical) PDGF-BB_{Zy} was iodinated and purified as described in Example 18.F. The concentrate was serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-AA, PDGF-BB_{Zy}, or PDGF-AB, and the mixtures were incubated for two hours at room temperature. Three hundred micrograms of unlabeled PDGF-BB was added to each sample mixture. The sample mixtures were added to 24-well plates containing confluent fetal foreskin fibroblast cells (AG1523, available from the Human Genetic Mutant Cell Repository, Camden, N.J.). The cells were incubated with the mixture for four hours at 4° C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held at 4° C. (PBS; Sigma, St. Louis, Mo.). Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the PDGFβ-R-SUC2 fusion protein was able to competitively bind all three isoforms of PDGF.

The PDGFβ-R produced from ZY400 [pBTL26] transformants was tested for cross reactivity to fibroblast growth factor (FGF) and transforming growth factor-β(TGF-β) using the competition assay essentially described above. Supernatant concentrates from ZY400[pBTL26] and ZY400 [JH50] (control) transformants were serially diluted in binding medium (Table 4). The dilutions were mixed with 7.9 ng of iodinated FGF or 14 ng of iodinated TGF-β, and the mixtures were incubated for two hours at room temperature. Fourteen micrograms of unlabeled FGF was added to each mixture containing labeled FGF, and 7 μg of unlabeled TGF-β was added to each mixture containing labeled TGF-β. The sample mixtures were added to 24-well plates containing confluent human dermal fibroblast cells. (Human dermal fibroblast cells express both FGF receptors and TGFβ receptors.) The cells were incubated with the mixtures for four hours at 4° C. Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated FGF or TGF-β bound to the cells was determined. The results of these assays showed that the PDGFβ-R-SUC2 fusion protein did not cross react with FGF or TGF-β.

TABLE 4

Reagent Recipes

Binding Medium
500 ml Ham's F-12 medium
12 ml 1M HEPES, pH 7.4
5 ml 100×PSN (Penicillin/Streptomycin/Neomycin, Gibco)
1 g rabbit serum albumin

Western Transfer Buffer
25 mM Tris, pH 8.3
19 mM glycine, pH 8.3
20% methanol

Western Buffer A
50 ml 1M Tris, pH 7.4
20 ml 0.25 mM EDTA, pH 7.0
5 ml 10% NP-40
37.5 ml 4M NaCl
2.5 g gelatin

The Tris, EDTA, NP-40 and NaCl were diluted to a final volume of one liter with distilled water. The gelatin was added to 300 ml of this solution and the solution was heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the remainder of the first solution and stirred at 4° C. until cool. The buffer was stored at 4° C.

Western Buffer B
50 ml 1M Tris, pH 7.4
20 ml 0.25M EDTA, pH 7.0
5 ml 10% NP-40
58.4 g NaCl
2.5 g gelatin
4 g N-lauroyl sarcosine

The Tris, EDTA, NP-40, and the NaCl were mixed and diluted to a final volume of one liter. The gelatin was added to 300 ml of this solution and heated in a microwave until the gelatin was in solution. The gelatin solution was added back to the original solution and the N-lauroyl sarcosine was added. The final mixture was stirred at 4° C. until the solids were completely dissolved. This buffer was stored at 4° C.

2xLoading Buffer
36 ml 0.5M Tris-HCl, pH 6.8
16 ml glycerol
16 ml 20% SDS
4 ml 0.5% Bromphenol Blue in 0.5M Tris-HCl, pH 6.8

Mix all ingredients. Immediately before use, add 100 μl β-mercaptoethanol to each 900 μl dye mix

EXAMPLE 11

Construction and Expression of PDGF Receptor Analogs From BHK Cells

A. Construction of pBTL114 and pBTL115

The portions of the PDGF α-receptor extracellular domain present in pBTL14 and pBTL15 were placed in a mammalian expression vector. Plasmids pBTL14 and pBTL15 were digested with Eco RI to isolate the 1695 bp and 1905 bp SUC2 signal-PDGFβ-R-BAR1 fragments. The 1695 bp fragment and the 1905 bp fragment were each ligated to Zem229R that had been linearized by digestion with Eco RI.

The vector Zem229R was constructed as shown in FIG. 10 from Zem229. Plasmid Zem229 is a pUC18-based

expression vector containing a unique Bam HI site for insertion of cloned DNA between the mouse metallothionein-1 promoter and SV40 transcription terminator and an expression unit containing the SV40 early promoter, mouse dihydrofolate reductase gene, and SV40 transcription terminator. Zem229 was modified to delete the Eco RI sites flanking the Bam HI cloning site and to replace the Bam HI site with a single Eco RI cloning site. The plasmid was partially digested with Eco RI, treated with DNA polymerase I (Klenow fragment) and dNTPs, and religated. Digestion of the plasmid with Bam HI followed by ligation of the linearized plasmid with a Bam HI-Eco I adapter resulted in a unique Eco RI cloning site. The resultant plasmid was designated Zem229R.

The ligation mixtures were transformed into *E. coli* strain RR1. Plasmid DNA was prepared and the plasmids were subjected to restriction enzyme analysis. A plasmid having the 1695 bp pBTL14 fragment inserted into Zem229R in the correct orientation was designated pBTL14 (FIG. 9). A plasmid having the 1905 bp pBTL15 fragment inserted into Zem229R in the correct orientation was designated pBTL115 (FIG. 9).

B. Expression of Secreted PDGF α-Receptor Analogs in tk^{13} ts13 BHK Cells

Plasmids pBTL114 and pBTL115 were each transfected into tk^{13}-ts13 cells using calcium phosphate precipitation (essentially as described by Graham and van der Eb, *J. Gen. Virol.* 36: 59–72, 1977). The transfected cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum, 1×PSN antibiotic mix (Gibco 600-5640), 2.0 mM L-glutamine. The cells were selected in 250 nM methotrexate (MTX) for 14 days, and the resulting colonies were screened by the immunofilter assay (McCracken and Brown, *Biotechniques*, 82–87, March/April 1984). Plates were rinsed with PBS or No Serum medium (DMEM plus 1×PSN antibiotic mix). Teflon® mesh (Spectrum Medical Industries, Los Angeles, Calif.) was then placed over the cells. Nitrocellulose filters were wetted with PBS or No Serum medium, as appropriate, and placed over the mesh. After six hours incubation at 37° C., filters were removed and placed in Wester buffer A (Table 4) overnight at room temperature. The filters were developed using the antibody PR7212 and the procedure described in Example 8. The filters showed that conditioned media from pBTL114-transfected and pBTL115-transfected BHK cells bound the PR7212 antibody indicating the presence of biologically active secreted PDGFβ-R.

EXAMPLE 12

Expression of PDGF β-receptor Analogs in Cultured Mouse Myeloma Cells

A. Construction of pICμPRE8

The immunoglobulin μ heavy chain promoter and enhancer were subcloned into pIC19H to provide a unique Hind III site 3' to the promoter. Plasmid pμ (Grosschedl and Baltimore, *Cell* 41: 885–897, 1985) was digested with Sal I and Eco RI to isolate the 3.1 kb fragment comprising the μ promoter. Plasmid pIC19H was linearized by digestion with Eco RI and Xho I. The μ promoter fragment and the linearized pIC19H vector fragment were joined by ligation. The resultant plasmid, designated pICμ3, was digested with Ava II to isolate the 700 bp μ promoter fragment. The 700 bp fragment was blunt-ended by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. Plasmid pIC19H was linearized by digestion with Xho I, and the adhesive ends were filled in by treatment with DNA polymerase I (Klenow fragment) and deoxynucleotide triphosphates. The blunt-ended Ava II fragment was ligated with the blunt-ended, linearized pIC19H, and the ligation mixture was transformed into *E. coli* JM83. Plasmid DNA was prepared from the transformants and was analyzed by restriction digest. A plasmid with a Bgl II site 5' to the promoter was designated pICμPR1(–). Plasmid pICμPR1(–) was digested with Hind III and Bgl II to isolate the 700 bp μ promoter fragment. Plasmid pIC19R was linearized by digestion with Hind III and Bam HI. The 700 bp promoter fragment was joined with the linearized pIC19R by ligation. The resultant plasmid, designated pICμPR7, comprised the μ promoter with an unique Sma I site 5' to the promoter and a unique Hind III site 3' to the promoter.

The immunoglobulin heavy chain μ enhancer (Gillies et al., *Cell* 33: 717–728, 1983) was inserted into the unique Sma I site to generate plasmid pICμPRE8. Plasmid pJ4 (obtained from F. Blattner, Univ. Wisconsin, Madison, Wis.), comprising the 1.5 kb Hind III-Eco RI μ enhancer fragment in the vector pAT153 (Amersham, Arlington Heights, Ill.), was digested with Hind III and Eco RI to isolate the 1.5 kb enhancer fragment. The adhesive ends of the enhancer fragment were filled in by treatment with T4 DNA polymerase and deoxynucleotide triphosphates. The blunt-ended fragment and pICμPR7, which had been linearized by digestion with Sma I, were joined by ligation. The ligation mixture was transformed into *E. coli* RR1. Plasmid DNA was prepared from the transformants, and the plasmids were analyzed by restriction digests. A plasmid comprising the μ enhancer and the A promoter was designated pICμPRE8 (FIG. 7).

B. Construction of pSDL114

The DNA sequence encoding the extracellular domain of the PDGF α-receptor was joined with the DNA sequence encoding the human immunoglobulin light chain constant region. The PDGF β-receptor extracellular domain was obtained from mpBTL22, which comprised the Eco RI-Hind III fragment from pBTL22 (Example 8.A.) cloned into Eco RI-Hind III cut M13mp18. Single stranded DNA was prepared from a mpBTL22 phage clone, and the DNA was subjected to in vitro mutagenesis using the oligonucleotide ZC1886 (Table 1) and the method described by Kunkel (*Proc. Natl. Acad. Sci. USA* 82: 488–492, 1985). A phage clone comprising the mutagenized PDGFβ-R with a donor splice site (5' splice site) at the 3' end of the PDGFβ-R extracellular domain was designated pBTLR-HX (FIG. 7).

The native PDGFβ-R signal sequence was obtained from pPR5. Plasmid pPR5, comprising 738 bp of 5' coding sequence with an Eco RI site immediately 5' to the translation initiation codon, was constructed by in vitro mutagenesis of the PDGFβ-R cDNA fragment from RP51 (Example 1). Replicative form DNA of RP51 was digested with Eco RI to isolate the 1.09 kb PDGFβ-R fragment. The PDGFβ-R fragment was cloned into the Eco RI site of M13mp18. Single stranded template DNA was prepared from a phage clone containing the PDGFβ-R fragment in the proper orientation. The template DNA was subjected to in vitro mutagenesis using oligonucleotide ZC1380 (Sequence ID Number 8; Table 1) and the method described by Zoller and Smith (*Meth. Enzymol.* 100: 468–500, 1983). The mutagenesis resulted in the placement of an Eco RI site immediately 5' to the translation initiation codon. Mutagenized phage clones were analyzed by dideoxy sequence analysis. A phage clone containing the ZC1380 mutation was selected, and replicative form (RF) DNA was prepared from the phage clone. The RF DNA was digested with Eco RI and Acc I to isolate the 0.63 kb fragment. Plasmid pR-RXI (Example 1) was digested with Acc I and Eco RI to isolate the 3.7 kb



fragment. The 0.63 kb fragment and the 3.7 kb fragment were joined by ligation resulting in plasmid pPR5 (FIG. 7).

As shown in FIG. 7, the PDGFβ-R signal peptide and part of the extracellular domain were obtained from plasmid pPR5 as a 1.4 kb Eco RI-Sph I fragment. Replicative form DNA from phage clone pBTLR-HX was digested with Sph I and Hind III to isolate the approximately 0.25 kb PDGFβ-R fragment. Plasmid pUC19 was linearized by digestion with Eco RI and Hind III. The 1.4 kb Eco RI-Sph I PDGFβ-R fragment, the 0.25 kb Sph I-Hind III fragment from PBTLR-HX and the Eco RI-Hind III cut pUC19 were joined in a three-part ligation. The resultant plasmid, pSDL110, was digested with Eco RI and Hind III to isolate the 1.65 kb PDGFβ-R fragment.

Plasmid pICHuCκ3.9.11 was used as the source of the human immunoglobulin light chain gene (FIG. 7). The human immunoglobulin light chain gene was isolated from a human genomic library using an oligonucleotide probe (5'TGT GAC ACT CTC CTG GGA GTT A 3'; Sequence ID Number 32), which was based on a published human kappa C gene sequence (Hieter et al., *Cell* 22: 197–207, 1980). The human light chain (kappa) constant region was subcloned as a 1.1 kb Sph I-Hinf I genomic fragment of the human kappa gene, which has been treated with DNA polymerase DNA I (Klenow Fragment) to fill in the Hinf I adhesive end, into Sph I-Hinc II cut pUC19. The 1.1 kb human kappa constant region was subsequently isolated as a 1.1 kb Sph I-Bam HI fragment that was subcloned into Sph I-Bgl II cut pIC19R (Marsh et al., ibid.). The resultant plasmid was designated pICHuCκ3 9.11. Plasmid pICHuCκ3 9.11 was digested with Hind III and Eco RI to isolate the 1.1 kb kappa constant region gene. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGFβ-R fragment, the 1.1 kb human kappa constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL112, was digested with Bam HI and Cla I to isolate the 2.75 kb fragment. Plasmid pμPRE8 was linearized with Bgl II and Cla I. The 2.75 kb fragment and the linearized pμPRE8 were joined by ligation. The resultant plasmid was designated pSDL114 (FIG. 7).

Plasmid pSDL114 was linearized by digestion with Cla I and was cotransfected with Pvu I-digested p416 into SP2/0-Ag14 (ATCC CRL 1581) by electroporation using the method essentially described by Neumann et al. (*EMBO J.* 1: 841–845, 1982). (Plasmid p416 comprises the Adenovirus 5 70 ori, SV40 enhancer, Adenovirus 2 major late promoter, Adenovirus 2 tripartite leader, 5' and 3' splice sites, the DHFR^r cDNA, the SV40 polyadenylation signal and pML-1 (Lusky and Botchan, *Nature* 293: 79–81, 1981) vector sequences.) Transfectants were selected in growth medium containing methotrexate.

Media from drug resistant clones were tested for the presence of secreted PDGF β-receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml polyclonal goat anti-human kappa chain (Cappel Laboratories, Melvern, Pa.) diluted in phosphate buffered saline (PBS; Sigma) overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the well were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well and the wells were incubated for one hour at 4° C. One hundred microliters of chromophore (100 μl ABTS (2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt; Sigma)+1 μl 30% H_2O_2+12.5 ml citrate/phosphate buffer (9.04 g/l citric acid, 10.16 g/l Na_2HPO_4)) was added to each well, and the wells were incubated to thirty minutes at room temperature. The samples were measured at 405 nm. The results of the assay showed that the PDGFβ-R analog secreted by the transfectants contained an immunoglobulin light chain.

Spent media from drug resistant clones was also tested for the presence of secreted PDGF β-receptor analogs by immunoprecipitation. Approximately one million drug resistant transfectants were metabolically labeled by growth in DMEM medium lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μCi ^{35}S-cysteine. Media was harvested from the labeled cells and 250 Al of the spent media was assayed by immunoprecipitation with the anti-PDGF β-receptor antibody PR7212 to detect the presence of metabolically labeled PDGF β-receptor analogs. PR7212, diluted in PBS, was added to the media to a final concentration of 2.5 μg per 250 μl spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to the PR7212/media mixtures. The immunocomplexes were precipitated by the addition of 50 Al 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at −70° C. The results of the immunoprecipitation showed that the PDGF β-receptor analog secreted by the transfectants was bound by the anti-PDGF β-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF β-receptor analog secreted by the transfectants contained both the PDGF β-receptor ligand-binding domain and the human light chain constant region.

C. Cotransfection of pSDL114 With an Immunoglobulin Heavy Chain

Plasmid pSDL114 was cotransfected with pϕ5V$_H$huC$_γ$1M-neo, which encodes a neomycin resistance gene expression unit and a complete mouse/human chimeric immunoglobulin heavy chain gene expression unit.

Plasmid pϕ5V$_H$huC$_γ$1M-neo was constructed as follows. The mouse immunoglobulin heavy chain gene was isolated from a lambda genomic DNA library constructed from the murine hybridoma cell line NR-ML-05 (Serafini et al., *Eur. J. Nucl. Med.* 14: 232, 1988) using an oligonucleotide probe designed to span the $V_H/D/β_H$ junction (5' GCA TAG TAG TTA CCA TAT CCT CTT GCA CAG 3'; Sequence ID Number 33). The human immunoglobulin gamma-1 C gene was isolated from a human genomic library using a cloned human gamma-4 constant region gene (Ellison et al., *DNA* 1: 11–18, 1981). The mouse immunoglobulin variable region was isolated as a 5.3 kb Sst I-Hind III fragment from the original phage clone and the human gamma-1 C gene was obtained from the original phage clone as a 6.0 kb Hind III-Xho I fragment. The chimeric gamma-1 C gene was created by joining the VH and CH fragments via the common Hind III site and incorporating them with the *E. coli* neomycin resistance gene expression unit into pIC19H to yield pϕ5V$_H$huC$_γ$1M-neo.

Plasmid pSDL114 was linearized by digestion with Cla I and was co-transfected into SP2/0-Ag14 cells with Asp 718 linearized pϕ5V$_H$huC$_{α1}$1M-neo. The transfectants were selected in growth medium containing methotrexate and neomycin. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay.

The competition binding assay measured the amount of ^{125}I-PDGF left to bind to human dermal fibroblast cells after preincubation with the spent media from pSDL114-

pϕ5V$_H$huC$_γ$1M-neo transfected cells. The media were serially diluted in binding medium (Table 4). The dilutions were mixed with 0.5 ng of iodinated PDGF-BB or iodinated PDGF-AA, and the mixtures were incubated for two hours at room temperature. Three hundred micrograms of unlabeled PDGF-BB or unlabeled PDGF-AA was added to one tube from each series. The sample mixtures were added to 24 well plates containing confluent human dermal fibroblast cells. The cells were incubated with the mixture for four hours at 4° C. The supernatants were aspirated from the wells, and the wells were rinsed three times with phosphate buffered saline that was held a 4° C. (PBS; Sigma, St. Louis, Mo.). Five hundred microliters of PBS+1% NP-40 was added to each well, and the plates were shaken on a platform shaker for five minutes. The cells were harvested and the amount of iodinated PDGF was determined. The results of the competition binding assay showed that the protein produced from pSDL114-pϕ5V$_H$huC$_γ$1M-neo transfected cells was able to competitively bind PDGF-BB but did not bind PDGF-AA.

The PDGF β-receptor analog produced from a pSDL114-pϕ5V$_H$huC$_γ$1M-neo transfectant was assayed to determine if the receptor analog was able to bind PDGF-BB with high affinity. Eight and one half milliliters of spent media containing the PDGFβ-R analogs from a pSDL114-p5V$_H$huC$_γ$1M-neo transfectant was added to 425 μl of Sepharose Cl-4B-Protein A beads (Sigma, St. Louis, Mo.), and the mixture was incubated for 10 minutes at 4° C. The beads were pelleted by centrifugation and washed with binding medium (Table 4). Following the wash the beads were resuspended in 8.5 ml of binding media, and 0.25 ml aliquots were dispensed to 1.5 ml tubes. Binding reactions were prepared by adding iodinated PDGF-BB$_{yr}$ (Example 18 F.) diluted in DMEM+10% fetal calf serum to the identical aliquots of receptor-bound beads to final PDGF-BB$_{yr}$ concentrations of between 4.12 pM and 264 pM. Nonspecific binding was determined by adding a 100 fold excess of unlabeled BB to an identical set of binding reactions. Mixtures were incubated overnight at 4° C.

The beads were pelleted by centrifugation, and unbound PDGF-BB was removed with three washes in PBS. The beads were resuspended in 100 μl of PBS and were counted. Results of the assay showed that the PDGFβ-R analog was able to bind PDGF-BB with high affinity.

D. Construction of pSDL113

As shown in FIG. 8, the DNA sequence encoding the extracellular domain of the PDGF β-receptor was joined with the DNA sequence encoding a human immunoglobulin heavy chain constant region joined to a hinge sequence. Plasmid pSDL110 was digested with Eco RI and Hind III to isolate the 1.65 kb PDGFβ-R fragment. Plasmid pICHu$_γ$-1M was used as the source of the heavy chain constant region and hinge region. Plasmid pICHu$_γ$-1M comprises the approximately 6 kb Hind III-Xho I fragment of a human gamma-1 C gene subcloned into pUC19 that had been linearized by digestion with Hind III and Sal I. Plasmid pICHu$_γ$-1M was digested with Hind III and Eco RI to isolate the 6 kb fragment encoding the human heavy chain constant region. Plasmid pIC19H was linearized by digestion with Eco RI. The 1.65 kb PDGFβ-R fragment, the 6 kb heavy chain constant region fragment and the linearized pIC19H were joined in a three part ligation. The resultant plasmid, pSDL111, was digested with Bam HI to isolate the 7.7 kb fragment. Plasmid pμPRE8 was linearized with Bgl II and was treated with calf intestinal phosphatase to prevent self-ligation. The 7.7 kb fragment and the linearized pμPRE8 were joined by ligation. A plasmid containing the insert in the proper orientation was designated pSDL113 (FIG. 8).

Plasmid pSDL113 is linearized by digestion with Cla I and is cotransfected with Pvu I-digested p416 into SP2/0-Ag14 by electroporation. Transfectants are selected in growth medium containing methotrexate.

Media from drug resistant clones are tested for the presence of secreted PDGFβ-R analogs by immunoprecipitation using the method described in Example 12.B.

E. Cotransfection of pSDL113 With an Immunoglobulin Light Chain Gene

Plasmid pSDL113 is linearized by digestion with Cla I and was cotransfected with pICϕ5V$_κ$HuC$_κ$-Neo, which encodes a neomycin resistance gene and a mouse/human chimeric immunoglobulin light chain gene. The mouse immunoglobulin light chain gene was isolated from a lambda genomic DNA library constructed from the murine hybridoma cell line NR-ML-05 (Woodhouse et al., ibid.) using an oligonucleotide probe designed to span the V$_κ$/J$_κ$ junction (5' ACC GAA CGT GAG AGG AGT GCT ATA A 3'; Sequence ID Number 34). The human immunoglobulin light chain constant region gene was isolated as described in Example 12.B. The mouse NR-ML-05 immunoglobulin light chain variable region gene was subcloned from the original mouse genomic phage clone into pIC19R as a 3 kb Xba I-Hinc II fragment. The human kappa C gene was subcloned from the original human genomic phage clone into pUC19 as a 2.0 kb Hind III-Eco RI fragment. The chimeric kappa gene was created by joining the NR-ML-05 light chain variable region gene and human light chain constant region gene via the common Sph I site and incorporating them with the *E. coli* neomycin resistance gene into pIC19H to yield pICϕ5V$_κ$HuC$_κ$-Neo (FIG. 9).

The linearized pSDL113 and pICϕ5V$_κ$HuC$_κ$-Neo are transfected into SP2/0-Ag14 cells by electroporation. The transfectants are selected in growth medium containing methotrexate and neomycin.

F. Cotransfection of pSDL113 and pSDL114

A clone of SP2/0-Ag14 stably transfected with pSDL114 and p416 was co-transfected with Cla I-digested pSDL113 and Bam HI-digested pICneo by electroporation. (Plasmid pICneo comprises the SV40 promoter operatively linked to the *E. coli* neomycin resistance gene and pIC19H vector sequences.) Transfected cells were selected in growth medium containing methotrexate and G418. Media from drug-resistant clones were tested for their ability to bind PDGF-BB or PDGF-AA in a competition binding assay as described in Example 12.C. The results of the assay showed that the transfectants secreted a PDGF β-receptor analog which was capable of competitively binding PDGF-BB but did not detectably bind to PDGF-AA.

G. Cotransfection of pSDL114 With Fab

A clone of SP2/0-AG14 stably transfected with pSDL114 and p416 was transfected with the Fab region of the human gamma-4 gene (γ$_4$) in plasmid pϕ5V$_H$Fab-neo.

Plasmid pϕ5V$_H$Fab-neo was constructed by first digesting plasmid p24BRH (Ellison et al., *DNA* 1: 11, 1988) was digested with Xma I and Eco RI to isolate the 0.2 kb fragment comprising the immunoglobulin 3' untranslated region. Synthetic oligonucleotides ZC871 (Sequence ID Number 3; Table 1) and ZC872 (Sequence ID Number 4; Table 1) were kinased and annealed using essentially the methods described by Maniatis et al. (ibid.). The annealed oligonucleotides ZC871/ZC872 formed an Sst I-Xma I adapter. The ZC871/ZC872 adapter, the 0.2 kb p24BRH fragment and Sst I-Eco RI linearized pUC19 were joined in a three-part ligation to form plasmid pγ$_4$3'. Plasmid pγ$_4$3' was linearized by digestion with Bam HI and Hind III. Plasmid p24BRH was cut with Hind III and Bgl II to isolate

the 0.85 kb fragment comprising the $C_{\gamma}1$ region. The $p\gamma_4$3' fragment and the Hind III-Bgl II p24BRH fragment were joined by ligation to form plasmid $p\gamma_4$Fab. Plasmid $p\gamma_4$Fab was digested with Hind III and Eco RI to isolate the 1.2 kb fragment comprising γ_4Fab. Plasmid pICneo, comprising the SV40 promoter operatively linked to the *E. coli* neomycin resistance gene and pIC19H vector sequences, was linearized by digestion with Sst I and Eco RI. Plasmid $p\phi5V_H$, comprising the mouse immunoglobulin heavy chain gene variable region and pUC18 vector sequences, was digested with Sst I and Hind III to isolate the 5.3 kb V_H fragment. The linearized pICneo was joined with the 5.3 kb Sst I-Hind III fragment and the 1.2 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated $p\phi5V_H$Fab-neo (FIG. 10).

A pSDL114/p416-transfected SP2/0-AG14 clone was transfected with Sca I-linearized $p\phi5V_H$Fab-neo. Transfected cells were selected in growth medium containing methotrexate and G418. Media from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay as described in Example 12.C. The results of the assay showed that the PDGF β-receptor analog secreted from the transfectants was capable of competitively binding PDGF-BB.

H. Cotransfection of pSDL114 with Fab'

A stably transfected SP2/0-AG14 isolate containing pSDL114 and p416 was transfected with plasmid pWKI, which contained the Fab' portion of an immunoglobulin heavy chain gene. Plasmid pWKI was constructed as follows.

The immunoglobulin gamma-1 Fab' sequence, comprising the C_H1 and hinge regions sequences, was derived from the gamma-1 gene clone described in Example 12.C. The gamma-1 gene clone was digested with Hind III and Eco RI to isolate the 3.0 kb fragment, which was subcloned into Hind III-Eco RI linearized M13mp19. Single-stranded template DNA from the resultant phage was subjected to site-directed mutagenesis using oligonucleotide ZC1447 (Sequence ID Number 9; Table 1) and essentially the method of Zoller and Smith (ibid.). A phage clone was identified having a ZC1447 induced deletion resulting in the fusion of the hinge region to a DNA sequence encoding the amino acids Ala-Leu-His-Asn-His-Tyr-Thr-Glu-Lys-Ser-Leu-Ser-Leu-Ser-Pro-Gly-Lys (Sequence ID Number 31) followed in-frame by a stop codon. Replicative form DNA from a positive phage clone was digested with Hind III and Eco RI to isolate the 1.9 kb fragment comprising the C_H1 and hinge regions. Plasmid $p\phi5V_H$ was digested with Sst I and Hind III to isolate the 5.3 kb fragment comprising the mouse immunoglobulin heavy chain gene variable region. Plasmid pICneo was linearized by digestion with Sst I and Eco RI. The linearized pICneo was joined with the 5.3 kb Hind III-Sst I fragment and the 1.9 kb Hind III-Eco RI fragment in a three-part ligation. The resultant plasmid was designated pWKI (FIG. 10).

An SP2/0-AG14 clone stably transfected with pSDL114 and p416 was transfected with Asp 718-linearized pWKI. Transfected cells were selected by growth in medium containing methotrexate and G418. Media samples from transfected cells were assayed using the competition assay described in Example 12.C. Results from the assays showed that the transfected cells produced a PDGF β-receptor analog capable of competitively binding PDGF-BB.

EXAMPLE 13

Purification and Characterization of PDGF β-Receptor Analogs from Mammalian cells Co-transfected With pSDL.113 and pSDL.114

A. Purification of PDGF β-Receptor Analogs

The PDGF β-receptor analog was purified from conditioned culture media from a clone of transfected cells grown in a hollow fiber system. The media was passed over a protein-A sepharose column, and the column was washed sequentially with phosphate buffered saline, pH 7.2 (PBS; Sigma, St. Louis, Mo.) and 0.1M citrate, pH 5.0. The PDGF β-receptor analog was eluted from the protein-A column with 0.1M citrate pH 2.5 and immediately neutralized by the addition of Tris-base, pH 7.4. The eluate fractions containing PDGF β-receptor analog, as determined by silver stain, were pooled and chromatographed over an S-200 column (Pharmacia LKB Technologies, Inc., Piscataway, N.J.) equilibrated with PBS. The peak fractions from the S-200 column were pooled and concentrated on a centriprep-10 concentrator (Amicon). Glycerol (10% final volume) was added to the preparation and the sample frozen at −80° C. PDGF β-receptor analogs purified from pSDL114 +pSDL113 co-transfected cells were termed "tetrameric PDGF α-receptors".

B. Measurement of the Relative Binding Affinity of Tetrameric PDGF β-Receptor Analog by Soluble Receptor Assay

Purified tetrameric PDGF β-receptor analog was compared to detergent solubilized extracts of human dermal fibroblasts for ^{125}I-labeled PDGF-BB binding activity in a soluble receptor assay essentially as described by Hart et al. (*J. Biol. Chem.* 262: 10780–10785, 1987). Human dermal fibroblast cells were extracted at 20×10^6 cell equivalents per ml in TNEN extraction buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM PMSF, 10% glycerol). Two hundred and fifty thousand PDGF β-receptor-subunits per cell was used to calculate the tetrameric PDGF β-receptor analog number per volume of extract. This value has been previously published by Seifert et al. (*J. Biol Chem.* 264: 8771–8778, 1989). The PDGF β-receptor analog number was determined from the protein concentration of the PDGF β-receptor analog assuming an average molecular weight of 140 kDa for each immunoglobulin-PDGF β-receptor monomer, and four monomers per tetramer. Thus, each tetrameric molecule contains four receptor molecules.

Increasing amounts of either detergent solubilized extracts of human dermal fibroblast cells or purified PDGF β-receptor analog were incubated with 1 ng of ^{125}I-labeled PDGF-BB for one hour at 37° C. The sample was then diluted with 1 ml binding media and was added to monolayers of human dermal fibroblast cells grown in 24-well culture dishes. The samples were incubated for two hours at 4 ° C. The wells were washed to remove unbound, ^{125}I-labeled PDGF-BB. On half of a milliliter of extraction buffer (PBS+1% Nonidet P-40) was added to each well followed by a 5 minute incubation. The extraction mixtures were harvested and counted in a gamma counter.

The results showed that the PDGF β-receptor analog had the same relative binding affinity as solubilized PDGF β-receptor-subunit from mammalian cells in a solution phase binding assay.

C. Determination of the Binding Affinity of the PDGF β-Receptor Analog in a Solid Phase Format

The apparent dissociation constant KD(app) of the PDGF β-receptor analog was determined essentially as described by Bowen-Pope and Ross (*Methods in Enzymology* 109:

69–100, 1985), using the concentration of ^{125}I-labeled PDGF-BB giving half-maximal specific ^{125}I-labeled PDGF-BB binding. Saturation binding assays to determine the concentration of ^{125}I-labeled PDGF-BB that gave half-maximal binding to immobilized PDGF β-receptor analog were conducted as follows.

Affinity purified goat anti-human IgG, H- and L-chain (Commercially available from Cappel Labs) was diluted into 0.1M Na_2HCO_3, pH 9.6 to a concentration of 2 μg/ml. One hundred microliters of the antibody solution was coated onto each well of 96-well microtiter plates for 18 hours at 4° C. The wells were washed once with ELISA C buffer (PBS+0.05% Tween-20) followed by an incubation with 175 μl/well of ELISA B buffer (PBS+1% BSA+0.05% Tween-20) to block the wells. The wells were washed once with ELISA B buffer. One hundred microliters of 12.1 ng/ml or 24.3 ng/ml of tetrameric PDGF β-receptor analog protein diluted in ELISA B was added to each well and the plates were incubated for 2 hours at 37° C. Unbound protein was removed from the wells by two washes with ELISA C. ^{125}I-labeled PDGF-$BB_{2y,r}$ (Example 18.F.) was serially diluted into binding media (25 mM HEPES, pH 7.2, 0.25% rabbit serum albumin diluted in HAMs F-12 medium (GIBCO-BRL)), and 100 μl of the dilutions were added to the wells. The plates were incubated for two hours at room temperature. The unbound ^{125}I-labeled PDGF-BB was removed, and the wells were washed three times with binding media. Following the last wash, 100 μl of 0.1M citrate, pH 2.5 was added to each well. After five minutes, the citrate buffer was removed, transferred to a tube and counted in a gamma counter. The counts reflect counts of ^{125}I-labeled PDGF-$BB_{2y,r}$ bound by the receptor analog. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-$BB_{2y,r}$ was determined by a parallel assay wherein separate wells coated only with goat anti-human IgG were incubated with the ^{125}I-labeled PDGF-BB concentrations. Nonspecific binding was determined to be 2.8% of the total input counts per well and averaged 6% of the total counts bound.

Saturation binding assay on 12.1 and 24.3 ng/ml of tetrameric PDGF β-receptor analog gave half-maximal binding at 0.8 and 0.82 ng/ml ^{125}I-labeled PDGF-$BB_{2y,r}$ respectively. By Scatchard analysis (Scatchard, *Ann NY Acad. Sci* 51: 660–667, 1949) these values were shown to correspond to a KD(app) of 2.7×10^{-11} which agree with the published values for PDGF receptors on mammalian cells.

EXAMPLE 14

Solid Phase Ligand Binding Assay Using the PDGF β-Receptor Analog

A. Solid Phase Radioreceptor Competition Binding Assay

In a solid phase radioreceptor competition binding assay (RRA), the wells of 96-well microtiter plates were coated with 100 μl of 2 μg/ml affinity purified goat anti-human IgG (Cappel Labs) diluted in 0.1M Na_2HCO_3, pH 9.6. After an eighteen hour incubation at 4° C., the wells were washed once with ELISA C. The wells were blocked by incubation for 2 hours at 37° C. with 175 μl/well ELISA B. The wells were washed once with ELISA B then incubated for 2 hours at 37° C. with 50 ng/ml tetrameric PDGF β-receptor analog diluted in ELISA B. The unbound receptor was removed, and the test wells were incubated with increasing concentrations of serially diluted, unlabeled PDGF-BB (diluted in binding media. Following a two hour incubation at room temperature, the wells were washed three times with binding media. One hundred microliters of 5 ng/ml ^{125}I-labeled PDGF-$BB_{2y,r}$ (Example 18.F.) was added to each well, and the plates were incubated for an additional two hours at room temperature. The wells were washed three times with binding media followed by a 5 minute incubation with 100 μl/well of 0.1M citrate, pH 2.5. The samples were harvested and counted in a gamma counter.

Radioreceptor assay (RRA) competition binding curves were generated for PDGF β-receptor analog protein plated at 48.6 ng/ml. The sensitivity of the assays is 1 ng/ml of PDGF-BB, with 8 ng/ml giving 50% inhibition in ^{125}I-PDGF-BB binding, and a working range between 1 and 32 ng/ml of PDGF-BB. The values were similar to those obtained using monolayers of SK-5 cells in an RRA.

B. Use of Tetrameric PDGF β-Receptor Analogs As Antagonists for PDGF-Stimulated Mitogenesis

A tetrameric PDGF β-receptor analog, purified as described in Example 13, was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF β-receptor analog were mixed with 5 ng of PDGF. The mixtures were then added to cultures of mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of ^{3}H-thymidine, was determined essentially as described (Raines and Ross, *Methods in Enzymology* 109: 749–773, 1985, which is incorporated by reference herein). The tetrameric PDGF β-receptor analog demonstrated a dose response inhibition of PDGF-BB-stimulated ^{3}H-thymidine incorporation, while having essentially no effect on PDGF-AA- and PDGF-AB-stimulated ^{3}H-thymidine incorporation.

C. Binding of Tetrameric PDGF β-receptor Analog to Immobilized PDGF

A tetrameric PDGF β-receptor analog, purified as described in Example 13, was analyzed for its ability to bind to immobilized PDGF. PDGF-BB (100 ng/ml) was coated onto wells a 96-well microtiter plate, and the plates were incubated 18 hours at 4° C. followed by one wash with ELISA C buffer. The wells were incubated for 2 hours 37° C. with ELISA B buffer to block the wells. Increasing concentrations of ^{125}I-labeled tetrameric PDGF β-receptor analog, diluted in binding media, was added to the wells for two hours at room temperature. The wells were washed four times with ELISA C buffer to remove unbound receptor analog. One hundred microliters of 1M H_2SO_4 was added to each well and the plates were incubated for five minutes at room temperature. The solution was then harvested and transferred to tubes to be counted in a gamma counter. Nonspecific binding was determined to be less than 10% of the total counts bound.

A receptor competition binding assay was developed using this assay format. The assay was carried out as described above, and simultaneous to the addition of the ^{125}I-labeled tetrameric PDGF β-receptor analog, increasing amounts of PDGF-AA, AB or BB were added to the PDGF-BB coated wells. Under these conditions, only PDGF-BB was found to significantly block the binding of the labeled PDGF β-receptor analog to the immobilized PDGF-BB.

EXAMPLE 15

Construction and Expression of PDGFα-R Analogs in Cultured Mouse Myeloma Cells

A. Construction of an Optimized PDGFα-R cDNA

The PDGF αα-receptor coding region was optimized for expression in mammalian cells as follows. The 5' end of the cDNA was modified to include an optimized Kozak consensus translation initiation sequence (Kozak, *Nuc. Acids Res.* 12: 857–872, 1984) and Eco RI and Bam HI sites just

5' of the initiation methionine codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 (Sequence ID Numbers 23, 24, 25 and 26, respectively; Table 1) were designed to form, when annealed, an adapter having an Eco RI adhesive end, a Bam HI restriction site, a sequence encoding a Kozak consensus sequence 5' to the initiation methionine codon, a mammalian codon optimized sequence encoding amino acids 1–42 of FIG. 11A, and an Eco RI adhesive end that destroys the Eco RI site within the PDGFα-R coding sequence. The adapter also introduced a diagnostic Cla I site 3' to the initiation methionine codon. Oligonucleotides ZC2181, ZC2182, ZC2183 and ZC2184 were kinased, annealed and ligated. Plasmid pα17B was linearized by partial digestion with Eco RI. The linearized pα17B was ligated with the ZC2181/ZC2182/ZC2183/ZC2184 oligonucleotide adapter, and the ligation mixture was transformed into *E. coli*. Plasmid DNA prepared from the transformants was analyzed by restriction analysis and a positive clone having the oligonucleotide adapter in the correct orientation was digested with Eco RI and Pst I to isolate the 1.6 kb fragment. This fragment was subcloned into Eco RI+Pst I-linearized M13mp19. The resultant phage clone was designated 792-8. Single-stranded 792-8 DNA was sequenced to confirm the orientation of the adapter.

A fragment encoding the ligand-binding domain of the PDGF α-receptor (PDGFα-R) was then generated as follows. Restriction sites and a splice donor sequence were introduced at the 3' end of the PDGFα-R extracellular domain by PCR amplification of the 792–8 DNA and oligonucleotides ZC2311 and ZC2392 (Sequence ID Numbers 27 and 30, Table 1). Oligonucleotide ZC2311 is a sense primer encoding nucleotides 1470 to 1489 of FIG. 11B. Oligonucleotide ZC2392 is an antisense primer that encodes nucleotides 1759 to 1776 of FIG. 11B followed by a splice donor and Xba I and Hind III restriction sites. The 792–8 DNA was amplified using manufacturer recommended (Perkin Elmer Cetus, Norwalk, Conn.) conditions and the GeneAmp™ DNA amplification reagent kit (Perkin Elmer Cetus), and blunt-ended 329 bp fragment was isolated. The blunt-end fragment was digested with Nco I and Hind III and ligated with Sma I-digested pUC18. A plasmid having an insert with the Nco I site distal to the Hind III site present in the pUC18 polylinker was designated pUC18 Sma-PCR Nco HIII #13. The Hind III site present in the insert was not regenerated upon ligation with the linearized pUC18. Plasmid pUC18 Sma-PCR Nco HIII #13 was digested with Nco I and Hind III to isolate the 355 bp PDGFα-R containing fragment encoding PDGFαR. Oligonucleotides ZC2351 and ZC2352 (Table 1; Sequence ID Numbers 28 and 29) were kinased and annealed to form an Sst I-Nco I adapter encoding an internal Eco RI site and a Kozak consensus translation initiation site. The 355 bp Nco I-Hind III fragment, the ZC2351/ZC2352 adapter and a 1273 bp Nco I fragment comprising the extracellular domain of PDGF α-R derived from 792–8 were ligated with Hind III+SstI-digested pUC18 and transformed into *E. coli*. Plasmid DNA was isolated from the transformants and analyzed by restriction analysis. None of the isolates contained the 1273 bp Nco I fragment. A plasmid containing the Nco I-Hind III fragment and the ZC2351/ZC2352 adapter was designated pUC18 Hin Sst A Nco #46. Plasmid pUC18 Hin Sst A Nco #46 was linearized by digestion and joined by ligation with the 1273 bp Nco I fragment comprising the extracellular domain of the PDGFβ-R from clone α18 R-19. The ligations were transformed into *E. coli*, and plasmid DNA was isolated from the transformants. Analysis of the plasmid DNA showed that only clones with the Nco I fragment in the wrong orientation were isolated. A clone having the Nco I fragment in the wrong orientation was digested with Nco I, religated and transformed into *E. coli*. Plasmid DNA was isolated from the transformants and was analyzed by restriction analysis. A plasmid having the Nco I insert in the correct orientation was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb fragment comprising the extracellular domain of the PDGFα-R preceded by a consensus initiation sequence (Kozak, ibid.) and followed by a splice donor site.

B. Construction of pPAB7

The DNA sequence encoding the extracellular domain of the PDGFα-R was joined to the immunoglobulin μ enhancer-promoter and to a DNA sequence encoding an immunoglobulin light chain constant region. The immunoglobulin μ enhancer-promoter was obtained from plasmid pJH1 which was derived from plasmid PIC/μPRE8 (Example 12.A.) by digestion with Eco RI and Sst I to isolate the 2.2 kb fragment comprising the immunoglobulin enhancer and heavy chain variable region promoter. The 2.2 kb Sst I-Eco RI fragment was ligated with Sst I+Eco RI-linearized pUC19. The resulting plasmid, designated pJH1, contained the immunoglobulin enhancer and heavy chain variable region promoter immediately 5' to the pUC19 linker sequences. Plasmid pJH1 was linearized by digestion with Ssi I and Hind III and joined with the 1.6 kb partial Sst I-Hind III fragment containing the PDGFα-R extracellular domain sequences. The resulting plasmid having the immunoglobulin μ enhancer-promoter joined to the PDGFα-R extracellular domain was designated pPAB6. Plasmid pSDL112 was digested with Hind III to isolate the 1.2 kb fragment encoding the immunoglobulin light chain constant region (Cκ). The 1.2 kb Hind III fragment was ligated with Hind III-linearized pPAB6. A plasmid having the $_{\kappa}$ sequence in the correct orientation was designated pPAB7.

C. Construction of pPAB9

The partial Sst I-Hind III fragment encoding the extracellular domain of the PDGFα-R was joined to the immunoglobulin heavy chain constant region. For convenience, the internal Xba I site in plasmid pJH1 was removed by digestion with Xba I, blunt-ending with T4 DNA polymerase, and religation. A plasmid which did not contain the internal Xba I site, but retained the Xba I site in the polylinker was designated 11.28.3.6. Plasmid 11.28.3.6 was linearized by digestion with Sst I and Xba I. Plasmid pPAB6 was digested to completion with Hind III and partially digested with Sst I to isolate the 1.6 kb Sst I-Hind III fragment containing the PDGFα-R extracellular domain. Plasmid pφ5V$_{\mu}$huC$_{\gamma}$1M-neo (Example 12.C.) was digested with Hind III and Xba I to isolate the 6.0 kb fragment encoding the immunoglobulin heavy chain constant region (huC$_{\gamma}$1M). The Sst I-Hind III-linearized 11.28.3.6, the 1.6 kb Sst I-Hind III PDGFα-R fragment and the 6.0 kb Hind III-Xba I huC$_{\gamma}$1M fragment were ligated to form plasmid pPAB9.

D. Expression of pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Pvu I-linearized pPAB9 were cotransfected with Pvu I-linearized p416 into SP2/0-Ag14 cells by electroporation. Transfected cells were initially selected in growth medium containing 50 nM methotrexate and were subsequently amplified in a growth medium containing 100 μM methotrexate. Media from drug resistant clones were tested for the presence of secreted PDGF α-receptor analogs by enzyme-linked immunosorbant assay (ELISA). Ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml monoclonal antibody 292 1.8 which is specific for the PDGF α-receptor

diluted in phosphate buffered saline (PBS; Sigma] overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well, and the plates were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human IgG heavy chain antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well, and the plates were incubated for one hour at 4° C. One hundred microliters of chromophore (100 μl ABTS [2,2'-Azinobis(3-ethylbenzthiazoline sulfonic acid) diammonium salt; Sigma]+1 μl 30% H_2O_2 +12.5 ml citrate/phosphate buffer [9.04 g/l citric acid, 10.16 g/l Na_2HPO_4]) was added to each well, and the wells were incubated for 30 minutes at room temperature. The samples were measured at 405 nm. The results of the assay showed that the PDGF α-receptor analogs secreted by the transfectants contained an immunoglobulin heavy chain.

Analysis of spent media from transfected cells by Northern analysis, Western analysis and by radioimmunoprecipitation showed that the transfectants did not express a PDGF α-receptor analog from the pPAB7 construction. Transfectants were subsequently treated as containing only pPAB9.

Drug resistant clones were also tested for the presence of secreted PDGF α-receptor analogs by immunoprecipitation. For each clone, approximately one million drug resistant transfectants were grown in DMEM lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μCi ^{35}S-cysteine. The spent media was harvested from the labeled cells and 250 Al of medium from each clone was assayed for binding to the anti-PDGF α-receptor antibody 292.18. Monoclonal antibody 292.18 diluted in PBS was added to each sample to a final concentration of 2.5 μg per 250 Al spent media. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample, and the immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS). The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at -70 ° C. The results of the immunoprecipitation showed that the PDGF α-receptor analog secreted by the transfectants was bound by the anti-PDGF α-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF α-receptor analog secreted by the transfectants contained both the PDGF α-receptor ligand-binding domain and the human heavy chain.

Spent medium from drug-resistant clones were tested for their ability to bind PDGF in a competition binding assay essentially as described in Example 12.C. The results of the assay showed that the transfectants secreted a PDGF α-receptor analog capable of binding PDGF-AA. A clone containing the pPAB9 was designated 3.17.1.57.

E. Co-expression of pPAB7 and pPAB9 in Mammalian Cells

Bgl II-linearized pPAB7 and Bam HI-linearized pICneo were cotransfected into clone 3.17.1.57, and transfected cells were selected in the presence of neomycin. Media from drug resistant cells were assayed for the presence of immunoglobulin heavy chain, immunoglobulin light chain and the PDGF α-receptor ligand-binding domain by ELISA essentially as described above. Briefly, ninety-six well assay plates were prepared by incubating 100 μl of 1 μg/ml goat anti-human IgG Fc antibody (Sigma) or 100 μl of 1 μg/ml 292.18 overnight at 4° C. Excess antibody was removed by three washes with 0.5% Tween 20 in PBS. One hundred microliters of spent media was added to each well of each plate, and the plates were incubated for one hour at 4° C. Unbound proteins were removed by eight washes with 0.5% Tween 20 in PBS. One hundred microliters of peroxidase-conjugated goat anti-human IgG antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL)+0.5% Tween 20 in PBS) was added to each well of the plate coated with the anti-Fc antibody, and 100 μl of peroxidase-conjugated goat anti human kappa antibody (diluted 1:1000 in a solution containing 5% chicken serum (GIBCO-BRL) +0.5% Tween 20 in PBS) was added to each well of the plate coated with 292.18. The plates were incubated for one hour at 4° C. One hundred microliters of chromophore (100 μl ABTS [2,2'-Azinobis(3-ethylbenz-thiazoline sulfonic acid) diammonium salt; Sigma]+1 μl 30% H_2O_2+12.5 ml citrate/phosphate buffer [9.04 g/l citric acid, 10.16 g/l Na_2HPO_4]) was added to each well of each plate, and the plates were incubated to 30 minutes at room temperature. The samples were measured at 405 nm, the wavelength giving maximal absorbance of the chromogenic substrate, to identify clones having absorbances higher than background indicating the presence of immunoglobulin heavy chain. Clones that gave positive results in both ELISA assays (showing that the clones produced proteins containing heavy chain regions, light chain constant regions and the PDGF α-receptor ligand-binding region) were selected for further characterization.

Drug resistant clones that were positive for both ELISA assays were subsequently tested for the presence of secreted PDGF α-receptor analogs by immunoprecipitation. For each positive clone, approximately one million drug resistant transfectants were grown in DMEM lacking cysteine+2% calf serum for 18 hours at 37° C. in the presence of 50 μgCI ^{35}S-cysteine. The spent media was harvested from the labeled cells and 250 μl of medium from each clone was assayed for binding to monoclonal antibody 292.18. Monoclonal antibody 292.18 diluted in PBS was added to each sample to a final concentration of 2.5 μg. Five microliters of rabbit anti-mouse Ig diluted in PBS was added to each sample and the immunocomplexes were precipitated by the addition of 50 μl 10% fixed Staph A (weight/volume in PBS) The immunocomplexes were analyzed on 8% SDS-polyacrylamide gels followed by autoradiography overnight at –70° C. The results of the immunoprecipitation showed that the PDGF α-receptor analog secreted by the transfectants was bound by the anti-PDGF α-receptor antibody. The combined results of the ELISA and immunoprecipitation assays showed that the PDGF α-receptor analog secreted by the transfectants contained the PDGF α-receptor ligand-binding domain, the human heavy chain and the human light chain constant region. A clone that secreted a PDGF α-receptor analog that was positive for both the above-described ELISA assays and the immunoprecipitation assay was designated 5.6.2.1.

EXAMPLE 16

Purification and Characterization of PDGF α-Receptor Analogs

A. Purification of PDGF α-Receptor Analogs From Clone 3.17.1.57

The PDGF a-Receptor analog was purified from the conditioned culture media of clone 3.17.1.57 by cycling cell-conditioned medium over an immunoaffinity column composed of monoclonal antibody 292.18 bound to a CNBr-activated Sepharose 4B resin, which is specific for the PDGF α-receptor. The column was washed with PBA, then eluted with 0.1M citrate, pH 3.0. The peak column fractions containing the α-receptor were pooled, neutralized to pH 7.2 by the addition of 2M Tris, pH 7.4, then passed over a

protein-A Sepharose column. This column was washed sequentially with PBS, then with 0.1M citrate, pH 5.0. The PDGF α-receptor analog was then eluted with 0.1M citrate, pH 3.0. The peak eluate fractions were pooled, and glycerol was added to a final concentration of 10%. The sample was concentrated on a centriprep 10 concentrator (Ainicon). The PDGF α-receptor analog purified from clone 3.17.1.57 was termed a "dimeric PDGF α-receptor analog".

B. Purification of PDGF α-Receptor Analogs From Clone 5.6.2.1

The PDGF α-receptor analog was purified from the conditioned culture media of clone 5.6.2.1 by cycling cell-conditioned medium over the immunoaffinity column described above. The column was washed with PBS then eluted with 0.1M citrate, pH 3.0. The peak column fractions containing the α-receptor were pooled, neutralized to pH 7.2 by the addition of 2M Tris (what pH 7.4), then passed over a protein-A sepharose column. This column was washed sequentially with PBS then with 0.1M citrate, pH 5.0. The PDGF α-receptor analog was then eluted with 0.1M citrate, pH 3.0. The peak eluate fractions were pooled and glycerol was added to a final concentration of 10%. The sample was concentrated on a centriprep 10 concentrator. The PDGF α-receptor analogs purified from clone 5.6.2.1 was termed a "tetrameric PDGF α-receptor analog".

EXAMPLE 17

A. Use of the PDGF α-receptor Analogs in Ligand Binding Studies

Purified tetrameric PDGF α-receptor analog and purified dimeric PDGF α-receptor analog were compared to monolayers of a control cell line of canine kidney epithelial cells, which do not naturally express the PDGF α-receptor, transfected with the human PDGF α-receptor cDNA for ligand binding activity. The dissociation constant (Kd) of the receptor preparations was determined by saturation binding and subsequent Scatchard analysis.

Ligand binding of the purified PDGF α-receptor analogs was determined using a solid phase binding assay. Affinity-purified goat anti-human IgG was diluted to a concentration of 2 μg/ml in 0.1M Na_2HCO_3, pH 9.6 and 100 μl/well of the solution was used to coat 96-well microtiter plates for 18 hours at 4° C. Excess antibody was removed from the wells with one wash with ELISA C buffer (PBS, 0.05% Tween-20). The plates were incubated with 175 μl/well of ELISA B buffer (PBS, 1% BSA, 0.05% Tween-20) to block the wells, followed by two washes with ELISA C buffer. One hundred microliters of 50 ng/ml PDGF α-receptor analog (dimeric or tetrameric) diluted in ELISA buffer B was added to each well and the plates were incubated over night at 4° C. Unbound protein was removed from the wells with two washes with ELISA buffer B. ^{125}I-labeled PDGF-AA was serially diluted in binding media (Hams F-12, 25 mM HEPES pH 7.2, 0.25% rabbit serum albumin), and 100 μl of each dilution was added to the wells. The samples were incubated for two hours at room temperature. Unbound ^{125}I-labeled PDGF-AA was removed with three washes with binding media. One hundred microliters of 0.1M citrate, pH 2.5 was added to each well, and the plates were incubated for five minutes. After the incubation, the citrate buffer was removed and transferred to a tube for counting in a gamma counter. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-AA was determined by a parallel assay wherein separate wells coated only with goat anti-human IgG were incubated with the ^{125}I-labeled PDGF-AA samples.

A saturation binding assay was performed on alpha T-7 cells transfected with the PDGF α-receptor. The cells were grown to confluency in 24-well culture plates. The cells were washed one time with binding media. Iodinated PDGF-AA was serially diluted in binding media. One milliliter of each dilution was added to the wells, and the plates were incubated for 3 hours at 4° C. Unbound ^{125}I-labeled PDGF-AA was removed and the cells were washed three times with binding media. PBS containing 1% Triton X-100 was added to the cells for 5 minutes. The extracts were harvested and counted in a gamma counter. Nonspecific binding was determined at a single concentration of ^{125}I-labeled PDGF-AA using a 500-fold excess PDGF-BB.

The dissociation constants determined by Scatchard analysis (ibid.) of the saturation binding assays for the tetrameric PDGF α-receptor analog, dimeric PDGF α-receptor analog and the control cells (Table 5).

TABLE 5

Dissociation Constants for the Tetrameric PDGF α-Receptor, the Dimeric PDGF α-receptor and control cells Transfected with the PDGF α-receptor

Receptor	kD
Tetrameric PDGF α-receptor analog	1.6×10^{-12}
Dimeric PDGF α-receptor analog	8.51×10^{-11}
Control cells[PDGF α-receptor]	3.7×10^{-11}

A solid-phase competition binding assay was established using the tetrameric PDGF α-receptor analog. Ninety six-well microtiter plates were coated with goat anti-human IgG (2 μg/ml), the wells blocked with ELISA B buffer, 50 ng/ml of purified tetrameric PDGF α-receptor analog diluted in binding media was added, and the plates were incubated two hours at room temperature. Unbound receptor was removed and the wells were washed with binding media. The plates were incubated for two hours at room temperature with increasing concentrations of either PDGF-AA or PDGF-BB diluted in binding media. The wells were washed, then incubated for two hours at room temperature with 3 ng/ml ^{125}I-labeled PDGF-AA diluted in binding media. Unbound labeled PDGF-AA was removed, the wells were subsequently washed with binding media, and the bound, labeled PDGF-AA was harvested by the addition of 0.1M citrate, pH 2.5, as described for the saturation binding studies. PDGF-AB, PDGF-AA and PDGF-BB were found to compete for receptor binding with ^{125}I-PDGF-AA.

B. Use of Tetrameric PDGF α-Receptor Analogs as Antagonists for PDGF-Stimulated Mitogenesis.

A tetrameric PDGF α-receptor analog, purified as described in Example 16.B., was analyzed for the ability to neutralize PDGF-stimulated mitogenesis in mouse 3T3 cells. Increasing amounts of the purified tetrameric PDGF α-receptor analog were mixed with PDGF-AA, -AB or -BB ranging .6 to 5 ng. The mixtures were then added to cultures of confluent mouse 3T3 cells. The ability of the PDGF to stimulate a mitogenic response, as measured by the incorporation of ^{3}H-thymidine, was determined essentially as described (Raines and Ross, *Methods In Enzymology* 109: 749–773, 1985, which is incorporated by reference herein). The tetrameric PDGF α-receptor analog demonstrated a dose response inhibition of PDGF-stimulated ^{3}H-thymidine incorporation for all three isoforms of PDGF.

C. Inverse Ligand-Receptor Radioreceptor Assay

An inverse ligand-receptor radioreceptor assay was designed to screen for the presence of PDGF-BB, PDGF-BB binding proteins, PDGF-BB related molecules, and PDGF-β receptor antagonists in test samples. PDGF-BB (100 ng/ml) was coated onto the walls of 96-well microtiter plates, and

the plates were incubated at 4° C. for 16 hours. The wells were washed once with ELISA C buffer and then incubated with ELISA B buffer to block the nonspecific binding sites. To the wells were added 50 μl of either PDGF standard or a test sample and 50 μl of [125I]-labeled tetrameric PDGF β-receptor analog. The samples were incubated for one hour at room temperature. The wells were washed once with ELISA C buffer, and 0.1M citrate, pH 2.5 containing 1% NP-40 was added to each well to disrupt the ligand-receptor analog bond and elute the bound receptor analog. The acid wash was collected and counted in a gamma counter. The presence of PDGF or a molecule which mimics PDGF or otherwise interferes with the binding of the well-bound PDGF-BB with its receptor will cause a decrease in the binding of the radiolabeled tetrameric PDGF β-receptor. Using this assay, PDGF-BB was found to inhibit receptor binding while PDGF-AA and PDGF-AB caused no significant decrease in receptor binding.

EXAMPLE 18

Assay Methods ps A. Preparation of Nitrocellulose Filters for Colony Assay

Colonies of transformants were tested for secretion of PDGF β-receptor analogs by first growing the cells on nitrocellulose filters that had been laid on top of solid growth medium. Nitrocellulose filters (Schleicher & Schuell, Keene, N.H.) were placed on top of solid growth medium and were allowed to be completely wetted. Test colonies were patched onto the wetted filters and were grown at 30° C. for approximately 40 hours. The filters were then removed from the solid medium, and the cells were removed by four successive rinses with Western Transfer Buffer (Table 4). The nitrocellulose filters were soaked in Western Buffer A (Table 4) for one hour at room temperature on a shaking platform with two changes of buffer. Secreted PDGFβ-R analogs were visualized on the filters described below.

B. Preparation of Protein Blot Filters

A nitrocellulose filter was soaked in Western Buffer A (Table 4) without the gelatin and placed in a Minifold (Schleicher & Schuell, Keene, N.H.). Five milliliters of culture supernatant was added without dilution to the Minifold wells, and the liquid was allowed to pass through the nitrocellulose filter by gravity. The nitrocellulose filter was removed from the minifold and was soaked in Western Buffer A (Table 3) for one hour on a shaking platform at room temperature. The buffer was changed three times during the hour incubation.

C. Preparation of Western Blot Filters The transformants were analyzed by Western blot, essentially as described by Towbin et al. (*Proc. Natl. Acad. Sci. USA* 76: 4350–4354, 1979) and Gordon et al. (U.S. Pat. No. 4,452,901). Culture supernatants from appropriately grown transformants were diluted with three volumes of 95% ethanol. The ethanol mixtures were incubated overnight at −70° C. The precipitates were spun out of solution by centrifugation in an SS-24 rotor at 18,000 rpm for 20 minutes. The supernatants were discarded and the precipitate pellets were resuspended in 200 μl of dH_2O. Two hundred microliters of 2xloading buffer (Table 4) was added to each sample, and the samples were incubated in a boiling water bath for 5 minutes.

The samples were electrophoresed in a 15% sodium dodecylsulfate polyacrylamide gel under non-reducing conditions. The proteins were electrophoretically transferred to nitrocellulose paper using conditions described by Towbin et al. (ibid.). The nitrocellulose filters were then incubated in Western Buffer A (Table 4) for 75 minutes at room temperature on a platform rocker.

D. Processing the Filters for Visualization with Antibody

Filters prepared as described above were screened for proteins recognized by the binding of a PDGF β-receptor specific monoclonal antibody, designated PR7212. The filters were removed from the Western Buffer A (Table 4) and placed in sealed plastic bags containing a 10 ml solution comprising 10 μg/ml PR7212 monoclonal antibody diluted in Western Buffer A. The filters were incubated on a rocking platform overnight at 4° C. or for one hour at room temperature. Excess antibody was removed with three 15-minute washes with Western Buffer A on a shaking platform at room temperature.

Ten microliters biotin-conjugated horse anti-mouse antibody (Vector Laboratories, Burlingame, Calif.) in 20 ml Western Buffer A was added to the filters. The filters were re-incubated for one hour at room temperature on a platform shaker, and unbound conjugated antibody was removed with three fifteen-minute washes with Western Buffer A.

The filters were pre-incubated for one hour at room temperature with a solution comprising 50 μl Vectastain Reagent A (Vector Laboratories) in 10 ml of Western Buffer A that had been allowed to incubate at room temperature for 30 minutes before use. The filters were washed with one quick wash with distilled water followed by three 15-minute washes with Western Buffer B (Table 4) at room temperature. The Western Buffer B washes were followed by one wash with distilled water.

During the preceding wash step, the substrate reagent was prepared. Sixty mg of horseradish peroxidase reagent (Bio-Rad, Richmond, Calif.) was dissolved in 20 ml HPLC grade methanol. Ninety milliliters of distilled water was added to the dissolved peroxidase followed by 2.5 ml 2M Tris, pH 7.4 and 3.8 ml 4M NaCl. One hundred microliters of 30% H_2O_2 was added just before use. The washed filters were incubated with 75 ml of substrate and incubated at room temperature for 10 minutes with vigorous shaking. After the 10 minute incubation, the buffer was changed, and the filters were incubated for an additional 10 minutes. The filters were then washed in distilled water for one hour at room temperature. Positives were scored as those samples which exhibited coloration.

E. Processing the Filters For Visualization with an Anti-Substance P Antibody

Filters prepared as described above were probed with an anti-substance P antibody. The filters were removed from the Western Buffer A and rinsed with Western transfer buffer, followed by a 5-minute wash in phosphate buffered saline (PBS, Sigma, St. Louis, Mo.). The filters were incubated with a 10 ml solution containing 0.5M 1-ethyl-3-3-dimethylamino propyl carbodiimide (Sigma) in 1.0M NH_4Cl for 40 minutes at room temperature. After incubation, the filters were washed three times, for 5 minutes per wash, in PBS. The filters were blocked with 2% powdered milk diluted in PBS.

The filters were then incubated with a rat anti-substance P monoclonal antibody (Accurate Chemical & Scientific Corp., Westbury, N.Y.). Ten microliters of the antibody was diluted in 10 ml of antibody solution (PBS containing 20% fetal calf serum and 0.5% Tween-20). The filters were incubated at room temperature for 1 hour. Unbound antibody was removed with four 5-minute washes with PBS.

The filters were then incubated with a biotin-conjugated rabbit anti-rat peroxidase antibody (Cappel Laboratories, Malvern, Pa.) The conjugated antibody was diluted 1:1000 in 10 ml of antibody solution for 2 hours at room tempera-

ture. Excess conjugated antibody was removed with four 5-minute washes with PBS.

The filters were pre-incubated for 30 minutes at room temperature with a solution containing 50 μl Vectastain Reagent A (Vector Laboratories) and 50 μl Vectastain Reagent B (Vector Laboratories) in 10 ml of antibody solution that had been allowed to incubate for 30 minutes before use. Excess Vectastain reagents were removed by four 5-minute washes with PBS.

During the preceding wash step, the substrate reagent was prepared. Sixty milligrams of horseradish peroxidase reagent (Bio-Rad Laboratories, Richmond, Calif.) was dissolved in 25 ml of HPLC grade methanol. Approximately 100 ml of PBS and 200 μl H_2O_2 were added just before use. The filters were incubated with the substrate reagent for 10 to 20 minutes. The substrate was removed by a vigorous washing distilled water.

E. Iodination of PDGF-BB.

A PDGF-BB mutant molecule having a tyrosine replacing the phenylalanine at position 23 (PDGF-BB_{Y_2}) was iodinated and subsequently purified, using a purification method which produces 125I-labeled PDGF-BB with a higher specific activity than primary-labeled material and which was found to substantially decrease the nonspecific binding component. The PDGF-BB_{Y_2} was labeled using the Iodobead method (Pierce Chemical). The labeled protein was gel filtered over a C-25 desalting column (Pharmacia LKB Technologies) equilibrated with 10 mM acetic acid, 0.25% gelatin and 100 mM NaCl. The peak fractions were pooled and pH adjusted to 7.2 by the addition of Tris-base. The labeled mixture was chromatographed over an affinity column composed of PDGF β-receptor analog protein coupled to CnBr-activated Sepharose (Pharmacia LKB Technologies, Inc.). The column was washed with phosphate buffered saline and eluted with 0.1M citrate, pH 2.5 containing 0.25% gelatin. The peak eluate fractions were pooled and assayed by ELISA to determine the PDGF-BB concentration.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be evident that certain changes and modifications may be practiced within the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(1 1 1) NUMBER OF SEQUENCES: 36

(2) INFORMATION FOR SEQ ID NO:1:

(1) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4465 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(1 1) MOLECULE TYPE: cDNA

(1 1 1) HYPOTHETICAL: N

(1 v) ANTI-SENSE: N

(v 1) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens
(D) DEVELOPMENTAL STAGE: Adult
(F) TISSUE TYPE: Skin
(G) CELL TYPE: Fibroblasts

(v 1 1) IMMEDIATE SOURCE:
(B) CLONE: pR-ZX1

(1 x) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 354..3671
(D) OTHER INFORMATION:

(x 1) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCCAGGGCGG CCCCTCTGGC GGCTCTGCTC CTCCCGAAGG ATGCTTGGGG AGTGAGGCGA     120

AGCTGGGCGC TCCTCTCCCC TACAGCAGCC CCCTTCCTCC ATCCCTCTGT TCTCCTGAGC     180

CTTCAGGAGC CTGCACCAGT CCTGCCTGTC CTTCTACTCA GCTGTTACCC ACTCTGGGAC     240

CAGCAGTCTT TCTGATAACT GGGAGAGGGC AGTAAGGAGG ACTTCCTGGA GGGGGTGACT     300

GTCCAGAGCC TGGAACTGTG CCCACACCAG AAGCCATCAG CAGCAAGGAC ACC ATG        356
                                                          Met
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CGG CTT CCG GGT GCG ATG CCA GCT CTG GCC CTC AAA GGC GAG CTG CTG      484
Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu Leu
  1               5                  10                  15

TTG CTG TCT CTC CTG TTA CTT CTG GAA CCA CAG ATC TCT CAG GGC CTG      532
Leu Leu Ser Leu Leu Leu Leu Leu Glu Pro Gln Ile Ser Gln Gly Leu
             20                  25                  30

GTC GTC ACA CCC CCG GGG CCA GAG CTT GTC CTC AAT GTC TCC AGC ACC      580
Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser Thr
         35                  40                  45

TTC GTT CTG ACC TGC TCG GGT TCA GCT CCG GTG GTG TGG GAA CGG ATG      628
Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg Met
     50                  55                  60                  65

TCC CAG GAG CCC CCA CAG GAA ATG GCC AAG GCC CAG GAT GGC ACC TTC      676
Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr Phe
                 70                  75                  80

TCC AGC GTG CTC ACA CTG ACC AAC CTC ACT GGG CTA GAC ACG GGA GAA      724
Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly Glu
             85                  90                  95

TAC TTT TGC ACC CAC AAT GAC TCC CGT GGA CTG GAG ACC GAT GAG CGG      772
Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu Arg
        100                 105                 110

AAA CGG CTC TAC ATC TTT GTG CCA GAT CCC ACC GTG GGC TTC CTC CCT      820
Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu Pro
    115                 120                 125

AAT GAT GCC GAG GAA CTA TTC ATC TTT CTC ACG GAA ATA ACT GAG ATC      868
Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu Ile
130                 135                 140                 145

ACC ATT CCA TGC CGA GTA ACA GAC CCA CAG CTG GTG GTG ACA CTG CAC      916
Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu His
                150                 155                 160

GAG AAG AAA GGG GAC GTT GCA CTG CCT GTC CCC TAT GAT CAC CAA CGT      964
Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln Arg
            165                 170                 175

GGC TTT TCT GGT ATC TTT GAG GAC AGA AGC TAC ATC TGC AAA ACC ACC     1012
Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr Thr
        180                 185                 190

ATT GGG GAC AGG GAG GTG GAT TCT GAT GCC TAC TAT GTC TAC AGA CTC     1060
Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg Leu
    195                 200                 205

CAG GTG TCA TCC ATC AAC GTC TCT GTG AAC GCA GTG CAG ACT GTG GTC     1108
Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val Val
210                 215                 220                 225

CGC CAG GGT GAG AAC ATC ACC CTC ATG TGC ATT GTG ATC GGG AAT GAG     1156
Arg Gln Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn Glu
                230                 235                 240

GTG GTC AAC TTC GAG TGG ACA TAC CCC CGC AAA GAA AGT GGG CGG CTG     1204
Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg Leu
            245                 250                 255

GTG GAG CCG GTG ACT GAC TTC CTC TTG GAT ATG CCT TAC CAC ATC CGC     1252
Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile Arg
        260                 265                 270

TCC ATC CTG CAC ATC CCC AGT GCC GAG TTA GAA GAC TCG GGG ACC TAC     1300
Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr Tyr
    275                 280                 285

ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT GAA AAG GCC     1348
Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys Ala
290                 295                 300                 305

ATC AAC ATC ACC GTG GTT GAG AGC GGC TAC GTG CGG CTC CTG GGA GAG     1396
Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly Glu
                310                 315                 320
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GTG GGC ACA CTA CAA TTT GCT GAG CTG CAT CGG AGC CGG ACA CTG CAG      1364
Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu Gln
            325                 330                 335

GTA GTG TTC GAG GCC TAC CCA CCG CCC ACT GTC CTG TGG TTC AAA GAC      1412
Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys Asp
        340                 345                 350

AAC CGC ACC CTG GGC GAC TCC AGC GCT GGC GAA ATC GCC CTG TCC ACG      1460
Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser Thr
    355                 360                 365

CGC AAC GTG TCG GAG ACC CGG TAT GTG TCA GAG CTG ACA CTG GTT CGC      1508
Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val Arg
370                 375                 380                 385

GTG AAG GTG GCA GAG GCT GGC CAC TAC ACC ATG CGG GCC TTC CAT GAG      1556
Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His Glu
                390                 395                 400

GAT GCT GAG GTC CAG CTC TCC TTC CAG CTA CAG ATC AAT GTC CCT GTC      1604
Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro Val
            405                 410                 415

CGA GTG CTG GAG CTA AGT GAG AGC CAC CCT GAC AGT GGG GAA CAG ACA      1652
Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln Thr
        420                 425                 430

GTC CGC TGT CGT GGC CGG GGC ATG CCC CAG CCG AAC ATC ATC TGG TCT      1700
Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp Ser
    435                 440                 445

GCC TGC AGA GAC CTC AAA AGG TGT CCA CGT GAG CTG CCG CCC ACG CTG      1748
Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr Leu
450                 455                 460                 465

CTG GGG AAC AGT TCC GAA GAG GAG AGC CAG CTG GAG ACT AAC GTG ACG      1796
Leu Gly Asn Ser Ser Glu Glu Glu Ser Gln Leu Glu Thr Asn Val Thr
                470                 475                 480

TAC TGG GAG GAG GAG CAG GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG      1844
Tyr Trp Glu Glu Glu Gln Glu Phe Glu Val Val Ser Thr Leu Arg Leu
            485                 490                 495

CAG CAC GTG GAT CGG CCA CTG TCG GTG CGC TGC ACG CTG CGC AAC GCT      1892
Gln His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn Ala
        500                 505                 510

GTG GGC CAG GAC ACG CAG GAG GTC ATC GTG GTG CCA CAC TCC TTG CCC      1940
Val Gly Gln Asp Thr Gln Glu Val Ile Val Val Pro His Ser Leu Pro
    515                 520                 525

TTT AAG GTG GTG GTG ATC TCA GCC ATC CTG GCC CTG GTG GTG CTC ACC      1988
Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu Thr
530                 535                 540                 545

ATC ATC TCC CTT ATC ATC CTC ATC ATG CTT TGG CAG AAG AAG CCA CGT      2036
Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro Arg
                550                 555                 560

TAC GAG ATC CGA TGG AAG GTG ATT GAG TCT GTG AGC TCT GAC GGC CAT      2084
Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly His
            565                 570                 575

GAG TAC ATC TAC GTG GAC CCC ATG CAG CTG CCC TAT GAC TCC ACG TGG      2132
Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr Trp
        580                 585                 590

GAG CTG CCG CGG GAC CAG CTT GTG CTG GGA CGC ACC CTC GGC TCT GGG      2180
Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser Gly
    595                 600                 605

GCC TTT GGG CAG GTG GTG GAG GCC ACG GCT CAT GGC CTG AGC CAT TCT      2228
Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His Ser
610                 615                 620                 625

CAG GCC ACG ATG AAA GTG GCC GTC AAG ATG CTT AAA TCC ACA GCC CGC      2276
Gln Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala Arg
                630                 635                 640
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AGC AGT GAG AAG CAA GCC CTT ATG TCG GAG CTG AAG ATC ATG AGT CAC      2324
Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser His
                645                 650                 655

CTT GGG CCC CAC CTG AAC GTG GTC AAC CTG TTG GGG GCC TGC ACC AAA      2372
Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys
            660                 665                 670

GGA GGA CCC ATC TAT ATC ATC ACT GAG TAC TGC CGC TAC GGA GAC CTG      2420
Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp Leu
        675                 680                 685

GTG GAC TAC CTG CAC CGC AAC AAA CAC ACC TTC CTG CAG CAC CAC TCC      2468
Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His Ser
    690                 695                 700

GAC AAG CGC CGC CCG CCC AGC GCG GAG CTC TAC AGC AAT GCT CTG CCC      2516
Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu Pro
705                 710                 715                 720

GTT GGG CTC CCC CTG CCC AGC CAT GTG TCC TTG ACC GGG GAG AGC GAC      2564
Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser Asp
                725                 730                 735

GGT GGC TAC ATG GAC ATG AGC AAG GAC GAG TCG GTG GAC TAT GTG CCC      2612
Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val Pro
            740                 745                 750

ATG CTG GAC ATG AAA GGA GAC GTC AAA TAT GCA GAC ATC GAG TCC TCC      2660
Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser Ser
        755                 760                 765

AAC TAC ATG GCC CCT TAC GAT AAC TAC GTT CCC TCT GCC CCT GAG AGG      2708
Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu Arg
    770                 775                 780

ACC TGC CGA GCA ACT TTG ATC AAC GAG TCT CCA GTG CTA AGC TAC ATG      2756
Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr Met
785                 790                 795                 800

GAC CTC GTG GGC TTC AGC TAC CAG GTG GCC AAT GGC ATG GAG TTT CTG      2804
Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe Leu
                805                 810                 815

GCC TCC AAG AAC TGC GTC CAC AGA GAC CTG GCG GCT AGG AAC GTG CTC      2852
Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
            820                 825                 830

ATC TGT GAA GGC AAG CTG GTC AAG ATC TGT GAC TTT GGC CTG GCT CGA      2900
Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala Arg
        835                 840                 845

GAC ATC ATG CGG GAC TCG AAT TAC ATC TCC AAA GGC AGC ACC TTT TTG      2948
Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe Leu
    850                 855                 860

CCT TTA AAG TGG ATG GCT CCG GAG AGC ATC TTC AAC AGC CTC TAC ACC      2996
Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr Thr
865                 870                 875                 880

ACC CTG AGC GAC GTG TGG TCC TTC GGG ATC CTG CTC TGG GAG ATC TTC      3044
Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile Phe
                885                 890                 895

ACC TTG GGT GGC ACC CCT TAC CCA GAG CTG CCC ATG AAC GAG CAG TTC      3092
Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln Phe
            900                 905                 910

TAC AAT GCC ATC AAA CGG GGT TAC CGC ATG GCC CAG CCT GCC CAT GCC      3140
Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His Ala
        915                 920                 925

TCC GAC GAG ATC TAT GAG ATC ATG CAG AAG TGC TGG GAA GAG AAG TTT      3188
Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys Phe
    930                 935                 940

GAG ATT CGG CCC CCC TTC TCC CAG CTG GTG CTG CTT CTC GAG AGA CTG      3236
Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg Leu
945                 950                 955                 960
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TTG GGC GAA GGT TAC AAA AAG AAG TAC CAG CAG GTG GAT GAG GAG TTT      3284
Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu Phe
            965                 970                 975

CTG AGG AGT GAC CAC CCA GCC ATC CTT CGG TCC CAG GCC CGC TTG CCT      3332
Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu Pro
        980                 985                 990

GGG TTC CAT GGC CTC CGA TCT CCC CTG GAC ACC AGC TCC GTC CTC TAT      3380
Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu Tyr
    995                1000                1005

ACT GCC GTG CAG CCC AAT GAG GGT GAC AAC GAC TAT ATC ATC CCC CTG      3428
Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro Leu
1010                1015                1020                1025

CCT GAC CCC AAA CCC GAG GTT GCT GAC GAG GGC CCA CTG GAG GGT TCC      3476
Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly Ser
                1030                1035                1040

CCC AGC CTA GCC AGC TCC ACC CTG AAT GAA GTC AAC ACC TCC TCA ACC      3524
Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser Thr
            1045                1050                1055

ATC TCC TGT GAC AGC CCC CTG GAG CCC CAG GAC GAA CCA GAG CCA GAG      3572
Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro Glu
        1060                1065                1070

CCC CAG CTT GAG CTC CAG GTG GAG CCG GAG CCA GAG CTG GAA CAG TTG      3620
Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln Leu
    1075                1080                1085

CCG GAT TCG GGG TGC CCT GCG CCT CGG GCG GAA GCA GAG GAT AGC TTC      3668
Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser Phe
1090                1095                1100                1105

CTG TAGGGGGCTG GCCCCTACCC TGCCCTGCCT GAAGCTCCCC CCCTGCCAGC           3721
Leu

ACCCAGCATC TCCTGGCCTG GCCTGACCGG GCTTCCTGTC AGCCAGGCTG CCCTTATCAG    3781

CTGTCCCCTT CTGGAAGCTT TCTGCTCCTG ACGTGTTGTG CCCCAAACCC TGGGGCTGGC    3841

TTAGGAGGCA AGAAAACTGC AGGGGCCGTG ACCAGCCCTC TGCCTCCAGG GAGGCCAACT    3901

GACTCTGAGC CAGGGTTCCC CCAGGGAACT CAGTTTTCCC ATATGTAAGA TGGGAAAGTT    3961

AGGCTTGATG ACCCAGAATC TAGGATTCTC TCCCTGGCTG ACACGGTGGG GAGACCGAAT    4021

CCCTCCCTGG GAAGATTCTT GGAGTTACTG AGGTGGTAAA TTAACATTTT TTCTGTTCAG    4081

CCAGCTACCC CTCAAGGAAT CATAGCTCTC TCCTCGCACT TTTTATCCAC CCAGGAGCTA    4141

GGGAAGAGAC CCTAGCCTCC CTGGCTGCTG GCTGAGCTAG GGCCTAGCTT GAGCAGTGTT    4201

GCCTCATCCA GAAGAAAGCC AGTCTCCTCC CTATGATGCC AGTCCCTGCG TTCCCTGGCC    4261

CGAGCTGGTC TGGGGCCATT AGGCAGCCTA ATTAATGCTG GAGGCTGAGC CAAGTACAGG    4321

ACAGCCCCAG CCTGCAGCCC TTGCCCAGGG CACTTGGAGC ACACCCAGCC ATAGCAAGTG    4381

CCTGTGTCCC TGTCCTTCAG GCCCATCAGT CCTGGGGCTT TTTCTTTATC ACCCTCAGTC    4441

TTAATCCATC CACCAGAGTC TAGA                                           4465
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(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1104 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Arg Leu Pro Gly Ala Met Pro Ala Leu Ala Leu Lys Gly Glu Leu
 1               5                  10                  15
```

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Leu Leu Leu Ser Leu Leu Leu Leu Leu Gln Pro Gln Ile Ser Gln Gly
            20                  25                  30

Leu Val Val Thr Pro Pro Gly Pro Glu Leu Val Leu Asn Val Ser Ser
        35                  40                  45

Thr Phe Val Leu Thr Cys Ser Gly Ser Ala Pro Val Val Trp Glu Arg
    50                  55                  60

Met Ser Gln Glu Pro Pro Gln Glu Met Ala Lys Ala Gln Asp Gly Thr
65                  70                  75                  80

Phe Ser Ser Val Leu Thr Leu Thr Asn Leu Thr Gly Leu Asp Thr Gly
                85                  90                  95

Glu Tyr Phe Cys Thr His Asn Asp Ser Arg Gly Leu Glu Thr Asp Glu
            100                 105                 110

Arg Lys Arg Leu Tyr Ile Phe Val Pro Asp Pro Thr Val Gly Phe Leu
        115                 120                 125

Pro Asn Asp Ala Glu Glu Leu Phe Ile Phe Leu Thr Glu Ile Thr Glu
    130                 135                 140

Ile Thr Ile Pro Cys Arg Val Thr Asp Pro Gln Leu Val Val Thr Leu
145                 150                 155                 160

His Glu Lys Lys Gly Asp Val Ala Leu Pro Val Pro Tyr Asp His Gln
                165                 170                 175

Arg Gly Phe Ser Gly Ile Phe Glu Asp Arg Ser Tyr Ile Cys Lys Thr
            180                 185                 190

Thr Ile Gly Asp Arg Glu Val Asp Ser Asp Ala Tyr Tyr Val Tyr Arg
        195                 200                 205

Leu Gln Val Ser Ser Ile Asn Val Ser Val Asn Ala Val Gln Thr Val
    210                 215                 220

Val Arg Glu Gly Glu Asn Ile Thr Leu Met Cys Ile Val Ile Gly Asn
225                 230                 235                 240

Glu Val Val Asn Phe Glu Trp Thr Tyr Pro Arg Lys Glu Ser Gly Arg
                245                 250                 255

Leu Val Glu Pro Val Thr Asp Phe Leu Leu Asp Met Pro Tyr His Ile
            260                 265                 270

Arg Ser Ile Leu His Ile Pro Ser Ala Glu Leu Glu Asp Ser Gly Thr
        275                 280                 285

Tyr Thr Cys Asn Val Thr Glu Ser Val Asn Asp His Gln Asp Glu Lys
    290                 295                 300

Ala Ile Asn Ile Thr Val Val Glu Ser Gly Tyr Val Arg Leu Leu Gly
305                 310                 315                 320

Glu Val Gly Thr Leu Gln Phe Ala Glu Leu His Arg Ser Arg Thr Leu
                325                 330                 335

Gln Val Val Phe Glu Ala Tyr Pro Pro Pro Thr Val Leu Trp Phe Lys
            340                 345                 350

Asp Asn Arg Thr Leu Gly Asp Ser Ser Ala Gly Glu Ile Ala Leu Ser
        355                 360                 365

Thr Arg Asn Val Ser Glu Thr Arg Tyr Val Ser Glu Leu Thr Leu Val
    370                 375                 380

Arg Val Lys Val Ala Glu Ala Gly His Tyr Thr Met Arg Ala Phe His
385                 390                 395                 400

Glu Asp Ala Glu Val Gln Leu Ser Phe Gln Leu Gln Ile Asn Val Pro
                405                 410                 415

Val Arg Val Leu Glu Leu Ser Glu Ser His Pro Asp Ser Gly Glu Gln
            420                 425                 430

Thr Val Arg Cys Arg Gly Arg Gly Met Pro Gln Pro Asn Ile Ile Trp
        435                 440                 445
```

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```
Ser Ala Cys Arg Asp Leu Lys Arg Cys Pro Arg Glu Leu Pro Pro Thr
     450                 455                 460

Leu Leu Gly Asn Ser Ser Glu Glu Glu Ser Glu Leu Glu Thr Asn Val
 465                 470                 475                 480

Thr Tyr Trp Glu Glu Glu Glu Glu Phe Glu Val Val Ser Thr Leu Arg
                 485                 490                 495

Leu Glu His Val Asp Arg Pro Leu Ser Val Arg Cys Thr Leu Arg Asn
             500                 505                 510

Ala Val Gly Glu Asp Thr Glu Glu Val Ile Val Val Pro His Ser Leu
         515                 520                 525

Pro Phe Lys Val Val Val Ile Ser Ala Ile Leu Ala Leu Val Val Leu
     530                 535                 540

Thr Ile Ile Ser Leu Ile Ile Leu Ile Met Leu Trp Gln Lys Lys Pro
 545                 550                 555                 560

Arg Tyr Glu Ile Arg Trp Lys Val Ile Glu Ser Val Ser Ser Asp Gly
                 565                 570                 575

His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Thr
             580                 585                 590

Trp Glu Leu Pro Arg Asp Gln Leu Val Leu Gly Arg Thr Leu Gly Ser
         595                 600                 605

Gly Ala Phe Gly Gln Val Val Glu Ala Thr Ala His Gly Leu Ser His
     610                 615                 620

Ser Glu Ala Thr Met Lys Val Ala Val Lys Met Leu Lys Ser Thr Ala
 625                 630                 635                 640

Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Ser
                 645                 650                 655

His Leu Gly Pro His Leu Asn Val Val Asn Leu Leu Gly Ala Cys Thr
             660                 665                 670

Lys Gly Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Arg Tyr Gly Asp
         675                 680                 685

Leu Val Asp Tyr Leu His Arg Asn Lys His Thr Phe Leu Gln His His
     690                 695                 700

Ser Asp Lys Arg Arg Pro Pro Ser Ala Glu Leu Tyr Ser Asn Ala Leu
 705                 710                 715                 720

Pro Val Gly Leu Pro Leu Pro Ser His Val Ser Leu Thr Gly Glu Ser
                 725                 730                 735

Asp Gly Gly Tyr Met Asp Met Ser Lys Asp Glu Ser Val Asp Tyr Val
             740                 745                 750

Pro Met Leu Asp Met Lys Gly Asp Val Lys Tyr Ala Asp Ile Glu Ser
         755                 760                 765

Ser Asn Tyr Met Ala Pro Tyr Asp Asn Tyr Val Pro Ser Ala Pro Glu
     770                 775                 780

Arg Thr Cys Arg Ala Thr Leu Ile Asn Glu Ser Pro Val Leu Ser Tyr
 785                 790                 795                 800

Met Asp Leu Val Gly Phe Ser Tyr Gln Val Ala Asn Gly Met Glu Phe
                 805                 810                 815

Leu Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val
             820                 825                 830

Leu Ile Cys Glu Gly Lys Leu Val Lys Ile Cys Asp Phe Gly Leu Ala
         835                 840                 845

Arg Asp Ile Met Arg Asp Ser Asn Tyr Ile Ser Lys Gly Ser Thr Phe
     850                 855                 860

Leu Pro Leu Lys Trp Met Ala Pro Glu Ser Ile Phe Asn Ser Leu Tyr
```



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```
865                 870                 875                 880

Thr Thr Leu Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Trp Glu Ile
            885                 890                 895

Phe Thr Leu Gly Gly Thr Pro Tyr Pro Glu Leu Pro Met Asn Glu Gln
        900                 905                 910

Phe Tyr Asn Ala Ile Lys Arg Gly Tyr Arg Met Ala Gln Pro Ala His
    915                 920                 925

Ala Ser Asp Glu Ile Tyr Glu Ile Met Gln Lys Cys Trp Glu Glu Lys
930                 935                 940

Phe Glu Ile Arg Pro Pro Phe Ser Gln Leu Val Leu Leu Leu Glu Arg
945                 950                 955                 960

Leu Leu Gly Glu Gly Tyr Lys Lys Lys Tyr Gln Gln Val Asp Glu Glu
                965                 970                 975

Phe Leu Arg Ser Asp His Pro Ala Ile Leu Arg Ser Gln Ala Arg Leu
            980                 985                 990

Pro Gly Phe His Gly Leu Arg Ser Pro Leu Asp Thr Ser Ser Val Leu
        995                 1000                1005

Tyr Thr Ala Val Gln Pro Asn Glu Gly Asp Asn Asp Tyr Ile Ile Pro
    1010                1015                1020

Leu Pro Asp Pro Lys Pro Glu Val Ala Asp Glu Gly Pro Leu Glu Gly
1025                1030                1035                1040

Ser Pro Ser Leu Ala Ser Ser Thr Leu Asn Glu Val Asn Thr Ser Ser
                1045                1050                1055

Thr Ile Ser Cys Asp Ser Pro Leu Glu Pro Gln Asp Glu Pro Glu Pro
            1060                1065                1070

Glu Pro Gln Leu Glu Leu Gln Val Glu Pro Glu Pro Glu Leu Glu Gln
        1075                1080                1085

Leu Pro Asp Ser Gly Cys Pro Ala Pro Arg Ala Glu Ala Glu Asp Ser
    1090                1095                1100

Phe Leu
1105
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 57 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC871

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

ATTATACGCT CTCTTCCTCA GGTAAATGAG TGCCAGGGCC GGCAAGCCCC CGCTCCA 57

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid



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(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC872

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

CCGGGGAGCG GGGGCTTGCC GGCCCTGGCA CTCATTTACC TGAGGAAGAG AGAGCT 56

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 45 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC914

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CATGGGCACG TAATCTATAG ATTCATCCTT GCTCATATCC ATGTA 45

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC905

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCCT GGGCAGCC 38

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC905

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGCTGTCCT CTGCTTCAGC CAGAGGTCCT GGGCAGCC 38

(2) INFORMATION FOR SEQ ID NO:8:



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(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1380

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CATGGTGGAA TTCCTGCTGA T 21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1447

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGGTTGTGCA GAGCTGAGGA AGAGATGGA 29

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1453

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

AATTCATTAT GTTGTTGCAA GCCTTCTTGT TCCTGCTAGC TGGTTTCGCT GTTAA 55

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 55 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1454

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(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GATCTTAACA GCGAAACCAG CTAGCAGGAA CAAGAAGGCT TGCAACAACA TAATG 55

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1478

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATCGCGAGCA TGCAGATCTG A 21

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1479

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AGCTTCAGAT CTGCATGCTG CCGAT 25

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1774

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

AGCTGAGCGC AAATGTTGTG TCGAGTGCCC ACCGTGCCCA GCTTAGAATT CT 52

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 52 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear



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(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1777

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CTAGAGAATT CTAAGCTGGG CACGGTGGGC ACTCGACACA ACATTTGCGC TC 52

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1840

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GATCGGCCAC TGTCGGTGCG CTGCACGCTG CGCAACGCTG TGGGCCAGGA CACGCAGGAG 60

GTCATCGTGG TGCCACACTC CTTGCCCTTT AAGCA 95

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 95 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1847

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AGCTTGCTTA AAGGGCAAGG AGTGTGGCAC CACGATGACC TCCTGCGTGT CCTGGCCCAC 60

AGCGTTGCGC AGCGTGCAGC GCACCGACAG TGGCC 95

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 43 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1886

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:



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CCAGTGCCAA GCTTGTCTAG ACTTACCTTT AAAGGGCAAG GAG 43

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1892

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGCTTGAGCG T 11

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1893

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CTAGACGCTC A 11

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1894

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

AGCTTCCAGT TCTTCGGCCT CATGTCAGTT CTTCGGCCTC ATGTGAT 47

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 47 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC1895

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:22:

CTAGATCACA TGAGGCCGAA GAACTGACAT GAGGCCGAAG AACTGGA 47

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 66 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2181

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:23:

AATTCGGATC CACCATGGGC ACCAGCCACC CGGCGTTCCT GGTGTTAGGC TGCCTGCTGA 60

CCGGCC 66

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2182

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:24:

TGAGCCTGAT CCTGTGCCAA CTGAGCCTGC CATCGATCCT GCCAAACGAG AACGAGAAGG 60

TTGTGCAGCT A 71

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 69 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2183

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AATTTAGCTG CACAACCTTC TCGTTCTCGT TTGGCAGGAT CGATGGCAGG CTCAGTTGGC 60

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ACAGGATCA 69

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 68 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2184

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GGCTCAGGCC GGTCAGCAGG CAGCCTAACA CCAGGAACGC CGGGTGGCTG GTGCCCATGG 60

TGGATCCG 68

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 20 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2311

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:27:

TGATCACCAT GGCTCAACTG 20

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2351

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATTCCAC 10

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear



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(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2152

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:29:

ATTATACGCA TGGTGGAATT CGAGCT 26

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 41 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(v i i) IMMEDIATE SOURCE:
(B) CLONE: ZC2393

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:30:

ACGTAAGCTT GTCTAGACTT ACCTTCAGAA CGCAGGGTGG G 41

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 17 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pWK1

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:31:

Ala Leu His Asn His Tyr Thr Glu Lys Ser Leu Ser Leu Ser Pro Gly
1 5 10 15

Lys

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: Other nucleic acid

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:32:

TGTGACACTC TCCTGGGAGT TA 22

(2) INFORMATION FOR SEQ ID NO:33:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCATAGTAGT TACCATATCC TCTTGCACAG 30

(2) INFORMATION FOR SEQ ID NO:34:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

ACCGAACGTG AGAGGAGTGC TATAA 25

(2) INFORMATION FOR SEQ ID NO:35:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4054 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Homo sapiens

(vii) IMMEDIATE SOURCE:
(B) CLONE: p-alpha-17B

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 285..3471
(D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

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GCCCTGGGGA CGGACCGTGG GCGGCGCGCA GCGGCGGGAC GCGTTTTGGG GACGTGGTGG      60

CCAGCGCCTT CCTGCAGACC CACAGGGAAG TACTCCCTTT GACCTCCGGG GAGCTGCGAC     120

CAGGTTATAC GTTGCTGGTG GAAAAGTGAC AATTCTAGGA AAAGAGCTAA AAGCCGGATC     180

GGTGACCGAA AGTTTCCCAG AGCT ATG GGG ACT TCC CAT CCG GCG TTC CTG         231
                          Met Gly Thr Ser His Pro Ala Phe Leu
                           1               5

GTC TTA GGC TGT CTT CTC ACA GGG CTG AGC CTA ATC CTC TGC CAG CTT        279
Val Leu Gly Cys Leu Leu Thr Gly Leu Ser Leu Ile Leu Cys Gln Leu
 10                  15                  20                  25

TCA TTA CCC TCT ATC CTT CCA AAT GAA AAT GAA AAG GTT GTG CAG CTG        327
Ser Leu Pro Ser Ile Leu Pro Asn Glu Asn Glu Lys Val Val Gln Leu
             30                  35                  40
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AAT TCA TCC TTT TCT CTG AGA TGC TTT GGG GAG AGT GAA GTG AGC TGG        375
Asn Ser Ser Phe Ser Leu Arg Cys Phe Gly Glu Ser Glu Val Ser Trp
                45                  50                  55

CAG TAC CCC ATG TCT GAA GAA GAG AGC TCC GAT GTG GAA ATC AGA AAT        423
Gln Tyr Pro Met Ser Glu Glu Glu Ser Ser Asp Val Glu Ile Arg Asn
            60                  65                  70

GAA GAA AAC AAC AGC GGC CTT TTT GTG ACG GTC TTG GAA GTG AGC AGT        471
Glu Glu Asn Asn Ser Gly Leu Phe Val Thr Val Leu Glu Val Ser Ser
        75                  80                  85

GCC TCG GCG GCC CAC ACA GGG TTG TAC ACT TGC TAT TAC AAC CAC ACT        519
Ala Ser Ala Ala His Thr Gly Leu Tyr Thr Cys Tyr Tyr Asn His Thr
    90                  95                  100                 105

CAG ACA GAA GAG AAT GAG CTT GAA GGC AGG CAC ATT TAC ATC TAT GTG        567
Gln Thr Glu Glu Asn Glu Leu Glu Gly Arg His Ile Tyr Ile Tyr Val
                110                 115                 120

CCA GAC CCA GAT GTA GCC TTT GTA CCT CTA GGA ATG ACG GAT TAT TTA        615
Pro Asp Pro Asp Val Ala Phe Val Pro Leu Gly Met Thr Asp Tyr Leu
            125                 130                 135

GTC ATC GTG GAG GAT GAT GAT TCT GCC ATT ATA CCT TGT CGC ACA ACT        663
Val Ile Val Glu Asp Asp Asp Ser Ala Ile Ile Pro Cys Arg Thr Thr
        140                 145                 150

GAT CCC GAG ACT CCT GTA ACC TTA CAC AAC AGT GAG GGG GTG GTA CCT        711
Asp Pro Glu Thr Pro Val Thr Leu His Asn Ser Glu Gly Val Val Pro
    155                 160                 165

GCC TCC TAC GAC AGC AGA CAG GGC TTT AAT GGG ACC TTC ACT GTA GGG        759
Ala Ser Tyr Asp Ser Arg Gln Gly Phe Asn Gly Thr Phe Thr Val Gly
170                 175                 180                 185

CCC TAT ATC TGT GAG GCC ACC GTC AAA GGA AAG AAG TTC CAG ACC ATC        807
Pro Tyr Ile Cys Glu Ala Thr Val Lys Gly Lys Lys Phe Gln Thr Ile
                190                 195                 200

CCA TTT AAT GTT TAT GCT TTA AAA GCA ACA TCA GAG CTG GAT CTA GAA        855
Pro Phe Asn Val Tyr Ala Leu Lys Ala Thr Ser Glu Leu Asp Leu Glu
            205                 210                 215

ATG GAA GCT CTT AAA ACC GTG TAT AAG TCA GGG GAA ACG ATT GTG GTC        903
Met Glu Ala Leu Lys Thr Val Tyr Lys Ser Gly Glu Thr Ile Val Val
        220                 225                 230

ACC TGT GCT GTT TTT AAC AAT GAG GTG GTT GAC CTT CAA TGG ACT TAC        951
Thr Cys Ala Val Phe Asn Asn Glu Val Val Asp Leu Gln Trp Thr Tyr
    235                 240                 245

CCT GGA GAA GTG AAA GGC AAA GGC ATC ACA ATA CTG GAA GAA ATC AAA        999
Pro Gly Glu Val Lys Gly Lys Gly Ile Thr Ile Leu Glu Glu Ile Lys
250                 255                 260                 265

GTC CCA TCC ATC AAA TTG GTG TAC ACT TTG ACG GTC CCC GAG GCC ACG       1047
Val Pro Ser Ile Lys Leu Val Tyr Thr Leu Thr Val Pro Glu Ala Thr
                270                 275                 280

GTG AAA GAC AGT GGA GAT TAC GAA TGT GCT GCC CGC CAG GCT ACC AGG       1095
Val Lys Asp Ser Gly Asp Tyr Glu Cys Ala Ala Arg Gln Ala Thr Arg
            285                 290                 295

GAG GTC AAA GAA ATG AAG AAA GTC ACT ATT TCT GTC CAT GAG AAA GGT       1143
Glu Val Lys Glu Met Lys Lys Val Thr Ile Ser Val His Glu Lys Gly
        300                 305                 310

TTC ATT GAA ATC AAA CCC ACC TTC AGC CAG TTG GAA GCT GTC AAC CTG       1191
Phe Ile Glu Ile Lys Pro Thr Phe Ser Gln Leu Glu Ala Val Asn Leu
    315                 320                 325

CAT GAA GTC AAA CAT TTT GTT GTA GAG GTG CGG GCC TAC CCA CCT CCC       1239
His Glu Val Lys His Phe Val Val Glu Val Arg Ala Tyr Pro Pro Pro
330                 335                 340                 345

AGG ATA TCC TGG CTG AAA AAC AAT CTG ACT CTG ATT GAA AAT CTC ACT       1287
Arg Ile Ser Trp Leu Lys Asn Asn Leu Thr Leu Ile Glu Asn Leu Thr
                350                 355                 360
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GAG ATC ACC ACT GAT GTG GAA AAG ATT CAG GAA ATA AGG TAT CGA AGC      1335
Glu Ile Thr Thr Asp Val Glu Lys Ile Glu Glu Ile Arg Tyr Arg Ser
            365                 370                 375

AAA TTA AAG CTG ATC CGT GCT AAG GAA GAA GAC AGT GGC CAT TAT ACT      1383
Lys Leu Lys Leu Ile Arg Ala Lys Glu Glu Asp Ser Gly His Tyr Thr
        380                 385                 390

ATT GTA GCT CAA AAT GAA GAT GCT GTG AAG AGC TAT ACT TTT GAA GTG      1431
Ile Val Ala Gln Asn Glu Asp Ala Val Lys Ser Tyr Thr Phe Glu Leu
    395                 400                 405

TTA ACT CAA GTT CCT TCA TCC ATT CTG GAC TTG GTC GAT GAT CAC CAT      1479
Leu Thr Gln Val Pro Ser Ser Ile Leu Asp Leu Val Asp Asp His His
410                 415                 420                 425

GGC TCA ACT GGG GGA CAG ACG GTG AGG TGC ACA GCT GAA GGC ACG CCG      1527
Gly Ser Thr Gly Gly Gln Thr Val Arg Cys Thr Ala Glu Gly Thr Pro
                430                 435                 440

CTT CCT GAT ATT GAG TGG ATG ATA TGC AAA GAT ATT AAG AAA TGT AAT      1575
Leu Pro Asp Ile Glu Trp Met Ile Cys Lys Asp Ile Lys Lys Cys Asn
            445                 450                 455

AAT GAA ACT TCC TGG ACT ATT TTG GCC AAC AAT GTC TCA AAC ATC ATC      1623
Asn Glu Thr Ser Trp Thr Ile Leu Ala Asn Asn Val Ser Asn Ile Ile
        460                 465                 470

ACG GAG ATC CAC TCC CGA GAC AGG AGT ACC GTG GAG GGC CGT GTG ACT      1671
Thr Glu Ile His Ser Arg Asp Arg Ser Thr Val Glu Gly Arg Val Thr
    475                 480                 485

TTC GCC AAA GTG GAG GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT      1719
Phe Ala Lys Val Glu Glu Thr Ile Ala Val Arg Cys Leu Ala Lys Asn
490                 495                 500                 505

CTC CTT GGA GCT GAG AAC CGA GAG CTG AAG CTG GTG GCT CCC ACC CTG      1767
Leu Leu Gly Ala Glu Asn Arg Glu Leu Lys Leu Val Ala Pro Thr Leu
                510                 515                 520

CGT TCT GAA CTC ACG GTG GCT GCT GCA GTC CTG GTG CTG TTG GTG ATT      1815
Arg Ser Glu Leu Thr Val Ala Ala Ala Val Leu Val Leu Leu Val Ile
            525                 530                 535

GTG ATC ATC TCA CTT ATT GTC CTG GTT GTC ATT TGG AAA CAG AAA CCG      1863
Val Ile Ile Ser Leu Ile Val Leu Val Val Ile Trp Lys Gln Lys Pro
        540                 545                 550

AGG TAT GAA ATT CGC TGG AGG GTC ATT GAA TCA ATC AGC CCG GAT GGA      1911
Arg Tyr Glu Ile Arg Trp Arg Val Ile Glu Ser Ile Ser Pro Asp Gly
    555                 560                 565

CAT GAA TAT ATT TAT GTG GAC CCG ATG CAG CTG CCT TAT GAC TCA AGA      1959
His Glu Tyr Ile Tyr Val Asp Pro Met Gln Leu Pro Tyr Asp Ser Arg
570                 575                 580                 585

TGG GAG TTT CCA AGA GAT GGA CTA GTG CTT GGT CGG GTC TTG GGG TCT      2007
Trp Glu Phe Pro Arg Asp Gly Leu Val Leu Gly Arg Val Leu Gly Ser
                590                 595                 600

GGA GCG TTT GGG AAG GTG GTT GAA GGA ACA GCC TAT GGA TTA AGC CGG      2055
Gly Ala Phe Gly Lys Val Val Glu Gly Thr Ala Tyr Gly Leu Ser Arg
            605                 610                 615

TCC CAA CCT GTC ATG AAA GTT GCA GTG AAG ATG CTA AAA CCC ACG GCC      2103
Ser Gln Pro Val Met Lys Val Ala Val Lys Met Leu Lys Pro Thr Ala
        620                 625                 630

AGA TCC AGT GAA AAA CAA GCT CTC ATG TCT GAA CTG AAG ATA ATG ACT      2151
Arg Ser Ser Glu Lys Gln Ala Leu Met Ser Glu Leu Lys Ile Met Thr
    635                 640                 645

CAC CTG GGG CCA CAT TTG AAC ATT GTA AAC TTG CTG GGA GCC TGC ACC      2199
His Leu Gly Pro His Leu Asn Ile Val Asn Leu Leu Gly Ala Cys Thr
650                 655                 660                 665

AAG TCA GGC CCC ATT TAC ATC ATC ACA GAG TAT TGC TTC TAT GGA GAT      2247
Lys Ser Gly Pro Ile Tyr Ile Ile Thr Glu Tyr Cys Phe Tyr Gly Asp
                670                 675                 680
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TTG GTC AAC TAT TTG CAT AAG AAT AGG GAT AGC TTC CTG AGC CAC CAC      2295
Leu Val Asn Tyr Leu His Lys Asn Arg Asp Ser Phe Leu Ser His His
                685                 690                 695

CCA GAG AAG CCA AAG AAA GAG CTG GAT ATC TTT GGA TTG AAC CCT GCT      2343
Pro Glu Lys Pro Lys Lys Glu Leu Asp Ile Phe Gly Leu Asn Pro Ala
            700                 705                 710

GAT GAA AGC ACA CGG AGC TAT GTT ATT TTA TCT TTT GAA AAC AAT GGT      2391
Asp Glu Ser Thr Arg Ser Tyr Val Ile Leu Ser Phe Glu Asn Asn Gly
        715                 720                 725

GAC TAC ATG GAC ATG AAG CAG GCT GAT ACT ACA CAG TAT GTC CCC ATG      2439
Asp Tyr Met Asp Met Lys Gln Ala Asp Thr Thr Gln Tyr Val Pro Met
    730                 735                 740                 745

CTA GAA AGG AAA GAG GTT TCT AAA TAT TCC GAC ATC CAG AGA TCA CTC      2487
Leu Glu Arg Lys Glu Val Ser Lys Tyr Ser Asp Ile Gln Arg Ser Leu
                750                 755                 760

TAT GAT CGT CCA GCC TCA TAT AAG AAG AAA TCT ATG TTA GAC TCA GAA      2535
Tyr Asp Arg Pro Ala Ser Tyr Lys Lys Lys Ser Met Leu Asp Ser Glu
            765                 770                 775

GTC AAA AAC CTC CTT TCA GAT GAT AAC TCA GAA GGC CTT ACT TTA TTG      2583
Val Lys Asn Leu Leu Ser Asp Asp Asn Ser Glu Gly Leu Thr Leu Leu
        780                 785                 790

GAT TTG TTG AGC TTC ACC TAT CAA GTT GCC CGA GGA ATG GAG TTT TTG      2631
Asp Leu Leu Ser Phe Thr Tyr Gln Val Ala Arg Gly Met Glu Phe Leu
    795                 800                 805

GCT TCA AAA AAT TGT GTC CAC CGT GAT CTG GCT GCT CGC AAC GTC CTC      2679
Ala Ser Lys Asn Cys Val His Arg Asp Leu Ala Ala Arg Asn Val Leu
810                 815                 820                 825

CTG GCA CAA GGA AAA ATT GTG AAG ATC TGT GAC TTT GGC CTG GCC AGA      2727
Leu Ala Gln Gly Lys Ile Val Lys Ile Cys Asp Phe Gly Leu Ala Arg
                830                 835                 840

GAC ATC ATG CAT GAT TCG AAC TAT GTG TCG AAA GGC AGT ACC TTT CTG      2775
Asp Ile Met His Asp Ser Asn Tyr Val Ser Lys Gly Ser Thr Phe Leu
            845                 850                 855

CCC GTG AAG TGG ATG GCT CCT GAG AGC ATC TTT GAC AAC CTC TAC ACC      2823
Pro Val Lys Trp Met Ala Pro Glu Ser Ile Phe Asp Asn Leu Tyr Thr
        860                 865                 870

ACA CTG AGT GAT GTC TGG TCT TAT GGC ATT CTG CTC TGG GAG ATC TTT      2871
Thr Leu Ser Asp Val Trp Ser Tyr Gly Ile Leu Leu Trp Glu Ile Phe
    875                 880                 885

TCC CTT GGT GGC ACC CCT TAC CCC GGC ATG ATG GTG GAT TCT ACT TTC      2919
Ser Leu Gly Gly Thr Pro Tyr Pro Gly Met Met Val Asp Ser Thr Phe
890                 895                 900                 905

TAC AAT AAG ATC AAG AGT GGG TAC CGG ATG GCC AAG CCT GAC CAC GCT      2967
Tyr Asn Lys Ile Lys Ser Gly Tyr Arg Met Ala Lys Pro Asp His Ala
                910                 915                 920

ACC AGT GAA GTC TAC GAG ATC ATG GTG AAA TGC TGG AAC AGT GAG CCG      3015
Thr Ser Glu Val Tyr Glu Ile Met Val Lys Cys Trp Asn Ser Glu Pro
            925                 930                 935

GAG AAG AGA CCC TCC TTT TAC CAC CTG AGT GAG ATT GTG GAG AAT CTG      3063
Glu Lys Arg Pro Ser Phe Tyr His Leu Ser Glu Ile Val Glu Asn Leu
        940                 945                 950

CTG CCT GGA CAA TAT AAA AAG AGT TAT GAA AAA ATT CAC CTG GAC TTC      3111
Leu Pro Gly Gln Tyr Lys Lys Ser Tyr Glu Lys Ile His Leu Asp Phe
    955                 960                 965

CTG AAG AGT GAC CAT CCT GCT GTG GCA CGC ATG CGT GTG GAC TCA GAC      3159
Leu Lys Ser Asp His Pro Ala Val Ala Arg Met Arg Val Asp Ser Asp
970                 975                 980                 985

AAT GCA TAC ATT GGT GTC ACC TAC AAA AAC GAG GAA GAC AAG CTG AAG      3207
Asn Ala Tyr Ile Gly Val Thr Tyr Lys Asn Glu Glu Asp Lys Leu Lys
                990                 995                1000
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GAC TGG GAG GGT GGT CTG GAT GAG CAG AGA CTG AGC GCT GAC AGT GGC      3255
Asp Trp Glu Gly Gly Leu Asp Glu Gln Arg Leu Ser Ala Asp Ser Gly
            1005                1010                1015

TAC ATC ATT CCT CTG CCT GAC ATT GAC CCT GTC CCT GAG GAG GAG GAC      3303
Tyr Ile Ile Pro Leu Pro Asp Ile Asp Pro Val Pro Glu Glu Glu Asp
        1020                1025                1030

CTG GGC AAG AGG AAC AGA CAC AGC TCG CAG ACC TCT GAA GAG AGT GCC      3351
Leu Gly Lys Arg Asn Arg His Ser Ser Gln Thr Ser Glu Glu Ser Ala
    1035                1040                1045

ATT GAG ACG GGT TCC AGC AGT TCC ACC TTC ATC AAG AGA GAG GAC GAG      3399
Ile Glu Thr Gly Ser Ser Ser Ser Thr Phe Ile Lys Arg Glu Asp Glu
1050                1055                1060                1065

ACC ATT GAA GAC ATC GAC ATG ATG GAC GAC ATC GGC ATA GAC TCT TCA      3447
Thr Ile Glu Asp Ile Asp Met Met Asp Asp Ile Gly Ile Asp Ser Ser
                1070                1075                1080

GAC CTG GTG GAA GAC AGC TTC CTG TAACTGGCGG ATTCGAGGGG TTCCTTCCAC     3501
Asp Leu Val Glu Asp Ser Phe Leu
            1085

TTCTGGGGCC ACCTCTGGAT CCCGTTCAGA AAACCACTTT ATTGCAATGC GGAGGTTGAG    3551

AGGAGGACTT GGTTGATGTT TAAAGAGAAG TTCCCAGCCA AGGGCCTCGG GGAGCGTTCT    3621

AAATATGAAT GAATGGGATA TTTTGAAATG AACTTTGTCA GTGTTGCCTC TTGCAATGCC    3691

TCAGTAGCAT CTCAGTGGTG TGTGAAGTTT GGAGATAGAT GGATAAGGGA ATAATAGGCC    3761

ACAGAAGGTG AACTTTGTGC TTCAAGGACA TTGGTGAGAG TCCAACAGAC ACAATTTATA    3831

CTGCGACAGA ACTTCAGCAT TGTAATTATG TAAATAACTC TAACCAAGGC TGTGTTTAGA    3901

TTGTATTAAC TATCTTCTTT GGACTTCTGA AGAGACCACT GAATCCATCC TGTACTTCCC    3971

TCTTGAAACC TGATGTAGCT GCTGTTGAAC TTTTTAAAGA AGTGCATGAA AAACCATTTT    4041

TGAACCTTAA AAGGTACTGG TACTATAGCA TTTTGCTATC TTTTTTAGTG TTAAAGAGAT    4111

AAAGAATAAT AAG                                                       4134
```

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1089 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: protein

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:36:

```
Met Gly Thr Ser His Pro Ala Phe Leu Val Leu Gly Cys Leu Leu Thr
 1               5                  10                  15

Gly Leu Ser Leu Ile Leu Cys Gln Leu Ser Leu Pro Ser Ile Leu Pro
            20                  25                  30

Asn Glu Asn Glu Lys Val Val Gln Leu Asn Ser Ser Phe Ser Leu Arg
        35                  40                  45

Cys Phe Gly Glu Ser Glu Val Ser Trp Gln Tyr Pro Met Ser Glu Glu
    50                  55                  60

Glu Ser Ser Asp Val Glu Ile Arg Asn Glu Glu Asn Asn Ser Gly Leu
65                  70                  75                  80

Phe Val Thr Val Leu Glu Val Ser Ser Ala Ser Ala Ala His Thr Gly
                85                  90                  95

Leu Tyr Thr Cys Tyr Tyr Asn His Thr Gln Thr Glu Glu Asn Glu Leu
            100                 105                 110

Glu Gly Arg His Ile Tyr Ile Tyr Val Pro Asp Pro Asp Val Ala Phe
        115                 120                 125
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Val Pro Leu Gly Met Thr Asp Tyr Leu Val Ile Val Glu Asp Asp Asp
130 135 140

Ser Ala Ile Ile Pro Cys Arg Thr Thr Asp Pro Glu Thr Pro Val Thr
145 150 155 160

Leu His Asn Ser Glu Gly Val Val Pro Ala Ser Tyr Asp Ser Arg Glu
165 170 175

Gly Phe Asn Gly Thr Phe Thr Val Gly Pro Tyr Ile Cys Glu Ala Thr
180 185 190

Val Lys Gly Lys Lys Phe Gln Thr Ile Pro Phe Asn Val Tyr Ala Leu
195 200 205

Lys Ala Thr Ser Glu Leu Asp Leu Glu Met Glu Ala Leu Lys Thr Val
210 215 220

Tyr Lys Ser Gly Glu Thr Ile Val Val Thr Cys Ala Val Phe Asn Asn
225 230 235 240

Glu Val Val Asp Leu Gln Trp Thr Tyr Pro Gly Glu Val Lys Gly Lys
245 250 255

Gly Ile Thr Ile Leu Glu Glu Ile Lys Val Pro Ser Ile Lys Leu Val
260 265 270

Tyr Thr Leu Thr Val Pro Glu Ala Thr Val Lys Asp Ser Gly Asp Tyr
275 280 285

Glu Cys Ala Ala Arg Gln Ala Thr Arg Glu Val Lys Glu Met Lys Lys
290 295 300

Val Thr Ile Ser Val His Glu Lys Gly Phe Ile Glu Ile Lys Pro Thr
305 310 315 320

Phe Ser Gln Leu Glu Ala Val Asn Leu His Glu Val Lys His Phe Val
325 330 335

Val Glu Val Arg Ala Tyr Pro Pro Pro Arg Ile Ser Trp Leu Lys Asn
340 345 350

Asn Leu Thr Leu Ile Glu Asn Leu Thr Glu Ile Thr Thr Asp Val Glu
355 360 365

Lys Ile Glu Glu Ile Arg Tyr Arg Ser Lys Leu Lys Leu Ile Arg Ala
370 375 380

Lys Glu Glu Asp Ser Gly His Tyr Thr Ile Val Ala Gln Asn Glu Asp
385 390 395 400

Ala Val Lys Ser Tyr Thr Phe Glu Leu Leu Thr Gln Val Pro Ser Ser
405 410 415

Ile Leu Asp Leu Val Asp Asp His His Gly Ser Thr Gly Gly Gln Thr
420 425 430

Val Arg Cys Thr Ala Glu Gly Thr Pro Leu Pro Asp Ile Glu Trp Met
435 440 445

Ile Cys Lys Asp Ile Lys Lys Cys Asn Asn Glu Thr Ser Trp Thr Ile
450 455 460

Leu Ala Asn Asn Val Ser Asn Ile Ile Thr Glu Ile His Ser Arg Asp
465 470 475 480

Arg Ser Thr Val Glu Gly Arg Val Thr Phe Ala Lys Val Glu Glu Thr
485 490 495

Ile Ala Val Arg Cys Leu Ala Lys Asn Leu Leu Gly Ala Glu Asn Arg
500 505 510

Glu Leu Lys Leu Val Ala Pro Thr Leu Arg Ser Glu Leu Thr Val Ala
515 520 525

Ala Ala Val Leu Val Leu Leu Val Ile Val Ile Ile Ser Leu Ile Val
530 535 540

Leu Val Val Ile Trp Lys Gln Lys Pro Arg Tyr Glu Ile Arg Trp Arg
545 550 555 560



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Val Ile Glu Ser Ile Ser Pro Asp Gly His Glu Tyr Ile Tyr Val Asp
565 570 575

Pro Met Gln Leu Pro Tyr Asp Ser Arg Trp Glu Phe Pro Arg Asp Gly
580 585 590

Leu Val Leu Gly Arg Val Leu Gly Ser Gly Ala Phe Gly Lys Val Val
595 600 605

Glu Gly Thr Ala Tyr Gly Leu Ser Arg Ser Gln Pro Val Met Lys Val
610 615 620

Ala Val Lys Met Leu Lys Pro Thr Ala Arg Ser Ser Glu Lys Gln Ala
625 630 635 640

Leu Met Ser Glu Leu Lys Ile Met Thr His Leu Gly Pro His Leu Asn
645 650 655

Ile Val Asn Leu Leu Gly Ala Cys Thr Lys Ser Gly Pro Ile Tyr Ile
660 665 670

Ile Thr Glu Tyr Cys Phe Tyr Gly Asp Leu Val Asn Tyr Leu His Lys
675 680 685

Asn Arg Asp Ser Phe Leu Ser His His Pro Glu Lys Pro Lys Lys Glu
690 695 700

Leu Asp Ile Phe Gly Leu Asn Pro Ala Asp Glu Ser Thr Arg Ser Tyr
705 710 715 720

Val Ile Leu Ser Phe Glu Asn Asn Gly Asp Tyr Met Asp Met Lys Gln
725 730 735

Ala Asp Thr Thr Gln Tyr Val Pro Met Leu Glu Arg Lys Glu Val Ser
740 745 750

Lys Tyr Ser Asp Ile Gln Arg Ser Leu Tyr Asp Arg Pro Ala Ser Tyr
755 760 765

Lys Lys Lys Ser Met Leu Asp Ser Glu Val Lys Asn Leu Leu Ser Asp
770 775 780

Asp Asn Ser Glu Gly Leu Thr Leu Leu Asp Leu Leu Ser Phe Thr Tyr
785 790 795 800

Gln Val Ala Arg Gly Met Glu Phe Leu Ala Ser Lys Asn Cys Val His
805 810 815

Arg Asp Leu Ala Ala Arg Asn Val Leu Leu Ala Gln Gly Lys Ile Val
820 825 830

Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Met His Asp Ser Asn
835 840 845

Tyr Val Ser Lys Gly Ser Thr Phe Leu Pro Val Lys Trp Met Ala Pro
850 855 860

Glu Ser Ile Phe Asp Asn Leu Tyr Thr Thr Leu Ser Asp Val Trp Ser
865 870 875 880

Tyr Gly Ile Leu Leu Trp Glu Ile Phe Ser Leu Gly Gly Thr Pro Tyr
885 890 895

Pro Gly Met Met Val Asp Ser Thr Phe Tyr Asn Lys Ile Lys Ser Gly
900 905 910

Tyr Arg Met Ala Lys Pro Asp His Ala Thr Ser Glu Val Tyr Gln Ile
915 920 925

Met Val Lys Cys Trp Asn Ser Glu Pro Glu Lys Arg Pro Ser Phe Tyr
930 935 940

His Leu Ser Glu Ile Val Glu Asn Leu Leu Pro Gly Gln Tyr Lys Lys
945 950 955 960

Ser Tyr Glu Lys Ile His Leu Asp Phe Leu Lys Ser Asp His Pro Ala
965 970 975

Val Ala Arg Met Arg Val Asp Ser Asp Asn Ala Tyr Ile Gly Val Thr



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                980                 985                 990
Tyr Lys Asn Glu Glu Asp Lys Leu Lys Asp Trp Glu Gly Gly Leu Asp
            995                1000                1005
Glu Glu Arg Leu Ser Ala Asp Ser Gly Tyr Ile Ile Pro Leu Pro Asp
        1010                1015                1020
Ile Asp Pro Val Pro Glu Glu Glu Asp Leu Gly Lys Arg Asn Arg His
    1025                1030                1035                1040
Ser Ser Gln Thr Ser Glu Glu Ser Ala Ile Glu Thr Gly Ser Ser Ser
                1045                1050                1055
Ser Thr Phe Ile Lys Arg Glu Asp Glu Thr Ile Glu Asp Ile Asp Met
            1060                1065                1070
Met Asp Asp Ile Gly Ile Asp Ser Ser Asp Leu Val Glu Asp Ser Phe
        1075                1080                1085
Leu
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We claim:

1. A method for producing a secreted active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to a dimerizing protein heterologous to said non-immunoglobulin polypeptide;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion encoded by said DNA sequence; and

isolating said dimerized polypeptide fusion from said host cell.

2. The method of claim 1 wherein the dimerized polypeptide fusion is a homodimer.

3. The method of claim 1 wherein the dimerizing protein comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

4. The method of claim 1 wherein the dimerizing protein comprises an immunoglobulin light chain constant region.

5. The method of claim 1 wherein the dimerizing protein comprises an immunoglobulin heavy chain constant region.

6. The method of claim 5 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin hinge region.

7. The method of claim 5 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin variable region.

8. The method of claim 5 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

9. A method for producing a secreted, biologically active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA, and optionally encoding an immunoglobulin hinge or variable region joined in proper reading frame to said immunoglobulin heavy chain constant region;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a biologically active dimerized polypeptide fusion encoded by said first and second DNA sequences; and

isolating said biologically active dimerized polypeptide fusion from said host cell.

10. The method of claim 9 wherein said second DNA sequence further encodes an immunoglobulin hinge region and wherein said hinge region is joined to said immunoglobulin heavy chain constant region.

11. The method of claim 9 wherein said second DNA sequence further encodes an immunoglobulin variable region and wherein said variable region is joined upstream of and in proper reading frame with said immunoglobulin heavy chain constant region domain.

12. The method of claim 9 wherein said host cell is a fungal cell or a cultured mammalian cell.

13. The method of claim 9 wherein said host cell is a cultured rodent myeloma cell line.

14. The method of claim 9 wherein said non-immunoglobulin polypeptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531, a polypeptide comprising the amino acid sequence of FOGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of FIGS. 11A–11 D (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524.

15. A method for producing a secreted, biologically active dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence fol-

lowed downstream by and in proper reading frame with a first DNA sequence encoding a non-immunoglobulin polypeptide requiring dimerization for biological activity joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4, and optionally encoding an immunoglobulin hinge or variable region joined in proper reading frame to said immunoglobulin heavy chain constant region;

introducing into said host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding an immunoglobulin light chain constant region, and optionally encoding immunoglobulin variable region joined in proper reading frame to said immunoglobulin heavy chain constant region;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a biologically active dimerized polypeptide fusion encoded by said first and second DNA sequences; and

isolating said biologically active dimerized polypeptide fusion from said host cell.

16. The method of claim 15 wherein said first DNA sequence further encodes an immunoglobulin hinge region and wherein said hinge region is joined to said immunoglobulin constant region.

17. The method of claim 15 wherein said second DNA sequence further encodes an immunoglobulin variable region and wherein said variable region is joined upstream of and in proper reading frame with said immunoglobulin light chain constant region domain.

18. The method of claim 15 wherein said host cell is a fungal cell or a cultured mammalian cell.

19. The method of claim 15 wherein said host cell is a cultured rodent myeloma cell line.

20. The method of claim 15 wherein said non-immunoglobulin polypeptide requiring dimerization for biological activity is selected from the group consisting of a polypeptide comprising the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to lysine, number 531 a polypeptide comprising the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine, number 29, to methionine, number 441, and a polypeptide comprising the amino acid sequence of FIGS. 11A–11D (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524.

21. A method for producing a secreted receptor analog, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to at least one secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a ligand-binding domain of a receptor requiring dimerization for biological activity joined to a dimerizing protein heterologous to said ligand-binding domain, and optionally an immunoglobulin hinge or variable region to said dimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a receptor analog encoded by said DNA sequence; and

isolating said receptor analog from said host cell.

22. The method of claim 21 wherein the dimerizing protein comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

23. The method of claim 21 wherein the dimerizing protein comprises an immunoglobulin light chain constant region.

24. The method of claim 21 wherein the dimerizing protein comprises an immunoglobulin heavy chain constant region.

25. The method of claim 24 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin hinge region.

26. The method of claim 24 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin variable region.

27. The method of claim 26 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

28. A method for producing a secreted PDGF receptor analog, comprising:

introducing into a cultured rodent myeloma cell a first DNA construct comprising a mouse V_H promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of FIGS. 11A–11D (Sequence ID Numbers 35 and 36) from glutamine, number 24 to glutamic acid, number 524, joined to an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_H4 joined to an immunoglobulin hinge region;

introducing into said cultured rodent myeloma cell a second DNA construct comprising a mouse $V_κ$ promoter operatively linked to a PDGF receptor signal sequence followed downstream of and in proper reading frame with a DNA sequence encoding the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2) from isoleucine number 29, to lysine, number 531, joined to an immunoglobulin light chain constant region;

growing said cultured rodent myeloma cell in an appropriate growth medium under physiological conditions to allow the secretion of a PDGF receptor analog encoded by said first and second DNA sequences; and

isolating said PDGF receptor analog from said cultured myeloma cell.

29. A method for producing a secreted active multimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a DNA sequence encoding a non-immunoglobulin polypeptide requiring multimerization for biological activity joined to a multimerizing protein selected from the group consisting of: an immunoglobulin heavy chain or light chain constant region, and optionally encoding an immunoglobulin hinge or variable region joined to said multimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a multimerized polypeptide fusion encoded by said DNA sequence; and

isolating said multimerized polypeptide fusion from said host cell.

30. The method of claim 29 wherein said multimerized polypeptide fusion is a tetramer comprising four polypeptide

fusions each having a non-immunoglobulin polypeptide joined to a multimerizing protein.

31. The method of claim 29 wherein the multimerizing protein comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

32. The method of claim 29 wherein the multimerizing protein comprises an immunoglobulin light chain constant region.

33. The method of claim 29 wherein the multimerizing protein comprises an immunoglobulin heavy chain constant region.

34. The method of claim 33 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin hinge region.

35. The method of claim 33 wherein the immunoglobulin heavy chain constant region is joined to an immunoglobulin variable region.

36. The method of claim 35 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

37. A method for producing a secreted active heteromultimeric polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a first polypeptide fusion comprising a first non-immunoglobulin polypeptide joined to a first multimerizing protein selected from the group consisting of: an immunoglobulin heavy chain or light chain constant region, and optionally encoding an immunoglobulin hinge or variable region joined to said first multimerizing protein, and a second DNA construct comprising a transcriptional promoter operatively linked to a secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide fusion comprising a second non-immunoglobulin polypeptide joined to a second multimerizing protein selected from the group consisting of: an immunoglobulin heavy chain or light chain constant region, and optionally encoding an immunoglobulin hinge or variable region joined to said second multimerizing protein;

growing said host cell in an appropriate growth medium under physiological conditions to allow secretion of said heteromultimeric polypeptide fusion encoded by said DNA sequences; and

isolating said heteromultimeric polypeptide fusion from said host cell.

38. The method of claim 37 wherein the first and second non-immunoglobulin polypeptides each comprise an amino acid sequence selected from the group consisting of (A) the amino acid sequence of FIGS. 1A–1D (Sequence ID Numbers 1 and 2), and (B) the amino acid sequence of FIGS. 11A–11D (Sequence ID Numbers 35 and 36).

39. The method of claim 37 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3, and C_HA of a γ, α, ε, μ, or δ class immunoglobulin heavy chain.

40. The method of claim 37 wherein the first multimerizing protein is different from the second multimerizing protein.

41. The method of claim 40 wherein the first and second non-immunoglobulin polypeptides are the same.

42. The method of claim 37 wherein said multimerized polypeptide fusion comprises a first polypeptide fusion comprising a first receptor or receptor ligand binding domain requiring multimerization for activity joined to a first immunoglobulin constant region and a second polypeptide fusion comprising a second receptor or receptor ligand binding domain requiring multimerization for activity joined to a second immunoglobulin constant region.

43. The method of claim 37 wherein said heteromultimeric polypeptide fusion is a heterotetramer comprising four polypeptide fusions each having a non-immunoglobulin polypeptide joined to a multimerizing protein.

44. The method of claim 37 wherein said heteromultimeric polypeptide fusion comprises a first polypeptide fusion having a first non-immunoglobulin polypeptide joined to a first immunoglobulin constant region and a second polypeptide fusion having a second non-immunoglobulin polypeptide fused to a second immunoglobulin constant region different from the first immunoglobulin constant region.

45. The method of claim 44 wherein the first multimerizing protein comprises an immunoglobulin heavy chain constant region and the second multimerizing protein comprises an immunoglobulin light chain constant region.

46. The method of claim 37 wherein the first and second multimerizing proteins each comprise an immunoglobulin heavy chain constant region or an immunoglobulin light chain constant region.

47. The method of claim 46 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region joined to an immunoglobulin hinge region.

48. The method of claim 46 wherein one of said multimerizing proteins comprises an immunoglobulin heavy chain constant region joined to an immunoglobulin variable region.

49. The method of claim 48 wherein the immunoglobulin variable region is selected from the group consisting of V_H, Vκ, and Vλ.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 5,843,725
DATED : December 1, 1998
INVENTOR(S) : Andrzej Z. Sledziewski et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 103, claim 15,
Line 7, the term "or variable region" should be deleted.
Lines 7-8, the term "in proper reading frame" should be deleted.
Line 17, the term "heavy" should read -- light --.

Signed and Sealed this

First Day of January, 2002

Attest:

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office

EXHIBIT 3

EXHIBIT 3
REDACTED IN ITS ENTIRETY

EXHIBIT 4

EXHIBIT 4
REDACTED IN ITS ENTIRETY

EXHIBIT 5

EXHIBIT 5
REDACTED IN ITS ENTIRETY

EXHIBIT 6

A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells

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CTLA-4, expressed by activated T cells, transduces an inhibitory signal. We show here that PCR amplification of the coding sequence of CTLA-4 in nonstimulated human T lymphocytes results in the amplification of two transcripts of 650 and 550 bp. Sequencing shows that the larger form codes for membrane CTLA-4 and the 550-bp transcript is a spliced variant in which exon 2 coding for the transmembrane region is deleted. This spliced cDNA has been named CTLA-4delTM. The splicing induces a frame shift which results in the addition of 22 extra amino acids before a translational termination. Activation of T cells with phorbol 12-myristate 13-acetate plus ionomycin or anti-CD3 plus anti-CD28 monoclonal antibodies induces a suppression of CTLA-4delTM mRNA expression associated with a preferential expression of the membrane CTLA-4 mRNA, showing that CTLA-4delTM mRNA expression is restricted to nonactivated T cells. A soluble immunoreactive form of CTLA-4 was detected in the serum of 14/64 healthy subjects. These results suggest that nonstimulated T cells may constitutively produce a soluble form of CTLA-4 which may have an important role in the regulation of immune homeostasis.

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Key words: CTLA-4 / Alternative splicing / Human T lymphocyte

1 Introduction

The generation of an efficient antigen-specific T cell response is initiated by signals delivered via the CD3/TCR complex and requiers co-stimulatory signals via cell surface molecules expressed by T cells [1]. Among the co-stimulatory signals, the interaction between CD80 and/or CD86 expressed by APC and CD28 constitutively expressed by T cells is crucial [2]. It is responsible for the proliferation, cytokine production and acquisition of effector functions by T cells [3–5]. Blocking this interaction using neutralizing anti-CD28 or anti-CD86 mAb induces T cell anergy [6] and inhibits the development of helper and cytotoxic T cells [7–9]. Once activated, T cells also express another ligand for CD80/CD86, CTLA-4 or CD152 [10–12]. CTLA-4 and CD28 are homologous and belong to the Ig superfamily. Their binding motifs to CD80 are located in the complementarity-determining region (CDR)1- and CDR3-like domains [13, 14] and involve at least the MYPPPY motif but CTLA-4 binds to CD80/CD86 with 20- to 50-fold higher affinity than does CD28 [15]. In contrast to CD28 which delivers an activation signal to T cells, CTLA-4 transduces an inhibitory signal to T cells [16–18]. The role of CTLA-4 was clearly evidenced in CTLA-4-deficient mice which present massive lymphoproliferation and multiorgan tissue destruction [19, 20]. Anti-CTLA-4 mAb increased *in vivo* the antigen-specific T cell proliferation [21] and allowed rejection of established tumors [22].

In this study, reverse transcriptase (RT)-PCR analysis shows that nonactivated human peripheral blood T lymphocytes express an alternatively spliced form of CTLA-4 mRNA. The splicing induces the deletion of the transmembrane region, resulting in the production of a soluble form of CTLA-4 in transfected cells. Interestingly, a soluble form of CTLA-4 was detected in the sera of healthy individuals.

[I 19836]

The two first authors constibuuuted equally to this work

Abbreviations: CDR: Complementarity-determining region **RT:** Reverse transcriptase

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2 Results

2.1 Identification of spliced variants of CTLA-4 mRNA

The amplification by PCR of the human CTLA-4 coding sequence revealed the constitutive expression by non-activated T cells of two transcripts whose sizes are approximatively 650 and 550 bp (Fig. 1 A). Both cDNA fragments were subcloned in the TA cloning vector and sequenced. The larger form (650 bp) showed complete homology with the published CTLA-4 sequence (data not shown) [23]. The smaller cDNA fragment has a deletion from nucleotide 456 (starting from ATG codon) to nucleotide 563. According to the genomic organization of the human CTLA-4 gene [24], this cDNA is generated by alternative splicing of the CTLA-4 mRNA of which the exon encoding the transmembrane region (exon 2) is spliced out (Fig. 1 b). The deletion causes a frame shift resulting in 22 extra amino acids before a translational termination at position 523 (Fig. 1 b). This spliced transcript has been named CTLA-4delTM. The expression of the two CTLA-4 mRNA variants has been observed in 10/10 healthy subjects tested. CTLA-4delTM has a predicted molecular mass of 20 kDa.

2.2 CTLA-4delTM mRNA is expressed only in nonactivated T cells

CTLA-4 mRNA expression is increased following stimulation [25–27]. Thus, we have evaluated the influence of T cell activation on CTLA-4 *vs.* CTLA-4delTM mRNA expression. An 8-h stimulation with PMA plus ionomycin or anti-CD3 plus anti-CD28 mAb resulted in the inhibition of CTLA-4delTM mRNA (Fig 2). As expected [25–27], activation increased membrane CTLA-4 mRNA expression. These data suggest that CTLA-4delTM mRNA expression is down-regulated by activation.

2.3 Recombinant CTLA-4delTM is produced in a soluble monomeric form

Membrane CTLA-4 is expressed as a homodimer; the covalent dimerization is mediated by a disulfide bond at cysteine residue 120 [28]. The alternative splicing of CTLA-4 mRNA results in the loss of this cysteine residue, suggesting that CTLA-4delTM could be produced as monomer. This hypothesis has been confirmed by Western blotting analysis showing that recombinant CTLA-4delTM expressed in COS cells has an apparent molecular mass of 23 kDa under non-reducing conditions (Fig 3 a). As previously reported, recombinant membrane CTLA-4 has an apparent molecular mass of 45 kDa under nonreducing conditions (Fig. 3 a). The deletion of exon 2 results in the lack of the transmembrane domain, thereby suggesting that CTLA-4delTM could be produced as a soluble protein by transfected cells. A soluble protein reactive with anti-CTLA-4 mAb was detected by ELISA in the cell-free supernatant of CTLA-4delTM-transfected cells but not in the supernatants of membrane CTLA-4-transfected cells and mock-transfected cells (Fig 3 b).

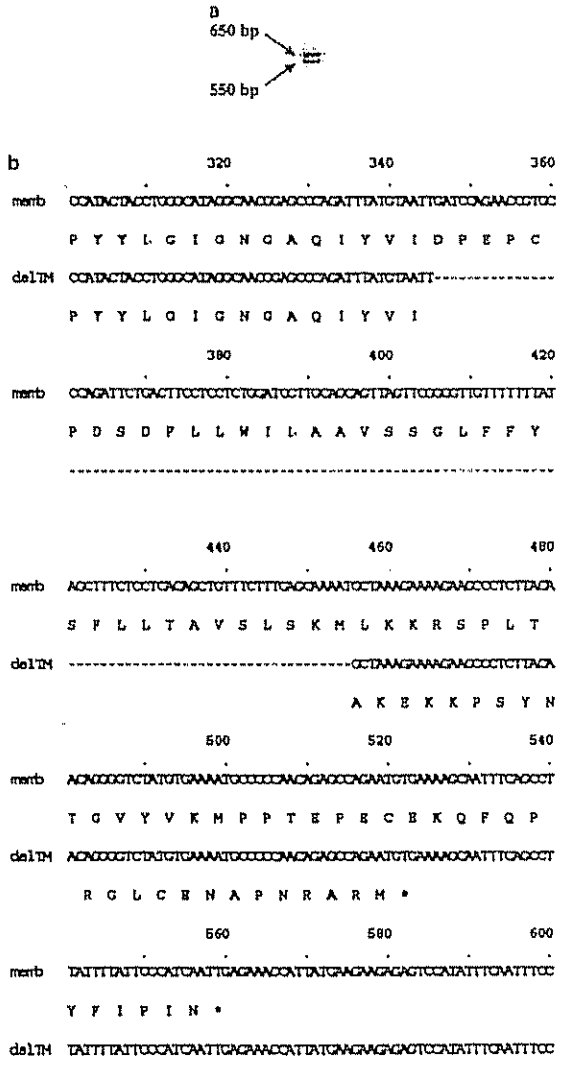
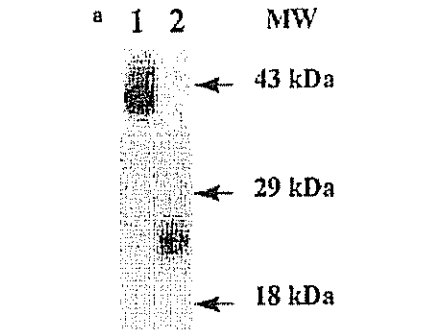


Figure 1. Identification of an alternatively spliced variant of CTLA-4. (a) RT-PCR amplification of CTLA-4 mRNA. RT-PCR was performed as described in Sect. 4.2. (b) Alignment of 3' nucleic acid sequences of membrane CTLA-4 and CTLA-4delTM. The deduced amino acid sequence of CTLA-4delTM is also shown. The stop codon is indicated by an asterisk.



b

	Soluble CTLA-4
Mock	< 1 ng/ml
Membrane CTLA-4	< 1 ng/ml
CTLA-4delTM	55 ± 8 ng/ml

Figure 2. Recombinant CTLA-4delTM is produced in a soluble monomeric form. (a) Recombinant CTLA-4delTM is produced in a monomeric form. COS cells were transfected with membrane CTLA-4 and CTLA-4delTM cDNA ligated into the pCDNA3.1$^+$ vector. CTLA-4 expression was evaluated by Western blotting under nonreducing conditions. (b) Recombinant CTLA-4delTM is produced as a soluble molecule. The production of an immunoreactive form of CTLA-4 was evaluated by ELISA in the concentrated culture supernatant of mock-transfected cells or COS cells transfected with membrane CTLA-4 or CTLA-4delTM. Results are expressed in ng/ml (mean ± SD, $n = 5$).

These data suggest that CTLA-4delTM could be expressed in a soluble monomeric form by nonactivated peripheral blood T cells.

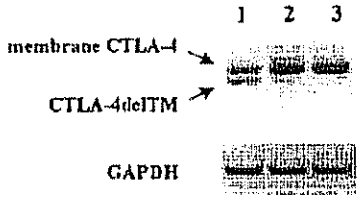


Figure 3. Regulation of CTLA-4 mRNA expression in activated T cells. CTLA-4 mRNA expression was evaluated in human T cells either unstimulated (lane 1) or stimulated with PMA plus ionomycin for 6 h (lane 2) or anti-CD3 plus anti-CD28 mAb for 18 h (lane 3). RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA.

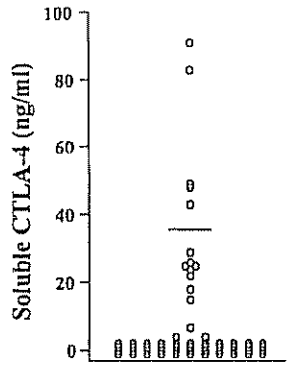


Figure 4. A soluble immunoreactive form of CTLA-4 is detected in the serum of healthy subjects. The concentration of soluble CTLA-4 was evaluated by ELISA using recombinant CTLA-4-Fc as a standard. Results are expressed in ng/ml.

2.4 A soluble immunoreactive form of CTLA-4 is detected in the serum of healthy subjects

We then evaluated whether soluble CTLA-4 could be detected by ELISA in the serum of healthy subjects. A soluble immunoreactive form of CTLA-4 was detected in the freshly isolated serum of 14 out of 64 healthy subjects (36 ± 8 ng/ml, mean ± SEM, ranging from 3 to 90 ng/ml) (Fig. 4).

3 Discussion

Nonstimulated human T cells constitutively express membrane CTLA-4 transcript [29]. Here we describe the identification of an additional spliced variant named CTLA-4delTM which lacks both transmembrane and intracellular domains. The splicing, resulting in the deletion of the exon 2 which encodes the transmembrane domain and the cytoplasmic tail of CTLA-4 [24], occurs as for the Ig genes [30]. The recombinant molecule is expressed as a soluble monomeric protein. Interestingly, a soluble immunoreactive form of CTLA-4 can be found in the serum of healthy subjects.

The Ig-like structure of CTLA-4 is maintained by two intrachain disulfide bonds between Cys^{21}–Cys^{92} and Cys^{45}–Cys^{68} [28]. These cysteine residues are conserved in CTLA-4delTM, suggesting that the extracellular conformation is maintained. The binding domains of CTLA-4 to CD80/CD86 are located in the CDR1- and CDR3-like domains [14, 15]. As these domains are conserved in CTLA-4delTM, it is tempting to speculate that its ability to bind CD80/CD86 could be unaffected. Membrane CTLA-4 is a homodimer [28]. The dimerization implies

the formation of a disulfide bond at C120 and requires both transmembrane and intracellular domains [28]. Moreover, the intracytoplasmic tail of membrane CTLA-4 presents the motif TVLM which is reponsible for its accumulation in the endosomes of activated T cells [31, 32]. The deletion of exon 2 results in the loss of both transmembrane and intracellular domains (including C120), suggesting that CTLA-4delTM could be produced as a soluble monomer. This hypothesis was confirmed by Western blotting and ELISA showing that recombinant CTLA-4delTM is a soluble monomeric molecule. The monomeric CTLA-4-Fc has reduced binding affinity and shows rapid dissociation from CD80/CD86 compared with dimeric CTLA-4 [28, 33]; therefore one can assume that the affinity of soluble CTL4-4delTM could be reduced compared with membrane CTLA-4.

A soluble immunoreactive forms of CTLA-4 was detected in the serum of healthy subjects. This unexpected results suggests a potentially important role for soluble CTLA-4 in the regulation of immune homeostasis. This role may be dependent on the time when soluble CTLA-4 is present. Indeed, blocking the interaction of B7 molecules with their ligands could interfere either at the initiation [34–36] or during the outcome of the immune response [22]. The administration of CTLA-4-Ig fusion protein before the onset of disease prevents the initiation of Th-mediated pathologies such as lupus [37], EAE [38], celiac disease [39] or collagen-induced arthritis [40]. On the contrary, blocking the CTLA-4-B7 interaction using neutralizing anti-CTLA-4 mAb has been shown useful to induce tumor rejection in a mouse model [23] while it exacerbates EAE when injected during the development of the pathology [41]. As a consequence, soluble CTLA-4 could either inhibit or exacerbate the immune response if present during the initiation (blocking of CD28-B7 interaction) or at latter stages (blocking the CTLA-4-B7 interaction) of the immune response, respectively. Upon activation, T cells selectively express membrane CTLA-4, showing that soluble CTLA-4 may be produced only by nonactivated T cells. This result suggest that soluble CTLA-4 could act essentially in a paracrine manner to regulate the immune response.

4 Materials and methods

4.1 Isolation and activation of human T cells

T cells were isolated from heparinized venous blood from healthy subjects. Briefly, PBMC were isolated by centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and T cells were purified by rosetting with sheep erythrocytes. The purity of T cells was > 95 %, as assessed by FCM analysis using a FITC-labeled anti-CD3 mAb (Immunotech, Marseille, France) (data not shown). T cells were cultured in complete medium [RPMI 1640 medium supplemented with 5 % FCS and antibiotics (all from Life technologies, Cergy Pontoise, France)] and were either unstimulated or stimulated with 10 ng/ml anti-CD3 mAb (clone OKT3; ATCC, Manassas, VA) plus 10 ng/ml immobilized anti-CD28 mAb (Immunotech) for 24 h or stimulated for 6 h with 10 ng/ml PMA plus 1 μg/ml ionomycin (both from Calbiochem, La Jolla, CA).

4.2 Cloning and sequencing of the CTLA-4 mRNA variants

Total RNA was extracted using Trizol reagent (Life Technologies) and polyA$^+$ RNA was purified using oligo-dT-coated magnetic beads (Dynal, Oslo, Norway) according to the manufacturer's recommendations. The single-stranded cDNA was synthesized using 1 μg of polyA$^+$ RNA by reverse transcription using an oligo-dT primer (Pharmacia). PCR reactions were performed with cDNA corresponding to 10 ng of polyA$^+$ RNA, Taq polymerase (Clontech, Palo Alto, CA) and primers designed to amplify the entire coding sequence of CTLA-4: 5'-ATGGCTTGCCTTGGATTTC-AGCGGCACAAGG-3' and 5'-TCAATTGATGGGAATAAAA-TAAGGCTGAAATTGC-3' (predicted size: 672 bp). PCR reaction was as follows: 94 °C for 5 min, 30 cycles 94 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min followed by a final extension at 72 °C for 5 min. The amplified fragments were separated on 1 % agarose gel and visualized by ethidium bromide. After excision from the gel, each amplified cDNA fragment was subcloned into a TA cloning vector (pCRII; In Vitrogen, Leek, The Netherlands). Sequencing was performed using the ABI-PRISM Dye Terminator Cycle Ready Reaction kit (Perkin Elmer, Foster City, CA). The expression of CTLA-4 mRNA in activated *vs.* nonstimulated human T cells was evaluated by PCR using the primers mentioned above. RNA integrity and cDNA synthesis was verified by amplifying glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA (5'-TCCACCACCCTGTTGCTGTA-3' and 5'-ACCACAGTCCATGCCATCAC-3').

4.3 Production of a recombinant soluble CTLA-4 protein

The membrane CTLA-4 and CTLA-4delTM cDNA were subcloned in the vector and used to transfect COS cells. COS cells (ATCC) were cultured in DMEM medium supplemented with 10 % FCS, L-glutamine and antibiotics (all from Life Technologies) and transfected with the vectors using the calcium phosphate method (5prime-3prime, Boulder, CO). The production of a soluble form of CTLA-4 was evaluated by ELISA in the cell-free supernatants of transfected cells. Briefly, 96-well plates (Nunc) were coated with the anti-CTLA-4 mAb (clone BNI3; Pharmingen, San Diego, CA) at 1 ng/100 μl/well in 0.1 M phosphate buffer, pH 4.0 (16 h at 4 °C) before incubation for 2 h at room temperature with PBS/BSA 1 %. After washing, plates were incubated for

16 h at 4 °C with concentrated culture supernatant (200 µl/well). Bound CTLA-4 was detected with biotin-labeled anti-CTLA-4 mAb 8H5 (Pharmingen) followed by incubation with streptavidin-biotinylated-horseradish peroxidase (used at 1/5000; Amersham, Amersham, GB). The specificity and concentration of soluble CTLA-4 was determined using commercially available recombinant CTLA-4-Ig (Ancell, Bayport, MN).

4.4 Western blotting analysis

Western blotting analysis was performed with COS cells transfected with CTLA-4 or CTLA-4delTM cDNA. Cells were washed in ice-cold PBS before lysis in 10 mM phosphate buffer, pH 7.4 containing 0.5 % NP40 (Sigma) and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Proteins from 5×10^6 cells were electrophoretically separated on a 10 % polyacrylamide gel under nonreducing conditions and then transferred onto a nitrocellulose membrane (Bio-Rad, Ivry sur Seine, France). After saturation, membranes were incubated with an anti-CTLA-4 mAb (Pharmingen). After washing, membranes were incubated with peroxidase-labeled anti-mouse IgG Ab (DAKO) and bound Ab were detected using the enhanced chemiluminescence (ECL) system (Amersham).

4.5 Detection of soluble CTLA-4 in human serum

Soluble CTLA-4 was evaluated in the serum of healthy volunteers by ELISA, as described above. The concentration of soluble CTLA-4 was estimated by using chimeric CTLA-4-Ig as standard.

4.6 Statistical analysis

Statistical significance was determined using the Student's *t*-test.

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EXHIBIT 7

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A Native Soluble Form of CTLA-4[1,2]

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CTLA-4 is an immunoregulatory receptor expressed on the surface of activated T and B lymphocytes. The counterreceptors for CTLA-4 are the B7 family molecules. We describe alternatively spliced mRNAs expressed in hematolymphoid tissues of humans, mice, and rats that lack the transmembrane domain coded by exon 3 of the CTLA-4 gene. These alternate transcripts were detected by RT-PCR in B cells and resting T cells of both the CD4 and the CD8 phenotype. Activation of human blood mononuclear cells with PHA or anti-CD3 + anti-CD28 monoclonal antibodies appears to effect a decrease in the amount of the alternative transcript relative to the full-length transcript. Recombinant sCTLA-4 is a B7-binding protein and has immunomodulatory effects as measured by inhibition of the mixed leukocyte response. Human serum contains immunoreactive material consistent with a native soluble form of CTLA-4. © 2000 Academic Press

Key Words: T lymphocytes; comparative immunology; mRNA.

INTRODUCTION

CTLA-4[4] is a lymphocyte cell surface receptor originally discovered in a search for molecules having a role in T cell cytotoxicity (1, 2). Although originally identified as a cytolytic T-cell-associated molecule, CTLA-4 expression has been detected in both activated CD4 and CD8 T cell populations as well as in B cells with the use of monoclonal antibodies (3, 4). CTLA-4 is a member of the immunoglobulin superfamily and is highly homologous to another T cell surface receptor, CD28 (1, 2, 5, 6). Both CD28 and CTLA-4 bind the same ligands, namely, members of the B7 family of molecules expressed on APC (7, 8). Expression of CTLA-4 is highly activation-dependent. CTLA-4 is not detected on resting T cells but is induced during T cell activation (3, 9, 10). With activated T cells, maximal surface CTLA-4 protein expression is ~2–3% of surface CD28 (9). Recombinant forms of CTLA-4 bind CD80 (B7.1) and CD86 (B7.2) with higher avidity (>20-fold) than soluble CD28 (9, 11). Thus, CTLA-4 is a high-avidity, low-abundance receptor for B7 molecules.

Much of the study of the interactions of CTLA-4 and its B7 counterreceptors has relied on the use of a recombinant soluble form of CTLA-4 (CTLA4-Ig). CTLA4-Ig is a fusion protein consisting of the extracellular domain of CTLA-4 and the human IgG heavy chain constant region gene (8). Numerous reports have documented the usefulness of CTLA4-Ig as an immunoregulatory agent. For example, CTLA4-Ig inhibits alloreactivity both in the context of the mixed leukocyte reaction (8, 12, 13) and *in vivo* in experimental cardiac allografts (12, 14) and pancreatic islet xenografts (15). CTLA4-Ig is also reported to modify the course of several animal models of human disease including murine lupus (16), experimental autoimmune encephalomyelitis (17), allergic contact dermatitis (18), and autoimmune anti-glomerular basement membrane glomerulonephritis (19). In addition, CTLA4-Ig also inhibits cellular and humoral responses to conventional antigens such as keyhole limpet hemocyanin and sheep red blood cells (20) and pigeon cytochrome c (21).

We have reported the molecular cloning and the nucleotide sequence of the full-length rat CTLA-4 gene (22). During the course of these experiments, in addition to the expected target CTLA-4 transcript, distinct smaller amplification products were also observed following RT-PCR of RNA from a variety of lymphoid

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[2] The nucleotide and predicted amino acid sequence data reported in this paper have been submitted to the GenBank sequence database and have been assigned the following accession numbers: Mouse sCTLA-4, U90270; Rat sCTLA-4, U90271; Rat intron/exon boundaries, AF012711, AF012712, and AF012713; Human sCTLA-4, U90273.

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[4] Abbreviations used: APC, antigen presenting cells; BMC, bone marrow cells; CTLA-4, cytotoxic T lymphocyte-associated molecule-4; EIA, enzyme immunoassay; KLH, keyhole limpet hemocyanin; PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; SA, splice acceptor; SD, splice donor; sCTLA-4, soluble CTLA-4; RT-PCR, reverse transcriptase-PCR; TM, transmembrane.

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tissues from humans, mice, and rats. This report describes the nucleotide and the predicted amino acid sequence of these alternative transcripts. In addition, we show that the variant transcript is most abundant in cells from bone marrow, blood, and lymph nodes, whereas the transmembrane form of the molecule is the predominant, and perhaps the only species, expressed in the adult thymus. We also report the expression and immunoregulatory properties of recombinant sCTLA-4 and present data that suggest that a native soluble form of sCTLA4 is present in human serum.

MATERIALS AND METHODS

Tissue samples and cells. Female ACI rats (150–200 g) and Balb/c mice were obtained from Harlan Sprague–Dawley (Indianapolis, IN). Human blood samples were obtained from healthy volunteers. Human adult lymph node and spleen samples and neonatal thymus were obtained at autopsy. Mononuclear cells were obtained from spleen and blood by standard Ficoll–Hypaque density gradient centrifugation. Other organs and tissues were frozen and stored in liquid nitrogen until used for RNA extraction. Human PBMCs were activated for 48 h *in vitro* by culture in RPMI 1640 medium containing 10% FBS, 5 μg/mL Con A, and 10 nM PMA (from Sigma Chemical Co., St. Louis, MO). Rat $CD4^+$ and $CD8^+$ splenic lymphocytes were isolated by dual rounds of affinity chromatography (Cellect immunocolumns, Biotex Labs, Edmonton, Alberta) according to the manufacturer's instructions. B cells were negatively selected by dual passage over a T cell chromatography column followed by immunomagnetic positive selection with the use of anti-CD45RA/B (clone OX-33). The purity of these suspensions was examined by flow cytometry using anti-CD4 and anti-CD8 monoclonal antibodies (clones OX-35 and OX-8, respectively). Fewer than two fluorescence events per 200,000 ungated cells were present in these enriched populations (not shown) demonstrating the high enrichment of the cells used for RT-PCR amplification. The antibodies used in these studies were purchased from Pharmingen (San Diego, CA).

RT-PCR. Total cellular RNA was extracted by the guanidine thiocyanate method using kit-supplied reagents (RNAgents, Promega Biotec, Madison, WI). Single-strand cDNA was generated from 1 μg total cellular RNA with the use of Superscript II preamplification reagents (Life Technologies, Bethesda, MD) according the manufacturer's instructions. PCR was carried out in 25-μL volumes of 1× PCR buffer (60 Tris–HCl (pH 9.0), 15 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$) containing 1/10 the contents of the reverse transcription reaction, 0.2 mM each dNTP, 0.5 μM each primer, and 0.05 U/μL Taq DNA polymerase (Promega, Madison, WI). The reactions were subjected to 20–35 amplification cycles on a Perkin–Elmer Cetus thermal cycler. The amplification cycle profile was 95°C denaturation for 1 min, followed by primer annealing at 60°C and extension at 72°C for 1 min each. PCR products were separated by gel electrophoresis and analyzed with a Gel-Doc 1000 system equipped with Molecular Analyst software (Bio-Rad Instruments).

Oligonucleotide primers. Primers were designed with the aid of commercially available software (Primer Designer; S&E Software, State Line, PA) from previously published sequences of rat (22), mouse (1), and human (5) CTLA-4 genes. The following are the 5′ → 3′ sequences of the sense (s) and antisense (as) primers used in these studies: RCTLA4-57s = CATGGCTTGTCTTGGACTCC; RCTLA4-781as = GCCTCAGCTTTTAGAGACTG; HCTLA4-4s = TTCCTGAAGACCTGAACACC; HCTLA4-766as = AGAATTGCCTCAGCTCTTGG; RCTLA4-366s = TGACACCGGACTGTACTTCT; HCTLA4-341s = TCACTATCCAAGGACTGAGG; RHCTLA4-635as = TCACATTCTGGCTCTGTTGG. The size of the amplification products from cDNA derived from the transmembrane (TM) and the variant transcript described in this communication are as follows. 57s/781as rat primer set yields a 724-bp TM fragment and a 614-bp sCTLA-4 fragment; 4s/766as human primer set yields a 762-bp TM fragment and a 652-bp sCTLA-4 fragment. The 366s/635as primer set yields TM and sCTLA-4 fragments of 269 and 159 bp, respectively, using rat or mouse cDNA as a template. The 341s/635as primer set yields a TM product of 294 bp and a sCTLA-4 product of 184 bp with human cDNA as a template. Note that the 635as oligonucleotide is used as a common anti-sense primer for rat, mouse, and human cDNAs, as this sequence is derived from the highly conserved cytoplasmic domain of CTLA-4. All primers were synthesized by Operon Technologies (Alameda, CA).

Cloning and sequencing. PCR product was cloned into the pCR3 vector using a TA cloning kit (Invitrogen Corp., CA). PCR products as well as cloned inserts were sequenced bidirectionally by dideoxy cycle sequencing using the above-described PCR primers and vector primers, respectively.

Antibodies. Rabbit anti-human CTLA-4 antibodies were generated by standard methods in New Zealand White rabbits immunized with KLH-conjugated peptides. The peptides were synthesized and conjugated by Research Genetics (Huntsville, AL). Antiserum 8K was raised to the novel carboxyl-terminal sequence of the predicted sCTLA-4 protein (KPSYNRGLCENAPNRARM). This amino acid sequence shares no significant similarity with proteins cataloged on the GenBank database, and the 8K antiserum is not reactive with commercially available CTLA4-Ig. The anti-CTLA4 monoclonal antibody (clone BNI3) used in inhibition experiments was obtained from Pharmingen.

sCTLA-4 expression Recombinant baculovirus clones containing the human sCTLA-4 gene were generated by standard cloning methods. Viral stocks were generated by cotransfection of recombinant sCTLA-4 in pBlueBacHis expression vector with the pBlueBac transfer vector (Invitrogen, Carlsbad, CA) into the SF9 insect cell line. The High-Five insect cell line was used as the host for expression experiments. Control baculovirus containing the chloramphenicol acetyl transferase gene (CAT) was prepared and tested in parallel in all baculovirus expression experiments. His fusion proteins (CAT and sCTLA-4) were purified by Ni column chromatography before use in functional assays.

Fusion proteins B7.1 and B7.2 immunoglobulin fusion proteins were generated by cloning the extracellular domains of these cDNAs into the signal-pIG vector (Novagen, Madison, WI). For this purpose, we used reverse transcription PCR of Raji cell (ATCC, Rockville, MD) RNA using the following sense and antisense primers:

B7.1s = AAGCTTGGTCTTTCTCACTTCTGTTCAG;
B7.1as = GGATCCGCATCAGGAAAATGCTCTTGC;
B7.2s = GCTAGCGCTCCTCTGAAGATTCAAGCTT;
B7.2as = GGATCCGCCGGGGGAGGCTGAGGGTCCTCA.

The antisense primers contain 3′ *Bam*HI sites, and the B7.1 and B7.2 sense primers contain a 5′ *Hin*dIII and *Nhe*I site, respectively, to provide for directional cloning into the signal pIG vector. Cos-7 cells were transfected with the use of Cellfectin (Life Technologies, Bethesda, MD), and neomycin-resistant cell lines were generated by stepwise selection in G418 up to 1 mg/mL of active drug. Cell culture supernatants were collected in serum-free medium and tested in a sandwich ELISA for B7 and human IgG1 epitopes. Positive culture supernatants were pooled and purified by protein A chromatography (Pierce Immunochemical, Rockville, IL). Each fusion protein was reactive with CTLA4-Ig (Ancell, Bayport, MN) as determined by an enzyme immunoassay (not shown). SDS–PAGE analysis of the fusion proteins showed major products of approximately 150 kDa under nonreducing conditions and products of 74 and 83 kDa for B7.1–Ig and B7.2–Ig, respectively, when analyzed under reducing conditions. Each fusion protein demonstrated T cell costimulation in conjunction with fluid phase OKT3 as measured by a PBMC proliferation assay (not shown). The Muc–Ig fusion protein was expressed from a commercially available (Novagen) plasmid following transfection in CHO cells as described above.

Enzyme immunoassays A modified sandwich EIA was used for determination of binding of B7–Ig fusion proteins to recombinant sCTLA-4. For this purpose, wells of a 96-well microtiter plate were coated overnight with approximately 200 ng/well of the appropriate B7–Ig fusion protein. The wells were washed with 0.5% Tween 20 in PBS, pH 7.6, and then blocked with Superblock (Pierce Immunochemical). One hundred microliters of the test samples was applied to the wells, and the plates were incubated for 30 min at room temperature and then washed to remove the unbound sample. One hundred microliters of a 1:500 dilution of the rabbit anti-human sCTLA-4 (8K) antiserum was then added, and the plates were incubated for 1 h, washed, and reacted for 30 min with 100 μL of a 1:50,000 dilution of affinity-purified HRP-conjugated goat anti-rabbit IgG. After being washed, the reactions were developed with the use of 3, 3′, 5, 5′-tetramethyl benzidine substrate (Turbo TMB ELISA, from Pierce). Absorbance was read at 450 nm on a Molecular Devices microplate spectrophotometer.

Mixed leukocyte reactions (MLR) Two-way MLR was used to test recombinant sCTLA-4 for functional activity. The cells used in these experiments were from a panel of previously HLA typed laboratory volunteers and were selected to provide two HLA-DR mismatches. PBMCs were isolated by standard Ficoll–Hypaque density gradient centrifugation, and equal numbers of cells (100,000) were cocultured in tissue culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 μg/mL gentamicin, and 50 μM 2-mercaptoethanol) containing graded dilutions of sCTLA-4 test sample. Triplicate cultures were set up in 0.2-mL volumes in round-bottom 96-well microculture plates and were incubated for 5 days at 37°C and 5% CO_2 in air. The cultures were pulsed with 0.5 μCi tritiated thymidine and harvested 18 h later. Isotope incorporation was measured on a Packard Tri-Carb 2000 scintillation counter.

Western blotting Western blotting was used to detect recombinant and native (serum) sCTLA-4. Proteins were separated by 10–20% gradient PAGE in a discontinuous buffer system on a Mini-Protean system (Bio-Rad). The separated components were electroblotted onto PVDF membranes. The blots were washed with 0.15 M NaCl, 0.05 M Tris (TBS), pH 7.5, with 0.3% Tween 20 and reacted with a 1:100 dilution of the 8K antiserum for 1 h at room temperature, washed, and then reacted with reporter antibody (HRP-conjugated anti-rabbit IgG) for 1 h. The blots were then developed with the use of a commercially available chemiluminesence detection kit (BMB, Indianapolis, IN) according to the manufacturers instructions. Control blots were stained as described above but with the addition of a preimmune serum from the individual rabbit used for antibody production.

RESULTS

In order to characterize the rat and human CTLA-4 mRNAs, we isolated cellular RNA from splenocytes of ACI rats and lymph nodes from humans. The CTLA-4 mRNA transcripts were then analyzed by RT-PCR am-

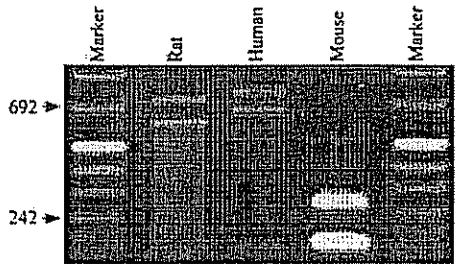


FIG. 1. Gel electrophoresis of RT-PCR products of rat, human, and mouse mRNAs with CTLA-4 primers. Rat and human PCR products are derived from spleen cDNAs amplified with primers designed to detect the coding region of CTLA-4. Mouse products are from spleen cDNA amplified with primers that flank the CTLA-4 TM region. The upper bands in each lane represent amplification products of the CTLA-4 TM transcript, the lower bands are amplifications of the sCTLA-4 transcript.

plification with the use of oligonucleotide primers that flanked the ATG initiation codons and the translation termination codons. The amplified products were expected to encompass the entire translation region as well as a portion of the 5′ untranslated region of the CTLA-4 gene and to be 724 and 762 bp for rat and human, respectively. In addition to the anticipated full-length CTLA-4 cDNA, a distinct smaller 614-bp amplification product was also observed in rats, and a 652-bp fragment was detected in humans (Fig. 1). We designated these transcripts *sCTLA-4* and refer to them as such hereafter. Screening of the cloned PCR product also revealed recombinant clones of similar sizes, and restriction enzyme analysis showed the absence of two restriction enzyme sites (based on the nucleotide sequences of full-length CTLA-4) within the transmembrane region of CTLA-4 (data not shown). Similar findings were also observed upon RT-PCR of RNA from mouse splenocytes when amplified with primers that flank the transmembrane region of CTLA-4. These data suggest that these molecules represent alternative transcripts of the CTLA-4 molecule that lack a transmembrane domain.

Nucleotide sequencing of these cDNAs proved this assumption to be correct. The nucleotide and predicted amino acid sequence of the mature human sCTLA-4 molecule is presented in Fig. 2. The predicted sCTLA-4 molecule (minus the leader peptide) is a 137 amino acid polypeptide and is 49 amino acids shorter than the full-length molecule. When compared to their full-length counterparts, rat and human molecules have identical nucleotide and predicted amino acid sequences spanning their entire extracellular domains, but the sCTLA-4 form represents a deletion of the entire transmembrane domain. The nucleotide sequences of the "cytoplasmic" domains of each species are identical to their full-length counterparts with the exception of the first codon. The net effect of this is that the deletion changes the reading frame of the molecule and, thus, the amino acid sequence of the vestige of the cytoplasmic domain of the sCTLA-4. A comparison of the rat sCTLA-4 molecule to its transmembrane counterpart is presented in Fig. 3. This alternative transcript is also found in mouse splenocyte RNA and has

```
  M  H  V  A  Q  P  A  V  V  L  A  S  S  R  G  I  A  S  F  V -20
ATGCACGTGGCCCAGCCTGCTGTGGTACTGGCCAGCAGCCGAGGCATCGCCAGCTTTGTG
************************************************************

  C  E  Y  A  S  P  G  K  A  T  E  V  R  V  T  V  L  R  Q  A -40
TGTGAGTATGCATCTCCAGGCAAAGCCACTGAGGTCCGGGTGACAGTGCTTCGGCAGGCT
************************************************************

  D  S  Q  V  T  E  V  C  A  A  T  Y  M  M  G  N  E  L  T  F -60
GACAGCCAGGTGACTGAAGTCTGTGCGGCAACCTACATGATGGGGAATGAGTTGACCTTC
************************************************************

  L  D  D  S  I  C  T  G  T  S  S  G  N  Q  V  N  L  T  I  Q -80
CTAGATGATTCCATCTGCACGGGCACCTCCAGTGGAAATCAAGTGAACCTCACTATCCAA
************************************************************

  G  L  R  A  M  D  T  G  L  Y  I  C  K  V  E  L  M  Y  P  P -100
GGACTGAGGGCCATGGACACGGGACTCTACATCTGCAAGGTGGAGCTCATGTACCCACCG
************************************************************

  P  Y  Y  L  G  I  G  N  G  T  Q  I  Y  V  I  A  K  E  K  K -120
CCATACTACCTGGGCATAGGCAACGGAACCCAGATTTATGTAATTGCTAAAGAAAAGAAG
*********************************************

  P  S  Y  N  R  G  L  C  E  N  A  P  N  R  A  R  M  -
CCCTCTTACAACAGGGGTCTATGTGAAAATGCCCCCAACAGAGCCAGAATGTGA
```

FIG. 2. The nucleotide and predicted amino acid sequence of the human sCTLA-4 transcript (***), "extracellular" V domain; (—), coding region of remnants of cytoplasmic domain. Bold amino acids represent predicted N-linked glycosylation sites

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MACLGLQRYKTHLQLPSRTWPFGVLLSLLFIPIFSEAIQVTQPSVVLASSHGVASFPCEYASSHN
MACLGLQRYKTHLQLPSRTWPFGVLLSLLFIPIFSEAIQVTQPSVVLASSHGVASFPCEYASSHN
^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^^

TDEVRVTVLRQTNDQVTEVCATTFTVKNTLGFLDDPFCSGTFNESRVNLTIQGLRAADTGLYFCK
TDEVRVTVLRQTNDQVTEVCATTFTVKNTLGFLDDPFCSGTFNESRVNLTIQGLRAADTGLYFCK
************************************************************

VELMYPPPYFVGMGNGTQIYVI                                        AKEKKS
VELMYPPPYFVGMGNGTQIYVIDPEPCPDSDFLLWILAAVSSGLFFYSFLVTAVSLNRTLKKRSP
**********************^^^^^^^^^^^^^^^^^^^
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   I  Y  V  I↓                (≈0.5 Kb)              ↓D  P  E  P
ATTTATGTCATCGgtaagcagagccattcc.........ttttgtgttgcagATCCAGAACCA
   I  Y  V  I                  INTRON A

  C  P  D  S  D  F  L  L  W  I  L  A  A  V  S  S  G  L  F  F  Y
TGCCCAGATTCAGACTTCCTCCTTTGGATCCTTGCTGCAGTTAGTTCGGGGTTGTTTTTCTAC

  S  F  L  V  T  A  V  S  L  N  R  T↓        (≈1.1 Kb)
AGTTTCCTGGTCACCGCTGTTTCTTTGAACAGGACGgtgagtgt.........tcttttgtg
                                              INTRON B
         ↓ L  K  K  R  S  P.............
ttttttggcagCTAAAGAAAAGAAGTCCT
           A  K  E  K  K  S.............
```

FIG. 5. The intron\exon boundaries of genomic DNA of the rat CTLA-4 gene ↓ Indicates location of nucleotide at intron\exon boundary Uppercase nucleotides denote exons; lowercase denotes introns Splices between the SD\SA sites of both introns A and B produces the transcript encoding the transmembrane form of the molecule (amino acid sequence shown above nucleotide sequence) A splicing event between the SD site of intron A and the SA site of intron B produces the soluble form of CTLA-4 The amino acid sequence of sCTLA-4 is shown below the nucleotide sequence

tron A donor site to the intron B acceptor site would accurately yield the sCTLA-4 transcript The net effect of this splicing event is a 110-bp deletion corresponding to the entire transmembrane domain of the CTLA-4 molecule. The A → B splicing event also introduces a frameshift mutation within the "cytoplasmic" domain of sCTLA-4 (the alternative reading frames of each are illustrated in Fig. 5). Thus, although the nucleotide sequences of these molecules are identical downstream of the splice sites, the change in reading frame produces an amino acid tail that is unique to the sCTLA-4 molecule. This tail is 22 amino acids long and 12 amino acids shorter than that of the transmembrane form of CTLA-4 due to the introduction of a TGA termination codon at nucleotide position 523 of the rat sCTLA-4 gene. The genomic structure of the human CTLA-4 gene is highly similar (5), and examination of the sequences of the SD\SA sites predicts the possible presence of the described sCTLA-4 transcript.

In order to examine the tissue distribution of the TM and sCTLA-4 transcripts, we used RT-PCR with primers that flank the TM region of CTLA-4. Because the two transcripts compete for the same oligonucleotide primers during PCR, the relative levels of each transcript can be estimated from their fluorescent intensities on an ethidium-bromide-stained gel The results of a typical experiment are shown in Fig 6 Both the TM and the sCTLA-4 transcripts were detected in lymph node, spleen, and peripheral blood in both humans and rats (Fig. 6A). The TM transcript appears to be the only transcript expressed in the thymus of adult rats, whereas the sCTLA-4 transcript is the only mRNA species detected in bone marrow cells The expression of both CTLA-4 transcripts appears to be exclusive to the hematolymphoid system: neither the TM nor the sCTLA-4 transcripts were detected in a wide variety of nonlymphoid tissues including adrenal, brain, eye, heart, kidney, liver, lung, ovary, pancreas, salivary gland, seminal vesicles, skeletal muscle, testes, and thyroid (data not shown).

We next attempted to determine the distribution of the TM and sCTLA-4 transcripts within lymphocyte subsets. As demonstrated in Fig. 6B, both the TM and the sCTLA-4 transcripts are expressed in the CD4 and CD8 subsets of T cells, as well as B lymphocytes. The relative amounts of the TM and sCTLA-4 transcripts appear to differ between the CD4 and CD8 T cell subsets. For example, nearly identical amounts of the TM and sCTLA-4 products are obtained following RT-PCR of $CD4^+$ T cells, whereas there appears to be nearly 2.5-fold more TM product than sCTLA-4 product among $CD8^+$ T cells. This skewed distribution of CTLA-4 transcripts among the CD4 and CD8 populations produces a TM:sCTLA-4 ratio of approximately 1.5:1 when analyzed as unfractionated T cells The TM

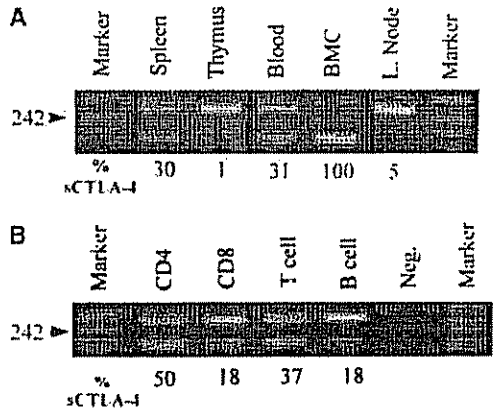


FIG. 6. The tissue distribution of the sCTLA-4 transcript in rats. Gels of RT-PCR products of RNA from various rat hematolymphoid tissues using primers that surround the transmembrane region of rat CTLA-4 (A) Unfractionated tissues; (B) fractionated splenic mononuclear subsets The upper band is approximately 269 bp and corresponds to products of the transcript that encodes the TM form of CTLA-4; the lower band is approximately 159 bp. which is consistent with products of the sCTLA-4 transcript Arrow indicates a marker band of 242 bp; "Neg" lane is product from RT-PCR reaction mix minus cDNA

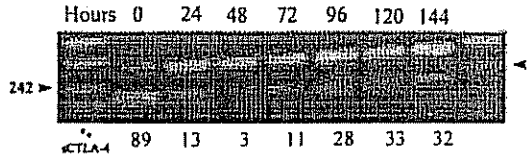


FIG. 7. Effects of activation status on the relative levels of TM vs sCTLA-4 mRNA. Human PBMCs were activated for the indicated time periods as described under Materials and Methods and harvested for RT-PCR analysis using primers that span the TM region of the CTLA-4 gene. The arrow indicates a 242-bp marker; the upper band represents the product of the TM CTLA-4 transcript; the lower band indicates a 184-bp product of the sCTLA-4 transcript. The results are representative of three separate experiments on different blood donors.

CTLA-4 species is the predominant transcript among B cells, representing 71% of the total CTLA-4 amplification product in this experiment. By contrast, the relative amount of TM and sCTLA-4 transcripts among unfractionated T cells is 59% vs 41%, respectively. Similar data were obtained in a duplicate set of experiments.

Because the mRNA and protein product of the CTLA-4 gene are reported to be expressed on activated but not resting T cells, we attempted to determine the relative levels of CTLA-4 TM vs sCTLA-4 on resting and activated human PBMCs. The results of a representative experiment are shown in Fig. 7. Although both transcripts are detected in resting cells, the sCTLA-4 transcript appears to be the predominant one. This ratio is reversed by 24 h postactivation, and by 48 h full-length CTLA-4 is almost the exclusive mRNA transcript. From 72 to 120 h following activation, the sCTLA-4 transcript appears to increase relative to CTLA-4 TM, although it does not exceed about 2/3 of the total CTLA-4 mRNA. Similar results were obtained upon activation of either rat or human PBMC with anti-CD3 + anti-CD28 (not shown). Several human and mouse T and B cell lines (including EL-4, EL-4-IL2, Jurkat, HPB-MLT, Hut-78, and SP2-0/Ag-14) were negative when tested for expression of the sCTLA-4 transcript.

Recombinant sCTLA-4 was expressed in a baculovirus system for the purpose of determining functional activity. Recombinant material obtained by infection of the High-Five insect cell line with the baculovirus expression plasmid contained immunoreactive sCTLA-4 which demonstrated specific binding to each of the B7.1 and B7.2 immunoglobulin fusion proteins, but minimal binding to a control protein (Fig. 8). Partial inhibition of B7:CTLA-4 interactions could be demonstrated when the binding assays were performed in the presence of monoclonal antibody to CTLA-4. Recombinant sCTLA-4 showed specific binding to Raji and Daudi cells (known to express B7 molecules) as well as CHO transfectants of the transmembrane forms of B7.1 and B7.2 by an indirect immunofluoresence assay (not shown).

We next examined the immunoregulatory properties of recombinant sCTLA-4 by determining its effect on the MLR. The data presented in Fig. 9 show that sCTLA-4 inhibits the MLR in a dose-dependent manner and is capable at high concentrations of complete inhibition of cellular proliferation in this system. The inhibition of the MLR by sCTLA-4 could be partially reversed by the addition of anti-CTLA-4 monoclonal antibody, and recombinant sCTLA4 also inhibited the costimulatory properties of a CHO cell line that expresses a full-length B7.1 gene in a manner similar to CTLA4–Ig (data not shown). Taken together, the data support the notion that sCTLA-4 encodes a receptor with functional properties including its ability to bind the B7 family of molecules and, as a consequence, inhibit B7:CD28 interactions that enhance cellular immune responses to alloantigen.

Finally, we attempted to determine whether a circulating form of CTLA-4 was present in human serum. For this purpose, Western blots of human serum were probed with the antiserum (8K) generated against the carboxyl-terminal fragments of the sCTLA-4 polypeptide. As shown in Fig. 10, this antiserum reacts predominantly with a polypeptide species of approximately 23 kDa, which is consistent with the predicted size based on the amino acid sequence and predicted N-linked glycosylation pattern of native sCTLA-4. This immunoreactive species was identified in 11 of 20 serum samples from patients with endocrine autoimmune disease. A more detailed description of these findings is published elsewhere (26). Some serum samples revealed a larger immunoreactive species of ap-

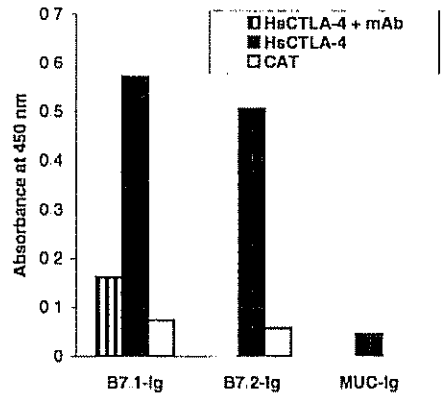


FIG. 8. Recombinant sCTLA-4 is a B7-binding protein. B7.1–, B7.2– or Muc18–immunoglobulin fusion proteins were immobilized to microplate wells, recombinant proteins were added, and CTLA-4 immunoreactive material was detected as outlined under Materials and Methods. CAT is chloramphenicol acetyl transferase expressed in a baculovirus system and is used here as a negative control. The data represent five replicates in two separate experiments. Muc18–Ig is an irrelevant fusion protein was used to determine the specificity of B7–Ig:CTLA-4 binding. In select experiments, we added anti-CTLA4 monoclonal antibody (500 ng/mL final concentration) and sCTLA-4 simultaneously in order to examine inhibition of the B7:sCTLA-4 interaction.

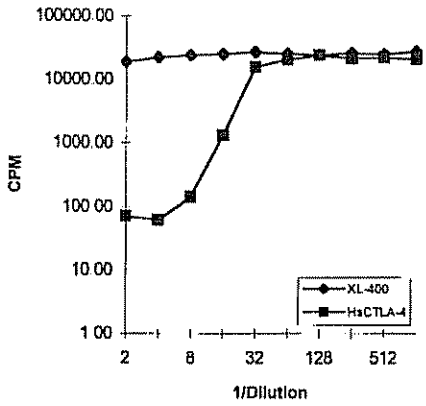


FIG. 9. Immunoregulatory properties of sCTLA-4. Inhibition of two-way MLR. HsCTLA-4 is recombinant CTLA-4 derived from baculovirus expression and partially purified by Ni-resin chromatography; XL-400 culture supernatant was derived from mock-infected insect cells. The data represent the mean of quadruplicate samples. Initial dilutions of proteins used in these assays are approximately 250 ng/mL. Similar results were obtained in three separate experiments from a variety of donor/recipient combinations.

proximately 75–80 kDa that was observed under both reducing and nonreducing conditions. The nature of this species is not known and was not investigated further. No immunoreactive material was detected on blots probed with preimmune sera from individual animals used for the immunization procedure, even when used 50-fold more concentrated than that from immune rabbits. Immunoprecipitation experiments using a monoclonal antibody (BNI3 described under Materials and Methods section) to capture CTLA-4 immunoreactive material from serum and developed with the 8K antiserum also showed the same 23-kDa species (not shown). Thus, the material present in human serum appears to have epitopes derived from the extracellular domain of the classic CTLA-4 protein as well as those believed to be unique to the sCTLA-4 polypeptide. Western analysis of recombinant sCTLA-4 shows two major immunoreactive species of approximately 25 and 19 kDa. The 25-kDa species is consistent with the expected size of the fully glycosylated recombinant protein. The origin of the 19-kDa species is not clear, but is consistent with a hypoglycosylated form of the recombinant protein. This smaller species was not observed following expression of sCTLA-4 in CHO cells (not shown) and is thought to represent a variant due to expression in insect cells.

DISCUSSION

Soluble forms of receptors are produced through proteolytic cleavage of membrane-bound receptors, as in the case of the tumor necrosis factor (27) and the interleukin-2 receptors (28), or by translation of alternative spliced mRNA as found in the IL-4 (29), IL-7 (30), and Fas (31) receptors. Secreted receptors represent truncated forms of the membrane-bound receptors because of the introduction of a frameshift or stop codons. These soluble receptors can bind ligand in a similar manner as their membrane-bound counterparts and as a result may play an important role in the regulation of receptor activity (31, 32). The data presented in this report suggest that, like many other receptors with immunoregulatory functions, CTLA-4 exists in a truncated form. When compared to the transmembrane form of the CTLA-4 molecule, the sCTLA-4 transcript appears to be more predominant in resting cells than in activated cells. This finding may explain why this mRNA species has not been previously identified, as CTLA-4 is widely considered an activation antigen and, thus, most studies of CTLA-4 expression are performed on activated cells.

We think it unlikely that the sCTLA-4 mRNA species represents an artifact that was amplified during PCR. First, cellular activation appears to regulate the relative levels of each CTLA-4 transcript, as the sCTLA-4 variant appears to be the predominant transcript in resting peripheral blood mononuclear cells, whereas the TM form is the predominant species by 24–48 h postactivation (see Fig. 7). Second, the relative levels of each transcript appear to vary by lymphocyte subset. For example, among T cells the relative levels of each CTLA-4 mRNA species appear to be similar; whereas the TM species is the predominant one within B cells. Third, no other variant transcripts or clones (such as transcripts lacking an extracellular region) were identified in numerous PCR experiments and during the screening of several hundred recombinant plasmids. Perhaps more importantly, lymphoid tissues appear to express varying relative amounts of each transcript. For example, the TM form appears to be the only transcript expressed in the thymus, but the sCTLA-4 transcript is the predominant species in bone marrow cells. It is thus difficult to envision a scenario by which errors in splicing would be differentially reg-

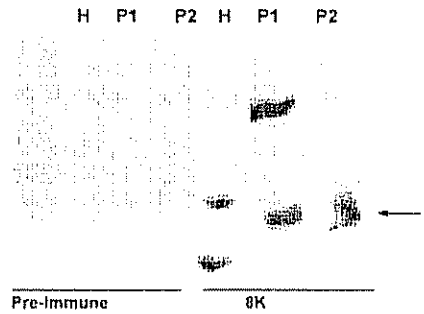


FIG. 10. Western blot of recombinant sCTLA-4 and human serum proteins. Lane marked H indicates the recombinant His-sCTLA-4 fusion protein. P1 and P2 refer to different samples used in these experiments. The left side of the blot was probed with a preimmune serum from the same animal used to produce the 8K antiserum used on the right side of the blot as underlined. The arrow indicates an ~23-kDa band of CTLA-4 immunoreactive material.

ulated in different lymphoid compartments as well as by activation status.

Our data suggest that a native soluble form of CTLA-4 is expressed in humans. Western blotting experiments with an anti-peptide antiserum designed to be reactive with the polypeptide encoded by the variant transcript described here (but not the transmembrane form of the molecule) reveal immunoreactive material in human serum consistent with the size of the predicted sCTLA-4 molecule. Our observation that the sCTLA-4 transcript was not detectable in T cell lines is perhaps not surprising given that it appears to be downregulated in activated cells. The fact that this transcript lacks the entire membrane-spanning domain of the classical CTLA-4 molecule suggests that it encodes a native soluble form of the CTLA-4 molecule. Because the amino acid residues that are thought to be critical to B7 molecule binding (namely the MYPPPY motif) are intact, we predict that expression of this molecule should encode a protein capable of binding the B7 family of molecules. In support of this, we show that recombinant sCTLA-4 is a B7 binding molecule and demonstrate that it has immunoregulatory properties *in vitro*. Furthermore, we show that human serum contains circulating material that is immunoreactive with an antibody raised against an epitope within the putative sCTLA-4 molecule, but not the transmembrane species.

Our understanding of the functional consequences of the interactions of the B7 family of molecules with the coreceptors CD28 and CTLA-4 has become clearer over recent years. The consequences of B7 molecule binding of the membrane forms of CD28 and CTLA-4 appear to be opposing (33). It is well established that B7:CD28 interactions provide a major costimulatory signal to T cell activation (34, 35). The emerging notion of B7:CTLA-4 binding is that CTLA-4 triggering provides a negative signal to T cells (36–38) and in fact may lead to apoptosis and/or a state of immunologic anergy (39–42). In this context, one can envision a dual role for the candidate sCTLA-4 molecule. On one hand, sCTLA-4 may bind B7 expressed on APC and thus interfere with B7:CD28 mediated costimulation of T cell responses. On the other hand, sCTLA-4 may also be capable of interfering with B7:CTLA-4 interactions, thereby blocking the negative signal imparted via the TM form of CTLA-4. In this regard, it is interesting that sCTLA-4 does not appear to be expressed within the thymus, an organ in which negative selection of autoreactive T cells occurs.

Our data also raise the possibility that recombinant sCTLA-4 could be used to regulate B7:CD28 interactions. For example, CTLA4–Ig has been highly successful in regulating the *in vivo* and *in vitro* immune response to a wide variety of antigens (20, 21, 43). Expression of the sCTLA-4 gene may produce a natural soluble form of CTLA-4 with similar useful immunoregulatory properties.

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EXHIBIT 8

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Soluble CTLA-4 in autoimmune thyroid diseases: Relationship with clinical status and possible role in the immune response dysregulation

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Thyroid disease;
Autoimmunity

Abstract CTLA-4 molecule, expressed by activated T and B lymphocytes, transduces an inhibitory signal. Increasing evidence showed *CTLA-4* gene as an important susceptibility locus for autoimmune endocrinopathies and other autoimmune disorders. The aim is to evaluate the augmented sCTLA-4 serum levels in different autoimmune thyroid diseases when compared with normal donors or with non-autoimmune hyperthyroidism and to investigate the functional activities and suggest the possible pathogenetic role of sCTLA-4. We demonstrate the presence of a soluble form of CTLA-4 in 59/90 sera from patients with autoimmune thyroid diseases (both Graves' disease and autoimmune thyroiditis). sCTLA-4 levels were not related to specific clinical manifestations, such as clinical thyroid status (hypo- or hyperthyroidism), circulating thyroid hormones, or other clinical features (ophthalmopathy). sCTLA-4 production does not seem to be affected by disease evolution during time. We showed that sCTLA-4 from sera of patients with thyroid autoimmunity is able to bind its physiological ligands CD80/CD86 and displays functional activities on different *in vitro* systems (T-cell proliferation induced by specific soluble antigens, bi-directional mixed lymphocyte reaction). In conclusion, we demonstrate an increment of sCTLA-4 in serum of patients with autoimmune thyroid diseases. Its possible pathogenetic role during autoimmune processes can be speculated: sCTLA-4 can specifically inhibit the early T-cell activation by blocking the interaction of CD80/CD86 with the co-stimulatory receptor CD28. Conversely, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86, in later T lymphocytes activation phase, causing a reduction of inhibitory signaling
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Introduction

Effective T-cell activation requires a primary signal delivered by the antigenic peptide presented by major histocompatibility complex molecules and a non-specific signal (co-stimulation signal) mediated by CD28 interaction with B7

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family members on antigen-presenting cells [1]. Cytotoxic T lymphocyte associated gene-4 (CTLA-4) was initially described as a classical type I glycoprotein on the surface of activated T cells [2]. CTLA-4 is a member of the Ig gene superfamily and along with its homologue, CD28, which is a B7 binding protein [3,4]. The emerging opinion about the function of CTLA-4 is that it represents a negative regulator of T-cell activation. Thus, ligation of CTLA-4 on the T-cell surface initiates a cascade of biochemical events that attenuate an ongoing immune response [5]. CTLA-4 specific antibodies, which were cross-linked by a second antibody or immobilized on beads, have been found to prevent activation [6–8]. The most convincing data that support such a role for CTLA-4 come from experiments in which the *CTLA-4* gene is inactivated by a construction of CTLA-4 knockout mice [9,10]. These mice demonstrate profound polyclonal lymphoproliferative disorders that infiltrate most major organ systems and die a few weeks after birth. The majority of animals have increased levels of IgG: this fact illustrates the role of CTLA-4 on humoral immune responses as well [11,12].

A role for CTLA-4 in autoimmune diseases is suggested by the observation that blockade of B7/CTLA-4 interaction via administration of anti-CTLA-4 mAb exacerbates animal models of autoimmune diseases such as experimental autoimmune encephalomyelitis [13] and type-1 diabetes (T1D) [14].

A search for genetic markers that segregate with Graves' disease revealed an association with CTLA-4 polymorphisms [15]. Such association was further substantiated in various ethnic populations, as well as in other endocrine autoimmune disease [16–20]. More recently, Ueda et al. [21] analyzed the fine polymorphism of *CTLA-4* gene in patients with various autoimmune disease, including ATD and they found some possible associations.

Oaks and Hallett [22] submitted the nucleic acid sequences of alternate transcripts of *CTLA-4* in man, mouse, and rat that lacked transmembrane encoding regions. More recently, Magistrelli et al. [23] described the same transcript and detected immunoreactive material in human serum demonstrating the presence of a native soluble form of CTLA-4 (sCTLA-4). In addition, the presence of high concentration of sCTLA-4 was observed in sera patients with ATD (mainly Graves' disease) [24].

Finally, recent reports show that sCTLA-4 levels are augmented in patients with autoimmune diseases, such as type-1 diabetes [25], diffuse cutaneous systemic sclerosis [26], systemic lupus erythematosus [27], and myasthenia gravis [28]. In addition, plasma levels of sCTLA-4 are increased in patients with allergic asthma [29].

The present study has been conceived to further evaluate the immunopathological roles of sCTLA-4. In particular, we have verified the increasing of sCTLA-4 in different autoimmune thyroid diseases, analyzing the possible differences related to clinical manifestations. Finally, we have investigated the functional activities of sCTLA-4 in the serum of these patients.

Materials and methods

Serum samples

Serum samples were obtained from 90 patients with autoimmune thyroid diseases (ATD) (16 males, 74 females, age range 22–60) after informed consent. Fifty-nine of them had Hashimoto's thyroiditis (HT), 31 Graves' disease (GD). The very large majority of ATD patients had detectable levels of autoantibodies to thyroid peroxidase (86 out of 90), 51 out of 90 had also autoantibodies to thyroglobulin. The diagnosis was formulated on the basis of commonly accepted clinical and laboratory criteria, including thyroid ultrasonography, serum TSH and free thyroid hormone levels, anti-thyroid peroxidase antibodies, and anti-TSH receptor antibodies and thyroid scintigraphy (for GD). Patients with HT were either mild hypothyroid (raise TSH with low–normal free thyroxine) or euthyroid under L-thyroxine replacement. Patients with GD were hyperthyroid (from mild to severe) and were studied before or immediately after starting anti-thyroid therapy: 26 of them had clinically apparent Graves' ophthalmopathy.

From some GD patients serum samples collected at different time points, namely before, 1, and 3 months after radioiodine therapy, were available. In these patients euthyroidism or post-treatment hypothyroidism was documented, as well as thyroid gland volume reduction (by ultrasonography).

For control, we collected sera from 20 patients with non-autoimmune hyperthyroidism (toxic adenoma or multinodular toxic goiter) and from 45 age- and sex-matched blood donors.

Free thyroid hormone concentrations were assayed by routine ELISA methods, as well as TSH (3rd generation) and thyroid peroxidase and thyroglobulin autoantibodies.

ELISA

Specific ELISA kits were used for measuring serum sCTLA-4 levels (Bender Med System, Milano, Italy), according to the manufacturer's protocol. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was < 10% for any reported value. The lowest sensitivity threshold is 0.1 ng/ml.

The analytical response was linear approximately between 0.162 and 1.200 of absorbance values (corresponding to 0.1–50 ng/ml) as assessed by serial dilution test using a strongly positive serum (data not shown).

As control, for a subgroup of patients ELISA kits specific for sCD28, sCD80 and sCD86 were also used (Bender manufacturer's protocol).

Western blotting

Western blotting was used to detect serum sCTLA-4. Proteins were separated by 10–20% gradient PAGE in a discontinuous buffer system on a Mini-Protean system (Bio-Rad, Segrate, Milano, Italy). The separated components were electroblotted onto PVDF membranes. The blots were washed with 0.15 M NaCl, 0.05 M Tris (TBS), pH 7.5, with 0.3% Tween 20 and reacted with a 1:100 dilution of the anti-CTLA-4 mAb (clone 14D3, IgG2a, eBiosciences, San Diego, CA, USA) for 1 h at room temperature, washed, and then reacted with reporter antibody (HRP-conjugated anti-rabbit IgG) for 1 h. The blots were then developed by the use of a commercially available chemiluminescence detection kit (BMB, Indianapolis, IN) according to the manufacturer's instructions.

Evaluation of immunophenotype

A B-cell line transformed by Epstein–Barr virus (B-EBV) (5×10^5), obtained as previously described [8], was preincubated with different sera from patients selected for their sCTLA-4 positivity or negativity. In addition, B-EBV cells were incubated with Fc-blocking mAb (KD1, IgG1, anti-CD16) to prevent non-specific binding of labeled mAb and then stained with FITC-conjugated anti-CD80 (clone HIB19, Becton Dickinson, Milano, Italy) and anti-CD86 (clone G18–145). Cells not pretreated with serum were used as positive control of CD80/CD86 expression. After staining, cells were analyzed, and fluorescence signals were collected in log mode using a FACScalibur (Becton Dickinson). CTLA-4 Ig (provided by A. Lanzavecchia, Institute for Research in Biomedicine, Bellinzona, Switzerland) was utilized as a positive control of molecule reacting with CD80 and CD86 ligands.

Mixed leukocyte reactions (MLR)

Two-way MLR was used to test sCTLA-4 for functional activity. The cells used in these experiments were from a panel of previously HLA typed laboratory volunteers and were selected to provide two HLA-DR mismatches. PBMCs were isolated by standard Ficoll-Hypaque density gradient centrifugation, and equal numbers of cells (100,000) were cocultured in tissue culture medium (RPMI 1640 supplemented with 10% human AB serum, 10 mg/ml gentamicin, and 50 mM 2-mercaptoethanol) containing graded dilutions of sCTLA-4 test sample. Sera were added at different time points as indicated. Triplicate cultures were set up in 0.2-ml volumes in round-bottom 96-well microculture plates and were incubated for 5 days at 37 °C and 5% CO_2 in air. The cultures were pulsed with 0.5 mCi ^{3}H-thymidine and harvested 18 h later. Isotope incorporation was measured on a Beckman β-counter. For tests with *Candida* bodies (Ca), Ca-specific CD4$^+$ T-cell lines were generated from total PBMCs by repeated stimulation with autologous irradiated PBMCs pulsed with the appropriate antigen, as previously described [30].

Depletion of sCTLA-4 protein from sera

In order to understand the effective role played by serum sCTLA-4 avoiding the effect related to other serum proteins, we performed an MLR assay using sera previously depleted of sCTLA-4. To this end we incubated the sera from different patients (2 positive and 2 negative after ELISA testing) for 3 h at room temperature on ELISA plates coated with the capture mAb anti-sCTLA-4 (included in the ELISA kit). In order to avoid any specific binding to the plastic, plates were blocked (with PBS buffer containing 5 g/l of bovine serum albumin and 0.5 ml/l of Tween 20) and fixed (with PBS buffer containing 15% sucrose) before adding the sera. This procedure (suggested by the manufacturer) was repeated twice before utilizing these sera in an MLR assay as specified before. As a control we used sera not subjected to the depletion procedure.

Statistical analysis

Statistical analysis was performed using the Mann–Whitney *U*-test for comparison of sCTLA-4 levels by using the GraphPad Prism4 software 4.0 (GraphPad Software Inc., CA, USA). Differences in cell proliferation between control and mAb-treated cells were observed. To assess their statistical significance, the Student's *t* test was used with a level of $p < 0.05$.

Results

Serum sCTLA-4 levels in ATD patients

To determine whether a circulating form of CTLA-4 was present in human serum, we utilized a sensitive ELISA. Fig. 1A shows combined data from the ELISA on patients with ATD and normal apparently healthy volunteers. sCTLA-4 was virtually undetectable in the very large majority of healthy volunteers as defined by the detection limit of 0.1 ng/ml for this assay. Six serum samples out of 45 from healthy controls

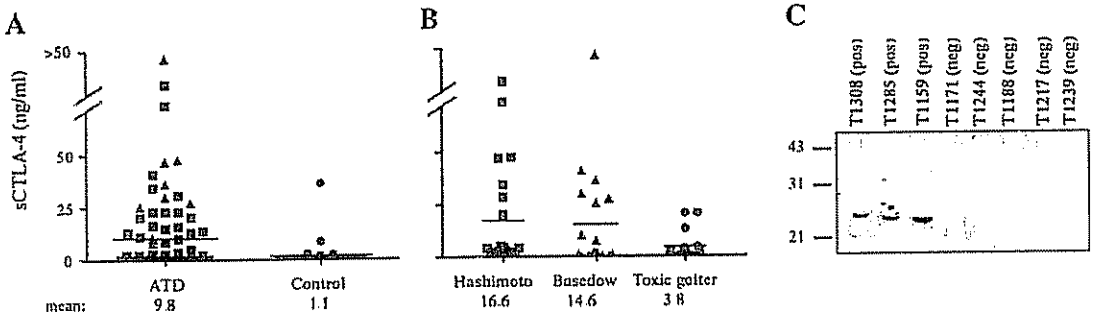


Figure 1 A soluble form of CTLA-4 is found in serum of patients with ATD. (A) The concentration of sCTLA-4 was evaluated by ELISA on sera collected from ATD patients ($n=90$; squares HT, triangles GD) and a healthy donor group as a control ($n=45$). Results are expressed as ng/ml. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. (B) Results of sCTLA-4 ELISA on 24 selected sera of untreated hyperthyroid GD, 20 sera of HT patients, and 20 control sera of patients with non-autoimmune hyperthyroidism (toxic adenoma or multinodular toxic goiter). Results are expressed in ng/ml. Each sample was diluted 1:10 and tested in triplicate. Deviation between triplicates was <10% for any reported value. (C) Immunoprecipitation of sCTLA-4 was performed on sera from a representative group of ATD patients. T1308, T1285, and T1159 were resulted positive during ELISA analysis; T1171, T1244, T1188, T1217, and T1239 were sera negative for sCTLA-4. Arrow marks 23-kDa species. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively.

Table 1 Sera collected from the same patients at different time points after therapy showed a comparable amount of sCTLA-4

Time points	Patient sCTLA-4 evaluation (ng/ml)					
	T105	T77	T56	T52	T23	T155
T_0	37.6	36.4	27.4	<0.1	<0.1	<0.1
T_1	27.0	46.9	9.4	<0.1	0.3	0.6
T_2	30.6	47.1	16.1	0.2	0.3	0.3

ELISA was performed on sera collected from the same patients at different time points after radioiodine therapy (T_0: before radioiodine therapy, T_1: 1 month and T_2: 2 months after therapy). Of note, patients that were positive for the soluble spliced form (T105, T77 and T56) maintain the ability to produce it over time, irrespective of the kinetic of thyroid function. On the other hand, patients unable to produce sCTLA-4 at the beginning of the therapy (T52, T23, and T155) have virtually no detection of this protein at any time points.

had detectable sCTLA-4 (from 0.1 to 36.1 ng/ml). By contrast, 59 out of 90 patients (65%) with ATD had detectable circulating CTLA-4 levels in the range of 0.1–>50 ng/ml ($p<0.05$) (Fig. 1A). Of note, there were no differences between patients with HT and GD. In addition, sCTLA-4 concentration was significantly higher in patients with hyperthyroid GD than in controls with non-autoimmune hyperthyroidism ($p<0.05$) (Fig. 1B). There was no obvious relationship between the sex and age of the patient and levels of sCTLA-4. Moreover, no relationship was found in GD patients between sCTLA-4 levels and degree of hyperthyroidism (free T4 or T3 concentrations) or severity of ophthalmopathy. Finally, there was no relationship between thyroid autoantibodies and sCTLA-4 levels (data not shown).

In addition, Western blot of human serum was probed with specific anti-CTLA-4 mAb. As shown in Fig. 1C, the three different samples resulted positive by ELISA analyses (T1308, T1285, and T1159) were characterized by a band of approximately 23 kDa, which is consistent with previous reports [22–24]. These species were not evident when sera which resulted negative during ELISA were plotted (T1171, T1244, T1188, T1217, and T1239).

Of interest, the analysis of the sera collected from the same patient at different time points after radioiodine therapy showed that the ability to produce sCTLA-4 is not related to thyroid functions. In particular, patients who were positive for the soluble spliced form maintained their ability to produce it over time. On the other hand, patients unable to produce sCTLA-4 at the time of therapy resulted negative during the follow-up (Table 1).

sCTLA-4 from serum of autoimmune patients is able to bind CD80/CD86

In order to verify the capability of sCTLA-4 to recognize its specific ligands (CD80/CD86) and to avoid the possibility that the soluble molecule is the result of a degradation or shedding process, we attempted to block the binding of specific mAb to CD80/CD86 present on B-EBV cell targets. Pretreatment of B-EBV cell lines with sera from autoimmune patients characterized by the presence of sCTLA-4 led to a significant reduction of staining by anti-CD80 or anti-

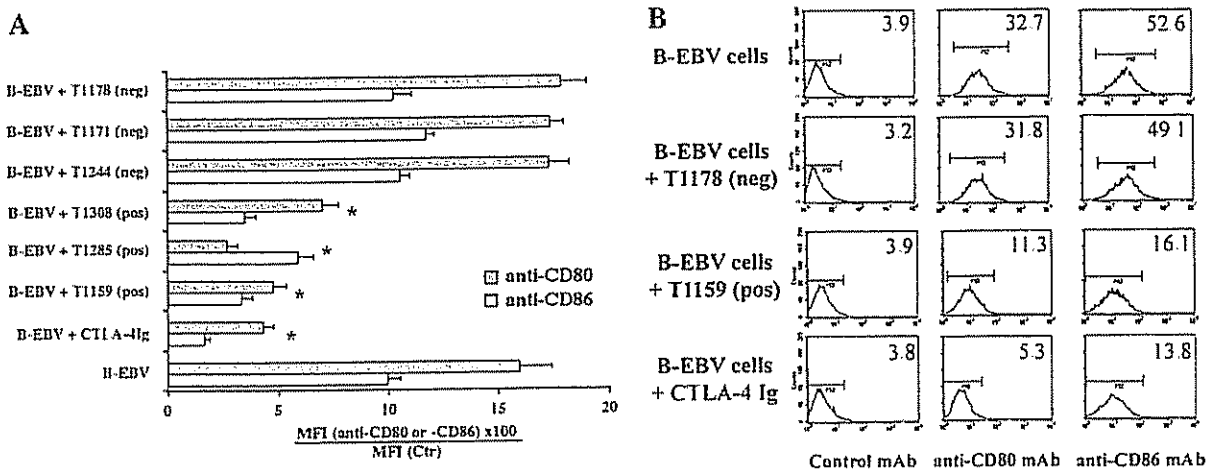


Figure 2 sCTLA-4 from sera of ATD patients is able to interact with its natural ligands. (A) Data from three independent experiments showing the capability of sCTLA-4 to inhibit anti-CD80/anti-CD86 mAb immunoreactivity. The test was performed utilizing a B-EBV cell line expressing both CD80 and CD86 molecules. Six different sera from patients with ATD (T1308, T1285, and T1159 positive and T1171, T1244, and T1188 negative for sCTLA-4) were preincubated with B-EBV cells prior to stain cells with mAb to CD80 and CD86. The addition of CTLA-4 Ig fusion protein was utilized as positive control of interference of specific mAb binding to CD80 and CD86. The graphic plots the ratio between the Mean Fluorescence Intensity (MFI) of the cells stained with specific mAb (anti-CD80 or -CD86) and the MFI of the negative control (B-EBV no treated with sera). The averages ± standard deviations (error bars) of three different experiments are shown. * Indicates samples that are significantly different ($p<0.05$) from the control (B-EBV). (B) Representative data from an experiment to test whether sCTLA-4 was capable of inhibiting anti-CD80/anti-CD86 mAb immunoreactivity. The test was performed as previously described.

CD86 mAb (T1308, T1285, and T1159). These results were obtained on three independent serum samples and ranged from 17 to 61% and from 56 to 85% of inhibition, respectively, for CD80 and CD86 (Fig. 2). A negligible inhibition (<5%) was observed when sera negative for sCTLA-4 were tested. CTLA-4 Ig, which reduced the staining of B-EBV cell lines by anti-CD80 or anti-CD86 mAb, was utilized as a positive control. These data show that the CTLA-4 immunoreactive material present in human serum represents an intact functional receptor for CD80/CD86 ligands.

sCTLA-4 from serum of autoimmune patients is able to inhibit both mixed leukocyte reactions (MLR) and proliferation of antigen-specific T-cell clones in a dose dependent fashion

We next examined the immunoregulatory properties of sCTLA-4 by determining its effect on two-way MLR. The data presented in Fig. 3A show that sCTLA-4 had significant inhibitory effect, similarly to that one obtained by the addition of CTLA-4 Ig. Moreover, we verified the dose effect

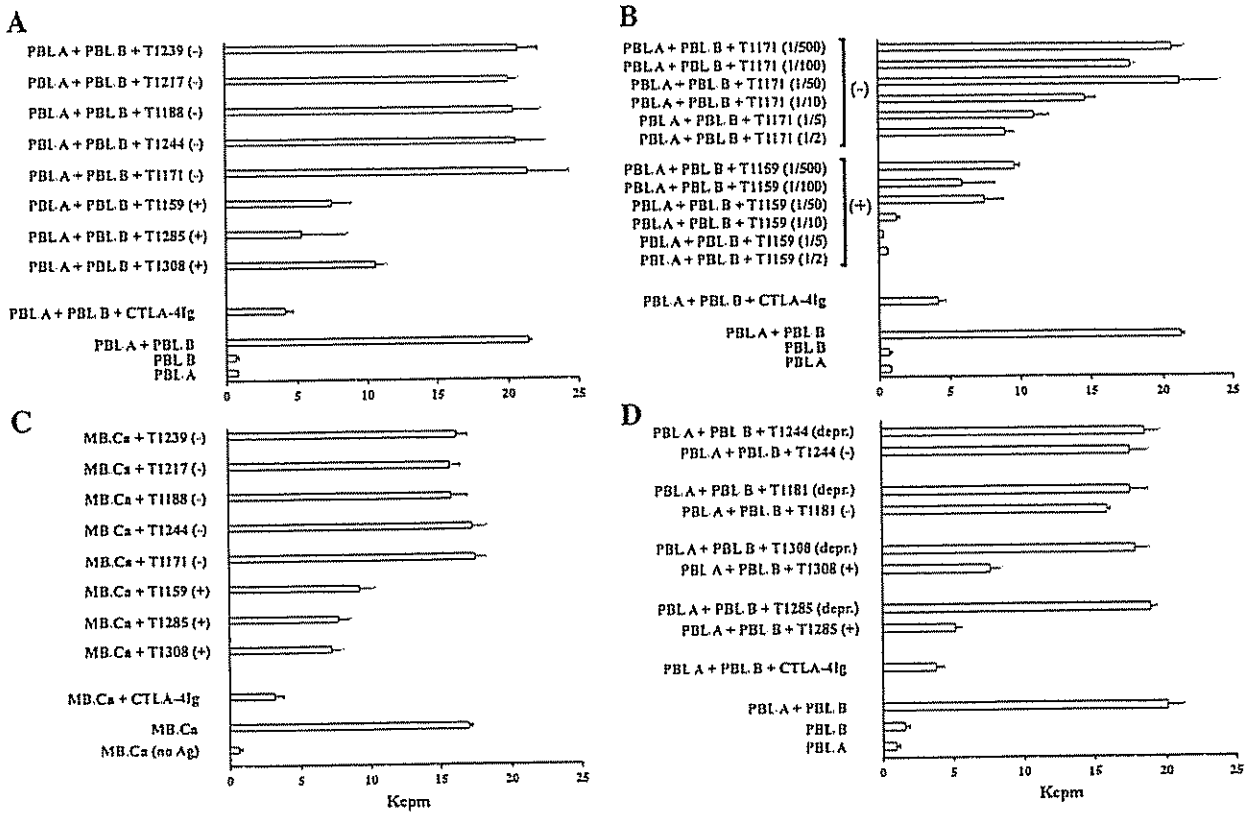


Figure 3 Immunoregulatory properties of sCTLA-4. (A) sCTLA-4 containing sera from ATD patient (T1308, T1285 and T1159) are able to inhibit significatively a two-way MLR. CTLA-4 Ig fusion protein is a positive control of inhibition of proliferation. Sera from ATD patients negative for sCTLA-4 (T1171, T1244, T1188, T1244, and T1239) do not affect the two-way MLR. The data represent the mean of quadruplicate samples. Initial dilutions of proteins used in these assays are approximately 0.1 ng/ml. The averages ± standard deviations (error bars) of three different experiments are shown. Similar results were obtained in three separate experiments from a variety of donor/recipient combinations. Positive or negative sera are indicated by a alphanumeric code followed by (+) and (−) respectively. (B) Analysis of the dose effect of 2 different sera (T1159 and T1171) respectively positive and negative for sCTLA-4. The inhibition rate depends on serum concentration of sCTLA-4. CTLA-4 Ig fusion protein is a positive control of inhibition of proliferation. The data represent the averages ± standard deviations (error bars) of quadruplicate samples. Positive or negative sera are indicated by a alphanumeric code followed by (+) and (−) respectively. (C) Similar inhibitory effect of sCTLA-4 was observed in an antigen-specific ^{3}H-thymidine incorporation assay. The proliferation of an antigen-specific T-cell clone (specific for *Candida albicans* bodies, MB.Ca) induced by PBMCs pulsed with the antigen was reduced by the addition of sera which resulted positive for sCTLA-4, whereas the negative ones had no effect. The averages ± standard deviations (error bars) of three different experiments are shown. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively. (D) The inhibitory effect on T lymphocytes proliferation is related to sCTLA-4 and is not due to a different serum protein. sCTLA-4-deprived sera (T1285 and T1308) are ineffective on MLR ^{3}H-thymidine incorporation. Controls are the same sera before the treatment with the capture mAb specific for sCTLA-4 (see Materials and methods). The averages ± standard deviations (error bars) of three different experiments are shown. Positive or negative sera are indicated by an alphanumeric code followed by (+) and (−) respectively. The abscissa indicate the incorporation of ^{3}H-thymidine during the last 18 h of culture. Isotope incorporation was measured on a Beckman β-counter (Kcpm = 1000 cpm).

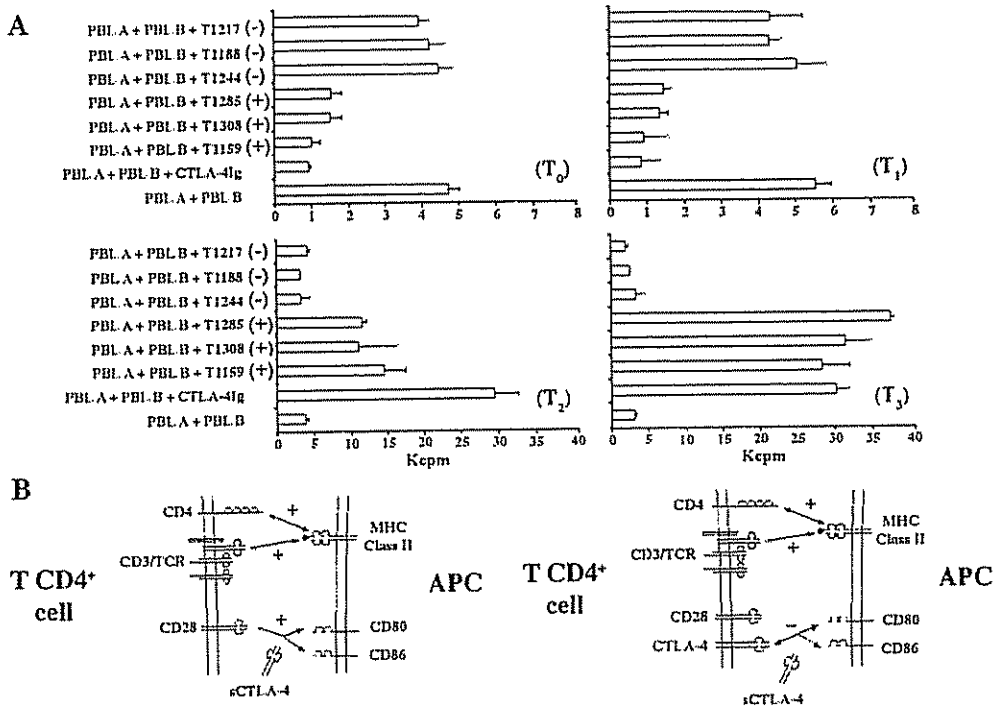


Figure 4 sCTLA-4 from serum of autoimmune patients is able to control MLR in a dynamic way. (A) The inhibitory effect on T lymphocytes proliferation due to sCTLA-4 is related to the activation state at which the soluble protein is added. When sCTLA-4-positive sera or CTLA-4 Ig fusion protein are added to the two-way MLR at time 0 (T_0, from the beginning of the T lymphocyte activation) or at time 1 (T_1, 24 h later) the result is a decrease of proliferative ability. On the contrary, when sCTLA-4 and CTLA-4 Ig were added at times 2 and 3 (T_2, T_3, respectively after 2 and 3 days from the beginning of the test) ^{3}H-thymidine incorporation rate is increased. The averages±standard deviations (error bars) of three different experiments are shown. (B) A model explaining the different role played by sCTLA-4 on T-cell activation. T lymphocyte activation requires two signals: signal 1, provided by antigen–TCR engagement and signal 2 (co-stimulatory), mainly provided by the interaction of the co-stimulatory molecule, CD28, with its ligands CD80/CD86, on antigen-presenting cells. CTLA-4 is another co-stimulatory molecule also binding to CD80/CD86 but providing a negative signal to T-cell activation. The altered levels of sCTLA-4 could lead to either blockade of available CD80/CD86 molecules leading to a decreased stimulatory signal from CD28 engagement during the priming of immune response (left) or to an inability of membrane-bound CTLA-4 to engage with CD80/CD86 and hence less negative signaling (right) later during T lymphocyte activation. The abscissa indicates the incorporation of ^{3}H-thymidine during the last 18 h of culture. Isotope incorporation was measured on a Beckman β-counter (Kcpm = 1000 cpm).

of 2 different sera (namely T1159 and T1171) respectively positive and negative for sCTLA-4 ELISA. As shown in Fig. 3B, the inhibition rate depends on serum concentration of sCTLA-4. A similar inhibitory effect of sCTLA-4 was observed in an antigen-specific ^{3}H-thymidine incorporation assay. The proliferation of an antigen-specific T-cell clone (specific for *Candida* bodies, MB.Ca) induced by PBMCs pulsed with the antigen was reduced by the addition of sCTLA-4-positive sera, whereas the negative ones had no effect (Fig. 3C).

The inhibitory effect on T lymphocytes proliferation is related to sCTLA-4 and is not due to a different serum protein

In Fig. 3D we show an assay similar to that one described in Fig. 3A, where the sera utilized were previously deprived of sCTLA-4 proteins as described in Materials and methods section. Controls were the same sera before depletion. The results show that the depleted sera (T1285 and T1308) are ineffective on MLR ^{3}H-thymidine incorporation. This strongly suggests a direct role of the soluble form of CTLA-4 in regulating lymphocytes proliferation and rules out the intervention of putative different serum molecules.

sCTLA-4 from serum of autoimmune patients is able to control MLR in a dynamic way

It remains unknown how alterations of sCTLA-4 levels may contribute in the development of autoimmunity. It has been speculated that a reduction in sCTLA-4 level could lead to the reduction in the blocking of CD80/CD86 molecules and sustained T-cell activation through CD28 [26]. However, higher levels of sCTLA-4 could compete with membrane-bound CTLA-4 for CD80/CD86-binding sites and cause a reduction of inhibitory signals. In an attempt to clarify this point we planned experiments of two-way MLR where the ATD sera were added at different time points after the activation. As shown in Fig. 4A, the addition of sCTLA-4-

Table 2 Sera collected from the ATD patients and controls showed a comparable amount of sCD28

	Patient sCD28 evaluation (ng/ml)	
	Range	Mean ± SD
Hashimoto patients (n=11)	4.1–315.3	153.9 ± 120.62
Basedow patients (n=11)	5.7–292.8	108.3 ± 111.88
Toxic goiter (n=10)	1.3–239.1	81.7 ± 88.24
Normal donors (n=9)	0.4–172.6	103.2 ± 76.47

ELISA was performed on selected sera collected from ATD patients (Hashimoto's thyroiditis, and Graves' disease) and from the control groups (non-autoimmune hyperthyroidism – toxic adenoma or multinodular toxic goiter- and age- and sex-matched blood donors).

positive sera (T1159, T1308, and T1285), as well as the control CTLA-4 Ig, resulted in a reduction of proliferation when added at the beginning (T_0) and after 24 h (T_1) of the test. The opposite effect was measurable when sera were added after 48 h (T_2) and 72 h (T_3). Sera negative for sCTLA-4 protein (T1244, T1188 and T1217) were ineffective on thymidine incorporation.

In our model (Fig. 4B), altered levels of soluble CTLA-4 could lead to either blockade of available CD80/CD86 co-stimulatory molecules with decreased co-stimulatory signal from CD28 engagement (if sCTLA-4 is increased during the early activation process) or to an inability of membrane-bound CTLA-4 to engage with CD80/CD86 and hence to reduced inhibitory signals (if sCTLA-4 levels are increased during the late activation phase).

Evaluation of other soluble molecules belonging to the B7 family in sera from ATD patients

In order to better understand the pivotal role of sCTLA-4 and to investigate the possible involvement of other soluble B7 family members, we performed specific ELISA tests. We found undetectable amount of sCD80 and sCD86 both in the ATD patient group and in the control ones (data not shown). On the other hand, sCD28 was detectable in both groups (Table 2). However, the concentrations of sCD28 were widely dispersed in both ATD and control groups. Statistical analyses for sCD28 did not evidence any significative correlation when comparing the amount of sCD28 detected by ELISA and the pathological state of the donors.

Discussion

The regulation of immune responses is the outcome of a balance between positive signals that trigger them and inhibitory mechanisms that prevent excessive clonal expansion and autoimmunity. Therefore, a prevalence of activation should render T cells responsive to antigens, whereas a prevalence of inhibition should lead to T-cell anergy. The majority of the data on the inhibitory function exerted by CTLA-4 have been gathered by studies of proliferation or cytokine production of T lymphocytes analyzing the interaction between CTLA-4 and CD80/CD86 at the membrane level.

Several papers [22–24] have recently described an alternate transcript of the *CTLA-4* gene that encodes a protein that lacks a transmembrane region and likely represents a native soluble form of CTLA-4. The data herein reported confirm and extend the original findings of Oaks et al. [24], who detected sCTLA-4 in the serum of about half of a group of ATD patients. Consistently, we found detectable serum sCTLA-4 in 59 out of 90 ATD patients. Furthermore, we evaluated the possible relationship of sCTLA-4 levels with single ATD, GD, or HT, but no differences were found: accordingly, no relationships were observed between sCTLA-4 and specific clinical features (namely, severity of ophthalmopathy) among GD patients. Finally, there was no correlation between sCTLA-4 and clinical thyroid status (eu-, hypo-, or hyperthyroidism) or circulating thyroid hormones or TSH levels. It is likely that the presence of sCTLA-4 in the serum in the majority of ATD patients is linked to autoimmunity in a broad sense and not to specific clinical manifestations. As well, the levels of sCTLA-4 in a single patient do not seem related to disease evolution during time: this is suggested by our findings on sera of GD patients withdrawn before and after radioiodine treatment. In fact the ability to produce sCTLA-4 persists after therapy, despite profound changes in thyroid function, volume, and structure. On the other hand, radioiodine *per se* does not induce sCTLA-4 release in patients that were negative before treatment. It is known that radioiodine treatment of GD may transiently result in an increased activation of the autoimmune process (specifically, TSH-receptor antibodies increase), probably due to massive self-antigens release [31]. This further supports that the ability to produce sCTLA-4 is genetically determined, and not merely dependent to immune activation. This would be consistent with the hypothesis that increased sCTLA-4 levels in ATD are "constitutive", possibly related to the genetic control of sCTLA-4 synthesis [21,24]: however no correlation between sCTLA-4 levels and the well known CT-60 A/G SNP was observed [25]. Definitive evidence for, or against the genetic regulation of sCTLA-4, might derive from longitudinal studies starting from disease onset.

The present work provides evidence about the possible functional role of sCTLA-4. The fact that CTLA-4 immunoreactivity can be blocked by its known ligands (namely, CD80 and CD86) and by the synthetic receptor (CTLA-4 Ig) supports the notion that sCTLA-4 is a functional receptor. Its ability to interfere with T-cell proliferation in MLR or following specific soluble antigen stimulation in a dose-dependent manner supports its possible immunoregulatory action *in vivo*, like other soluble receptors [32–37]. In addition, the absence of the soluble form of CD80 and CD86 and the lack of correlation with the pathological state of the amount of soluble CD28 in the analyzed sera support the hypothesis that the functional effects of serum from autoimmune patients are principally due to the presence of sCTLA-4. The effect of sCTLA-4 binding to CD80/CD86 molecules might depend on the activation status of the cells involved. For example, on resting cells, sCTLA-4 may block CD80/CD86–CD28 interactions, thereby interfering with T-cell co-stimulation. On the other hand, inhibition of CD80/CD86–CTLA-4 interactions on activated T cells (in these conditions the transmembrane form of the molecule is selectively expressed) may prevent down-regulation of T-cell responses.

The experiments performed in the present study suggest that sCTLA-4 present in ATD sera is able to specifically enhance T-cell proliferation induced by MLR when added after the initial phase, i.e. in the presence of already activated T cells. Thus, sCTLA-4 might indirectly act as an enhancer of the autoimmune response considering that in ATD intrathyroid T lymphocytes are mainly composed by activated cells [38] and that, as shown in previous studies, molecules of the B7 family are expressed within the thyroid by different cell types, including follicular cells, in ATD [39].

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EXHIBIT 9

A new member of the immunoglobulin superfamily—CTLA-4

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The immunoglobulin superfamily is a group of proteins, each made of one or several domains sharing key structural features with either the variable (V) or the constant (C) immunoglobulin domains[1,2]. It includes such functionally important members as the immunoglobulins themselves, major histocompatibility complex (MHC) class I and class II and T-cell receptor (TCR) molecules. Several members of this superfamily are expressed on lymphocytes where they are membrane-bound and capable of interactions with other members of the family, thus taking part in cell-cell recognition. In screening mouse cytolytic-T-cell-derived cDNA libraries, we came across cDNA clones defining a sequence, CTLA-4, which could encode a 223-amino-acid protein clearly belonging to the immunoglobulin superfamily. It consists of one V-like domain flanked by two hydrophobic regions, one of which has a structure suggestive of membrane anchoring. CTLA-4 is mainly expressed in activated lymphocytes and is coinduced with T-cell-mediated cytotoxicity in inducible models of this process. The mouse *ctla-4* gene maps to band C of chromosome 1.

We previously reported the construction of a subtracted cytolytic T-cell(Tc)-derived complementary DNA library of about 8,300 clones, from which three distinct cytolytic T-cell-associated sequences, CTLA-1, CTLA-2 and CTLA-3, were isolated by conventional differential screenings[3]. CTLA-1 and CTLA-3 encode distinct serine-esterases, also detected, using similar approaches, as CCP-1 (ref. 4) and H factor[5], respectively; CTLA-2 encodes a previously undescribed protein product (F.D. *et al.*, in preparation). Further screening of the same subtracted library with a subtracted (Tc−B) cDNA probe allowed the detection of 113 additional clones which had not detectably hybridized with a total Tc-derived cDNA probe. These clones were subjected to successive rounds of screening with several other Tc cDNA probes subtracted against messenger RNA derived from either the B lymphoma M12.4.1, or the EL4 thymoma, or thymocytes. The positive, thus still apparently Tc-specific, clones were then depleted of copies of CTLA-1, -2 and -3 by probing with a pool of inserts representative of these families. The inserts of the 17 cDNA clones left at that stage were used as probes on Northern blots of a panel of RNAs. Only one clone, M17G7, survived this ultimate screening for Tc specificity in that it detected a 2-kilobase (kb) transcript in RNA from the original KB5C20 Tc clone and not from other, non-Tc cells. The M17G7 cDNA insert defines CTLA-4.

A study of the tissue distribution of the CTLA-4 2-kb transcript (Fig. 1) revealed its presence in each of the Tc or Tc-containing populations tested (two clones: KB5C20 and A15.1.17, albeit at a low level for the latter; and three *in vitro* or *in vivo* primary populations: mixed leucocyte culture cells, concanavalin-A-activated blasts and allo-sensitized peritoneal exudate lymphocytes). In addition, like the CTLA-1, -2 and -3 transcripts[3], the CTLA-4 transcript was coinduced with cytotoxicity in two systems: on incubation of the constitutively growing cytolytic hybridoma PC60 (ref. 6) with interleukin-containing supernatants and on incubation of concanavalin-A-pulsed thymocytes with an interleukin-2-containing supernatant. A weaker signal was detected in lipopolysaccharide(LPS)-activated blasts (the significance of which is unclear due to the likely heterogeneity of this population) and non-induced PC60 cells, and a very weak one in non-activated thymocytes (Fig. 1) and in non-experimentally-activated peripheral lymphocytes, that is column-purified or unpurified normal spleen cells (data not shown). EL4, the T helper hybridoma T14-117, NK-cell-

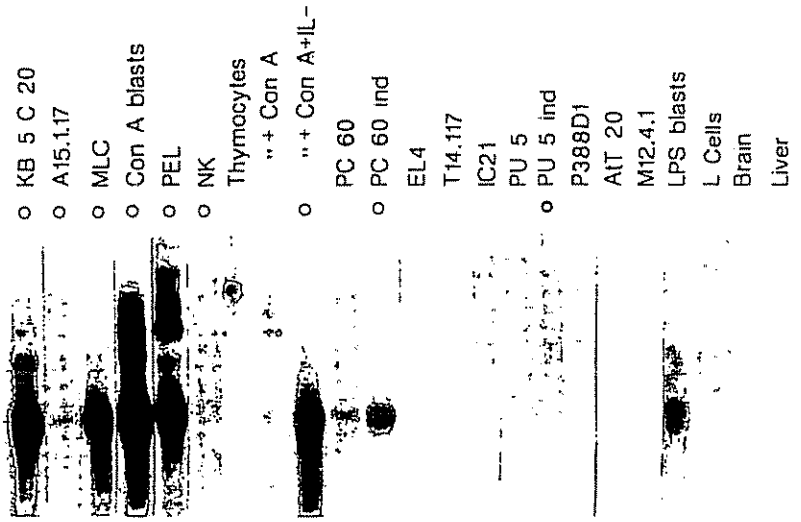


Fig. 1 Specificity pattern of CTLA-4 RNA transcripts. Northern blots were prepared with RNA from the following cells: KB5C20, a B10.BR anti-H-2K^b Tc clone[16]; A15.1.17, an A.Th anti-A.TL Tc clone[17]; MLC cells, A/J spleen cells stimulated for five days *in vitro* with C57BL/6 irradiated spleen cells; Con A blasts, a mixture of C57BL/6 spleen cells stimulated for two or three days *in vitro* with concanavalin A (1.5 μg ml^{-1}); PEL, nylon-wool purified BALB/c anti-EL4 peritoneal exudate cells[18]; NK, nylon-wool purified Swiss *nu/nu* spleen cells; thymocytes, from C57BL/6 mice; thymocytes plus Con A, the latter incubated for 24 h with Con A (5 μg ml^{-1}); thymocytes plus Con A and IL-2, the latter further incubated for five days in the presence of IL-2-containing supernatant from PMA-stimulated EL4 C116 cells; PC60, a mouse × rat hybridoma[6]; PC60ind, the latter induced with interleukin-containing supernatants[19]; EL4, a C57BL/6 thymoma; T14 117, a hybridoma between a *T*-helper cell clone and the BW5147 thymoma[20]; IC-21, PU5 and P388D1, macrophage cell lines; PU-5ind, PU-5 activated by a 24-h incubation with supernatant with supernatant from restimulated KB5C20 cells; AtT20, a neurosecretory cell line; M12.4.1, a B lymphoma[21]; LPS blasts, C57BL/6 spleen cells grown for three days with lipopolysaccharide (5 μg ml^{-1}); L cells, a C3H fibroblast line; brain and liver cells from C57BL/6 mice. The Northern blots (with about the same amount of RNA per lane according to ethidium bromide staining of gels, not shown) were probed with the radiolabelled 300-bp insert from the CTLA-4 cDNA clone M17G7, isolated from a first, subtracted, KB5C20 cDNA library[3]. Positive lanes show a main band corresponding to a transcript of about 2 kb, but often also higher bands corresponding to longer transcripts. In parallel, the same cells had been tested for cytotoxity on EL4, RDM4 and YAC target cells in the absence or in the presence of Con A (10 μg ml^{-1}); those found positive in a 4-h or in a 20-h ^{51}Cr release test are labelled O or O, respectively; the others were negative. All methods were exactly as described before[3].

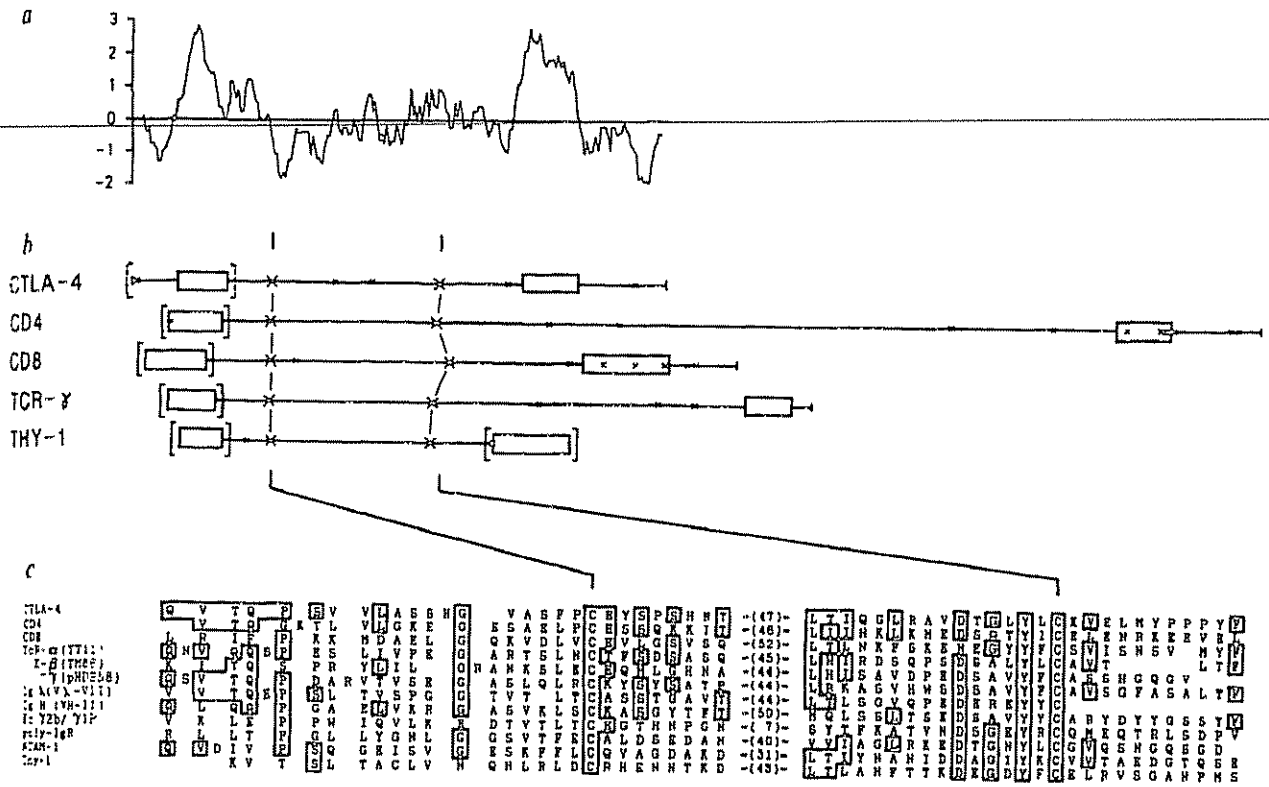


Fig. 3. *a*, Hydropathicity plot[28] of CTLA-4, with a window of 11 residues. *b*, Aligned overall primary protein structures of CTLA-4, CD4 (L3T4), CD8 (Lyt-2), γ-chain of the T-cell receptor (TcR)and Thy-1. Boxes, long hydrophobic residues; brackets, peptides known to be cleaved off the mature proteins; stippled brackets, putative CTLA-4 leader sequence; crosses, cysteine residues (note the two unusual extra cysteines in the V-like domain of CTLA-4). *c*, Alignment of the sequences, around the two cysteines involved in the immunoglobulin fold, of CTLA-4, CD4 (ref. 29), CD8 (ref. 30), T-cell receptor α-chain[31], β-chain[32] and γ-chain[33], human V_λ chain[34] (Ig λ), mouse V_H chain[35] (Ig H), Fcγ receptor[14,36] (Fc γ2b/γ1R), polyimmunoglobulin (Ig) receptor[37], the first domain of murine N-CAM[38] and Thy-1 (ref. 1). Boxed residues are identified in CTLA-4 and three or more members of the immunoglobulin superfamily (conservative changes like Phe 56 are not boxed).

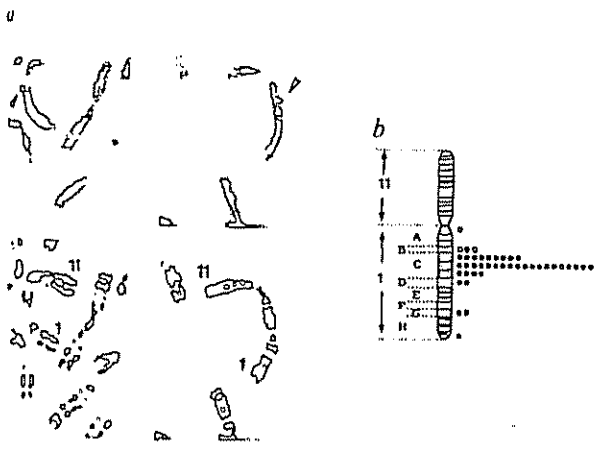


Fig. 4 Localization of the *ctla-4* gene to mouse chromosome 1 by *in situ* hybridization with the CTLA-4 probe F41F4. *a*, Two partial WMP mouse metaphases, showing the specific site of hybridization to chromosome 1. Top, arrowheads indicate silver grains on Giemsa-stained chromosomes, after autoradiography. Bottom, chromosomes with silver grains were subsequently identified by R-banding. Note the homogeneously staining region[39] in the C–D band interface of chromosome 1. *b*, Diagram of WMP mouse Rb(1; 11) chromosome, indicating the distribution of labelled sites. In the 123 metaphases examined, there were 250 silver grains associated with chromosomes and 41 of these (16.4%) were localized on chromosome 1. On this chromosome, 78% of the grains mapped to the C band (for banding nomenclature, see ref. 40), with a maximum in the middle part of this band.
Methods. *In situ* hybridizations with probe F41F4 were done using metaphase spreads from a male WMP mouse in which all the autosomes (except 19) are in the form of robertsonian translocations (given by J. L. Guénet, Pasteur Institute). All methods were as indicated before[3,41,42]. The specific activity of the probe was 6×10^7 d.p.m. μg^{-1}, its final concentration was 25 ng ml^{-1}, and the slides were exposed for 18 days at 4 °C.

plasmic hinge of many membrane receptors[9]; and the rest of a hydrophilic putative cytoplasmic tail. The 5′ flanking region has the location and hydrophobicity of a leader sequence, but is unusual in its overall length and also perhaps in the basic Arg-Arg-Tyr-Lys stretch at positions 7–10. This leader sequence would be cleaved off between the Ser at position 35 and the Glu at position 36 (taking into account the CTLA-4 hydrophobicity pattern and the site of cleavage for other members of the immunoglobulin superfamily); alternatively, it may stay uncleaved[10]. Antibodies should be of help in determining the location and mode of insertion into the membrane, the precise tissue distribution and possibly the functional (maybe interactive) role of the CTLA-4 protein product.

In a preliminary study of the corresponding gene(s), the Southern blot patterns (shown) were consistent with there being only one copy of the *ctla-4* gene per mouse haploid

genome. Multiplicity of gene copies cannot therefore account for the several CTLA-4 transcripts longer than the 2-kb one, detected in Northern blots of, in particular, concanavalin A blasts and peritoneal exudate lymphocytes (Fig. 1). In agreement with the Southern blot hybridization patterns, by *in situ* hybridization only one main location was found for the *ctla-4* gene, on band C of mouse chromosome 1 (Fig. 4). The genes for the IgG1/IgG2b FcR-Ly-17 alloantigen and for M1s map apparently more distally on the same chromosome[11-14].

Altogether, compared to other currently known members[15] of the immunoglobulin superfamily, the CTLA-4 protein product has in common with Thy-1, CD4 (L3T4), CD8 (Lyt 2) and CD3 (T3)ε-chain a single V-like domain most probably expressed at the cell membrane in certain lymphocyte subpopulations. CTLA-4, however, is peculiar in that it is mainly found in activated lymphocytes. The regulation of transcription of the *ctla-4* gene, and also the function of the CTLA-4 protein, seem particularly interesting areas for future studies.

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Recombinant human lipocortin 1 inhibits thromboxane release from guinea-pig isolated perfused lung

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The guinea-pig perfused isolated lung, used in conjunction with the cascade superfusion system to measure the release of thromboxane A_2 (TXA_2), is a simple and convenient model for assessing the inhibition by glucocorticoids of eicosanoid formation[1–8]. Dexamethasone inhibits the release of TXA_2 from the lung when it is stimulated by agents such as RCS-RF[2] of leukotrienes,[4,8] but not when bradykinin or arachidonic acid are used[1–7]. Using this model we have shown that the glucocorticoids suppress eicosanoid generation by cells through the induction of a family of phospholipase A_2-inhibitory proteins[5,6] now termed the 'lipocortins'[9]. Recently the primary structure of one form of lipocortin has been elucidated and the human gene cloned[10]. Lipocortin 1 is a polar monomeric protein with anti-phospholipase properties *in vitro*[10,11] and we now report that when infused into guinea-pig lung preparations this protein has the same inhibitory profile as the glucocorticoids but with a more rapid onset of action. This is the first demonstration that eicosanoid formation can be inhibited by a recombinant phospholipase inhibitory protein applied extracellularly.

Bolus injections of leukotriene C_4 (LTC_4), 1–10 ng, elicited dose-dependent release of TXA_2 from the guinea-pig perfused lungs (equivalent to ~10–50 ng of the TXA_2 agonist U44619) but had no direct action on the isolated tissues. In 32 out of 32 experiments, in which lipocortin was infused into the lung at concentrations of 0.4–4.0 μg ml^{-1} (10^{-8} M to 10^{-7}), inhibition of TXA_2 release was detected. The latency and final degree of inhibition achieved depended upon the concentration of lipocortin, the duration of infusion and the dose of LTC_4. Table 1 summarizes mean data obtained from 18 matched experiments in which different combinations of LTC_4 and lipocortin were tested. Despite variations in potency between batches of recombinant lipocortin, a general dose-response trend is apparent. The top two tracings in Fig. 1*a* show examples from two experiments where low and high doses of LTC_4 were used. Lipocortin at 0.4 μg ml^{-1} immediately and completely abolished the release of TXA_2 triggered with 2 ng of LTC_4. Recovery was complete ~70 min after infusion was terminated. With 10 ng of LTC_4 a more graded type of inhibition was observed. In the latter case infusions of 0.4 μg ml^{-1} lipocortin produced inhibition, but this was not as extensive in magnitude and was slower in onset.

In 4 experiments, a sham preparation of lipocortin was tested against 2, 3 and 10 ng of LTC_4 (see Table 1 with Fig. 1*a*). In no case was inhibition of more than 10% observed with the sham preparation (this is within the normal variation of the tissue response) and where small effects were seen, they were not sustained (Fig. 1*a*). A number of other controls were examined. Boiled lipocortin was without effect. Similarly, 40 infusions of 10 μg ml^{-1} BSA (Sigma) or 1 μg ml^{-1} superoxide dismutase (Sigma, bovine kidney) were negative. *Escheri*

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EXHIBIT 10

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Binding Stoichiometry of the Cytotoxic T Lymphocyte-associated Molecule-4 (CTLA-4)

A DISULFIDE-LINKED HOMODIMER BINDS TWO CD86 MOLECULES*

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CD28 and CTLA-4 are homologous T cell receptors of the immunoglobulin (Ig) superfamily, which bind B7 molecules (CD80 and CD86) on antigen-presenting cells and transmit important costimulatory signals during T cell activation. Here we have investigated the subunit structure of CTLA-4 and the stoichiometry of its binding to B7 molecules. We demonstrate CTLA-4 is a homodimer interconnected by one disulfide bond in the extracellular domain at cysteine residue 120. Each monomeric polypeptide chain of CTLA-4 contains a high affinity binding site for B7 molecules; soluble CTLA-4 and CD86 form complexes containing equimolar amounts of monomeric CTLA-4 and CD86 (*i.e.* a 2:2 molecular complex). Thus, CTLA-4 and probably CD28 have a receptor structure consisting of preexisting covalent homodimers with two binding sites. Dimerization of CTLA-4 and CD28 is not required for B7 binding, nor is it sufficient to trigger signaling.

Intercellular interactions between T lymphocytes and antigen-presenting cells (APC)[1] generate T cell costimulatory signals which regulate T cell responses to antigen in an antigen nonspecific manner (1). Costimulatory signals determine the magnitude of T cell responses, and whether an encounter with antigen activates or inactivates subsequent responses to antigen (2). T cell activation in the absence of costimulation results in aborted or anergic T cell responses (3). A key costimulatory signal(s) is provided by engagement of T cell receptors CD28 and CTLA-4 with B7 molecules on APC (4). CD28 and CTLA-4 are both members of the immunoglobulin superfamily (IgSF) having single variable-like domains in their extracellular regions (5). These molecules share sequence identity, most notably clustered in their CDR3-like regions (6). Both CD28 and CTLA-4 bind the same natural ligands, CD80 (formerly known as B7-1) and CD86 (formerly known as B7-2 or B70) (7, 8). Engagement of CD28 by B7 molecules delivers a strong costimulatory signal (4, 9), whereas engagement of CTLA-4 in different systems triggers either a weak costimulatory signal (10, 11) or a negative signal (12).

Binding interactions between CD28, CTLA-4, and B7 molecules have been studied with recombinant-soluble immunoglobulin fusion proteins of these molecules. CTLA4Ig bound to CD80 with ~20-fold higher avidity than did the analogous CD28Ig (13). Amino acid residues responsible for the increased binding activity of CTLA4Ig to CD80 were localized to the CDR-1 and COOH-terminally extended CDR3-like regions (6). CD80 and CD86 share only ~25% amino acid sequence identity in their extracellular domains, but show very similar equilibrium binding to CD28Ig or CTLA4Ig (14).

A fundamental uncertainty regarding the binding interactions of CTLA-4·CD28 and B7 molecules is their binding stoichiometry. CD28 is a disulfide-linked homodimer, whereas the subunit structure of CTLA-4 has been controversial. Some studies have suggested that CTLA-4 is also a disulfide-linked homodimer (10, 13, 15), but another suggested that it exists as a monomer (16). It is currently unknown whether dimerization is required for binding activity of CD28 and or CTLA-4, as it is for many IgSF members (17). In this study, we have determined the binding stoichiometry of CTLA-4 for B7 molecules. We show that CTLA-4 is a disulfide-linked homodimer containing two binding sites for its ligands.

MATERIALS AND METHODS

mAbs, Ig Fusion Proteins, and Flow Cytometry—Murine mAbs 9.3 (anti-CD28), G10-1 (anti-CD8), and G17-2 (anti-CD4) and $F(ab')_2$ fragments of mAb G19-4 (anti-CD3) were provided by Dr. J. Ledbetter of Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle. Murine mAb L6 (anti-human lung tumor antigen) and its chimeric derivative (human Fc) were from Drs. K. E. and I. Hellström also of Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle. mAb 11D4 (anti-CTLA-4) has been described previously (10). mAb 7H4 (anti-CTLA-4) was prepared in the same manner as other anti-CTLA-4 mAbs (10); mAb 7H4 binds CTLA4Ig, but does not block its binding to CD80. Human CTLA4Ig, CD28Ig, CD80Ig, CD86Ig, CD80 mIg, CD86 mIg fusion proteins were prepared as described (14). For immunostaining, cells were removed from their culture vessels by incubation in PBS containing 10 mM EDTA. CHO cells ($1–10 \times 10^6$) were first incubated with mAbs or Ig fusion proteins in DMEM containing 10% fetal bovine serum (FBS), then washed and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse or anti-human Ig second step reagents (Tago, Burlingame, CA). Cells were washed and analyzed on a FACScan (Becton Dickinson).

Cell Culture—Anti-CD3-activated T cell blasts were prepared essentially as described (10), except that culture flasks were coated with $F(ab')_2$ fragments (10 μg/ml) of anti-CD3 mAb G19-4 instead of intact mAb. Peripheral blood mononuclear cells were isolated from heparinized blood and cultured on anti-CD3 $F(ab')_2$-coated flasks for 3 days at 37 °C in RPMI 1640 containing 10% FCS, 2 mM glutamine, and pen-strep (100 units/ml penicillin, 75 units/ml streptomycin). Retroviral vectors encoding CD28 and C4–28 (extracellular and transmembrane

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[1] The abbreviations used are: APC, antigen-presenting cell; IgSF, immunoglobulin superfamily; mAb, monoclonal antibody; DMEM, Dulbecco's modified Eagle's medium; Ig, immunoglobulin; FBS, fetal bovine serum; IPA, immobilized protein A; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; Ch, chymotrypsin.

regions of human CTLA-4 fused to the cytoplasmic domain of CD28) will be described elsewhere.[2] 3T3 cells were transduced with these vectors, selected for neomycin resistance, and cultured in DMEM containing 10% FBS, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and pen-strep. COS cells were maintained in DMEM containing 10% FBS

Iodination and Immunoprecipitation—Cells were harvested and washed with PBS. Cell surface iodination using lactoperoxidase was performed as described (18). Surface-labeled cells were washed three times with PBS and solubilized in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 1% Nonidet P-40, 0.25% deoxycholate, 10 μg/ml each of aprotinin and leupeptin) for 30 min at 4 °C. Detergent-insoluble material was then removed by centrifugation (13,000 × *g* for 15 min at 4 °C) and the supernatant was used for immunoprecipitation. Solubilized membrane proteins were also iodinated using lactoperoxidase. Cells (2×10^6 for COS cells and 150×10^6 for peripheral blood lymphocytes) were resuspended in (5 or 10 ml, respectively) hypotonic buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 10 μg/ml each of aprotinin and leupeptin) and incubated on ice for 10 min. Cells were then lysed (>95% lysis) using a tight fitting Dounce homogenizer. The homogenate was transferred to a clean tube containing 0.1 volume of 10 mM Tris-HCl, pH 7.5, 0.5 mM $MgCl_2$, 1.5 M NaCl, and debris was removed by centrifugation at 800 × *g* for 5 min. The supernatant was removed, and EDTA was added to a final concentration of 5 mM. Membranes were collected by centrifugation at 125,000 × *g* for 45 min. The membrane pellet was solubilized in lysis buffer on ice for 30 min, and detergent-insoluble material was removed by centrifugation. Soluble proteins were then iodinated using lactoperoxidase, and unreacted ^{125}I was removed by passage over a column of Sephadex G-25 (Pharmacia Biotech Inc.), equilibrated with lysis buffer. The labeled protein extract was then used for immunoprecipitation analysis.

Immunoprecipitation Analysis—^{125}I-Labeled lysates were incubated at 4 °C with an irrelevant antibody (2 μg) and 50-μl packed volume immobilized protein A (IPA, r-protein A-Sepharose, Repligen, Cambridge, MA). IPA was incubated before use for ~1 h in a solution of 5% bovine serum albumin in PBS and washed in lysis buffer. Sepharose beads were removed by centrifugation, and aliquots of the supernatant ($1–2 \times 10^6$ cpm) were mixed with mAbs or Ig fusion proteins and incubated at 4 °C for 1–2 h. Bovine serum albumin-blocked IPA was added (50 μl packed volume), and incubation at 4 °C was continued for an additional 1–2 h. Samples treated with mAb 11D4 or its isotype controls (mouse IgG1) were incubated with a "bridging" antibody (rabbit anti-mouse IgG, Sigma) at 50 μg/ml for 1–2 h at 4 °C before addition of protein A-Sepharose. Beads were collected by centrifugation, washed four times with PBS containing 0.5% Nonidet P-40, twice with PBS alone, and immunoprecipitated proteins solubilized in SDS-PAGE sample buffer. When labeled lysates from anti-CD3-stimulated T lymphocytes were used, CD28 was removed from solution by immunoprecipitation with anti-CD28 mAb 9.3 before addition of CD80Ig. For immunoblotting analysis, aliquots of conditioned medium or samples of purified protein (1 μg) were incubated with 5 μg of human Ig (negative control), CD80Ig, or CD86Ig for 1 h at 4 °C. Ig-containing proteins were then precipitated with 50 μl of IPA. Precipitates were washed three times with PBS, bound proteins were eluted by addition of concentrated SDS-PAGE sample buffer, and samples were analyzed by SDS-PAGE.

Preparation of Recombinant Fragments of CTLA-4—Soluble extracellular domain of CTLA-4 was expressed in COS cells by introducing a stop codon into CTLA-4 cDNA after Asp^{124} by polymerase chain reaction-directed mutagenesis (6). For CTLA4X, the OMCTLA-4 (13) expression plasmid was used as template, the forward primer GAGGTGATAAAGCTTCACCAATGGGTGTACTGCTCACACAG was chosen to match sequences in the vector, and the reverse primer, GTGGTGTATTGGTCTAGATCAATCAGAATCTGGGCACGGTTC, corresponded to the last seven amino acids in the extracellular domain of CTLA-4 followed by a stop codon (TGA). $CTLA4X_{C120S}$ was constructed in similar fashion, except that the reverse primer specified a C120S mutation. Polymerase chain reaction products were digested with *HindIII*/*XbaI* and directionally subcloned into the expression vector πLN (a gift of Dr. A. Aruffo, Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle). Each construct was verified by DNA sequencing.

Expression and Purification of CTLA-4 Fragments—CTLA4X and $CTLA4X_{C120S}$ were produced by transiently transfected COS cells (18). Recombinant proteins were purified by CTLA-4 mAb affinity chromatography from transfected COS cell conditioned medium. Anti-CTLA-4 mAb 11D4 was coupled to cyanogen bromide (CNBr)-activated Sepharose 4B (8 mg of mAb/ml of resin) as suggested by the manufacturer (Pharmacia Biotech Inc.). COS cell conditioned medium was collected, filter-sterilized, and applied to the affinity resin by gravity flow. Following application of conditioned medium, the resin was washed with 5 bed volumes of PBS. Bound CTLA-4 fragments were eluted with 0.05 M trisodium phosphate, pH 11.5. Fractions (0.5 ml) were collected and immediately neutralized by addition of 0.2 ml of 2 M Tris-HCl, pH 6.8. CTLA-4-containing fractions were identified by capture immunoassay (10) and/or A_{280}. Peak fractions were pooled, concentrated using a Centriprep 10 (Amicon, Beverly, MA), and filter-sterilized. Recoveries of recombinant proteins were ~50–90% based on immunoreactivity in the capture immunoassay. Extinction coefficients of 1.0 and 1.1 ml/mg were experimentally determined by amino acid analysis for purified CTLA4X and $CTLA4X_{C120S}$ preparations, respectively. M_r = 13,400, corresponding to the predicted peptide mass of CTLA4X, was assumed for calculation of molar concentrations. Where indicated, concentrated conditioned medium from transfected COS cells was used as a source of recombinant CTLA-4X. This was concentrated ~30-fold using a Centriprep 10 before fractionation.

Preparation of Thrombin Fragments—CTLA4Ig or CD86Ig fusion proteins (0.5 mg/ml in Tris-HCl buffer, pH 7.4–7.5) were digested with purified bovine thrombin (Armour Pharmaceutical Corp., Kankakee, IL) at a final concentration of 100 units/ml, at 37 °C for 16–24 h. After cleavage, thrombin was removed by twice incubating the protein solution with 0.10 packed bed volume of washed Affi-Gel Blue (Bio-Rad). Ig Fc-containing fragments were removed by twice incubating the protein solution with 0.20 packed bed volume of washed IPA. The resulting solution contained protein fragments CTLA4t and CD86t. The final recovery of purified fragments was ~10–40%. Extinction coefficients of 1.3 and 1.2 ml/mg were experimentally determined by amino acid analysis for purified CTLA4t and CD86t preparations, respectively. M_r values of 13,900 and 25,800, corresponding to the predicted peptide mass of a single chain of CTLA4t and CD86t, respectively, were assumed for calculation of molar concentrations.

Gel Permeation Chromatography—Two columns were used for gel permeation chromatography. CTLA4X preparations were analyzed using a TSK-GEL G2000 SW_{XL} column (7.8 × 300 mm, Tosohaas, Montgomeryville, PA). The column was equilibrated in PBS containing 0.02% NaN_3 at a flow rate of 1 ml/min. Concentrated COS cell medium containing CTLA4X (0.2 ml) was fractionated by gel permeation chromatography, and fractions of 0.5 ml were collected and used for analysis. In other experiments, purified protein fragments were analyzed on a Waters 300 SW column equilibrated with PBS, containing 10 mM Tris-HCl, pH 7.4, and 0.01% NaN_3, at a flow rate of 0.35 ml/min. Essentially the same results were obtained with both systems.

Protein Sequencing—Protein sequencing methods have been described previously (21). CTLA4t was first cleaved with cyanogen bromide (CNBr), and the peptides were purified. CNBr peptides were further digested with trypsin or chymotrypsin in the presence of 1 mM iodoacetamide and purified by reversed-phase HPLC. Automated sequence analysis was performed in a pulsed-liquid protein sequencer, and mass spectra were recorded on a Biolon 20 plasma desorption mass spectrometer (Applied Biosystems, Inc., Foster City, CA).

SDS-PAGE and Immunoblotting—SDS-PAGE was performed on Tris/glycine gels. In some cases, performed gels (Novex, San Diego, CA) were used. Analytical gels were stained with Coomassie Blue, and images of wet gels were obtained by digital scanning. Gels containing ^{125}I-labeled proteins were dried and subjected to autoradiography. For immunoblotting, separated proteins were electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with Specimen Diluent (Genetic Systems Corp., Redmond, WA) and probed with anti-CTLA-4 mAb 7H4 diluted in Specimen Diluent. Bound mAb was detected using horseradish peroxidase-conjugated goat anti-mouse Ig (Biosource International, Camarillo, CA) and ECL reagents (Amersham Corp.).

Competitive Binding Assays—Binding assays were performed on immobilized recombinant B7Ig fusion proteins (14). Individual wells in 96-well plates (Immulon 2; Dynatech laboratories, Inc., Chantilly, VA) were coated for 16–24 h with 50 ng/well goat $F(ab')_2$ anti-mouse immunoglobulin antibodies (Tago). Wells were then washed, blocked with Ig binding medium (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10% FBS, 0.1% NaN_3), and coated with CD80 mIg or CD86 mIg at 1 μg/ml. CTLA4Ig was radiolabeled with ^{125}I using Bolton-Hunter reagent (14), diluted in Ig binding medium, and mixed with solutions of competitor proteins diluted in PBS. Aliquots of the mixtures (50 μl) were then added to duplicate B7Ig-coated wells. Binding was allowed to proceed for ~4 h at 23 °C, wells were washed, and bound radioactivity was measured by γ counting.

[2] H. Leung and P. S. Linsley, manuscript in preparation.

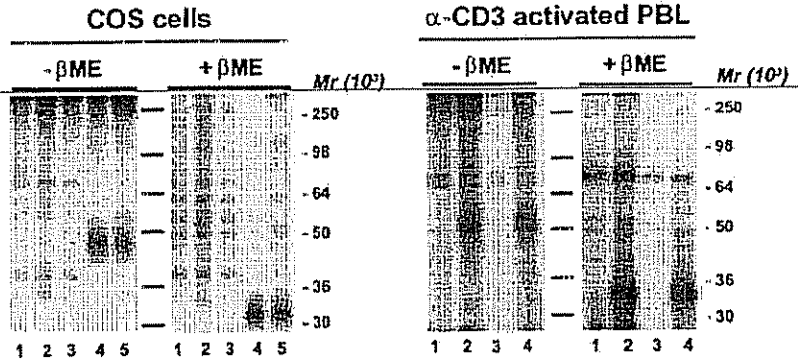


FIG. 1. **CTLA-4 contains an intermolecular disulfide bond.** *Left panel*, immunoprecipitation from COS cells. COS cells were transfected with the OMCTLA-4 expression plasmid. Forty-eight h post-transfection, cells were removed from their culture dishes and cell surface-labeled with ^{125}I using lactoperoxidase. ^{125}I-Labeled cells were then solubilized with non-ionic detergent solution, subjected to immunoprecipitation analyses with: CD5Ig (4 μg) (*lanes 1*), control mAb G17-2 (anti-CD4, 2 μg) (*lanes 2*), control mAb G10-1 (anti-CD5, 2 μg) (*lanes 3*), CD80Ig (4 μg) (*lanes 4*), and mAb 11D4 (anti-CTLA-4, 2 μg) (*lanes 5*). Samples were analyzed by SDS-PAGE (10% gel) under nonreducing (−β-mercaptoethanol (*βME*)) or reducing +β-mercaptoethanol (+*βME*) conditions. Migration positions of standards are indicated. *Right panel*, immunoprecipitation from anti-CD3-activated peripheral blood T cells. Membranes were prepared from anti-CD3-activated human mononuclear peripheral blood cells. Detergent extracts of these membranes were labeled with ^{125}I and subjected to immunoprecipitation analysis. Labeled extracts were precleared of CD28 by immunoprecipitation with mAb 9.3 before addition of the following (4 μg each): control mAb L20 (*lanes 1*); mAb 11D4 (anti-CTLA-4) (*lanes 2*); control Ig, chimeric mAb (lanes 3); CD80Ig (lanes 4). SDS-PAGE (10% gel) was run under nonreducing (−*βME*) or reducing (+*βME*) conditions.

RESULTS

Cell Surface CTLA-4 Is a Disulfide-linked Homodimer—Various studies on the subunit structure of CTLA-4 have employed different serological reagents and different cell types (10, 13, 15, 16). It was unclear how these differences contributed to the reported variation in the subunit structure of CTLA-4. We wished to determine the subunit structure of biologically active CTLA-4 transfected COS cells and on activated T cells. As shown in Fig. 1, both specific anti-CTLA-4 mAb and CD80Ig immunoprecipitated from surface-labeled COS cells a M_r ~ 46,000 protein under nonreducing conditions and a M_r ~ 30,000 protein under reducing conditions. Similar results were obtained with activated T cells; a M_r ~ 50,000 protein was obtained under nonreducing conditions and a M_r ~ 33,000 protein under reducing conditions. Thus, regardless of the cell source, CTLA-4, as recognized by specific mAb or soluble natural ligand, migrated more rapidly under reducing conditions. This was consistent with CTLA-4 being a disulfide-linked homodimer or being linked to an unlabeled protein. Evidence supporting the first of these possibilities was provided in subsequent experiments (see Table I).

Preparation and Characterization of Soluble Recombinant Fragments of the Extracellular Domains of CTLA-4 and CD86—Since biologically active CTLA-4 was a homodimer, we wished to determine whether one or two CTLA-4 chains comprise a binding site for B7 molecules. We therefore prepared soluble fragments of CTLA-4 and CD86 extracellular domains. Two strategies were employed, as shown schematically in Fig. 2. The characterization of these fragments is presented below (Fig. 3 and Table I).

We first prepared proteolytic fragments of CTLA4Ig and CD86Ig which retained binding activity. Preliminary examination of the Ig hinge sequence showed that it contained the sequence 126E-P-K128 which is similar to a thrombin cleavage site in actin (22). We determined that this nonclassical thrombin site was suitable for producing active fragments of CTLA-4, CD86, CTLA4t, and CD86t. As a second strategy, we introduced an in frame stop codon into a cDNA construct encoding CTLA-4, proximal to the transmembrane domain. The resulting cDNA was then expressed in COS cells and, yielding a mixture of monomeric and dimeric proteins, CTLA4X. These proteins were purified on an anti-CTLA-4 mAb affinity column as described under "Materials and Methods." Analysis of purified preparations by gel permeation chromatography indicated that purified preparations contained ~80% monomeric CTLA4X and ~20% dimeric CTLA4X. To improve the yield of monomeric CTLA4X, we later mutated the cysteine residue which mediates the interchain disulfide bond in CTLA-4 to a serine (see below). This mutant protein, CTLA4X$_{(C120S)}$, was expressed in COS cells and purified.

TABLE I
Disulfide-linked peptides from CTLA4t

CTLA4t was fragmented with CNBr and peptides were isolated by gel permeation chromatography and identified by amino acid sequence analysis. CNBr peptide M-(98–128) was subfragmented with chymotrypsin (Ch) and the peptides were purified by reversed-phase HPLC. An intermolecular disulfide bond (C120-C120) in one of these fragments was confirmed by mass spectral analysis as described in the text. A high molecular weight CNBr fragment was subfragmented with trypsin (T), cystine-containing peptides were isolated and identified by amino acid sequence analysis. Peptide T-(55–83) was sequenced only as indicated. These analyses permitted determination of two intramolecular disulfide bonds (Cys^{21}–Cys^{92} and Cys^{48}–Cys^{66}) as described in the text. Numbers are from the amino terminus of mature CTLA4t.

Designation	Peptide sequence	Disulfide bond assignment
Ch-(114–128)	^{114}VIDPEPXPDSDQEPK^{128}	Interchain, Cys^{120}–Cys^{120}
T-(16–28)/T-(86–97)	^{16}GIASFVXEYASPGK^{28}/ ^{86}DTGLYIXKVELM^{97}	Intrachain, Cys^{21}–Cys^{92}
T-(39–53)/T(55–83)	^{39}QADSQVTEVXAATYM^{53}/ ^{55}GNELTFLDDSIXTGT . . .	Intrachain, Cys^{48}–Cys^{66}

These fragments of CTLA-4 and CD86 were characterized by SDS-PAGE (Fig. 3A). Under nonreducing conditions, CTLA4Ig gave a major M_r ~ 100,000 protein and a minor protein of M_r ~ 50,000 (*lane 1*). The minor protein was not seen in all preparations and comigrated with the M_r ~ 50,000 protein found under reducing conditions (13). CD86Ig migrated as a diffuse M_r ~ 75,000 protein (*lane 2*) which was not affected by reduction (data not shown). Thus, as shown previously, CTLA4Ig migrated during SDS-PAGE as a disulfide-linked homodimer of M_r ~ 50,000 subunits, whereas CD86Ig did not contain an interchain disulfide bond. Since the Ig hinge disulfides were mutated to serine residues, dimerization of CTLA4Ig results from a disulfide bond in CTLA-4 (13). CD86 does not contain an interchain disulfide bond, hence, CD86Ig was not disulfide linked.

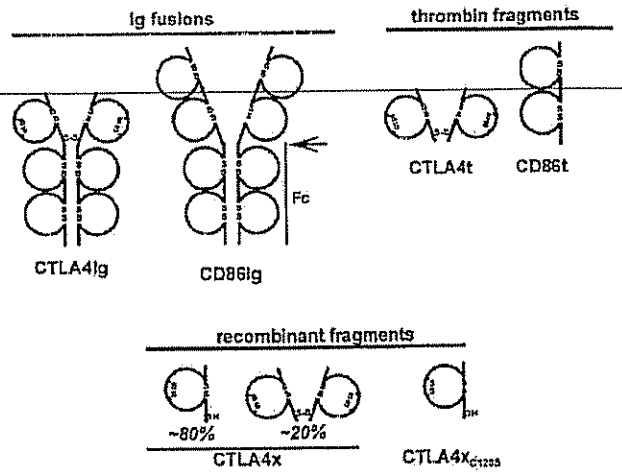


FIG. 2. **Schematic representation of Ig fusions and proteolytic and recombinant fragments used in this study.** Shown are cartoon structures of CTLA4Ig, CD86Ig, CTLA4t, CD86t, CTLA4X, and CTLA4X$_{C120S}$. Preparation of these molecules and elucidation of their subunit structures are discussed in the text. The relative positions of inter- and intracellular disulfide bonds in CTLA-4-related structures was determined as described in Table I; the disulfide bonds in CD86 were inferred from homology to other members of the IgSF. The Fc domains of CTLA4Ig and CD86Ig are indicated. The *arrow* indicates the site of thrombin cleavage in CTLA4Ig. The relative proportions of monomeric and dimeric CTLA4X were estimated from gel permeation chromatography profiles of purified preparations.

An intermolecular disulfide bond was also present in CTLA4t. CTLA4t (prepared from CTLA4Ig analyzed in lane 1) contained a major dimeric M_r ~ 38,000 protein and a minor monomeric M_r ~ 22,000 protein (*lane 3*). The presence of monomeric CTLA4t reflects the presence of monomeric CTLA4Ig in the starting preparation (*lane 1*). Another preparation of CTLA4t contained only the dimeric M_r ~ 38,000 protein when analyzed under nonreducing conditions (*lane 11*). Analysis of CTLA4t preparations after reduction revealed only the monomeric M_r ~ 22, 000 protein (*lane 8*). CTLA4X preparations contained a minor dimeric M_r ~ 38,000 protein and a major monomeric M_r ~ 22,000 protein (*lane 4*); reduction of this preparation gave a M_r ~ 22,000 protein (*lane 9*). The proportion of monomeric CTLA4X in these preparations could be enriched by gel permeation chromatography (*lane 5*). This enriched preparation was stable for several weeks when reanalyzed, indicating that monomeric CTLA4X did not readily reform dimeric molecules. CTLA4X$_{C120S}$ migrated as a monomeric M_r ~ 22,000 protein (*lane 12*). CD86t migrated as a M_r ~ 57,000 protein (*lane 6*); as with the parental molecule, CD86Ig, the mobility of CD86t was unaffected by reduction (data not shown).

Fragments of CTLA-4 and CD86 were further characterized by gel permeation chromatography (Fig. 3*B*). The elution times of CTLA-4 fragments corresponded closely to M_r values obtained by SDS-PAGE. The CTLA4t preparation analyzed by SDS-PAGE in Fig. 3*A*, *lane 1*, gave a major protein of M_r ~ 40,000 and a minor high molecular weight protein when analyzed by gel permeation chromatography (Fig. 3*B*, *panel a1*). The minor high molecular weight protein was not observed in another preparation which had not been concentrated after final purification (*panel b1*); reduction of both preparations before chromatography gave a M_r ~ 30,000 protein (*panels a2* and *b2*). CTLA4X eluted primarily as a M_r ~ 30,000 peak, with a shoulder (*panel a3*). CTLA4X$_{C120S}$ eluted as a M_r ~ 30,000 protein (*panel b3*) whose elution time was unaffected by reduction (*panel b4*). Thus, CTLA4t behaved as a disulfide-linked dimer in solution, whereas CTLA4X$_{C120S}$ and the major protein in CTLA4X were monomeric. As shown in *panel a4*, CD86t eluted larger (M_r ~ 140,000) than its M_r determined by SDS-PAGE (M_r ~ 57,000). The elution time of CD86t was unaffected

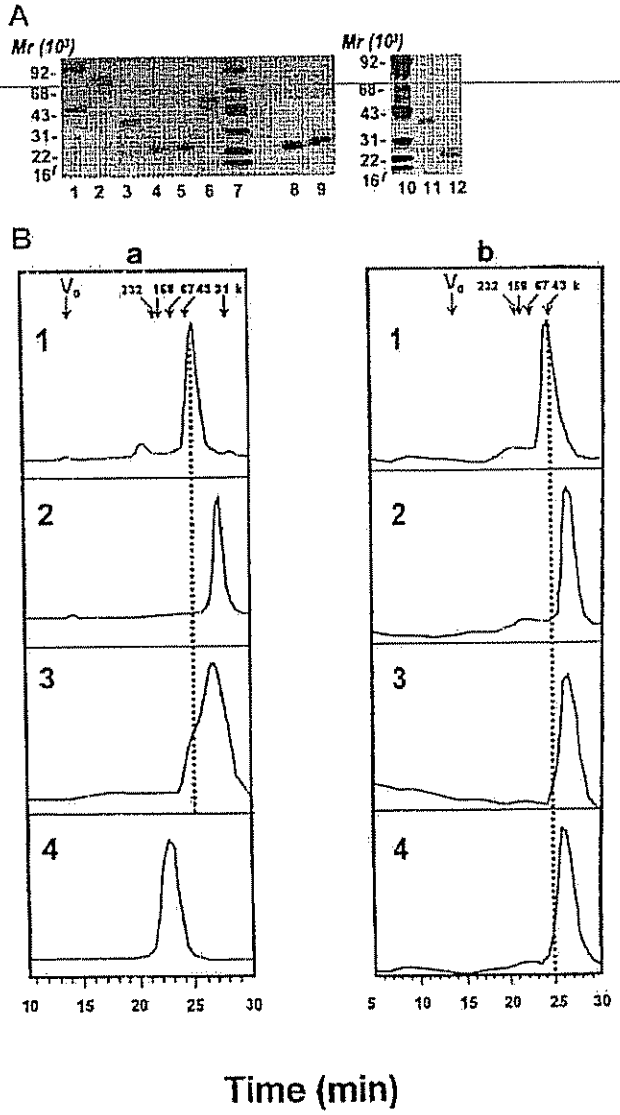


FIG. 3. **Characterization of Ig fusions and proteolytic and recombinant fragments.** Purified recombinant Ig fusion proteins, thrombin fragments and soluble receptors were analyzed by SDS-PAGE (*A*) or gel permeation chromatography (*B*). *A*, SDS-PAGE analysis. Samples (~1 μg) of each of the indicated proteins were analyzed on 4–20% (*lanes 1–9*) or 5–15% (*lanes 10–12*) acrylamide gels under nonreducing (*lanes 1–7, 10–12*) or reducing (*lanes 8, 9*) condition: *lane 1*, CTLA4Ig; *lane 2*, CD86Ig; *lane 3*, CTLA4t; *lane 4*, CTLA4X; *lane 5*, CTLA4X, which had been enriched for monomeric protein by gel permeation chromatography; *lane 6*, CD86t; *lane 7*, molecular weight markers; *lane 8*, CTLA4t; *lane 9*, CTLA4X; *lane 10*, molecular weight markers; *lane 11*, CTLA4t (a different preparation from that shown in *lane 1*); and *lane 12*, CTLA4X$_{C120S}$. *B*, gel permeation chromatography. Samples (~3–4 μg each) of the indicated proteins were analyzed by gel permeation chromatography on a Waters 300 SW column. *Panel a1*, CTLA4t; *panel a2*, CTLA4t, following reduction with 10 mM dithiothreitol at 23 °C for 30 min; *panel a3*, CTLA4X; *panel a4*, CD86t. *Panel b1*, CTLA4t (a different preparation than *panel a1*); *panel b2*, CTLA4t following reduction with dithiothreitol; *panel b3*, CTLA4X$_{C120S}$; *panel b4*, CTLA4X$_{C120S}$ following reduction. *Arrows* indicate elution positions of standards having the indicated molecular weights: V_0, void volume; catalase, M_r = 232, 000; aldolase, M_r = 158,000; bovine serum albumin, M_r = 67,000; ovalbumin, M_r = 43.000; carbonic anhydrase, M_r = 31,000.

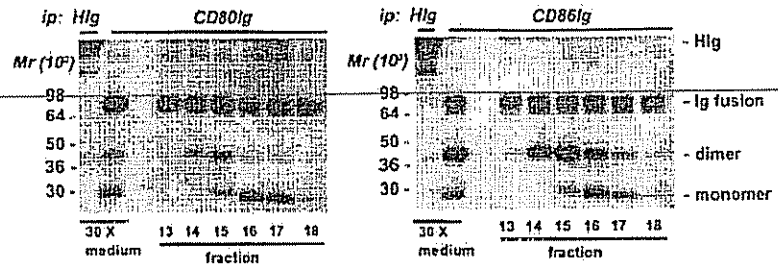


FIG. 4. **Monomeric extracellular domain of CTLA-4 binds CD80 and CD86.** COS cells were transfected with the CTLA4x expression plasmid, conditioned medium was collected and concentrated approximately 30-fold. A sample of concentrated conditioned medium (0.2 ml) was fractionated by gel permeation chromatography on a TSK-Gel G2000 SW_{XL} column at 1 ml/min and fractions (0.5 ml) were collected. In parallel column runs, aldolase, M_r = 158,000, eluted at 6.5 min (*fraction 13*); bovine serum albumin, M_r = 67,000, at 6.8 min (*fraction 14*); and ovalbumin, M_r = 43,000, at 7.4 min (*fraction 15*). Aliquots of concentrated conditioned medium (0.02 ml) or the indicated fractions (0.25 ml) were then subjected to immunoprecipitation analysis with human Ig (*Hlg*, negative control), CD80Ig, or CD86Ig. Precipitates were analyzed by SDS-PAGE (8–16% gel) under nonreducing conditions and CTLA-4-related proteins were detected by immunoblotting analysis. Migration positions of molecular weight (M_r) standards are indicated on the *left* and on the *right*, migration positions of Hlg, CD80Ig and/or CD86Ig, and CTLA4x dimer and monomer. Hlg, CD80Ig, and CD86Ig reactivities in this assay represent cross-reaction of the horseradish peroxidase conjugate with human Ig. This figure is representative of three experiments using concentrated culture medium and two experiments using affinity-purified CTLA4x.

by reduction and denaturation with 4 M guanidine HCl (data not shown and Fig. 6). This treatment disrupted the high avidity interaction between CTLA-4 and CD86, suggesting that it likewise would have disrupted interactions between subunits of CD86. Therefore, the larger apparent size of CD86t in solution is probably not due to oligomerization, but to aberrant migration during gel permeation chromatography, possibly resulting from its high degree of glycosylation (19, 20).

Determination of the Disulfide Bonding Pattern in CTLA4t—CTLA4t was further characterized by protein sequencing to identify amino and carboxyl termini and disulfide bonding patterns. The amino terminus of CTLA4t was determined by automated Edman degradation (21). Sequence analysis revealed an amino terminus corresponding to M1 (M27 of the precursor sequence), in agreement with the predicted site of signal peptide processing (13). Carboxyl-terminal sequence analysis was performed to determine the site of thrombin cleavage. CTLA4t was first fragmented with CNBr, and the peptides were purified by gel permeation chromatography. The carboxyl-terminal CNBr fragment was identified by sequence analysis. This peptide CNBr-(98-*X*) was subsequently subfragmented with chymotrypsin (Ch), and the amino acid sequence of the most carboxyl-terminal fragment (Ch-(114-*X*)) was determined. The sequence of this peptide terminated at residue Lys^{128}, suggesting that this residue was the carboxyl terminus. Fragment Ch-(114–128) was subjected to mass spectral analysis which confirmed the carboxyl-terminal residue as K128. This was the predicted thrombin cleavage site.

The disulfide bonding pattern of CTLA4t was determined (Table I). The position of the intermolecular disulfide bridge in CTLA4t was determined by further analysis of fragment Ch-(114–128). Mass spectral analysis of the peptide was performed in the presence or absence of dithiothreitol. This analysis revealed that the mass ($(M + H)^+$) of Ch-(114–128) under nonreducing and reducing conditions was 3336.4 and 1669.2, respectively. The change in mass of the peptide following reduction demonstrates that this peptide contained one intermolecular bond at Cys^{120}. The experimental mass values were in good agreement with the predicted masses of this peptide under nonreducing and reducing conditions, 3336.6 and, 1668.8, respectively.

The intramolecular disulfide bonding pattern was determined by Edman degradation of cystine-containing peptides. Sequence analysis located the positions of the purified CNBr peptides in the CTLA-4 sequence. Edman degradation of a large molecular weight CNBr fragment gave simultaneously three amino-terminal sequences beginning at Met^1 (or His^2), Gly^{55}, and Asp^{86} in equimolar amounts. These results suggested that the large molecular weight CNBr fragment consisted of three peptides interlinked by two disulfide bonds: CNBr-(1(2)-53)-CNBr-(55–85)-CNBr-(86–97). To confirm this, the large molecular weight peptide was subfragmented with trypsin, and the disulfide-containing peptides were purified by reversed-phase HPLC and identified by Edman degradation. Two major cystine-containing tryptic peptides (T) were obtained: T-(15–28)-T-(86–97) and T-(39–53)-T-(55–83). These results demonstrate the existence of two intramolecular disulfide bonds between Cys^{21}–Cys^{92} and Cys^{48}–Cys^{66}. The first of these pairs is the disulfide bond characteristic of the Ig fold. The C48-C66 disulfide bond is unusual in the Ig superfamily, but did not appear to be artifactual, since disulfide interchange during purification of fragments could be excluded by the lack of carboxamidomethylated cysteines in the fragments. This bond was also predicted in molecular modeling studies of CTLA-4, since these residues were adjacent in the model (6).

Monomeric CTLA-4 Binds CD80 and CD86—To determine if monomeric CTLA-4 contained an intact binding site for B7 molecules, we compared B7 binding properties of monomeric and dimeric forms of CTLA-4. Concentrated conditioned medium from CTLA4X-transfected COS cells was subjected to immunoprecipitation analysis with CD80Ig and CD86Ig. Immunoprecipitated proteins were separated by SDS-PAGE and different forms of CTLA-4 were identified by immunoblotting analysis. As shown in Fig. 4, CD80Ig and CD86Ig immunoprecipitated both monomeric and dimeric forms of CTLA-4 from solution. Fractionation of CTLA4X preparations by gel permeation chromatography separated monomeric and dimeric forms of CTLA-4. Both forms were immunoprecipitated with CD80Ig and CD86Ig. Identical results were obtained when purified CTLA4X was used for analysis. Thus, both monomeric and dimeric forms of CTLA-4 bound CD80 and CD86. However, the relative amounts of monomeric and dimeric forms of CTLA-4 precipitated by CD80Ig and CD86Ig were different (Fig. 4). CD80Ig precipitated more monomeric CTLA-4, whereas CD86Ig precipitated more dimeric CTLA-4.

The relative avidities of monomeric and dimeric CTLA-4 were compared by competitive binding assay in the experiment shown in Fig. 5. CTLA4Ig was labeled with ^{125}I and bound to CD80Ig and CD86Ig which had been immobilized on plastic wells. CTLA4t competed similar to CTLA4Ig (EC_{50} ~4 *versus* ~8 nM for CTLA4Ig *versus* CTLA-t inhibition of CD80Ig binding and ~8 *versus* ~10 nM for CD86Ig). $CTLA4X_{C120S}$ competed for ^{125}I-CTLA4Ig binding with an EC_{50} ~300 nM for CD80Ig and ~900 nM for CD86Ig. Monomeric CTLA-4 was a more

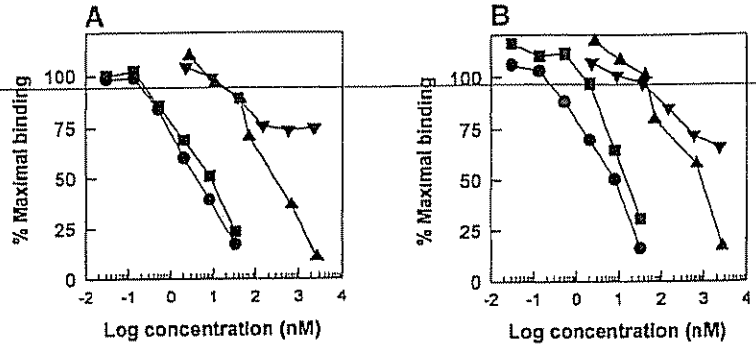


FIG. 5. **Comparison of the avidities of dimeric and monomeric CTLA-4.** Ninety-six-well plates were coated with CD80Ig (*A*) or CD86Ig (*B*). ^{125}I-Labeled CTLA4Ig (final concentration, 0.6 nM) was mixed with unlabeled CTLA4Ig (*circles*), CTLA4t (*squares*), CTLA4X$_{C120S}$ (*triangles*), or CD28Ig (*inverted triangles*), to the indicated final concentrations. The mixtures were then added to B7-coated wells and binding was measured. Binding in the presence of competitor is expressed as a percentage of binding in wells containing no competitor (30,000 cpm for CD80Ig and 16,000 cpm for CD86Ig). Concentrations of competitors were determined assuming the following M_r values per chain or binding site): CTLA4Ig, M_r = 39,000; CTLA4t, M_r = 13,900; CTLA4X$_{C120S}$, 13,400; CD28Ig, M_r = 42,000; and chimeric L6 mAb, M_r = 75,000. Addition of chimeric mAb L6 inhibited ^{125}I-CTLA4Ig binding by <10% over a concentration range of 6–13,000 nM. This experiment was repeated twice with identical results, and similar results were also observed using partially purified monomeric preparations of CTLA4X.

effective competitor than multimeric (18) CD28Ig (EC_{50} >~10,000 nM). The apparent plateau in binding inhibition by CD28Ig was not seen in other experiments. Control Ig did not show significant competition in this assay at concentrations >13,000 nM. Thus, monomeric CTLA-4 was a less avid competitor for ^{125}I-CTLA4Ig binding than was dimeric CTLA-4, but was more effective than multivalent CD28Ig.

CTLA-4 Homodimer Binds Two CD86 Molecules—To determine the stoichiometry of CTLA-4·B7 complexes, we analyzed the size of complexes formed with monomeric and dimeric CTLA-4 and the composition of the complex formed with dimeric CTLA-4 (Fig. 6). Addition of increasing concentrations of CD86t to a fixed concentration of CTLA4X monomer (*A*) or CTLA4t (*B*) resulted in formation of larger complexes. Higher concentrations of CD86t were required to force complete complex formation with monomeric CTLA-4X than with dimeric CTLA4t, consistent with the lower avidity of monomeric CTLA-4 (Fig. 5). Complexes formed with monomeric CTLA-4X eluted ~1 min later than complexes formed with dimeric CTLA4t. The elution times of these complexes were faster than the largest molecular weight standard, so it was not possible to accurately estimate their molecular mass; however, a change in elution time of 1 min in the range of standards analyzed corresponded to M_r ~ 70,000. To determine the composition of complexes between dimeric CTLA4t and CD86t, larger amounts of these molecules were mixed and complexes were subjected to preparative gel permeation chromatography (*C*). The elution profile of this mixture gave a more clearly resolved complex peak than seen in the experiment shown in *B*, and in addition, another late eluting peak. The identity of the late eluting peak was not determined (it was not seen when smaller amounts of these proteins were analyzed, see Fig. 3), although it eluted similar to monomeric CTLA4t. A peak corresponding to free CD86t was observed, indicating that it was present in excess. The leading edge (19–20 min elution time) of the high molecular weight complex peak was collected and analyzed for the relative amounts of CD86t and CTLA4t in two ways. In the first, an aliquot of the complex was dissociated with 4 M guanidine HCl and 10 mM dithiothreitol and reanalyzed by gel permeation chromatography (*D*). Peaks corresponding to CD86t and monomeric CTLA-4 were clearly resolved. Absorbance at 214 nm is proportional to the number of peptide bonds, and hence, to peptide mass. Thus, comparison of integrated peak areas divided by calculated molecular masses permits an estimate of the relative molecular ratios of CD86t and CTLA4t in the complex. This analysis gave a ratio of CD86t to CTLA4t of 1.2:1. The same value was obtained when a separate aliquot of the complex was fractionated and analyzed.

The composition of the CD86t·CTLA-4 complex was also determined by subjecting it to NH_2-terminal amino acid sequencing. An aliquot corresponding to ~⅛ of the total isolated CD86t·CTLA4t complex was subjected to automated Edman degradation. Two sequences were obtained simultaneously, corresponding to the NH_2 termini of CD86 and CTLA-4. The initial yield of M1 from CTLA-4 was 38 pmol and L1 from CD86, 41 pmol. At position 3, 34 pmol of valine (CTLA-4) and 37 pmol of isoleucine (CD86) were determined. Thus, the average ratio of CD86t to CTLA4t determined by amino acid sequence analysis was 1.1:1, similar to the ratio determined by comparison of absorbance peaks.

It was important to determine if some of the CD86t present in the complex peak was due to contamination with free CD86, which was present in excess. Extrapolation of the free CD86t A214 peak (*C*; see also Fig. 3*B*) indicated that free CD86t in the 19–20-min fraction contributed <~3% of initial CD86t. Since ~1700 pmol of CD86t were used, then ~<50 pmol were present in fraction 19–20. The total amount of CD86t recovered was ~320 pmol (40 pmol at position 1 in ~1/8 total sample). Thus, <~16% (<50/320) of recovered CD86t could be attributed to excess free CD86t based on sequence analysis. This was similar to the 10–20% of CD86t recovered in excess of a 1:1 ratio, as measured by both absorbance and NH_2-terminal sequencing.

DISCUSSION

CTLA-4 expressed in transfected COS cells or in activated T cells exists primarily as a protein of M_r ~ 45,000–50,000 under nonreducing conditions and M_r ~ 30,000–33,000 under reducing conditions. The native dimeric form of CTLA-4 is preserved in soluble fragments of its extracellular domain by an intermolecular disulfide bond at C120. Mutation of this cysteine to serine abolished residual dimeric protein in CTLA4X preparations. Our data thus show that CTLA-4, like its homologue CD28, exists primarily as a disulfide linked homodimer.

Residue Cys^{120} is conserved in most CTLA-4·CD28 family homologues (6) but a recent report (23) showed that chicken CD28 lacks this cysteine residue and does not contain an intermolecular disulfide bond. This led to the proposal that chicken CD28 differs from other family members in being monomeric. However, it cannot be ruled out that chicken CD28 exists as a non-disulfide-linked dimer. Our data show that

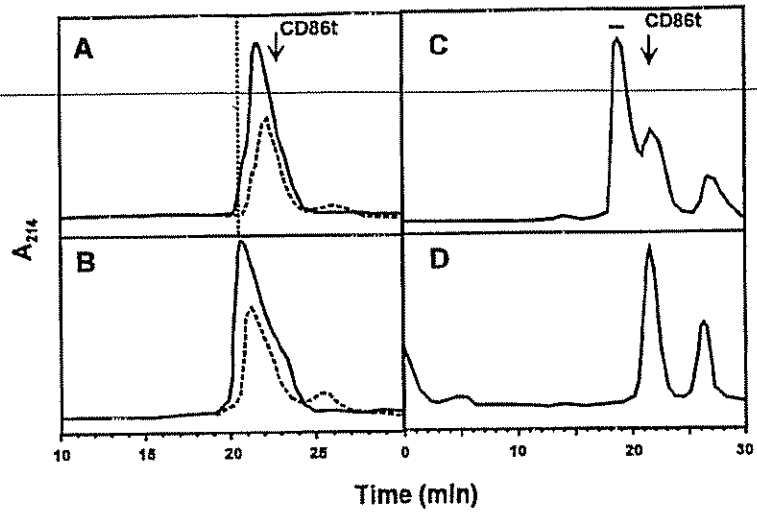


FIG. 6. **CTLA-4 homodimer binds two CD86 molecules.** *A*, formation of complexes between monomeric CTLA4X and CD86t. Aliquots (~3 μg, ~200 pmol) of CTLA4X, which had been enriched for monomeric protein by preparative gel permeation chromatography (see Fig. 3*A*), were incubated in a final volume of 0.1 ml with CD86t (7.4 μg, ~300 pmol, *dotted line*, or 15 μg, ~600 pmol, *solid line*) for 30 min at 23 °C before fractionation by gel permeation chromatography as in Fig. 3. The *arrow* indicates the elution position of CD86t and the *vertical dotted line* the elution position of CTLA4t/CD86t complexes. Monomeric CTLA4X elutes at 26 min. *B*, formation of complexes between CTLA4t and CD86t. Aliquots of CTLA4t (3.4 μg, ~250 pmol) were incubated in a final volume of 0.1 ml with CD86t (3.7 μg, ~140 pmol, *dotted line*, or 7.4 μg, ~300 pmol, *solid line*) for 30 min at 23 °C before fractionation by gel permeation chromatography. CTLA4t elutes at 25 min. *C*, preparative chromatography of complexes between CTLA4t and CD86t. Aliquots of CTLA4t (22 μg, ~1600 pmol) were incubated in a final volume of 0.2 ml with CD86t (44 μg, ~1700 pmol) for 30 min at 23 °C before fractionation by gel permeation chromatography. Complexes eluting with the *horizontal bar* (19–20 min) were collected and used in *D*. The *arrow* indicates the elution position of CD86t. The peak eluting at >26 min was not observed in other experiments in which lower concentrations were used. *D*, composition of complexes between CTLA4t and CD86t. An aliquot (~½ the total) of complexes eluting at the position of the *horizontal bar* in *C* was dissociated by treatment for ~30 min at 23 °C with 4 M guanidine HCl and 10 mM dithiothreitol and reanalyzed as in *B*. This experiment was repeated twice with identical results.

although full-length CTLA-4 is predominantly homodimeric, removal of the transmembrane and cytoplasmic domains (CTLA4X) leads to predominantly monomeric protein. Thus, the presence of C120 does not ensure dimerization. This suggests that dimerization of CTLA-4 and formation of the disulfide bond at Cys^{120} requires the transmembrane and/or cytoplasmic domains of the two chains.

Each chain of a CTLA-4 homodimer has a binding site for CD80 and CD86. Monomeric CTLA-4X has binding activity for CD80Ig and CD86Ig in immunoprecipitation analysis and monomeric $CTLA4X_{C120S}$ competes for binding of ^{125}I-labeled CTLA4Ig to CD80Ig and CD86Ig. Monomeric CTLA-4 had reduced binding activity when compared with dimeric CTLA-4. $CTLA4X_{C120S}$ was 30–90-fold less active at competing for ^{125}I-CTLA4Ig than dimeric CTLA4t. CD80Ig and CD86Ig differed in their preferences for monomeric and dimeric forms of CTLA-4. Monomeric CTLA-4 was preferentially immunoprecipitated by CD80Ig, whereas dimeric CTLA-4 was preferentially precipitated by CD86Ig. In competitive binding assays, dimeric CTLA4t was approximately equivalent at competing for ^{125}I-CTLA4Ig binding to CD80Ig and CD86Ig (EC_{50} ~8 *versus* 10 nM, respectively), whereas monomeric $CTLA4X_{C120S}$ was ~3-fold less effective at competing for binding to CD86Ig than to CD80Ig (EC_{50} ~300 *versus* 900 nM). Thus, CD86 binds less well to monomeric CTLA-4.

This difference may be due to differences in binding kinetics of CD80 and CD86. We previously showed that the equilibrium dissociation constants for CTLA4Ig binding to CD80 and CD86 were very similar (within 2-fold) but that CTLA4Ig dissociated more rapidly from CD86 than from CD80. We proposed that CD86 is a faster on/faster off counter receptor for CTLA-4 than is CD80. Since monomeric CTLA-4 binds less avidly than dimeric CTLA-4, dissociation differences between CD80 and CD86 may be more pronounced with monomeric CTLA-4. *In vivo*, this difference may be important where CD80 or CD86 expression levels are a limiting factor for interaction with CTLA-4. Occupancy of CTLA-4 would be suboptimal, and on average, less than two binding sites per molecule occupied. Since complexes of CD86 with a single CTLA-4 binding site would dissociate faster, the time of CTLA-4 receptor occupancy by CD86 would be less, and hence, effects on T cell costimulation would also be less. Thus, differences in costimulatory signals by CD80 and CD86 would be exaggerated at low expression levels. We previously noted that CHO cells transfected with CD80 and CD86 differed more in their adhesion to CD28 and in T cell costimulation when added at low numbers (14).

The complex of CD86t·CTLA4t formed in the presence of excess CD86t was isolated and determined to contain 1.1–1.2 mol of CD86t/mol of CTLA-4. Thus, one molecule of CD86t fragment bound per chain of CTLA4t or two molecules of CD86t per CTLA4t homodimer (*i.e.* a 2:2 stoichiometry). Since CD28 is homologous to CTLA-4, and is homodimeric, it likely also binds two B7 molecules.

The Ig domains of CD28 and CTLA-4 display little sequence similarity with other members of the IgSF, limited to a few conserved residues characteristic of the Ig fold (24). Despite sharing a common structural scaffold, members of the IgSF have diverse structures, utilize different combinations of Ig domains to form binding sites, and have distinct binding stoichiometries per molecule. In antibodies, variable regions from two different Ig domains combine to form an antigen binding site (25), and on per molecule basis, each Ig molecule contains two antigen binding sites (a 2:2 stoichiometry). With human growth hormone receptor, two molecules (two Ig domains) bind a single growth hormone molecule (human growth hormone receptor); the complex has a 2:1 stoichiometry (26). Thus, the number of Ig domains per binding site of human growth hormone receptor is the same as a Fab fragment, but the stoichiometries of the intact molecules is different. A single domain of CD2 binds its CD58 ligand and the binding stoichiometry on a

per molecule basis is thought to be 1:1 (27, 28). Finally, with CD8, a single Ig domain of each chain of a homodimer is believed to bind a major histocompatibility complex class I molecule; thus, on a per molecule basis, CD8/MHC class II complexes have a 2:2 stoichiometry (29, 30). The stoichiometry of CD8 has not been directly demonstrated, but is inferred from mutagenesis studies (29)

The binding stoichiometries of CTLA-4 and CD28 most closely resemble CD8. With both molecules each chain of a homodimer contains a binding site and the intact molecule has a 2:2 stoichiometry. However, there are significant differences in the way CD8 and CTLA-4·CD28 bind ligand. Different areas of the V(ariable)-like domains are utilized for binding, with the CDR-1 and CDR-2-like loops and β-strands A and B being involved in the binding site of each CD8 subunit (29), and the CDR-1 and CDR-3-like loops and β-strand G, at least in part, being involved in binding by CTLA-4 (26). Also, CD8 forms an Fv-like homodimer, which is stable even in the absence of a disulfide bond (30). In contrast, the two subunits of a homodimer of CTLA-4 have no particular affinity for each other, and monomeric CTLA-4 is stable in solution.

Ligand-induced receptor dimerization is a common theme of many signaling pathways. Dimerization of growth factor receptors permits receptor transphosphorylation (31) and facilitates recruitment of SH2-containing effector molecules (32, 33). The CD28 signaling pathway also involves tyrosine kinase activation and binding of SH2-containing effector molecules (9). However, CTLA-4 or CD28 signaling differ from growth factor receptor signaling in two ways: their dimeric states are preformed and they are inactive. Thus, with CTLA-4·CD28, dimerization *per se* is not sufficient to trigger signal transduction.

Why then are CTLA-4 and CD28 covalent homodimers having two binding sites? One possible clue to the answer is the fast kinetic off rates of B7 binding (14). Recent data[3] indicate that the $t_{1/2}$ of CTLA4Ig occupancy by CD80Ig and CD86Ig is ~11 and ~3 s, respectively, and that the $t_{1/2}$ of CD28Ig occupancy is also very short. These off rates are rapid compared with those known for growth factor/growth factor receptor interactions (34–36). Rapid receptor kinetic off rates favor disengagement of CD28/CTLA-4/B7 intercellular interactions, but discourage productive intracellular signaling. Homodimerization of CTLA-4 and CD28 should enhance signaling by increasing the time of receptor occupancy by ligand. Homodimeric CTLA-4 has increased binding avidity for B7 molecules, since the probability of rebinding ligand after its dissociation is increased. The effective time of receptor-ligand interactions would thereby be prolonged and receptor activation may be increased.

It is currently unknown whether signal transduction requires occupancy of one or both subunits of CTLA-4 and/or CD28 by B7 molecules. If occupancy of both subunits is required for signal transduction, monomeric B7 molecules on the APC must be brought into proximity such that both subunits of CTLA-4 CD28 can be occupied. However, unlike CTLA-4 and CD28, B7 molecules are not disulfide-linked. Thus for full occupancy of CTLA-4·CD28 to occur, B7 molecules must either preexist as noncovalent oligomers on the APC, or they must oligomerize as the result of binding to CTLA-4·CD28. Evidence for the former possibility was provided by immunofluorescence microscopy studies showing that that B7 molecules on human Langerhans cells assume a non-uniform distribution (37).

[3] W. Cosand, J. Emswiler, G. Leytze, J. Greene, and P. S. Linsley, unpublished data.

It is also possible that signaling through CTLA-4·CD28 requires occupancy of only one receptor subunit by a B7 molecule. If so, oligomers of B7 on APC (37) could cross-link different CTLA-4·CD28 homodimers. Early studies (4) showed that CD28 receptor cross-linking with mAbs enhanced signaling through this receptor, but that Fab fragments did not, suggesting that cross-linking of CTLA-4·CD28 receptors is an essential feature of their signaling pathways. The binding stoichiometry of CTLA-4·CD28 receptors may enhance CTLA-4·CD28 receptor cross-linking by B7 aggregates on APC.

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EXHIBIT 11

Coexpression and Functional Cooperation of CTLA-4 and CD28 on Activated T Lymphocytes

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Summary

T cell costimulation by molecules on the antigen presenting cell (APC) is required for optimal T cell proliferation. The B7 molecule on APC binds the T lymphocyte receptor CD28, triggering increased interleukin 2 (IL-2) production and subsequent T cell proliferation. CTLA-4 is a predicted T cell membrane receptor homologous to CD28, which also binds the B7 counter receptor, but whose distribution and function are unknown. Here we have developed monoclonal antibodies (mAbs) specific for CTLA-4 and have investigated these questions. mAbs were produced that bound CTLA-4 but not CD28, and that blocked binding of CTLA-4 to B7. CTLA-4 expression as measured by these mAbs was virtually undetectable on resting T cells, but was increased several hundred-fold during T cell activation. On activated lymphocytes, CTLA-4 was expressed equally on $CD4^+$ and $CD8^+$ T cell subsets and was coexpressed with CD25, CD28, and CD45RO. CTLA-4 expression was lower than that of CD28, reaching a maximum of ~1/30–50 the level of CD28. Despite its lower expression, CTLA-4 was responsible for much of the B7 binding by large activated T cells. Anti-CTLA-4 mAb 11D4 and anti-CD28 mAb 9.3 acted cooperatively to inhibit T cell adhesion to B7, and to block T cell proliferation in primary mixed lymphocyte culture. When coimmobilized with anti T cell receptor (TCR) mAb, anti-CTLA-4 mAbs were less effective than anti-CD28 mAb 9.3 at costimulating proliferation of resting or activated T cells. However, coimmobilized combinations of anti-CD28 and anti-CTLA-4 were synergistic in their ability to augment anti-TCR–induced proliferation of preactivated $CD4^+$ T cells. These results indicate that CTLA-4 is coexpressed with CD28 on activated T lymphocytes and cooperatively regulates T cell adhesion and activation by B7.

T lymphocyte activation requires occupancy of the TCR by antigenic peptides in the context of MHC molecules on the APC. Other T cell surface molecules interact with specific counter receptors on the APC to receive costimulatory signals that regulate the outcome of TCR occupancy (1, 2). T cell activation in vitro in the absence of costimulation results in development of antigen-specific unresponsiveness by clonal anergy (2, 3), or activation-induced cell death (4). The molecular basis for T cell costimulation is not clearly understood, but may involve several different T cell surface receptors and their counter receptors on the APC (5).

CD28 is a T cell surface receptor that delivers important costimulatory signals. CD28 mediates a T cell activation pathway that has been extensively studied using mAbs (6). These studies have led to the conclusion that CD28 is an important regulator of T cell cytokine production by transcriptional (7, 8) and posttranscriptional (9) mechanisms. More recent studies have suggested a role for CD28 in regulation of clonal anergy. mAbs to CD28 augmented T cell proliferation in the presence of costimulation-deficient APC (10), and reversed development of clonal anergy in T cell clones (11). A counter receptor for CD28 is B7 (12), a molecule first identified as a B lymphocyte activation antigen (13). B7 has been shown to costimulate proliferation of human (14–17) and murine T cells (18, 19).

CTLA-4 is a predicted T cell surface molecule that was identified by differential screening of a murine cytolytic-T cell cDNA library (20). CTLA-4 transcripts were found in T cell populations having cytotoxic activity, leading to the suggestion that CTLA-4 might function in the cytolytic response (20, 21). CTLA-4 bears structural homology to CD28 (22, 23), and genes for these molecules colocalize to the same chromosomal regions in human (24) and mouse (23, 25). Like CD28, CTLA-4 also binds the B7 counter receptor (26). CTLA4Ig, an immunoglobulin Fc fusion of the extracellular domain of CTLA-4, bound B7 with an avidity ~20-fold higher than an analogous CD28Ig fusion protein, and blocked alloantigen responses in vitro (26). CTLA4Ig administration

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in vivo suppresses antibody responses (27) and transplant rejection (28).

The normal function of CTLA-4 is unknown. It is unclear why distinct counter receptors for B7 have evolved. Until now, a protein product of the CTLA-4 gene has not been identified, and its cellular distribution and role in normal T cell immune responses are unknown. To address these problems, we have prepared mAbs specific for CTLA-4. In this study, we have used these reagents to address the distribution and function of CTLA-4 during T cell activation.

Materials and Methods

mAbs. Murine mAbs 9.6 (anti-CD2), G19-4 (anti-CD3), G17-2 and 66.1 (anti-CD4), G10.1 (anti-CD5), 9.3 (anti-CD28), and rat mAb 187.1 (antimurine Ig κ chain) have been described previously (29–32). mAb 4G7 (anti-CD19) was a gift of E. Engleman (Stanford University, Stanford, CA) and UCHL-1 (anti-CD45RO) was from P. Beverly (University College, London, UK). mAb WT31 (anti-TCR) was purchased from Becton Dickinson & Co. (San Jose, CA) and mAb M23 (antibreast tumor mucin) was acquired from Genetic Systems Corp. (Seattle, WA). mAb 7G7/B6 (anti-CD25) was obtained from the American Type Culture Collection (Rockville, MD).

Cell Culture. B7$^+$ CHO cells have been previously described (14, 26) and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS)[1], 0.2 mM proline, and 1 μM methotrexate. COS cells were maintained in DMEM supplemented with 10% FBS.

T cells were cultured in lymphocyte culture medium (RPMI 1640 medium containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin). PBL were isolated by centrifugation through lymphocyte separation medium (LSM; Organon Teknika Corp., Durham, NC). PHA-activated T cells were prepared by culturing PBL with 1 μg/ml PHA (Wellcome Diagnostic, Charlotte, NC) in lymphocyte culture medium for 6 d, then in lymphocyte culture medium lacking PHA for 1 d. Viable cells were collected by sedimentation through LSM before use. Enriched CD4$^+$ or CD8$^+$ populations were isolated by complement-mediated lysis of CD8$^+$ or CD4$^+$ T cells (labeled with mAbs G10.1 or 66.1, respectively) before PHA activation. The resulting CD4$^+$ culture was ~90% CD4$^+$, 2% CD8$^+$; and the CD8$^+$ culture, ~95% CD8$^+$ and 1% CD4$^+$, as judged by flow cytometry. In some experiments, PBL were also activated by immobilized anti-CD3 mAb G19-4. Plastic wells were coated with a solution of mAb G19-4 at 20 μg/ml in PBS for ~2 h at 37°C and washed with PBS before addition of PBL.

CD4$^+$ T cells were isolated from PBL by immunomagnetic negative selection (33) and cultured in lymphocyte culture medium. The selection procedure routinely yielded preparations of >95% CD4$^+$ cells. Alloantigen-primed CD4$^+$ T cells were generated from MLC of CD4$^+$ T cells with an irradiated (10,000 rad) EBV-transformed lymphoblastoid cell line from an unrelated donor (DR2/DR4). At weekly intervals, cells were refed with fresh irradiated lymphoblastoid cells and IL-2 (20 U/ml) as previously described (33). Viable cells were isolated before use by sedimentation through LSM. In some experiments, viable cells were rested for ~16–24 h in lymphocyte culture medium before use. Alloantigen-primed cells were cultured for 3–7 wk with weekly restimulation. CD4$^+$ T cells were also activated by coimmobilized anti-TCR mAb WT31 with intracellular adhesion molecule 1 receptor globulin (ICAM-1 Rg) fusion protein (33). Round bottomed 96-well microtiter plates were coated with goat anti-mouse and anti-human Ig, followed by mAb WT31 and ICAM Rg at 25 ng per well (33). CD4$^+$ T cells were added (5×10^4 per well) and cultured for 3 d at 37°C.

Plasmids and Transfections. Expression plasmids OMCD28 and OMCTLA-4 have been described (26). cDNA inserts from these plasmids were subcloned into the πLN expression vector (provided by Dr. A. Aruffo, Bristol-Myers Squibb Pharmaceutical Research Institute) and transfected into COS cells (14). Cell surface expression was analyzed 48–72 h after transfection. CTLA4 (mur)Ig was a genetic fusion identical to CTLA4Ig (26), except that the human Ig portion was replaced with the analogous fragment from murine Ig Cγ2a. Ig fusion proteins were purified from serum-free conditioned medium of transfected COS cells (14). Transfected COS cells were ^{125}I-surface labeled using lactoperoxidase, and H_2O_2, extracts were prepared and subjected to immunoprecipitation analysis as described (14). Cells cotransfected with OMCD28 and OMCTLA-4 were analyzed by two-color flow cytometry to determine the degree of coexpression of CD28 and CTLA-4. 34% of cells expressing CD28 also expressed CTLA-4, and 72% of cells expressing CTLA-4 expressed CD28.

mAb Production. BALB/c mice were primed subcutaneously with CTLA-4(mur)Ig (~50 μg/mouse) in Titermax adjuvant (CytRx Corp., Norcross, GA). Secondary and tertiary immunizations were given intraperitoneally in PBS, 3 d after the third immunization, the spleen was removed and spleen cells were fused (34) with murine myeloma Ag8.653. Hybrids were screened on day 11 by enzyme immunoassays (EIA) for antibodies that bound immobilized CTLA4Ig. EIA reagents were obtained commercially (Genetic Systems Corp.). Cells in positive wells were cloned by limiting dilution.

EIA. For hybridoma screening, microtiter plates (Immulon 2; Dynatech Laboratories, Inc., Chantilly, VA) were coated with 1 μg/ml CTLA4Ig for 16–24 h at 4°C. Wells were blocked for 1 h with sample diluent (Genetic Systems Corp.), then washed with PBS containing 0.05% Tween 20. Hybridoma supernatants were added and incubated at 23°C for 1 h. After washing, horseradish peroxidase (HRP)-conjugated goat anti-mouse Ig conjugate (Tago Inc., Burlingame, CA) was added and plates were again incubated for 1 h at 23°C. Finally, plates were washed thoroughly, and 3,3',5,5' tetramethylbenzidine (TMB) substrate (Genetic Systems Corp.) was added. For measurement of CTLA-4 and CD28 levels in cell extracts, microtiter plates were coated with mAb 11D4 or 9.3 at 1 μg/ml in PBS. Nonionic detergent extracts of cells were prepared as for immunoprecipitation studies (14) and were added to plates for 1 h at 23°C. Plates were washed, and biotinylated mAb 7F8 or 9.3 were added for CTLA-4 or CD28 determinations, respectively. After an additional 1-h incubation, plates were washed again before addition of a HRP-streptavidin (SA) conjugate (Jackson Immunoresearch Laboratories, Inc., West Chester, PA). Plates were again incubated for 1 h, then washed extensively, and finally, developed by addition of o-Phenylenediamine (OPD) substrate (Sigma Chemical Co., St. Louis, MO). Enzyme reactions proceeded for ~30 min, and were stopped by the addition of an equal volume of 1 N H_2SO_4. Absorbance at 450 nm (TMB) or 490 nm (OPD) were recorded on a microtiter plate reader (Genetic Systems Corp.). The amount of CTLA-4 or CD28 in each sample was determined

[1] *Abbreviations used in this paper:* EIA, enzyme immunoassay; FBS, fetal bovine serum; HRP, horseradish peroxidase; LFE, linear fluorescence units; LSM, lymphocyte separation medium; PE-SA, R-phycoerythrin-conjugated streptavidin.

from a standard curve using known amounts of purified recombinant CD28Ig (14) or CTLA4Ig (26) as standards. The linear range of the CTLA-4 EIA was ~0.2–10 ng/ml CTLA4Ig, and the CD28 EIA, ~20–1,000 ng/ml CD28Ig. The CTLA-4 EIA showed no detectable crossreactivity with CD28Ig, and the CD28 EIA showed no detectable crossreactivity for CTLA-4. Neither assay gave significant signals with extracts from cells that did not express CTLA-4 (by RNA blot analysis) or CD28 (by cell surface staining).

Immunostaining and Flow Cytometry. Cells were collected by sedimentation and incubated with saturating amounts of mAbs in DMEM containing 10% FBS for 1–2 h at 4°C. When indirect immunofluorescence was measured, binding was detected by addition of a FITC goat anti-mouse Ig reagent (Tago Inc.), or by R-PE–conjugated SA (PE-SA, Molecular Probes, Inc., Eugene, OR). Cells were then analyzed on a flow cytometer (Epics C; Coulter Electronics Inc., Hialeah, FL).

Cell Adhesion Assays. T cell adhesion to B7$^+$ CHO cells was measured by modification of a published procedure (12). Monolayers of B7$^+$ CHO cells were fixed in situ with 0.5% paraformaldehyde in PBS for 20 min at 23°C. Cells were then washed and incubated in lymphocyte culture medium for 1 h before addition of T cells. Alloactivated CD4$^+$ T cells were ^{51}Cr-labeled and preincubated for 1 h at 23°C with or without mAbs in lymphocyte culture medium. Labeled T cells were added to B7$^+$ CHO monolayers, and adhesion reactions were initiated by brief centrifugation to bring cells in contact. Background cation-dependent adhesion was minimized by addition of EDTA (10 mM) to lymphocyte culture medium. Adhesion was allowed to proceed for 1–2 h at 37°C. Nonadherent cells were removed by washing monolayers four times with lymphocyte culture medium. Adhesion was quantified by measuring monolayer bound radioactivity in a gamma counter.

RNA Blot Analysis. RNA was prepared from stimulated PHA blasts (~1–3 × 10^7/sample) by a rapid isolation procedure (35). RNA (10 μg) was fractionated on formaldehyde agarose gels, transferred, and crosslinked to zetaprobe (Bio-Rad Laboratories, Richmond, CA). Blots were prehybridized and hybridized with ^{32}P-labeled probes (36). CD28 probes were a 342-bp fragment corresponding to amino acids 4–117 of the predicted mature CD28 sequence; or full-length CD28 cDNA (37). The CTLA-4 probe was a 375-bp fragment corresponding to amino acids 1–125 of the predicted CTLA-4 protein sequence (22). The CD8 probe was a ~1.3-kb fragment of a CD8 cDNA clone provided by Dr. Jan Chalupny (Bristol-Myers Squibb Pharmaceutical Research Institute). The GAPDH probe has been previously described (14). DNA fragments were gel purified and ^{32}P-labeled using a random priming kit (Boehringer Mannheim Corp., Indianapolis, IN). Blots were sequentially hybridized with different ^{32}P-labeled probes. Between hybridizations, probe was stripped from the blots by boiling in a solution of SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.1% SDS.

MLC. PBL were prepared by density gradient centrifugation on Ficoll-Hypaque. The cells were resuspended in medium containing RPMI 1640, 25 mM Hepes, 1 U/ml penicillin, 1 μg/ml streptomycin, and 15% pooled human serum that had been heat inactivated at 56°C for 30 min. Responder and stimulator cells were unrelated individuals chosen so that there was at least one HLA class I and one HLA-DR antigen mismatched within each pair. Equal numbers (5 × 10^4 per well each) of responder and irradiated stimulator cells (3,000 rad) were cultured in the presence or absence of mAbs in round-bottomed 96-well plates. These were incubated at 37°C in a 5% CO_2 atmosphere. Assays were performed in triplicate. Cultures were pulsed with one μCi of [^{3}H]thymidine 18 h before harvesting.

Results

Regulation of Transcripts for CTLA-4. We first compared the expression and regulation of CTLA-4 transcripts in CD4$^+$ or CD8$^+$ T cells. CD4$^+$ and CD8$^+$ enriched lymphocyte populations were obtained by complement-mediated lysis of CD8$^+$ and CD4$^+$ T cells, respectively, and the resulting populations were activated with PHA and rested as described in Materials and Methods. Transcripts for CTLA-4 were detected at similar levels in RNAs from both CD4$^+$ and CD8$^+$ subsets. Low levels of two CTLA-4 transcripts of ~1.8 and ~0.6 kb were detected in CD4$^+$ and CD8$^+$ enriched cells (Fig. 1 *A*). The ~1.8-kb transcript was reduced in the resting CD4$^+$ enriched population shown in Fig. 1 *A*, but not in another experiment. Both transcripts were increased in CD4$^+$ and CD8$^+$ enriched populations by stimulation with crosslinked anti-CD28 mAb and with PMA. Crosslinked anti-CD2 mAb was less effective at inducing CTLA-4 transcripts. Levels of CTLA-4 transcripts in alloantigen-primed CD4$^+$ cells at various times after activation were examined in Fig. 1 *B*. CTLA-4 transcripts were absent from rested alloantigen reactive T cells, but accumulated during activation, reached a peak at ~24 h and thereafter decreased. Thus, CTLA-4 transcripts are expressed in both CD4$^+$ and CD8$^+$ T cells after activation by CD28 engagement, by PMA, or by alloantigen activation.

Preparation and Characterization of mAbs for CTLA-4. To aid in studies of the distribution and function of CTLA-4, we prepared mAbs specific for this molecule as described in Materials and Methods. We selected a panel of 14 anti-CTLA-4 mAbs, of which three (11D4, 10A8, and 7F8) were selected for further study. All three mAbs were of the IgG1 isotype. In initial characterization experiments, these three mAbs bound strongly to CTLA-4–transfected COS cells or immobilized CTLA4Ig, but not to CD28-transfected COS cells or immobilized CD28Ig. Studies of mAbs binding to a panel of mutant CTLA4Ig proteins indicated that epitopes for these mAbs are dependent upon protein conformation rather than contiguous amino acid sequence (data not shown). These mAbs also inhibited binding of CTLA4Ig to B7$^+$ CHO cells (Fig. 2 *A*), with mAb 10A8 requiring lower concentrations to block equivalently (half-maximal inhibition at ~0.03 μg/ml, as compared with 0.1–0.2 μg/ml for 11D4 and 7F8). This suggests that all three mAbs bind to biologically active (or "native") CTLA-4 at or near the binding site for B7. In other experiments, mAb 10A8 competed for binding of biotinylated 11D4 to CTLA-4, but mAb 7F8 did not. This indicates that mAbs 11D4 and 10A8 bind spatially related epitopes, whereas the epitope for 7F8 was distinct.

mAb 11D4 was also characterized by immunoprecipitation analysis (Fig. 2 *B*). COS cells were transfected with OMCD28, OMCTLA-4, or a mixture of the two plasmids, ^{125}I-surface labeled and subjected to immunoprecipitation analysis with different mAbs and Ig fusion proteins. B7Ig and mAb 9.3, but not mAb 11D4, immunoprecipitated an ~72,000-M_r protein (under nonreducing conditions) from CD28-transfected COS cells. This species is known from other experiments (14, 37) to be a disulfide-linked homodimer. Lesser

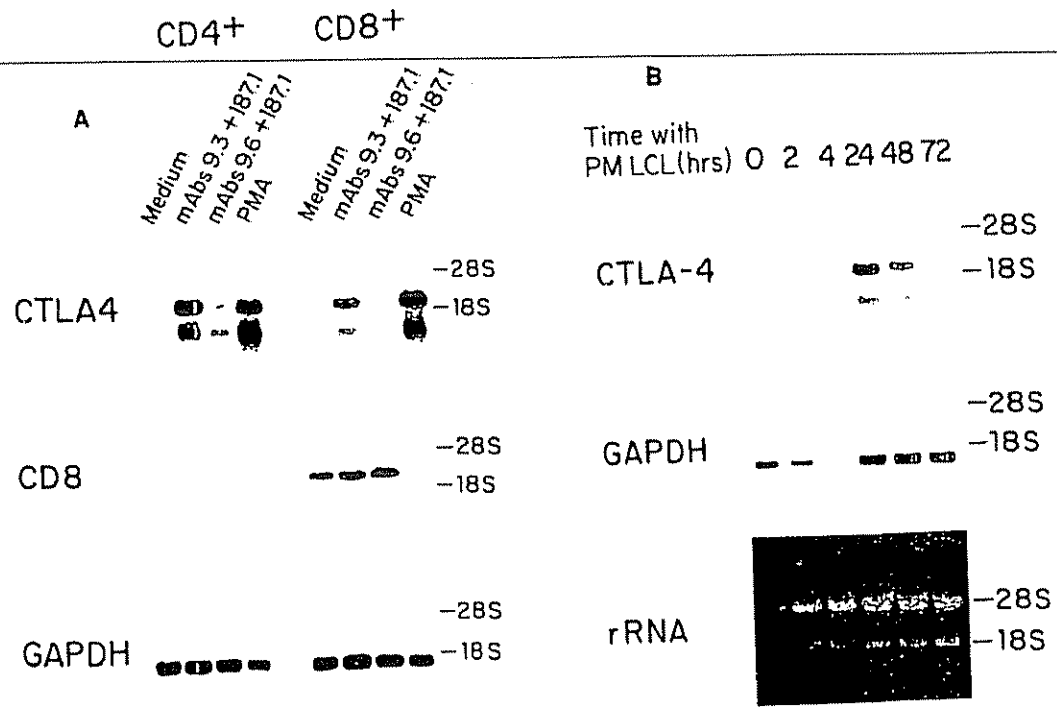


Figure 1. Regulation of CTLA-4 transcripts by T cell activation. (*A*) Regulation of CTLA-4 expression in different T cell subsets. PBL were depleted of $CD8^+$ or $CD4^+$ T cells and activated with PHA as described in Materials and Methods. Cells (2×10^7) were then stimulated for 4 h at 37°C with medium alone; with anti-CD28 mAb 9.3, or anti-CD2 mAb 9.6 at 2 μg/ml for 10 min at 37°C followed by mAb 187.1 (antimurine Ig κ chain) at 10 μg/ml; or with PMA (10 ng/ml). Cells were harvested, and RNA was extracted and analyzed by blot analysis. The blot was sequentially hybridized with ^{32}P-labeled probes for CTLA-4, CD8, or GAPDH, as described in Materials and Methods. Migration positions are noted of the 28S and 18S ribosomal RNA species visualized by ethidium bromide staining. (*B*) Regulation of CTLA-4 transcripts during alloactivation. Resting alloantigen-primed $CD4^+$ T cells (2×10^7) were collected and restimulated with irradiated lymphoblastoid cells. Cells were harvested at the indicated times, and RNA was extracted and analyzed by blot analysis as described in Fig 1 *A*. (*Bottom*) Ribosomal RNA bands visualized by ethidium bromide staining before transfer.

amounts of the nondisulfide-linked monomer (an ~37,000 M_r) were also precipitated with the mAb. The ~72,000-M_r protein found in the lane precipitated with CD5Ig was due to spillover and was not seen in other experiments. mAb 9.3 was more effective at immunoprecipitation than B7Ig, which agrees with the greater affinity of the mAb (14). B7Ig and mAb 11D4 immunoprecipitated primarily an ~40,000-M_r protein from cells transfected with CTLA-4. In another experiment, we found proteins immunoprecipitated by B7Ig migrated with ~27,000 M_r upon reduction. B7Ig immunoprecipitated ~72,000 and 40,000-M_r proteins from COS cells transfected with both CD28 and CTLA-4. The larger of these was immunoprecipitated by mAb 9.3, and the smaller by mAb 11D4. Thus, mAb 11D4 specifically immunoprecipitates CTLA-4 from mixtures with CD28. These experiments provided no evidence of disulfide-linked complexes between CD28 and CTLA-4.

Quantitation of CTLA-4 and CD28 Expression during Alloactivation. Initial attempts to use anti-CTLA-4 mAbs to stain T cells from peripheral blood suggested that CTLA-4 was expressed at low levels. To monitor CTLA-4 expression, we used mAbs to nonoverlapping epitopes on CTLA-4 to develop a sandwich EIA for this molecule as described in Materials and Methods. Biotinylated mAb 7F8 was used to detect CTLA-4 that had been immobilized by mAb 11D4. This assay could detect concentrations of CTLA4Ig of <0.2 ng/ml but gave no detectable signal at concentrations of CD28Ig of up to 10 μg/ml. CTLA-4 concentrations were then determined in lymphocyte cell extracts by comparison with a standard curve constructed with known concentrations of CTLA4Ig. Extracts of activated lymphocytes that gave transcripts for CTLA-4 (PHA-activated T cell blasts, and activated $CD4^+$ cytotoxic T cell clones) tested positive in the CTLA-4 sandwich EIA, but cells that lacked CTLA-4 transcripts were negative (resting $CD4^+$ T cell clones, lymphoblastoid cell lines, T cell leukemias Jurkat and CEM). Expression of CTLA-4 protein during activation of alloantigen-primed $CD4^+$ cells (Fig. 3) followed the time course of transcript expression (Fig. 1 *B*). CTLA-4 was undetectable in resting cells (< ~0.04 fmoles/10^6 cells or ~25 molecules/cell) and peak protein levels were measured at 1 d after activation (~10 fmoles/10^6 cells or ~6,000 molecules/cell). Thus, expression of CTLA-4 was induced ~>250-fold by alloactivation.

For comparison, CD28 levels in the same extracts were measured using a similar EIA (Fig. 3). In this case, mAb 9.3

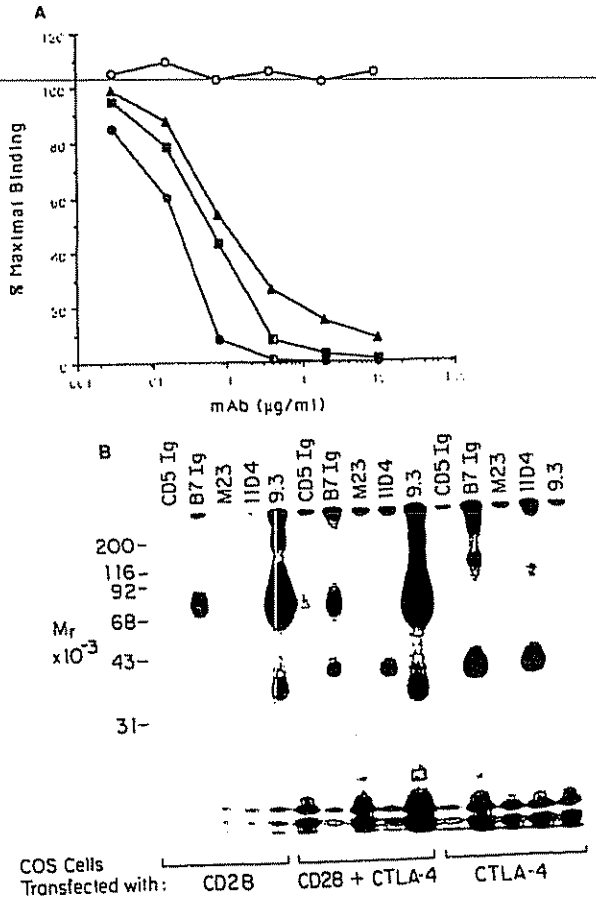


Figure 2. Characterization of anti-CTLA-4 mAbs. (*A*) Anti-CTLA-4 mAbs block binding of CTLA4Ig to B7. Biotinylated-CTLA4Ig (10 ng/ml final concentration) was incubated for 1 h at 23°C with the indicated concentrations of anti-CTLA-4 mAbs 7F8 (■), 10A8 (●), or 11D4 (▲); or with isotype control mAb M23 (○). B7+ CHO cells were removed from their culture vessel by incubation in PBS containing 10 mM EDTA, collected by centrifugation, incubated with the mAb/biotinyl-CTLA4Ig mixture for ~1 h at 23°C, and washed. Binding of biotinyl-CTLA4Ig was then detected with PE-SA, and cells were analyzed by flow cytometry. Mean fluorescence values were recorded and converted to linear fluorescence units (LFU). Background fluorescence (~2 LFU) was subtracted from all values. Values are expressed as percentage of maximal fluorescence in the absence of mAb (~13 LFU). (*B*) Immunoprecipitation of CTLA-4 from transfected COS cells. COS cells (~10^6) were transfected with equal amounts of expression plasmids OMCD28, OMCTLA-4, or a mixture of these two plasmids. 48 h after transfection, cells were detached from their culture dishes with EDTA, and subjected to cell surface iodination and immunoprecipitation analysis (14) with the indicated Ig molecules at 20 μg/ml. CD5Ig (human IgG1) and mAb M23 (murine IgG1) were used as controls. SDS-PAGE was performed on a 10–20% acrylamide gel run under nonreducing conditions. The migration of standard proteins is indicated.

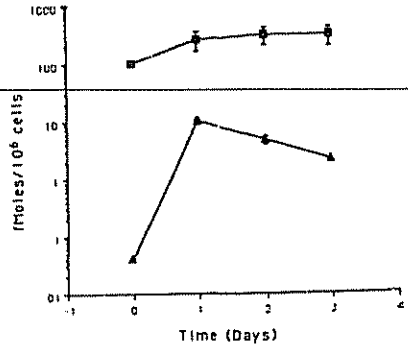


Figure 3. Ratio of CTLA-4 to CD28 increases during alloactivation of primed CD4+ cells. Resting, viable-primed CD4+ cells were restimulated with irradiated lymphoblastoid cells. At the indicated times, cells were collected, extracted with nonionic detergent (14), and amounts of CD28 (■) and CTLA-4 (▲) in the extract were measured by sandwich EIAs, as described in Materials and Methods. Shown are means and SD (*bars*) of measurements made with three serial dilutions that fell within the linear region of standard curves for each sample. The value for CTLA-4 at day 0 represents a maximal estimate since this measurement was below the linear portion of the standard curve.

was used as both capture and detecting mAb, and CD28Ig was used as standard. The ability of mAb 9.3 to both capture and detect CD28 probably reflects that this molecule is homodimeric. The CD28 sandwich EIA did not show reactivity with CTLA-4Ig at concentrations of up to 10 μg/ml, nor did it show reactivity with extracts from a lymphoblastoid cell line that does not express CD28. CD28 levels in extracts from antigen-primed CD4+ T cells also increased during activation, but less than those for CTLA-4. The ratio of CTLA-4 to CD28 therefore increased during alloactivation, reaching a maximum of ~1:30 between 1 and 2 d after activation.

Cell Surface Expression of CTLA-4 on Activated T Cells. Having determined the kinetics of CTLA-4 expression, we next investigated its surface expression. Preliminary experiments showed that when alloactivated, primed CD4+ T cells were stained with biotinylated mAb 11D4 and analyzed by flow cytometry, the intensity of staining with mAb 11D4 varied depending upon light scatter profile of the population analyzed. Staining was brighter on a subpopulation of larger activated cells. This staining was specific since unlabeled mAb 11D4 (but not an isotype control mAb) competed for staining by biotinylated mAb 11D4. The main population of activated cells and resting cells stained less intensely. Thus, surface expression of CTLA-4 was greater on larger, activated cells. Similar findings are demonstrated below in Fig. 5.

Surface expression of CTLA-4 was also detected on PBL activated with immobilized anti-CD3 mAb, and on purified CD4+ T cells activated with immobilized anti-CD3 mAb or with a coimmobilized combination of anti-TCR mAb and ICAM-1 Rg (33). Surface expression of CTLA-4 was not detected on resting cells, but became detectable after 2–4 d of activation. In all cases, staining was brighter on a population of larger, activated cells which comprised 5–20% of the total cell population, of which up to ~50% stained with mAb 11D4. When PBLs activated for 3 d with immobilized anti-CD3 were pulse labeled with BrdU and stained with FITC-labeled anti-BrdU mAb (38), the larger activated population had an ~two-fold higher percentage of stained cells (in S phase) than for the bulk population. No difference in BrdU incorporation was seen in 11D4 positive or negative

cells, indicating that CTLA-4 expression is not restricted to cells in S phase. The distribution of CTLA-4 on different T cell subsets was examined in the experiment shown in Fig. 4. Results presented are for the larger activated subpopulation, although qualitatively similar results were also seen when the total population was analyzed. CTLA-4 surface expression was detected on $CD4^+$ and $CD8^+$ cells. CTLA-4 was coexpressed with CD25, CD28, and CD45RO. Thus, CTLA-4 is expressed on a fraction of the activated $CD28^+$ T cells of both $CD4^+$ and $CD8^+$ subsets.

Cooperation of CTLA-4 and CD28 in Mediating Adhesion to B7. Since CTLA-4 and CD28 were coexpressed on T cells, it was important to determine the relative contributions of these molecules towards adhesion to B7. In previous studies (26), we showed that CTLA4Ig bound B7 with greater avidity than did CD28Ig, which suggested that CTLA-4 is a higher avidity receptor for B7 than is CD28. Expression of CTLA-4 on activated T lymphocytes might then be expected to increase their binding to B7. We took two approaches towards testing this possibility. In one approach, $CD4^+$ T cells from PBL were activated by coimmobilized anti-TCR plus ICAM-1 Rg and tested for their ability to bind biotinylated B7Ig (Fig. 5). Large, activated cells expressed more CTLA-4 and also expressed approximately twofold more CD28 than the smaller cell population. Comparison of mean fluorescence values indicated that large, activated cells expressed ~50-fold more CD28 than CTLA-4, assuming equivalent detection of the two mAbs. Large, activated cells also bound more B7Ig (Fig. 5). Binding of B7Ig to both large and small cell populations was stronger when the concentration of biotinylated B7Ig was increased, but for this experiment the concentration of B7Ig used was minimized so as to more easily compete for B7Ig binding with mAbs. B7Ig binding was blocked by both mAbs 11D4 and 9.3, but not by a control mAb of irrelevant specificity. Identical results were obtained when PBL were activated with immobilized anti-CD3 mAb before staining. These findings indicate that large activated cells bind more B7 than smaller cells, and that both CTLA-4 and CD28 contribute to this increased binding, despite the great difference in abundance of the two molecules.

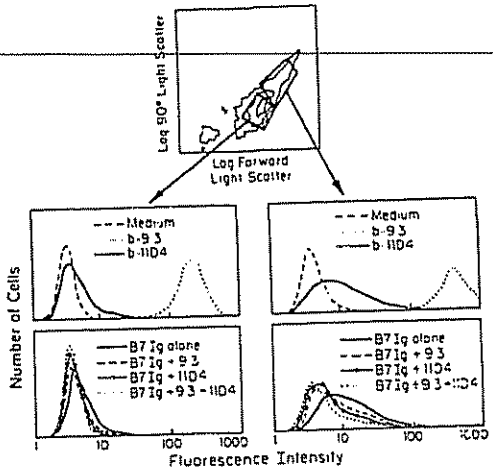


Figure 5. B7 binds better to T lymphocytes expressing the CTLA-4 counter receptor. $CD4^+$ T cells were activated by culturing for 3 d with coimmobilized anti-TCR mAb WT31 with ICAM-1 Rg fusion protein. Cells were collected and stained with medium alone, or with biotinylated mAbs 9.3 or 11D4 (*middle*) or with biotinylated B7Ig (*bottom*), all at 10 μg/ml. Where indicated, binding of biotinylated B7Ig was blocked by preincubation with an excess (100 μg/ml) of unlabeled mAbs 9.3, 11D4, or with 9.3 plus 11D4. B7Ig binding was detected by addition of PE-SA, and analyzed by flow cytometry. Control experiments showed that binding of biotinylated-B7Ig was blocked by an excess of unlabeled B7Ig, but not by an irrelevant human Ig, or by an irrelevant murine IgG1 mAb.

We also examined the relative contributions of CTLA-4 and CD28 on activated T cells towards binding B7 using a cellular adhesion assay. In these experiments, antigen-primed $CD4^+$ T cells were alloactivated, ^{51}Cr-labeled, and tested for their ability to adhere to $B7^+$ CHO cells in the presence of increasing amounts of anti-CD28 mAb 9.3 or a combination of anti-CD28 mAb 9.3 plus anti-CTLA-4 mAb 11D4 (Fig. 6). mAb 9.3 inhibited $CD4^+$ T cell adhesion to $B7^+$ CHO cells in a dose-dependent fashion. mAb 11D4 alone at 1 μg/ml did not significantly affect adhesion when compared with an irrelevant control mAb. The addition of 1 μg/ml mAb 11D4 to increasing concentrations of mAb 9.3 resulted in a shifting of the dose–response curve approximately fivefold to the left. Thus, mAb 11D4 was synergistic with mAb 9.3 at inhibiting adhesion of activated T cells to B7. Identical results were obtained in three different experiments. These data indicate that CTLA-4 and CD28 cooperatively mediate adhesion to B7.

Functional Cooperation between CTLA-4 and CD28 during T Cell Activation. The involvement of CTLA-4 and CD28 in costimulation during primary MLC was examined (Fig. 7). Anti-CD28 and anti-CTLA-4 mAbs were used alone and in combination to block involvement of these molecules in

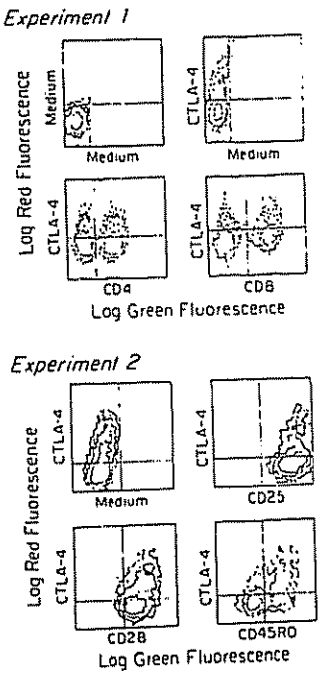


Figure 4. CTLA-4 expression by subsets of anti-CD3 activated T lymphocytes. PBL were activated by incubation with immobilized anti-CD3 mAb for 2 d at 37°C. Cells were collected and stained without or with biotinylated mAb 11D4. Cells were washed and then incubated with PE-SA without or with FITC-conjugated mAbs G17 2 (anti-*CD4*), 10.1 (anti-*CD8*), 7G7/B6 (anti-*CD25*), 9.3 (anti-*CD28*), or UCHL-1 (anti-*CD45RO*). Cells were then subjected to two-color analysis by flow cytometry. Shown are contour plots obtained from two independent experiments. Results shown are from larger activated cells, although qualitatively similar results were also obtained with smaller cells. Staining with biotinylated mAb 11D4 was inhibited by prior incubation of cells with excess unlabeled mAb 11D4, but not with an excess of isotype-matched control mAb.

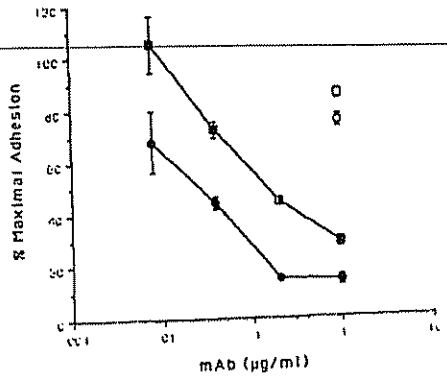


Figure 6. Anti-CD28 and anti-CTLA-4 mAbs cooperatively inhibit adhesion of activated $CD4^+$ cells to $B7^+$ CHO cells. Adhesion of alloactivated (24 h) ^{51}Cr-labeled $CD4^+$ T cells to fixed monolayers of $B7^+$ CHO cells was measured as described in Materials and Methods. T cells were incubated with 1 μg/ml anti-CTLA-4 mAb 11D4 (○) or isotype control mAb M23 (□); or with the indicated concentrations of mAb 9.3 mixed with 1 μg/ml mAbs 11D4 (●) or M23 (■) before initiation of adhesion. Adhesion was allowed to proceed for 1 h at 37°C, and nonadherent cells were removed by washing. Monolayers were solubilized by addition of 1 N NaOH, and bound radioactivity was measured with a gamma counter. Values are expressed as a percentage of adhesion measured in the absence of mAb (5,400 cpm, 3,800 cells, or ~6% of input cells).

proliferation during primary MLC. In these experiments, Fab fragments of mAb 9.3 were used since these are more efficient inhibitors of MLC (39). mAb 11D4 at 5 μg/ml did not inhibit primary MLC, but increased by ~10-fold the ability of various concentrations of Fab fragment of mAb 9.3 to inhibit MLC. These data suggest that CTLA-4 and CD28 cooperatively regulate T cell proliferative responses during MLC.

Experiments were performed to demonstrate effects of anti-CTLA-4 mAbs on T cell proliferation. We observed that,

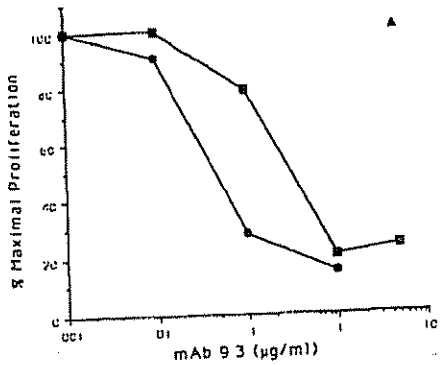


Figure 7. Anti-CD28 and anti-CTLA-4 mAbs cooperatively inhibit primary MLC. Primary MLC with HLA-DR mismatched responder and stimulator cells was performed in the presence of 5 μg/ml of anti-CTLA-4 mAb 11D4 alone (▲); or with the indicated concentrations of Fab fragment of anti-CD28 mAb 9.3 alone (■) or together with 5 μg/ml of mAb 11D4 (●). Means of cpm incorporation of three replicates were calculated. Data for each treatment are represented as a percentage of the peak proliferative response in untreated cultures (30,500 cpm).

in contrast to anti-CD28 mAb 9.3 (6), mAb 11D4 did not significantly augment anti-CD3 or PMA-induced proliferation of PBL during 3-d cultures. Since CTLA-4 expression is activation dependent, we also tested the ability of mAb 11D4 to augment proliferation of T cells that had been preactivated for 3 d with anti-TCR mAb and ICAM-1 Rg (33). Coimmobilization of anti-TCR mAb and anti-CD28 mAb 9.3 greatly increased proliferation of these cells (Fig. 8). Coimmobilized combinations of anti-TCR mAb and anti-CTLA-4 mAb 11D4 increased proliferation slightly. In some experiments, this combination of mAbs gave greater enhancement of proliferation, but always less than the combination of anti-TCR mAb plus anti-CD28 mAb. Coimmobilization of anti-TCR mAb plus a combination of mAbs 9.3 and 11D4 gave synergistic effects on proliferation. When anti-TCR mAb was omitted, proliferation was insignificant (data not shown), indicating that the proliferative effects of mAbs 11D4 and 9.3 also required TCR activation. Similar results were obtained with anti-CTLA-4 mAb 10A8. Thus, mAbs to CD28 and CTLA-4 were synergistic in their ability to costimulate preactivated $CD4^+$ T cells.

Discussion

CTLA-4 was identified as a transcript encoding a "cytotoxic T lymphocyte–associated" molecule (20). Here we have shown that CTLA-4 is expressed at similar levels on both $CD4^+$ and $CD8^+$ T cell subsets, and is coexpressed with the CD25 activation marker. CTLA-4 expression is regulated in similar fashion on both $CD4^+$ and $CD8^+$ T cell subsets. Expression of CTLA-4 is therefore not restricted to cytotoxic T cells, but rather more generally, to activated T lymphocytes. Our results also show that CTLA-4 and CD28 are not generally expressed on reciprocal subsets of T cells, as has been speculated (40). Instead, CTLA-4 was coexpressed with its homologue CD28 on a subset of activated T cells.

Cells that expressed the highest levels of CTLA-4 (detected by mAb 11D4) also bound more of the B7Ig fusion protein, partly as a result of CTLA-4 expression, since B7Ig binding

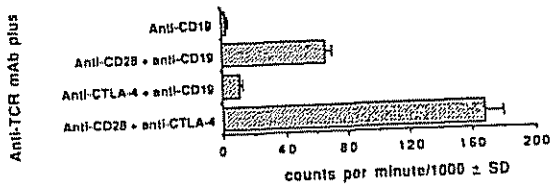


Figure 8. Anti-CTLA-4 and anti-CD28 mAbs synergistically costimulate proliferation of primed $CD4^+$ T lymphocytes. $CD4^+$ T cells from PBL were activated by culturing for 3 d with coimmobilized anti-TCR mAb WT31 with ICAM-1 Rg fusion protein as described in Fig. 5. Cells were then collected and cultured with 20 ng/well WT31 (anti-TCR) plus 40 ng/well negative control mAb 4G7 (anti-CD19); or 20 ng/well each of mAb 4G7 and mAbs 9.3 (anti-CD28) or 11D4 (anti-CTLA-4); or with 20 ng/well each of mAbs 9.3 and 11D4. Proliferation was measured by addition of [^{3}H]thymidine (1 μCi/well) during the final 16 h of a 3-d culture at 37°C. Shown are results from triplicate cultures (± SD). Wells from which anti-TCR mAb was omitted gave <1,000 cpm.

was partly blocked by mAb 11D4. Thus, although CTLA-4 was expressed much less than CD28 on this cell population (~50-fold less) it bound a significant fraction of the total B7Ig. This is consistent with CTLA-4 having higher avidity for B7 (26).

CTLA-4 is expressed much less abundantly than its homologue, CD28. Expression was induced several hundred-fold upon activation. This finding is consistent with earlier studies (20, 23) which demonstrated an increase in CTLA-4 transcript levels upon T cell activation Even at a maximum, CTLA-4 was expressed at only ~1/30–50 the levels of CD28, as estimated in measurements of total (intracellular plus cell surface) CTLA-4 (Fig. 3), and cell surface CTLA-4 (Fig. 5). The difference in expression between CD28 and CTLA-4 supports the earlier conclusion (14) that CD28 is the primary counter receptor for B7 on PHA-activated T cell blasts.

Taken together, these results indicate that CTLA-4 is an activation-regulated counter receptor of high avidity and low abundance. CD28, on the other hand, is expressed on both resting and activated cells, and has lower avidity for B7, but higher abundance. The overall degree of interaction between B7 costimulatory molecules on APC, and CD28 and CTLA-4 on activated T cells would be determined by the relative levels of the two T cell molecules. It is intriguing that the difference in expression levels between CD28 and CTLA-4 on activated cells (30–50-fold) is similar in magnitude to the difference in avidities of their respective Ig fusion proteins for B7 (~20-fold; reference 26). This suggests that the difference in avidities for B7 between these molecules is delicately balanced on activated T cells by differences in their levels of expression.

Combinations of mAbs to CD28 and CTLA-4 cooperatively inhibit adhesion of activated T cells to B7. Blocking CTLA-4 interactions with B7 therefore increased the ability of anti-CD28 mAbs to block CD28 interactions with B7. This suggests that CD28 and CTLA-4 cooperatively regulate adhesion to B7. Anti-CTLA-4 mAbs alone were poor inhibitors of T cell adhesion or proliferation in MLC even though these mAbs blocked binding of CTLA4Ig to B7. Anti-CTLA-4 mAbs were also relatively inefficient at promoting T cell activation when coimmobilized with anti-TCR mAb. CTLA-4 interactions with B7 therefore are neither necessary nor sufficient for T cell adhesion or activation by B7. Conversely, CD28 mAbs are good inhibitors of adhesion and proliferation in MLC (39), and are potent costimulators of T cell proliferation (6). This suggests that CD28–B7 interactions are essential for T cell activation by APC.

Our data suggest that one function of CTLA-4 on activated T cells may therefore be to promote effective interactions between CD28 and B7. Low abundance, high avidity CTLA-4 molecules appear to facilitate interactions and/or signals between higher abundance, lower avidity CD28 molecules and the B7 counter receptor. One model to explain these data would be in which CTLA-4 and CD28 exist on the T cell surface in microdomains, as has been demonstrated for other adhesion molecules (41). The inclusion of CTLA-4 in such microdomains might increase the probability of interaction between CD28 and B7 on the APC. Alternatively, B7 might exist in microdomains, as has been suggested by immunofluorescence studies of certain APC (Symington, F., W. Brady, and P. S. Linsley, unpublished observations). CTLA-4 might then bind to such a microdomain and thereby increase the probability that it would interact with CD28 on the T cell surface. A more complete understanding of the molecular mechanisms by which CTLA-4 facilitates T cell activation by CD28 will require careful investigation of the topography of CD28 and CTLA-4 on T cells, and of B7 on APC.

Anti-CTLA-4 mAbs were less effective costimulators of T cell proliferation than anti-CD28 mAb 9.3. We have also been unable to trigger intracellular calcium mobilization or increases in IL2 mRNA with anti-CTLA-4 mAbs under conditions that lead to clear signals with the anti-CD28 mAb 9.3 (Greene, J., N. R. Demle, and P. S. Linsley, unpublished observations). This may result in part from the lower expression of CTLA-4, but it may also indicate that signals transmitted by these two receptors are qualitatively different. The cytoplasmic domains of CD28 and CTLA-4 show somewhat limited sequence identity (26–29% for murine and human molecules; 23), possibly suggesting different interactions with signal-transducing molecules. It may also be significant that the cytoplasmic domains of murine and human CTLA-4 are identical (22, 23), whereas those from murine and human CD28 are only ~77% identical (40). This may reflect evolutionary pressure to conserve an essential signal-transducing function for CTLA-4. The anti-CTLA-4 mAbs we have described will facilitate future investigations on signal(s) transduced by this molecule.

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EXHIBIT 12

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Structural basis for co-stimulation by the human CTLA-4/B7-2 complex

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Regulation of T-cell activity is dependent on antigen-independent co-stimulatory signals provided by the disulphide-linked homodimeric T-cell surface receptors, CD28 and CTLA-4 (ref. 1). Engagement of CD28 with B7-1 and B7-2 ligands on antigen-presenting cells (APCs) provides a stimulatory signal for T-cell activation, whereas subsequent engagement of CTLA-4 with these same ligands results in attenuation of the response[1]. Given their central function in immune modulation, CTLA-4- and CD28-associated signalling pathways are primary therapeutic targets for preventing autoimmune disease, graft versus host disease, graft rejection and promoting tumour immunity[1,2]. However, little is known about the cell-surface organization of these receptor/ligand complexes and the structural basis for signal transduction. Here we report the 3.2-Å resolution structure of the complex between the disulphide-linked homodimer of human CTLA-4 and the receptor-binding domain of human B7-2. The unusual dimerization properties of both CTLA-4 and B7-2 place their respective ligand-binding sites distal to the dimer interface in each molecule and promote the formation of an alternating arrangement of bivalent CTLA-4 and B7-2 dimers that extends throughout the crystal. Direct observation of this CTLA-4/B7-2 network provides a model for the periodic organization of these molecules within the immunological synapse and suggests a distinct mechanism for signalling by dimeric cell-surface receptors.

The specificity of the T-cell response depends on the engagement of the T-cell receptor (TCR) with the cognate major histocompatibility complex (MHC)-peptide complex on the surface of APCs[3]. Effective regulation of this response requires a number of antigen-independent co-stimulatory signals, the most important of which are provided by the related T-cell surface molecules, CTLA-4 and CD28 (approximately 30% identity)[1]. Studies indicate that CTLA-4 both disrupts stimulatory signalling complexes by competing with CD28 for binding the B7 isoforms, and promotes the assembly of inhibitory signalling complexes[4]. To examine the structural basis for co-stimulation, we have solved the structure of the complex formed by the disulphide-linked homodimer of human CTLA-4 and the receptor-binding domain of human B7-2. The asymmetric unit contains two monomers of each CTLA-4 and B7-2, providing two independent views of the CTLA-4/B7-2 interface. Notably, crystal packing generates an alternating network of bivalent CTLA-4 and B7-2 homodimers. The CTLA-4 and B7-2 monomers are both two-layer β-sandwiches that display the chain topology characteristic of the immunoglobulin variable (V-type) domains present in antigen receptors such as the TCR β-chains and antibody V_L and V_H domains[5]. The front and back sheets of CTLA-4 contain strands A′GFCC′ and ABEDC″, respectively, whereas the front and back sheets of B7-2 are composed of AGFCC′C″ and BED strands, respectively. The individual CTLA-4 monomers in the present crystal structure are highly similar to the monomers observed in the murine CTLA-4 crystal structure[6] and the human CTLA-4 NMR structure[7], with root mean square (r.m.s.) deviations of 1.6 Å and 2.2 Å, respectively. Furthermore, the structure of the B7-2 monomer is similar to that observed for both unliganded B7-1 (ref. 8) and B7-2 (X.Z. and J.-C.D.S., unpublished results), displaying r.m.s. deviations of 2.3 Å and 0.8 Å, respectively.

The CTLA-4/B7-2 binding interface is formed by packing interactions between the front sheet of each molecule (Fig. 1a). CTLA-4 makes extensive contribution of residues from the FG loop (the complementary determining region 3 (CDR3)-like segment) and to a lesser extent the C and C′ strands. These segments pack against the G, F, C, C′ and C″ strands of B7-2, which form a shallow concave surface as a consequence of the natural twist of the sheet. The two independent CTLA-4/B7-2 interfaces have very similar atomic contacts, and bury 1,290-Å^2 (interface A) and 1,212 Å^2(interface B) of total accessible surface area. The size of this interface compares favourably with other complexes of V-type immunoglobulin domains, such as the heterophilic CD2/CD58 complex[9] and homophilic E-cadherin adhesion dimer[10] that bury 1,160 Å^{2}and 1,300 Å^2, respectively. Although the CD2/CD58 and E-cadherin interfaces are formed by packing of the strands residing on the front sheet of each interacting molecule, the CTLA-4/B7-2 interface is formed by the β-strands of B7-2 and surface loops on CTLA-4. In the crystal, the two CTLA-4/B7-2 interfaces show a difference in geometric complementarity, with shape correlation statistics (Sc values) of 0.66 and 0.58 for the A and B interfaces, respectively (see Methods). The differences in buried surface area and surface complementarity observed in the two CTLA-4/B7-2 interfaces arise from an approximate 13° rotational difference between the two CTLA-4 molecules relative to their respective B7-2 partners (see Methods).

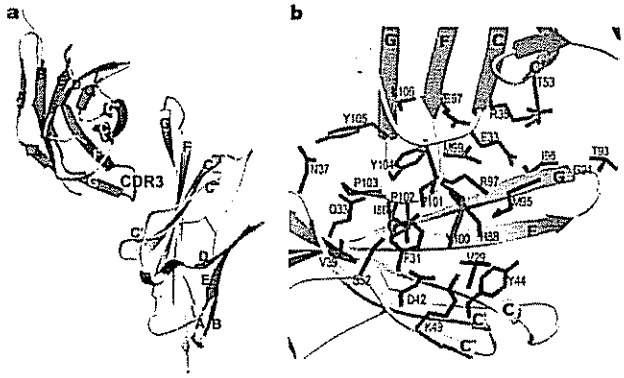


Figure 1 The CTLA-4/B7-2-binding interface. **a**, Ribbon diagram of the CTLA-4/B7-2 monomers that form the binding interface. For CTLA-4, the strands are labelled A (residues 4–8), A′ (residues 11–13), B (residues19–26), C (residues 33–42), C′ (residues 45–54), C″ (residues 56–60), D (residues 67–72), E (residues 75–81), F (residues 90–99) and G (residues 105–109; 112–115). The A′GFCC′ (front) and ABEDC″ (back) sheets are coloured pink and red, respectively. The CDR3-like loop of CTLA-4 is labelled. For B7-2, the strands are labelled A (residues 3–7), B (residues 9–11), C (residues 27–35), C′ (residues 38–45), C″ (residues 47–49), D (residues 60–64), E (residues 67–73), F (residues 81–91) and G (residues 94–108). The AGFCC′C″ (front) and BED (back) sheets are coloured dark blue and blue, respectively. All inter-sheet disulphides are shown in yellow. **b**, Detailed view of the human CTLA-4/B7-2 interface. The interface is formed by residues from the front sheets of CTLA-4 (CDR3 and the C and C′ strands) and a concave surface on B7-2 (the G, F, C, C′ and C″ strands and the CC′, C″D and FG loops).

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All subsequent discussions will refer to interface A.

The CTLA-4/B7-2 interface is stabilized by five hydrogen bonds and 28 van der Waals contacts involving a number of conserved residues, including the Met-Tyr-Pro-Pro-Pro-Tyr-Tyr sequence (residues 99–105) from CTLA-4 that corresponds to CDR3. As also observed in the murine CTLA-4 crystal structure[6], the three consecutive proline residues in the complex adopt an unusual *cis–trans–cis* conformation, which promotes numerous van der Waals interactions with Phe 31, Gln 33, Asn 37, Leu 38, Val 39, Glu 42 and Ser 52 from the C and C′ strands, and the CC′ and C″D loops of B7-2 (Fig. 1a, b). The three tyrosine residues flanking these prolines provide additional van der Waals contacts with Val 29, Phe 31, Gln 33, Tyr 44, Ile 86, His 88 and Met 95, and participate in a series of potential hydrogen bonds, involving the side-chain phenolic hydroxyls, with Asn 37, Glu 42, Lys 49 and Arg 97 of B7-2. Additional contacts involve Glu 33 and Arg 35 (C strand), Thr 53 (C′ strand) and Met 99 (CDR3) of CTLA-4 with residues 93–97 on the G strand of B7-2. An ionic interaction between Glu 97 of CTLA-4 and Arg 97 of B7-2 also contributes to the interface. These contacts are consistent with mutagenesis data, indicating that residues in CDR3 and the C strand of CTLA-4 (refs 7, 11) and the C and C′ strands of the B7 isoforms[12] contribute to the binding interface. In addition, the structure identifies a number of amino acids involved in the binding interface, including Thr 53 and Tyr 105 of CTLA-4 and residues on the G strand of B7, which have not been previously reported. Although the residues in the BC loop (CDR1) of CTLA-4 do not directly contact B7, CDR1 packs against CDR3 and may influence its detailed conformation. Such an indirect effect is consistent with mutagenesis data showing that alterations in CDR1 affect the affinity for the B7 isoforms[13]. The extensive sequence conservation present at the B7 binding sites of CTLA-4 and CD28 and the concordance of CTLA-4 and CD28 mutagenesis studies[11,14,15] suggest that CTLA-4 and CD28 form similar interfaces with the B7 isoforms.

The CTLA-4 dimer interface in the complex is formed by residues that are positioned carboxy terminal to the G strand and residues centred around the A′ strand, burying a total accessible surface area of 723 Å^2 (Fig. 2a, b). This newly discovered mode of dimerization results in a molecule that is substantially more extended than observed in other V-type dimers such as the CD8 homodimer[16] and the VαVβ and VLVH antigen receptor heterodimers[17,18], which form an extensive interface between their front sheets. Validation of this extended dimer is provided by the independent crystal structure of the unliganded, disulphide-linked human CTLA-4 dimer (J.-C.D.S. and X.Z., unpublished results), which exhibits the same dimer interface. Of note, comparison of the unliganded and liganded structures demonstrates that B7-2 binding does not induce any significant conformational reorganization in either the CTLA-4 monomer or the dimer interface, suggesting that unliganded CTLA-4 is poised in a 'pre-formed' productive binding mode. The organization of the human disulphide-linked CTLA-4 dimer places the B7-binding sites distal to the dimer interface and would be expected to support binding of two independent B7 molecules.

The residues that contribute to the human CTLA-4 dimer interface (Val 10, Leu 12, Ala 13, Ser 15, Tyr 115, Val 116, Ile 117, Asp 118, Pro 119 and Glu 120, as well as the disulphide linkage at Cys122) are nearly invariant in all known CTLA-4 sequences, which further supports the biological relevance of this interface (Fig. 2b, c). Although there is only modest sequence similarity between CTLA-4 and CD28 in this region, within the CD28 family there is considerable conservation, suggesting similar quaternary structures for both CTLA-4 and CD28 homodimers. Sequence divergence at this interface could provide a mechanism to prevent the formation

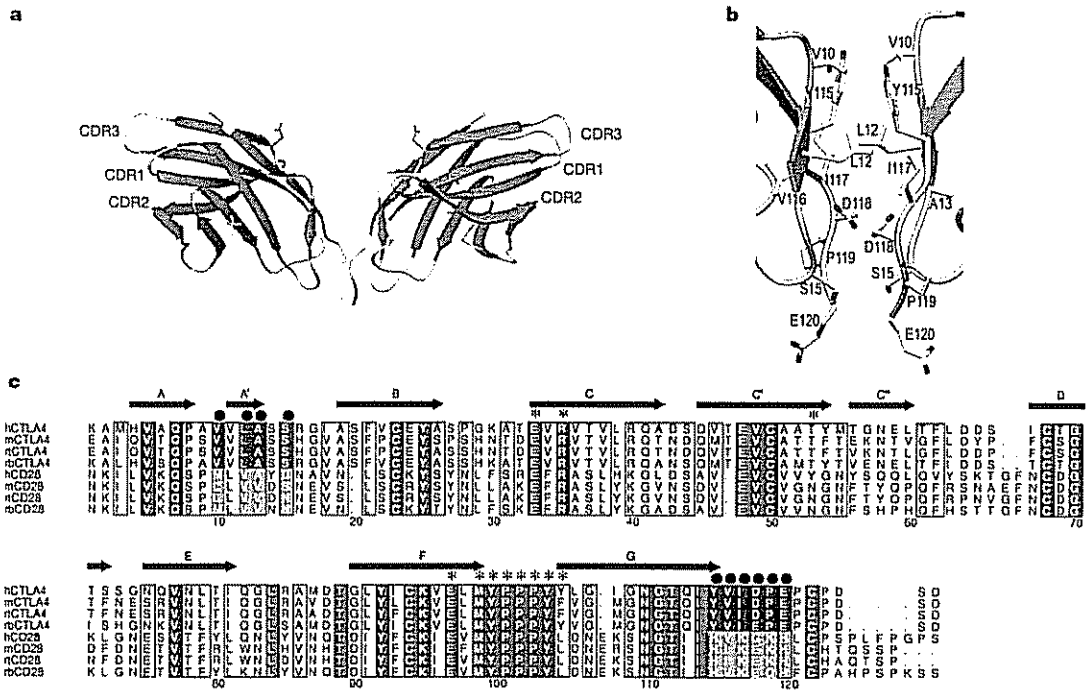


Figure 2 The disulphide-linked human CTLA-4 dimer. **a**, Ribbon diagram of the CTLA-4 dimer. The interface has contributions from residues C-terminal to the G strand and centred around the A′ strand. The CDR analogous regions (CDR1, CDR2 and CDR3) are labelled and potential glycosylation sites are highlighted in green. The observed organization places the B7-binding sites distal to the dimer interface. **b**, Detailed view of the CTLA-4 dimer interface. This same interface is also present in the unliganded human CTLA-4 disulphide-linked dimer (J.-C.D.S. and X.Z., unpublished results). **c**, Sequence alignment of the CTLA-4/CD28 family. Residues that participate in the human CTLA-4 dimer interface (filled circle) and B7-2-binding site (asterisk) are indicated. Residues with greater than 50% conservation are coloured in yellow, whereas those that are invariant are red. The residues in the human CTLA-4 dimer interface are coloured in blue, whereas the analogous residues in CD28 are green. H, human; m, murine; rt, rat; rb, rabbit. Strand colours are the same as in Fig. 1.

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of CTLA-4/CD28 heterodimers and is consistent with the failure to detect such heterodimers *in vivo*[19].

The B7-2 dimer interface is predominantly formed by the B, E and D strands and the BC, C″D and DE loops from the back sheet of each monomer (Fig. 3a, b) and buries a total accessible surface area of 1,405 Å^2, compared with 1,220 Å^2 in the B7-1 dimer interface[8]. This back-to-back packing arrangement is unusual for V-type dimers and has only been observed in the murine CTLA-4 (ref. 6) and human B7-1 non-covalent dimers[8]. Like CTLA-4, the B7-2 dimer observed in the crystal places the ligand-binding sites distal to the dimer interface, providing the potential for each B7-2 dimer to bind two CTLA-4 molecules. As with CTLA-4 and CD28, sequence divergence at the dimer interface may operate to prevent B7-1/B7-2 heterodimer formation (Fig. 3c).

The bivalent potential of both CTLA-4 and B7-2 is realized by the formation of a periodic assembly of dimeric molecules, characterized by a 103-Å repeat, that extends throughout the entirety of the crystal (Fig. 4). The direct observation of this periodic network immediately provides a model describing the assembly of these molecules at the T-cell/APC interface, and naturally suggests mechanisms that regulate the compartmentalization of signalling molecules at the immunological synapse. Such a network may promote the organized recruitment of inhibitory signalling molecules through interaction with either cytoplasmic or extracellular domains in CTLA-4 or B7, and may also decrease the local concentration of excitatory signalling molecules through simple steric crowding. The formation of an extended network may also act as a physical barrier to free diffusion that prevents entry and exit of molecules from the T-cell/APC interface. Given the similarities in their B7 recognition sites and modes of dimerization, some aspects of the organized assembly observed for CTLA-4 may also be operative in CD28-associated signalling.

Both B7 isoforms are composed of a membrane-distal receptor-binding domain and a membrane-proximal constant immunoglobulin domain. By modelling the complete B7 molecule into the observed crystallographic repeat, the maximum dimension of the CTLA-4/B7-2 complex is estimated to span about 100 Å, excluding the residues in B7-2 and CTLA-4 that connect the immunoglobulin domains to the membrane-spanning segments

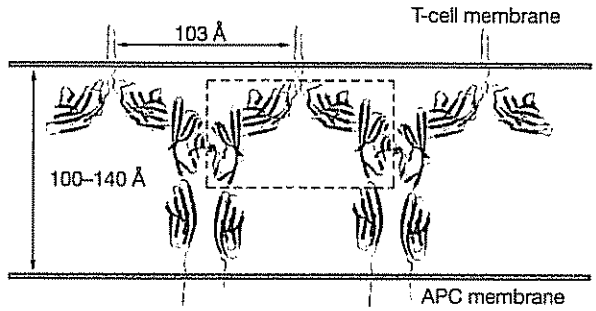


Figure 4 Molecular associations seen in the crystalline CTLA-4/B7-2 complex. The molecules in the dashed box represent the experimentally observed organization of CTLA-4 and the B7-2 receptor-binding domains within the crystal. The application of translational symmetry results in an alternating arrangement of bivalent dimers of CTLA-4 and B7-2 receptor-binding domains that extends throughout the entirety of the crystal. To model the interaction between CTLA-4 and the full-length B7 molecule, the membrane-proximal immunoglobulin domain from the human CD2 crystal structure was positioned on the basis of the known B7-1 crystal structure[8]. On the basis of the observed crystallographic repeat, this model predicts a characteristic spacing of 103 Å between adjacent CTLA-4 molecules and further suggests that the CTLA-4/B7-2 complex will impose a 100–140 Å spacing between the T cell and APC. The transmembrane and cytoplasmic regions are modelled to suggest that the periodic constraints of the extracellular domains are imposed on the cytoplasmic tails. Notably, all of the interfaces observed in the crystal are free of potential glycosylation sites that are present in CTLA-4 and B7-2. This model of association may extend to other members of this family, including CD28 and B7-1, as it is predicted that glycosylation would not preclude the formation of similar interfaces

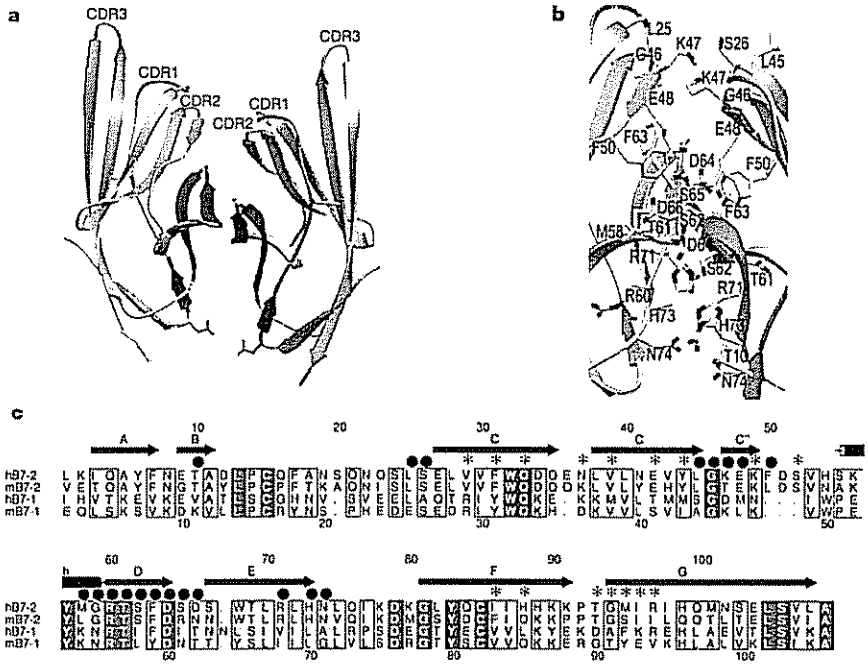


Figure 3 The human B7-2 dimer. **a**, Ribbon diagram of the B7-2 dimer. The interface is formed by the B, E, D, C′ and C″ strands, and the BC, C″D and DE loops. The CDR analogous regions (CDR1, CDR2 and CDR3) are labelled and the potential glycosylation site is highlighted in green. This organization places the CTLA-4-binding sites distal to the dimer interface. The colour scheme is the same as in Fig. 1b. **b**, Detailed view of the B7-2 dimer interface. A similar interface is present in the unliganded human B7-1 structure[11] (See Methods). **c**, Structure-based sequence alignment of the B7-1 and B7-2 variable-like domains. Residues that participate in the human B7-2 dimer interface (filled circle) and CTLA-4-binding site (asterisk) are indicated. The numbering scheme for B7-2 and B7-1 are shown above and below the sequence alignment, respectively. Residues with greater than 50% conservation are coloured in yellow, whereas those that are invariant are red. Sequence abbreviations as in Fig. 2c.

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(Fig. 4). This distance is consistent with the maximum dimensions of other receptor/ligand pairs that co-localize to the central region of the immunological synapse, including the MHC/TCR, CD2/CD58 and CD28/B7 complexes[20]. In contrast, the length of the CTLA-4/B7-2 complex is significantly smaller than that of molecular species, such as the LFA-1/ICAM-1 complex, that localize in the peripheral zone of the synapse[20]. Discrimination on the basis of molecular dimension may provide an efficient sorting mechanism for the compartmentalization observed at the immunological synapse, and is consistent with the disruption of the CD28/B7 complex through direct competition by CTLA-4.

We previously reported the structure of murine CTLA-4 (ref. 6) that formed a compact non-covalent dimer by means of packing of the back sheets, similar to that observed for the variable domains of B7-1 and B7-2. The Cα of the last, firmly anchored residue in each murine monomer is about 22 Å from its symmetry mate, a distance that could reasonably be spanned by the remaining five residues in each chain to accommodate the formation of the required disulphide bond. Geometric considerations, however, indicate that the organization of the murine dimer is incompatible with the formation of a CTLA-4/B7 assembly at the T-cell/APC interface, as imposition of the observed human CTLA-4/B7-2 interface on the murine dimer results in a near-orthogonal disposition of the CTLA-4 and B7 dimer axes, an arrangement that is not consistent with known membrane topologies. Thus, the structure reported for the non-covalent murine dimer would seem not to have an obvious function in CTLA-4 signalling.

The structure of the human CTLA-4/B7-2 complex provides insights into the signalling mechanisms used by dimeric cell-surface receptors. One classical mechanism is used by the human growth hormone[21] and fibroblast growth factor[22] receptors that transduce signals only on ligand-induced dimerization. Another mechanism is used by the erythropoetin[23] and insulin[24] receptors, in which preformed receptor dimers undergo a significant conformational rearrangement on ligand binding to trigger signalling. CTLA-4 is an obligate dimer owing to the interchain disulphide linkage, and the present work shows that no conformational changes or reorganization of the dimer interface occur on binding of B7. These observations indicate that CTLA-4 is poised in a pre-formed productive binding mode before ligand engagement, and suggest that signalling relies on the assembly of at least two CTLA-4 dimers, or perhaps an extended network of multiple CTLA-4 and B7 dimers as observed in the crystal. This is in contrast to the erythropoetin, insulin and growth factor receptors[21–24], which are competent to signal as isolated dimeric molecules. Thus, the combination of both a pre-formed productive binding mode for CTLA-4 and the unique form of ordered clustering characteristic of the CTLA-4/B7 ligand pair may represent a distinct signalling mechanism available to dimeric cell-surface receptors. □

Methods

Crystallization

The soluble extracellular domain of human CTLA-4 (residues 1–126) was expressed in *Escherichia coli*, purified and refolded from inclusion bodies to yield the dimeric species covalently linked by a disulphide bond between Cys 122 of each monomer. We confirmed the presence of the inter-chain disulphide by reducing and non-reducing SDS–PAGE of dissolved crystals. The CTLA-4-binding domain of human B7-2 (residues 1–109) was similarly purified and refolded. The covalent CTLA-4 dimer and B7-2 domain were mixed in a 1:1 stoichiometry (based on monomer) at a total protein concentration of 3 mg ml^{-1}. Crystals of the complex were grown by hanging-drop vapour diffusion by mixing 2 μl each of protein solution and crystallization buffer composed of 18% PEG 20000, 100 mM HEPES, pH 7.0, followed by equilibration against the same buffer.

Data collection and structure determination

Diffraction from these crystals was consistent with the monoclinic space group $P2_1$ ($a = 47.85$, $b = 54.56$, $c = 103.09$ and $\beta = 91.63°$, with two CTLA-4 monomers and two B7-2 monomers per asymmetric unit). Crystals were flash frozen at 100 K in mother liquor containing 15% glycerol. Data were collected at beamline X9B (National Synchrotron Light Source, Brookhaven National Laboratory) with 0.98 Å radiation, recorded on a 2 × 2 ADSC CCD detector and reduced with the HKL suite[25]. The R_{merge} was 12.0% for all data between 25–3.2 Å resolution (32.1% in the 3.3–3.2 Å shell) with overall completeness of 79% (79% in 3.3–3.2 Å shell). The average I/σ value is 6.6 for all data between 25–3.2 Å resolution (2.2 in the 3.3–3.2 Å shell). The structure was solved by molecular replacement, as implemented in CNS[26], using the 2.7-Å crystal structure of the B7-2 monomer (X.Z. and J.-C.D.S., unpublished results) and the murine CTLA-4 structure[6] as search models. Initially, the B7-2 monomer yielded two clear solutions related by approximate two-fold non-crystallographic symmetry, whereas the murine CTLA-4 monomer model yielded a single significant solution. Rigid-body refinement at 4.0 Å resolution resulted in the appearance of substantial density that was readily fit to a second CTLA-4 monomer by applying the non-crystallographic symmetry operator relating the two B7-2 monomers. After the removal of poorly ordered loops, rigid-body refinement at 4.0 Å resolution resulted in an R_{factor} and R_{free} of 37.2% and 40.7%, respectively. Modelling[27] and refinement at 3.2 Å resolution, including simulated annealing, bulk solvent correction and B-factor refinement[26], resulted in a final R_{factor} and R_{free} of 21.7% and 30.0%, respectively. The average B-factor for all atoms is 20.4 Å^2. The final model includes all residues except the two amino-terminal and five C-terminal residues in both CTLA-4 monomers, and residues 27–30 and 42–44 in one CTLA-4 monomer. The final model also has good geometry with 94.2% of residues falling in the most favourable and additionally allowed regions and only two residues (0.5%) in the disallowed region of the Ramachandran plot. r.m.s. bond lengths and r.m.s. bond angles are 0.008 Å and 1.3°, respectively, with all other stereochemical parameters comparable to or exceeding those of structures at similar resolution[28].

$R_{merge} = \Sigma|I_h - \langle I_h\rangle|/\Sigma I_h$, where $\langle I_h\rangle$ is the mean intensity for multiple measurements of symmetry-related reflection h, R_{factor} and $R_{free} = \Sigma_h||F(h)_{obs}| - |F(h)_{calc}||/\Sigma_h|F(h)_{obs}|$ for reflections in the working and test sets, respectively.

Structural analysis

Superposition of the two independent CTLA-4/B7-2 complexes in the asymmetric unit based on the B7-2 component revealed a 13° rotational difference between the two CTLA-4 molecules. The calculation was performed using the program FIT (G. Lu. http://bioinfo1.mbfys.lu.se/~guoguang/fit.html). The two independent CTLA-4 monomers that form the disulphide-linked dimer show close to a proper two-fold symmetry—being related by a rotation of 174.3° and a translation of 0.37 Å. The two monomers in the B7-2 dimer are not related by proper two-fold symmetry, as there is about a 175° rotation between the two monomers and about a 3.1 Å translation along the non-crystallographic symmetry axis, as calculated in FIT. The human B7-1 dimer shows perfect two-fold symmetry, as the molecular diad is coincident with a crystallographic two-fold axis[8]. Despite the deviation from perfect two-fold symmetry, most of the residues that contribute to the B7-2 interface occupy the same position in the primary sequence as those residues that form the B7-1 interface[8].

The shape correlation statistic (Sc) measures the geometric complementarity at protein–protein interfaces, with Sc = 1 corresponding to a perfect fit and Sc = 0 corresponding to totally uncorrelated surfaces[29]. Sc values in this study were calculated with the program SC[29] using default parameters. All buried surface areas were calculated with CNS[26] using a 1.4 Å probe. We prepared figures using the programs Setor[30] and ALSCRIPT[31]. We carried out structure-based sequence alignment of the B7 molecules with DALI[32].

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Acknowledgements

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Crystal structure of the B7-1/CTLA-4 complex that inhibits human immune responses

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Optimal immune responses require both an antigen-specific and a co-stimulatory signal. The shared ligands B7-1 and B7-2 on antigen-presenting cells deliver the co-stimulatory signal through CD28 and CTLA-4 on T cells. Signalling through CD28 augments the T-cell response, whereas CTLA-4 signalling attenuates it. Numerous animal studies[1,2] and recent clinical trials[3,4] indicate that manipulating these interactions holds considerable promise for immunotherapy. With the consequences of these signals well established, and details of the downstream signalling events emerging[5–7], understanding the molecular nature of these extracellular interactions becomes crucial. Here we report the crystal structure of the human CTLA-4/B7-1 co-stimulatory complex at 3.0 Å resolution. In contrast to other interacting cell-surface molecules, the relatively small CTLA-4/B7-1 binding interface exhibits an unusually high degree of shape complementarity. CTLA-4 forms homodimers through a newly defined interface of highly conserved residues. In the crystal lattice, CTLA-4 and B7-1 pack in a strikingly periodic arrangement in which bivalent CTLA-4 homodimers bridge bivalent B7-1 homodimers. This zipper-like oligomerization provides the structural basis for forming unusually stable signalling complexes at the T-cell surface, underscoring the importance of potent inhibitory signalling in human immune responses.

The extracellular domains of human soluble (s) CTLA-4, residues 3–125 (SWISSPROT numbering), and sB7-1, residues 1–214, were expressed in Chinese hamster ovary (CHO) cells. The complex containing fully glycosylated sCTLA-4 and sB7-1 was crystallized in the space group $C222_1$. In the crystal lattice, sCTLA-4 and sB7-1 monomers each associate as non-crystallographic, roughly two-fold symmetric homodimers; single copies of each homodimer together form the asymmetric unit (Fig. 1a). The association of the four monomers in the asymmetric unit is driven by the interaction of three surfaces: the surface mediating sB7-1 homodimerization; the CTLA-4 homodimer interface; and the receptor–ligand binding interface.

The structures of the uncomplexed sCTLA-4 (refs 8, 9) and sB7-1

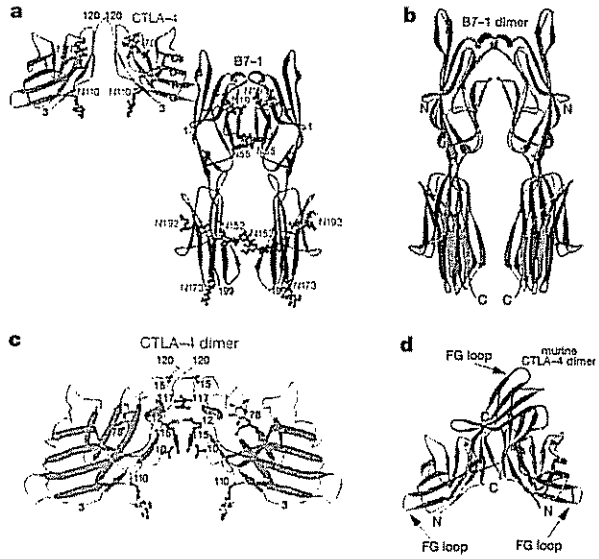


Figure 1 Structural comparison of sCTLA-4/sB7-1 complex with uncomplexed forms of sCTLA-4 and sB7-1. **a**, Ribbon diagram of the sCTLA-4/sB7-1 complex showing two sB7-1 (purple) and two sCTLA-4 (cyan) molecules in the asymmetric unit. Disulphide bonds (green) and sugar moieties (yellow) are also shown. β-sheets involved in the receptor–ligand interaction are labelled. Glycosylation sites on sCTLA-4 (Asn 78 and Asn 110) and on sB7-1 (Asn 19, Asn 55, Asn 152, Asn 173 and Asn 192) are all surface exposed. None of the ordered glycosides is involved in the receptor–ligand recognition. **b**, Superposition of the ligand-binding V-set domains of complexed (purple ribbons) and uncomplexed (cyan ribbons) forms of sB7-1. Apparent displacement of the membrane-proximal domains does not affect the interactions at the interdomain regions and is perhaps a result of differences in crystal packing contacts. **c**, Detailed view of the sCTLA-4 homodimer interface. Residues involved in hydrophobic interactions between the two monomers are shown in green. Two hydrogen bonds formed at the elbow of the homodimer are indicated as red dashed lines. Spatial proximity of the C termini (Glu 120) would enable a formation of disulphide bond between cysteines at positions 122. Asn 110 is the only strictly conserved glycosylation site in CTLA-4 and CD28. Located between the two monomers, *N*-glycans in these positions might stabilize the homodimer. **d**, Superposition of human sCTLA-4 dimer (cyan) on murine sCTLA-4 dimer (red). From this orientation, the contradiction between the two dimerization modes is evident. R.m.s. deviation between the two individual monomers is 1.5 Å for 114 Cα pairs. The FG loop of sCTLA-4 in the complex is displaced by 2.5 Å toward sB7-1. Figure prepared with RIBBONS[29].

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EXHIBIT 13

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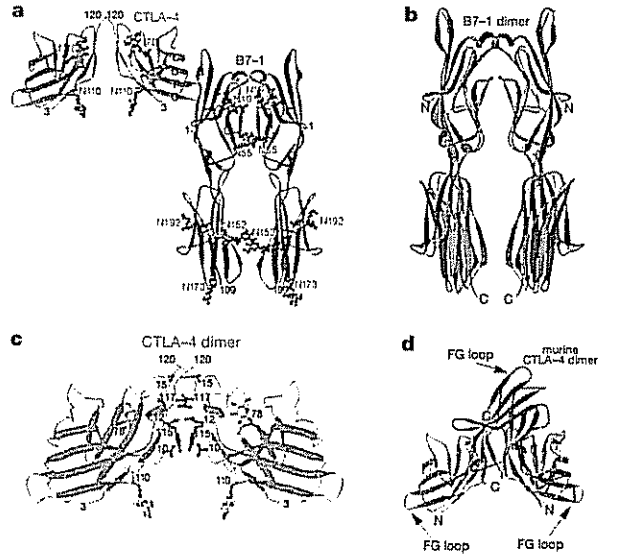


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(ref. 10) have shown that the two proteins comprise, respectively, a single V-set, and paired V-set and C1-set immunoglobulin superfamily (IgSF) domains. Overall, the sCTLA-4 and sB7-1 monomers do not show any significant conformational rearrangements on complex formation, apart from small local changes restricted to their binding sites. The glycosylated sB7-1 homodimer seen in the crystals of the complex is essentially identical to that observed in crystals of deglycosylated sB7-1 (ref. 10): the monomer–monomer interface is the same size (570 Å^2 per monomer; calculated using the program SURFACE[11]; probe radius 1.4 Å) and involves the same residues. When the V-set domains of the two sB7-1 homodimers are superimposed (Fig. 1b), the membrane-proximal C1-set domains are displaced by only 3 Å. Similar variation has been observed in individual crystals of sB7-1 (S.I. *et al.*, unpublished data), suggesting that these differences are more likely to result from variability in sB7-1 self-association than from the effects of ligand binding. sB7-1 homodimerization has been confirmed in solution using analytical ultracentrifugation methods[10].

Although the structure of the sCTLA-4 monomer is very similar to that of the murine sCTLA-4 monomer[8], the human and murine[8] sCTLA-4 homodimers observed in the two lattices are very different (Fig. 1c, d). Of the two structures, we consider that the sCTLA-4 homodimer present in the crystals of the complex represents the more plausible model for native CTLA-4. First, all of the interacting residues are strictly conserved, in contrast to the conservation of only four of the ten residues forming the murine dimer interface[8]. Second, the human sCTLA-4 homodimer is not precluded by glycosylation. If used as in human CTLA-4 (ref. 12), an *N*-linked glycan at the conserved Asn 78 glycosylation site would prevent murine-like homodimerization in all species. Third, the human homodimer allows co-ligation of sB7-1 molecules around an axis orthogonal to the membrane, maintaining the ~140 Å intermembrane distance thought to be a critical feature of the immunological synapse[5]. Last, the interchain disulphide bond will more readily form in the human sCTLA-4 homodimer. The 7.7 Å spacing between the Cα atoms of the last visible residue in each monomer (Pro 119) is more likely to be bridged by the three additional residues and by the disulphide bond in the human sCTLA-4 dimer than is the equivalent 30.1 Å distance in the murine structure. Consequently, the murine model for CTLA-4 dimerization and signalling proposed previously[8] is most probably not relevant to the formation of co-stimulatory complexes.

The human sCTLA-4 monomers interact through residues in the A and G β-strands (Val 10, Leu 12, Ser 15, Tyr 115, Ile 117, Glu 120), burying only 460 Å^2 of surface[11] per monomer. The interface is essentially hydrophobic except for reciprocal, interchain hydrogen bonds between the side chain of Ser 15 and the main-chain nitrogen of Glu 120 at the elbow of the homodimer (Fig. 1c). This weak interaction might explain why, in the absence of the interchain disulphide (Cys–Cys 122), sCTLA-4 is monomeric in solution (ref. 9; and Y.Z. and M.S., unpublished data). The amino acids mediating CTLA-4 dimerization are not conserved in CD28; however, the equivalent residues exhibit the same overall hydrophobicity, implying that CD28 might form similar homodimers.

Differences noted[8] between the murine structure and the human sCTLA-4 structure determined by NMR methods[9] are also seen in the complex crystals. In particular, the V-set domain has C″DEBA:GFCC′ rather than DEBA:GFCC′C″ topology, and the three consecutive prolines in the FG loop, MY^{101}PPP^{103}Y, adopt the *cis–trans–cis* rather than *trans–trans–cis* conformation seen in the NMR structure[9]. This has important implications for B7-1 binding (see below).

Mutational data have implicated the membrane-proximal domain of B7-1 in interactions with CTLA-4 and CD28 (ref. 13), but these effects must have been indirect as sCTLA-4 only contacts residues of the sB7-1 V-set domain. Receptor–ligand binding occurs through the GFCC′ face of the sCTLA-4 and sB7-1 V-set domains, with a roughly 90° angle between the interacting β-sheets (Fig. 2a). In this orientation, the FG, BC and C′C″ loops of sCTLA-4 extend across the base of the sB7-1 five-stranded β-sheet, burying a total of 1,255 Å^2 of solvent-accessible surface[11] (600 Å^2 from sB7-1 and 655 Å^2 from sCTLA-4). These values are at the low end of the range for protein–protein binding sites (600–900 Å^2)[14].

Orthogonal binding mediated by the GFCC′C″ face of the V-set IgSF domains of cell-surface molecules was first seen in rat and human sCD2 crystal contacts[15,16], and subsequently in the native complex of sCD2 and sLFA-3 (ref. 17) and in crystals of the coxsackie virus and adenovirus receptor[18]. It seems likely that this orthogonal binding mode will be a recurrent theme of V-set interactions. There is no compelling reason why such orthogonal interactions should have arisen through convergent evolution, but it is possible that these complexes bear the imprint of primordial IgSF interactions.

Interatomic contacts (85 total; van der Waals radius 3.9 Å) are made between 13 residues of CTLA-4 residues and 13 residues of

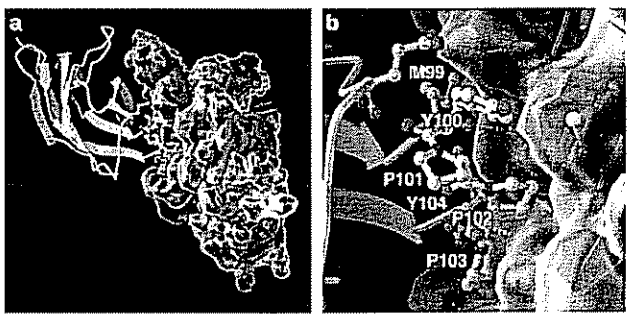


Figure 2 Overview of receptor–ligand interactions. **a**, Ribbon diagram showing orthogonal interaction between sCTLA-4 (cyan) and sB7-1 (purple) monomers. Also shown is the molecular surface representation (white transparent) of the ligand-binding domain of sB7-1 to emphasize the high geometric match between the two interacting surfaces. **b**, Direct receptor–ligand contacts. The ^{99}MYPPPY^{104} loop of sCTLA-4 is buried in a shallow depression of the sB7-1 GFCC′ surface. Colour coding is as in a. Three out of five hydrogen bonds formed across the β-sheets of the interacting domains are depicted as red dashed lines. Several other side chains on CTLA-4 and B7-1 (not shown) may contribute to the binding through appreciable, but not direct, contacts formed on the periphery of the binding interface. Figure prepared with BobScript[30].

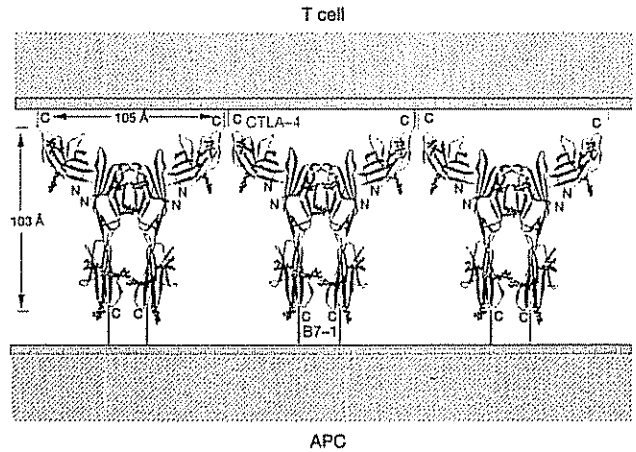


Figure 3 Molecular association of sCTLA-4 and sB7-1 in the crystal lattice. Shown are 'skewed zipper' arrays in which sCTLA-4/sB7-1 complexes would be evenly spaced along membrane surfaces with a separation of 105 Å. In the perpendicular direction, across membranes, ligated receptors would span 140 Å. Geometrically, sugar chains attached at Asn 173 on B7-1 (bottom) are close to the cell membrane, implying their potential involvement in interaction with the membrane, perhaps by stabilizing the orientation of the B7-1 dimers. APC, antigen-presenting cell. Figure prepared with RIBBONS[29].

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B7-1. Most of these are hydrophobic contacts, but there are also five hydrogen bonds. The FG loop of sCTLA-4, which contains the hydrophobic ^{99}MYPPPYY^{105} sequence that is strictly conserved in CTLA-4 and CD28, dominates the interaction and contributes 400 Å^2 of protein surface[11] to the binding interface (Fig. 2b). The FG loop makes hydrophobic contacts with a largely nonpolar surface of sB7-1 consisting of Tyr 31, Met 38, Thr 41, Met 43, Val 83, Leu 85, Ala 91, Phe 92 and Leu 97.

Mutations of the two central residues, Tyr 31 and Met 38, disrupt B7-1 binding to CTLA-4 and CD28 (ref. 13); the other residues forming this surface have not been tested. Five hydrogen bonds, including one between the charged residues Glu 33 on sCTLA-4 and Arg 29 on sB7-1 are likely to contribute to the specificity of binding (Fig. 2b). All residues in the ^{99}MYPPPYY^{105} sequence, except Pro 101, are in direct contact with sB7-1, and all alanine substitutions of these residues, including Pro 101, reduce or abolish binding to B7-1 (ref. 9). Analysis of the complex structure indicates that the crucial role of Pro 101 is to initiate the *cis–trans–cis* main-chain conformation of the FG loop, directing Tyr 100, Pro 102, Pro 103 and Tyr 104 towards interactions with sB7-1. At the core of the interface, Pro 102 of CTLA-4 and Tyr 31 of B7-1 participate in a stacking interaction (Figs 2b, 4) that is likely to be conserved both across species and in interactions involving CD28 and B7-2 (in B7-2 Tyr 31 is replaced by phenylalanine).

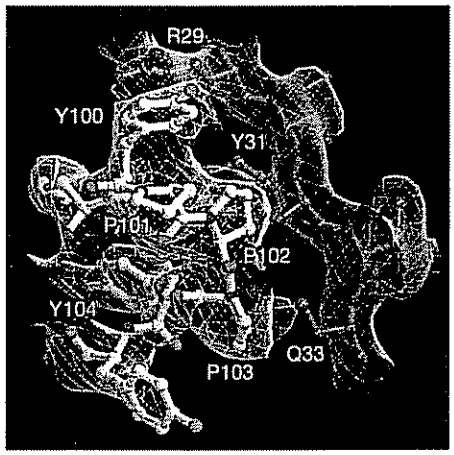


Figure 4 A 3 Å resolution electron-density map in the region of the receptor–ligand binding site. Electron density is from an annealed omit map calculated using the $3F_{obs} - 2F_{obs}$ amplitudes and model phases, with the CTLA-4 atoms excluded from the refinement and all calculations. The map is contoured at 1σ level. Colour coding is as in Figs 1 and 2.

Table 1 Statistics for data collection and refinement

Data collection		
Resolution range (Å)	20–3.0	3.11–3.0
Completeness (%)	99.2	94.1
Total observations	209,381	–
Unique reflections	38,815	3,636
Average $I/\sigma(I)$	19.1	3.0
R_{sym}*(%)	6.7	35.1
Model refinement		
Maximum resolution (Å)	3.0	
Number of reflections (free)	$F_{obs} > 2\sigma$ 31,410 (1,887)	$F_{obs} > 0\sigma$ 35,465
R_{work}/R_{free} †(%)	23.1/25.9	25.3/28.2
r.m.s. deviations		
Bonds (Å)	0.010	
Angles (°)	1.48	

*$R_{sym} = \Sigma|I_h - \langle I_h\rangle|/\Sigma I_h$, where $\langle I_h\rangle$ is the average intensity over symmetry equivalents. Numbers in the second column reflect statistics for the last resolution shell.
†$R_{work} = \Sigma||F_{obs}| - |F_{calc}||/\Sigma|F_{obs}|$. R_{free} is equivalent to R_{work}, but calculated for a randomly chosen 6% of reflections omitted from the refinement process.

Accordingly, the two binding surfaces exhibit a very high degree of shape complementarity. An algorithm[19] measuring the degree of geometric fit between two protein surfaces gives scores of 0.74–0.77 for the sCTLA-4/sB7-1 binding interface. This value is similar to those for constitutive oligomeric proteins (0.7–0.76) and much higher than scores for protein antigen–antibody interfaces (0.64–0.68) or interacting cell-surface molecules (0.45–0.58; ref. 17 and references therein). This degree of complementarity accounts for the enthalpy-driven nature of binding (R. O'Brien *et al*, personal communication), whereas the small interacting surfaces and slightly unfavourable entropy explain the very fast binding kinetics[12].

In the crystal lattice, the sCTLA-4 and sB7-1 homodimers pack together to form a periodic arrangement in which bivalent sCTLA-4 homodimers bridge bivalent sB7-1 homodimers (Fig. 3). The sB7-1 and shorter CTLA-4 homodimers associate orthogonally, thus generating a 'skewed zipper' arrangement. It has long been clear that CTLA-4 exists as a constitutive, bivalent homodimer, and the affinity of sB7-1 self-association (dissociation constant, K_d = 20–50 μM) indicates that B7-1 is also likely to exist as a dimer at the cell surface, albeit in dynamic monomer–dimer equilibrium[10]. We therefore expect oligomeric arrays that are similar, if not identical, to those seen in the crystals to form at the membrane interface between T cells and antigen-presenting cells.

Including the 'stalk' regions, the extracellular domains of the ligated receptors are expected to span a distance of ~140 Å between the opposing cell membranes, a distance compatible with that required by T-cell receptor (TCR)–MHC, natural killer cell inhibitory receptor (KIR)–MHC or CD2–CD58 intercellular interactions[5,17,20,21]. Overall, this arrangement is reminiscent of the 'cell-adhesion zipper' observed in the crystals of cadherins, which is thought to be a fundamental feature in adhesive interactions between cells expressing these molecules[22].

As far as is generally known, cell-surface molecules bind their ligands monovalently and with very low (micromolar) affinities[23]. The submicromolar affinity of B7-1 for CTLA-4 (K_d = 0.2–0.4 μM; ref. 12) is thus unusually high for interacting cell-surface molecules. Our structural analysis of the complex implies that potent B7-mediated inhibitory signalling is not based exclusively on the stability of association between individual homodimers, but rather that the counter-receptor oligomeric arrays will also strengthen the interaction between the opposing cells. The combination of sub-micromolar affinity and oligomeric, high-avidity binding is, to our knowledge, unique to CTLA-4/B7-1 interactions.

The amplification and attenuation of TCR signalling occurs in a specialized contact area on the cell surface, termed the immunological synapse[5,6], through the recruitment of receptors and their signalling complexes that effect the phosphorylation state of the TCR (ref. 7). Notably, inhibition of TCR signalling by CTLA-4 has been seen only to occur when both signals are delivered by the same cell surface[24]; however, other data suggest that the regulatory signals delivered by CD28 and CTLA-4 may not be strictly cell autonomous[25]. Regardless of the precise nature of the signals and mechanisms involved, it now seems that the inhibition of immune responses by CTLA-4 requires the formation of unusually stable complexes, the structure of which is revealed by the crystals of sCTLA-4/sB7-1. Whether such stable complexes are required for the efficient use of low-abundance CTLA-4 molecules at the T-cell surface, or enhance the potency of inhibitory CTLA-4 signalling by concentrating key effector molecules such as the phosphatase SHP-2 (ref. 7) under the immunological synapse, or both, remains to be established. □

Methods

Production and purification of B7-1 and CTLA-4

Constructs encoding the extracellular portions of human CTLA-4 or B7-1 were fused to an enterokinase cleavage sequence (DYKDDDDK) followed by the IgG1 CH2–CH3 domains, and expressed in CHO cells. In addition, the cysteine at position 122 of CTLA-4

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was mutated to serine. These were recovered from conditioned medium by protein-A chromatography, cleaved with enterokinase, further purified and confirmed to be monomeric by gel-filtration chromatography as described[26]. Appropriate binding characteristics for each protein were confirmed by surface plasmon resonance (Biacore) analysis and found to be consistent with published values[12]. For CTLA-4, the final cleaved protein comprised residues 3–125 (with the C122S mutation) followed by the octa-peptide DYKDDDDK at the carboxy terminus. For B7-1, the cleaved protein consisted of residues 1–214 with the DYKDDDDK octa-peptide again forming the C terminus.

Crystallization and data collection

The B7-1/CTLA4 complex was formed by incubating a 1:1.5 molar ratio of B7-1 and CTLA4 and purified using a TSK-3000SW size exclusion column (TosoHaas). The purified complex was buffer exchanged and concentrated to 10 mg ml^{-1} in 20 mM Tris-HCl, pH 8.0. Crystals were grown at 18 °C by the hanging drop method by combining 2 µl of protein solution with 2 µl of 14% PEG 8000, 200 mM magnesium acetate and 100 mM cacodylate, pH 6.3, and equilibrated against 1 ml of the same solution. Crystals appeared in 5 days and were soaked in 16% PEG 8000, 200 mM magnesium acetate, 20 mM Tris-HCl pH 8.0, 100 mM cacodylate, pH 6.3, 20% ethylene glycol for about 1 min before plunging into liquid nitrogen. Crystals belong to space group $C222_1$ with unit-cell dimensions $a = 88.5$, $b = 183.4$, $c = 230.8$ Å and a calculated solvent content of 58% for two B7-1 and two CTLA-4 molecules per asymmetric unit (adding 82,000 (82K) of glycosylation estimated from SDS–PAGE gel mobility). The 3.0 Å resolution data were collected from a single crystal at 100K at Beamline 5.0.2 at the Advanced Light Source using a Quantum 4 CCD. 725 0.2° rotation images were collected and reduced with HKL2000 (ref. 27) giving statistics outlined in Table 1.

Structure determination and refinement

The structure was solved by molecular replacement in CNS[28] using the crystallographic dimer observed in the human B7-1 structure as the search model[10]. After rigid body refinement of each B7-1 domain the R factor was 55.3%. Initial electron-density maps phased with B7-1 alone showed clear electron density for the two CTLA-4 molecules. The model of murine CTLA-4 monomer (PBD accession number 1dqt) was placed into electron density and was manually rebuilt in QUANTA (Molecular Simulations) to reflect the sequence of human CTLA-4. Non-conserved amino-acid residues and loop regions of CTLA-4 and B7-1, as well as sugar moieties, were entirely built into electron density. A composite annealed omit map was calculated to check the conformation of CTLA-4 and B7-1 loop regions. All refinement procedures were done in CNS and used data from 20.0–3.0 Å. A 2σ cut-off was applied during the refinement because of the anisotropy of the data. Six per cent of the reflections were randomly selected for generation of the R_{free} data set (see Table 1). The anisotropy of the data was corrected for also using CNS.

Tight non-crystallographic symmetry restraints were gradually relaxed during the refinement process resulting in a final model with R_{work} of 23.2% and R_{free} of 25.9%. Continuous electron density was seen for sugar moieties at four N-linked carbohydrate sites on CTLA-4 dimer (Asn 78 and Asn 110) and at ten glycosylation sites on B7-1 dimer (Asn 19, Asn 55, Asn 152, Asn 173 and Asn 192). No saccharides could be modelled at six other expected glycosylation sites on B7-1 (Asn 64, Asn 177 and Asn 198). The final model contains two B7-1 molecules (residues A1–A199 and B1–B199), two CTLA-4 molecules (residues C3–C120 and D3–D120), 20 monosaccharides and shows reasonable geometry: 98.9% of non-glycine φ and ψ angles lie in the allowed regions of the Ramachandran plot (77.6% in the most favoured regions and 21.4% in additionally allowed regions); six amino-acid residues (1.1%) have disallowed angles.

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Correspondence and requests for materials should be addressed to W.S.S. (e-mail: wsomers@genetics.com) or I.M. (e-mail: lmosyak@genetics.com). The coordinates have been deposited in the Protein Data Bank. The accession code is 1I84.

corrections

Genetic control and evolution of sexually dimorphic characters

Artyom Kopp, Ian Duncan, Dorothea Godt & Sean B. Carroll

Nature 408, 553–559 (2000).

The name of one author, Dorothea Godt, was omitted from the author list but is included above. She is affiliated with the Department of Zoology, University of Toronto, Canada. In addition, a gene name was misspelled: the correct spelling is bric à brac. □

Mitochondrial genome variation and the origin of modern humans

Max Ingman, Henrik Kaessmann, Svante Pääbo & Ulf Gyllensten

Nature 408, 708–712 (2000).

The complete mtDNA sequences upon which the analyses are based can be found at our website (http:/www.genpat.uu.se/mtDB/), dedicated to the analysis of complete mtDNA genomes of humans. The 53 sequences are also available in Genbank with the accession numbers AF346963–AF347015. □

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EXHIBIT 14

Structural mechanisms of costimulation

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Recent studies have highlighted the structural requirements for T cell costimulation and have revealed unusual modes of dimerization for the cytolytic T lymphocyte–associated antigen 4 (CTLA-4) costimulatory receptor and its B7 ligands. These distinctive quaternary structures potentially endow both receptor and ligand with bivalent binding properties, which suggests a number of mechanistic features relevant to signaling. These include the potential to form a highly ordered, alternating network of CTLA-4 and B7 homodimers that may represent the organization of these molecules and their associated signaling partners within the immunological synapse. Primary sequence and structural considerations suggest that some aspects of the organizational and mechanistic features associated with the CTLA-4–B7 complexes may extend to other members of the costimulatory receptor-ligand family. An examination of the signaling mechanisms within the costimulatory receptor-ligand family provides an excellent framework to consider the general principles that are relevant to cell surface receptor–mediated signaling events.

The engagement of cell surface receptors by their specific ligands results in the activation of signaling pathways through a wide range of mechanisms, including the multimerization of monomeric receptors by multivalent ligands[1] and ligand-induced structural changes that convert a receptor from an inactive conformation to one that is competent to signal[2,3]. More complex mechanisms have been suggested by studies demonstrating the potential to form receptor-ligand superclusters that show long-range periodic order[4–7]. Regardless of mechanism, the engagement of extracellular receptor-ligand pairs serves to control the formation and organization of multicomponent cytoplasmic assemblies required for signal transduction. For example, structural studies have revealed that the cytolytic T lymphocyte–associated antigen 4–B7 (CTLA-4–B7) receptor-ligand pair has the potential to form an extended organized array at the T cell–antigen presenting cell (APC) interface[4,5]. This suggests that CTLA-4 and the related family of costimulatory receptors use a distinctive strategy for regulating the adaptive immune response.

Biological roles of costimulatory molecules

The T cell response is dependent on signals that arise from two discrete receptor-ligand recognition events. The first signal results from interactions between T cell receptors (TCRs) on the surfaces of T cells and peptide–major histocompatibility complexes (pMHC) displayed on the surfaces of APCs. This interaction alone is not sufficient for complete T cell activation and, in the absence of a second costimulatory signal, may result in either apoptotic death or prolonged unresponsiveness of the responding T cell[8]. The engagement of a family of related costimulatory receptors with their cognate ligands provides the additional signals needed for efficient T cell activation; it also provides the negative signals required to attenuate the immune response and to maintain peripheral T cell tolerance to protect against autoimmunity[9–11] (**Fig. 1**).

The most extensively characterized T cell costimulatory receptors are CD28 and CTLA-4, which bind the B7-1 and B7-2 ligands on APCs. Together with signaling through the TCR, the engagement of CD28 by the B7 ligands leads to optimal T cell activation, which is characterized by T cell proliferation and cytokine secretion[8]. In contrast to the stimulatory activity of CD28, the interaction of B7 molecules with CTLA-4 provides inhibitory signals required for down-regulation of the response. CTLA-4 also plays a critical role in regulating both the T cell cycle and tolerance induction[11].

Although CD28-B7–mediated costimulation is essential for the activation of naïve T cells, it is usually not required for memory and effector T cell responses[12,13], suggesting more complex regulatory pathways exist that involve additional receptor-ligand interactions. This idea was supported by the identification of additional costimulatory receptor-ligand pairs, including inducible costimulator (ICOS)–B7-H[14–16] and PD-1–PD-L[17–20]. The interaction between the ICOS receptor, a CD28 and CTLA-4 homolog (24% and 17% identity, respectively), and B7-H, a B7 homolog (~20% sequence identity with B7-1 and B7-2), stimulates both $CD4^+$ and $CD8^+$ T cell responses. In contrast to the positive signal that the ICOS–B7-H interaction delivers to T cells, the engagement of PD-1 on T cells by its PD-L ligands present on APCs and other nonlymphoid cells is responsible for the delivery of inhibitory signals to the responding T cell[18]. These inhibitory signals are likely important for both the maintenance of self-tolerance and down-regulation of T cell activity at sites of immune activation[9,10].

Molecular organization at the immunological synapse

To facilitate a biologically optimal response, T cells must integrate a number of distinct extracellular and intracellular signals. This challenge is met, at least in part, through the formation of a highly dynamic interface between the T cell and APCs, termed the immunological synapse. Initial TCR engagement and signaling is followed by a

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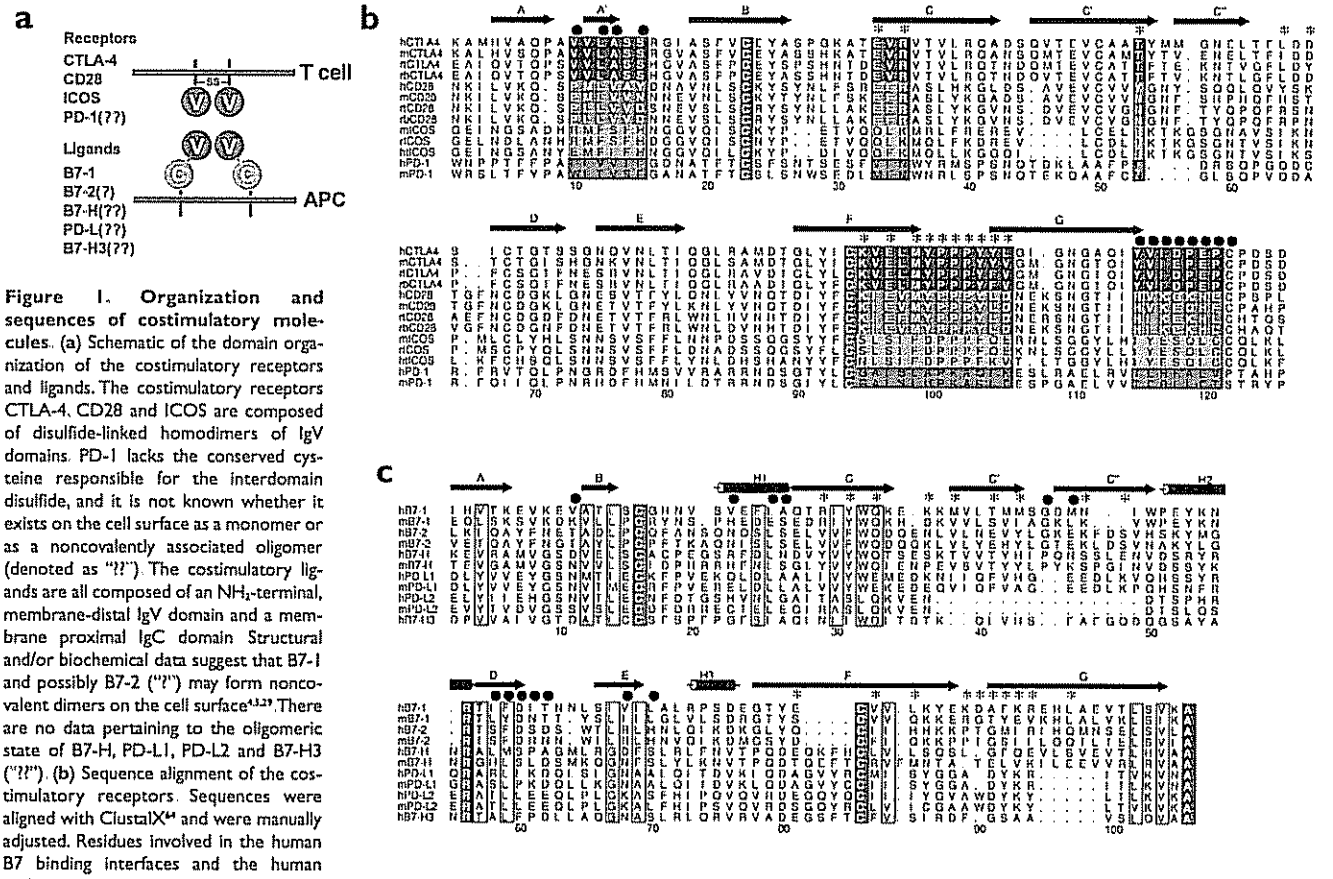


Figure 1. Organization and sequences of costimulatory molecules. (a) Schematic of the domain organization of the costimulatory receptors and ligands. The costimulatory receptors CTLA-4, CD28 and ICOS are composed of disulfide-linked homodimers of IgV domains. PD-1 lacks the conserved cysteine responsible for the interdomain disulfide, and it is not known whether it exists on the cell surface as a monomer or as a noncovalently associated oligomer (denoted as "??"). The costimulatory ligands are all composed of an NH_2-terminal, membrane-distal IgV domain and a membrane proximal IgC domain. Structural and/or biochemical data suggest that B7-1 and possibly B7-2 ("?") may form noncovalent dimers on the cell surface[4,5,29]. There are no data pertaining to the oligomeric state of B7-H, PD-L1, PD-L2 and B7-H3 ("??"). (b) Sequence alignment of the costimulatory receptors. Sequences were aligned with ClustalX[44] and were manually adjusted. Residues involved in the human B7 binding interfaces and the human CTLA-4 dimer interface are highlighted with asterisks and filled circles, respectively. Residues with significant conservation are highlighted in yellow and invariant cysteines in red. Residues for which there is experimental evidence or that are proposed to participate in ligand binding (that is, the MYPPPY loop) and homodimerization are highlighted for CTLA-4 (blue), CD28 (green), ICOS (pale blue) and PD-1 (pink). The following abbreviations are used for sequence identification: h, human; m, murine; rt, rat; rb, rabbit. (c) Sequence alignment of the costimulatory ligands. A structure-based sequence alignment of the B7-1 and B7-2 sequences was performed with Dali[45], and the remaining B7 family sequences were aligned with ClustalX[44] and were manually adjusted. Residues from B7-1 and/or B7-2 that contact CTLA-4 are highlighted by asterisks and residues that contribute to the B7-1 homodimer interface are denoted by filled circles. Residues with considerable conservation are highlighted in yellow, whereas invariant residues—including the two cysteines that form the hallmark disulfide—are highlighted in red. Given the weak sequence similarity between the different costimulatory family members, these alignments must be viewed with caution until additional structural information becomes available. These alignments were prepared with the program Alscript[46].

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remarkable reorganization and compartmentalization of signaling and adhesive molecules. The central zone of the mature synapse is characterized by the receptor-ligand pairs that control T cell activation; these include the TCR-CD3-pMHC, CD28-B7 and CD2-CD58 complexes and associated intracellular signaling molecules, such as Lck, Fyn and PKC-θ[21]. Surrounding the central zone is the peripheral zone, which is composed of large adhesion molecules and components of the actin cytoskeleton[21]. It was initially postulated that the clustering associated with synapse formation provided a mechanism for extended and sustained TCR signaling; however, we now know that TCR signaling precedes the establishment of the mature synapse, indicating that synapse formation is not directly involved in either the initiation or enhancement of TCR signaling[22]. A number of alternative functions for the synapse have been suggested, including the polarized secretion of cytokines, a role in TCR endocytosis and the promotion of costimulatory receptor-ligand engagement[22,23]. Major issues that remain are the identification of the structural and chemical mechanisms that facilitate this compartmentalization, and a description of the organization of the signaling and costimulatory molecules at the immunological synapse that serve to amplify or attenuate signals transduced through the TCR.

Costimulatory molecules share domain organization

The T cell costimulatory receptors CTLA-4, CD28, ICOS and PD-1 are composed of a single extracellular immunoglobulin variable (IgV) domain[24] (**Fig. 1**). The basic structural features of Ig domains are highlighted (**Fig. 2**). *In vivo*, CTLA-4, CD28 and ICOS are covalent homodimers, due to an interchain disulfide linkage formed by the equivalent of Cys^{122} in CTLA-4[25]. PD-1 lacks the equivalent of Cys^{122} and it is thus uncertain whether it functions *in vivo* as a monomer or as a noncovalent oligomer.

The costimulatory ligands B7-1, B7-2, B7-H and B7-H3, and the PD-L isoforms PD-L1 and PD-L2, also share a common domain structure[15,18,19,26,27], of a membrane proximal constant Ig (IgC) domain and a membrane distal IgV domain that is responsible for receptor binding and dimerization in B7-1 and B7-2 (**Figs. 1** and **2**). Analytical ultracentrifugation studies show that, in solution, the entire extracellular segment of B7-1 is in a rapid equilibrium between monomer and dimer with a very modest equilibrium dissociation constant of ~20–50 μM[28]. Gel filtration analysis of the B7-2 IgV domain does not show the presence of any dimer[29]; however, as described below, in the CTLA-4–B7-2 crystal structure[4], this domain displays a dimer interface similar to

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that observed in the B7-1 crystal structure[28]. The oligomeric state of the other costimulatory ligands has not yet been investigated.

Despite the apparently weak dimeric associations observed *in vitro*, on the cell surface the equilibria probably will favor homodimers of B7-1 and possibly B7-2. Although the oligomeric state of a macromolecule may be transient or not observable in solution, when confined to a two-dimensional lipid bilayer, even very weak interactions can result in substantial and biologically meaningful dimer formation. This is due to entropic factors that include reduced dimensionality, increased local concentration and constraints imposed by the plasma membrane that enforce molecular orientations promoting noncovalent association (that is, all molecules have their COOH-termini fixed in the membrane). Thus, to correctly interpret the biological roles and mechanisms of action of the costimulatory molecules, it is essential to establish their true *in vivo* oligomeric states.

IgV domains in the costimulatory receptors and ligands

Structural studies have detailed the features of a monomeric CTLA-4 variant in which Cys^{122}, the cysteine responsible for the intermolecular disulfide, has been modified to prevent covalent dimerization[5,30]. The crystal structure of the full-length B7-1 molecule revealed the overall organization of the individual Ig domains and defined the specific residues in the membrane distal IgV domains that contribute to the noncovalent dimer interface[28]. More recently, two independent crystallographic analyses, which used different protein expression strategies, provided the first structural description of the CTLA-4–B7 costimulatory complex[4,5]. With the use of bacterially expressed material, a 3.2-Å crystal structure of the complex formed by the disulfide-linked human CTLA-4 dimer and the IgV receptor–binding domain of human B7-2 was solved[4]. Another study, which used a Chinese hamster ovary (CHO) cell expression system, obtained the 3.0-Å crystal structure of glycosylated full-length B7-1 bound to a modified CTLA-4 in which Cys^{122} was mutated to Ser[5].

In general, IgV domains may be described as two layered β-sandwiches composed of a "top" and "bottom" β-sheet (**Fig. 2**)[31]. The front and back sheets of CTLA-4 contain strands A′GFCC′ and ABEDC″, respectively[32], whereas the front and back sheets of the B7 IgV domains are composed of strands AGFCC′C″ and BED, respectively[4,5,28]. The CTLA-4 monomer contains a disulfide linking the B and F strands, a hallmark feature of Ig domains, as well as a second disulfide that joins strands C′ and D[30]. These same disulfides are also invariant within the CD28 and ICOS families (**Fig. 1**), whereas PD-1 likely contains only the disulfide connecting the B and F strands (**Fig. 1**). All members of the B7 family contain the disulfide linkage between the B and F strands in each domain[33] (**Fig. 1**).

In the antigen receptors (TCRs and antibodies), the complementarity-determining regions CDR1, CDR2 and CDR3 are of particular functional importance, as they directly participate in the recognition of antigens. The heterodimers present in the antigen receptors (that is, $V_\alpha V_\beta$ and $V_H V_L$), as well as the homodimer formed by the CD8 IgV domain, all form as a result of packing between their front sheets[34–36]. This organization places CDR3 (the F-G loop) from each monomer in close proximity to one another at the dimer interface and results in molecules that are monovalent for their ligands (**Fig. 2**). As described below, the analog of CDR3 plays a major role in the biological function of the costimulatory receptors.

CTLA-4–B7 interface

Direct crystallographic analysis shows that CTLA-4–B7 binding interface is formed by orthogonal packing between the front sheets from each molecule and buries a total surface area of 1200–1300 $Å^2$ (**Fig. 2**). The interface is dominated by the interaction of the CDR3-analogous loop from CTLA-4, composed of the ^{99}Met-Tyr-Pro-Pro-Pro-Tyr-Tyr^{105} sequence (the so-called MYPPPY loop), with a concave surface on B7 formed predominately by the G, F, C, C′ and C″ strands (**Fig. 2**). Additional important residues are contributed from the C, C′ and F strands of CTLA-4. Of particular note is the unusual *cis-trans-cis* backbone conformation of the ^{101}Pro-Pro-Pro^{103} sequence in CTLA-4 that contributes to the significant geometric complementarity between CTLA-4 and the B7 isoforms (**Fig. 2**). Despite considerable sequence divergence in the B7 residues that contribute to the binding interface (**Fig. 1**), these structures demonstrate that CTLA-4 uses similar binding modes for the recognition of the two B7 isoforms. Importantly, the crystallographically observed interfaces are in good agreement with mutagenesis studies that implicated specific residues as directly contributing to the binding interface[37–39].

Sequence considerations indicate that the human CTLA-4–B7 structures may provide useful models for other members of the costimulatory receptor-ligand family. Though CTLA-4 and CD28 share only ~31% overall sequence identity, the extreme similarity of the CDR3-analogous loops (MYPPPYL in CD28, compared to MYPPPYY in CTLA-4), as well as the invariance of interface residues in the C and F strands, indicates that the CTLA-4–B7 structures provide realistic and useful models for understanding and manipulating the CD28-B7 binding interfaces (**Fig. 1**). In addition, the similarity of the CDR3-like loop (FDPPPFQ) and the highly conservative alterations in the C strands of ICOS suggest that the CTLA-4–B7 structures may represent the general features of the ICOS-B7-H binding interface (**Fig. 1** and **2**). PD-1 shows little conservation in these regions, making it difficult to accurately predict the ligand-binding site.

Dimeric features of CTLA-4 and costimulatory receptors

An essential functional feature revealed by the CTLA-4–B7-2 structure is that the disulfide-linked CTLA-4 dimer exhibits a "side-to-side" packing arrangement that places the B7 binding sites (CDR3) distal to the dimer interface, such that each CTLA-4 dimer can bind two independent B7 molecules (**Fig. 2**). The CTLA-4 dimer interface is formed by residues positioned COOH-terminal to the G strand and residues centered on the A′ strand and buries a rather modest total accessible surface area of 723 $Å^2$. This mode of dimerization results in a molecule that is substantially more extended than that observed for other V-type dimers such as the CD8 homodimer[34] and the $V_\alpha V_\beta$[35] and $V_H V_L$ antigen receptor heterodimers[36], all of which exhibit extensive interfaces involving their front sheets, resulting in the placement of the CDR3 loops at the dimer interface (**Fig. 2**).

Validation of this extended dimer is provided by the independent crystal structures of the CTLA-4–B7-1 complex[5] and the unliganded, disulfide-linked human CTLA-4 dimer (unpublished data), which show the same dimer interface. The presence of this unusual dimer in the CTLA-4–B7-1 complex is notable, as the CTLA-4 construct used in this study lacks Cys^{122}, which is responsible for the formation of the interchain disulfide bond and is monomeric in solution[5]. As suggested above, the observation of this noncovalent CTLA-4 dimer in the crystalline state reinforces the need for careful attention to the true oligomeric state of the costimulatory molecules on the cell surface.

The residues that contribute to the human CTLA-4 dimer interface—including the disulfide linkage at Cys^{122}—are nearly invariant in all known CTLA-4 sequences (**Fig. 1**), which further supports the biological relevance of the observed dimer interface (**Fig. 2**). Although there is only modest sequence similarity between CTLA-4 and CD28 in this region (**Fig. 1**), within the CD28 family there is considerable conservation, including the equivalent of Cys^{122}. This

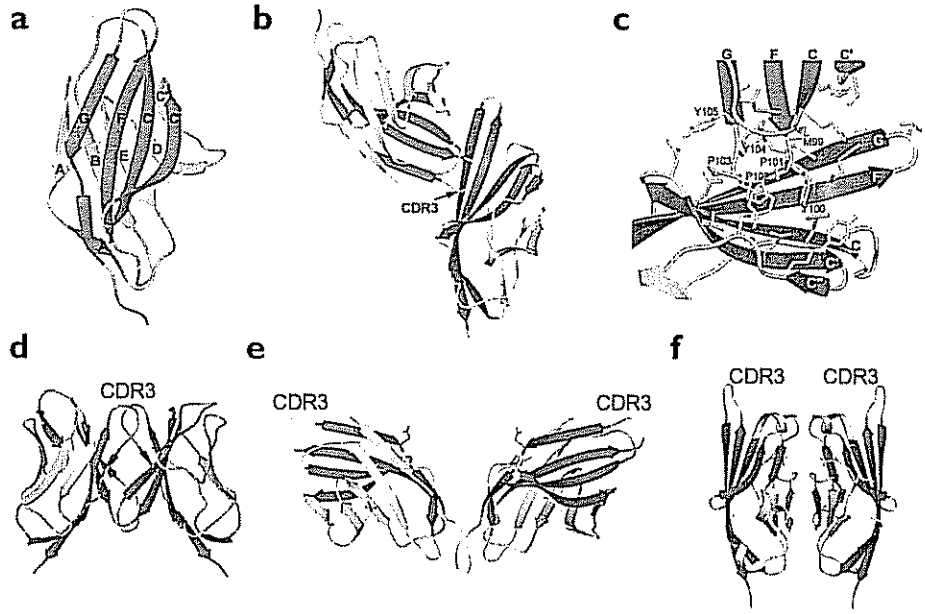


Figure 2. Structure of the CTLA-4–B7 complexes. (a) Fold of the human CTLA-4 monomer[4]. The front sheet (red) is composed of the A'GFCC' strands, whereas the back sheet (pink) is composed of the ABEDC'' strands. The disulfide joining the B and F as well as the C' and D strands are shown in yellow. (b) Ribbon diagram of the complex formed by CTLA-4 (red) and the B7-2 IgV domain (blue), which shows the orthogonal packing of the front sheet of each molecule as well as the prominent role played by CDR3 from CTLA-4 in forming the interface[4]. The front and back sheets of the B7 molecules are colored dark blue and light blue, respectively. (c) Detailed interactions at the CTLA-4–B7-2 binding interface, which shows specific atomic contacts[4]. The residues in the ^{99}MYPPPYY^{105} loop of CTLA-4 (red) are labeled. (d) Dimeric organization of the 2C TCR $V_\alpha V_\beta$ heterodimer[35], formed by packing of the front sheets (dark green) from each monomer. This packing, which is typical of the IgV superfamily, results in the placement of the two CDR3 loops in close proximity to one another at the dimer interface. (e) Ribbon diagram of the CTLA-4 homodimer that forms by side-to-side packing of the two monomers[4]. This interface has contributions from the A' strand and residues that follow the G strand. This mode of packing places CDR3 and the B7 binding sites distal to the dimer interfaces. The coloring scheme is as in Fig. 2a. Potential glycosylation sites are shown in green. (f) Ribbon diagram of the dimer interface formed by the B7-1 IgV domain homodimer[28]. The interface is formed by packing between the back sheets of each monomer with substantial contributions from the BED strands. This unusual back-to-back packing places the CTLA-4 binding surfaces distal to the dimer interfaces. The coloring scheme is as in Fig. 2b. Potential glycosylation sites are shown in green. This figure was prepared with the program Setor[47].

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suggests that CD28 homodimers may exhibit a quaternary structure similar, though not necessarily identical, to that of CTLA-4. Importantly, sequence divergence at this interface could provide a mechanism to prevent the formation of CTLA-4–CD28 heterodimers, consistent with the failure to detect such heterodimers *in vivo*[40]. In addition, based on the extensive sequence conservation within this region in the three available ICOS sequences (**Fig. 1**), it is plausible that the structure of the CTLA-4 dimer also provides a reasonable working model for the ICOS homodimer interface. Thus we suggest that CTLA-4, CD28 and ICOS will share some similarities in their modes of homodimerization and, as such, may all be predicted to bind two independent copies of their respective ligands. Again, it must be stressed that these hypotheses regarding oligomeric state and valency require direct experimental validation.

B7 isoform and costimulatory ligand's dimeric features

In the structures of the unliganded B7-1 molecule[28] and the CTLA-4–B7-1 complex[5], B7-1 forms an extensive interface primarily as a consequence of packing between the back sheets (strands DEB), which suggests a dimerization mode not previously observed for any other IgV domain. Despite considerable sequence divergence (**Fig. 1**), the B7-2 IgV domain exhibits a dimerization mode similar to that present in full-length B7-1[4,28]. These interfaces bury ~1200 Å^2 of surface area, with the B7-1 interface containing a higher proportion of hydrophobic residues than B7-2, consistent with the greater propensity of B7-1 to dimerize in solution. This organization of the B7 dimers has important biological implications: it places the ligand-binding sites distal to the dimer interface, which results in the potential for each B7 dimer to bind two independent CTLA-4 molecules (**Fig. 2**). As with CTLA-4 and CD28, sequence divergence at the dimer interface may provide a mechanism to prevent the formation of B7-1–B7-2 heterodimers. Due to the considerable sequence variation and lack of direct structural information, it is not possible to predict the oligomeric state or potential oligomerization interfaces for the other costimulatory ligands.

Receptor-ligand structure and costimulatory mechanisms

In the structures of the CTLA-4–B7-1[5] and CTLA-4–B7-2[4] complexes, both CTLA-4 and the B7 isoforms showed very unusual dimer interfaces that placed their respective ligand binding sites distal to the dimer interfaces, suggesting that these molecules might be bivalent. Most strikingly, in both crystal structures the bivalent potential of CTLA-4 and the B7 isoforms were realized by the formation of a periodic, alternating arrangement of CTLA-4 and B7 homodimers, characterized by a ~100-Å repeat that extended throughout the entirety of the crystals (**Fig. 3**). The direct observation of this periodic network provides a potential model for the assembly of these molecules at the T cell–APC interface and suggests several mechanisms that may operate to regulate the detailed organization and compartmentalization of these costimulatory molecules and their associated cytoplasmic signaling molecules at the immunological synapse (**Fig. 3**). The observation of nearly identical networks involving B7-1 and B7-2—in independent crystal structures (that is, different crystal forms, different constructs and different glycosylation states)—argues that the observed features are not a consequence of crystallization artifacts. In addition, it suggests that some aspects of this organization may be extend to other members of the costimulatory receptor–ligand family. Specifically, given the similarities in their ligand recognition sites and modes of dimerization (**Fig. 1**), some characteristics of the organized assembly observed for CTLA-4 may be operative in CD28- and ICOS-associated signaling. Uncertainties regarding the ligand binding sites and the oligomeric states of PD-1 and PD-L preclude a definitive statement about the organization of the PD-1–PD-L complexes.

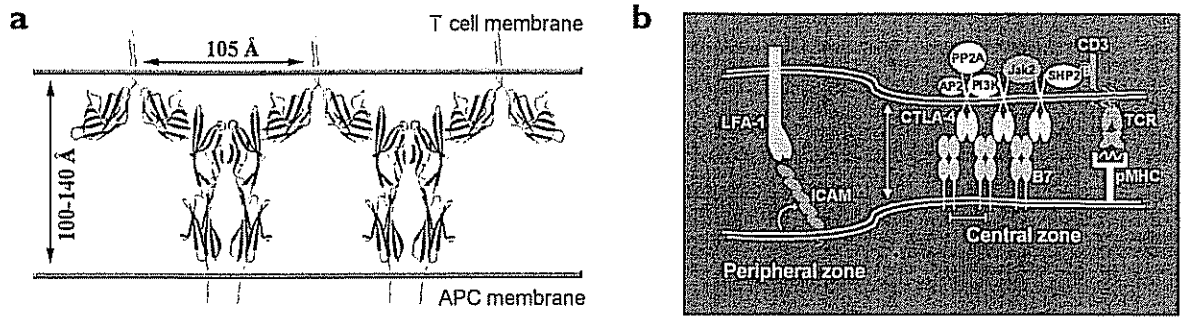


Figure 3. Extended periodic arrays formed by the CTLA-4–B7 complexes. (a) Lattice-like network formed by the CTLA-4–B7-1 complex[5]. This model is very similar to that described for the CTLA-4–B7-2 complex. Both models have very similar organizational features, with inter-dimer spacings of ~100 Å and maximal extents of ~140 Å, which are compatible with the dimensions of other molecules that localize to the central zone of the immunological synapse. Importantly, the differences in glycosylation state (that is, bacterial expression *versus* mammalian expression) suggest that glycosylation is not required to support the CTLA-4–B7 binding interaction. Primary sequence and structural considerations suggest that some features of these organized arrays may extend to other costimulatory receptor-ligand pairs. This figure was prepared with the program Setor[47]. (b) Schematic of the immunological synapse. The central zone contains the TCR-pMHC complex as well as costimulatory receptors (such as CTLA-4) and ligands (B7-1 and B7-2). The peripheral zone is occupied by the substantially larger adhesion proteins and cytoskeletal components. The assembly of the costimulatory receptor-ligand pairs potentially results in both the recruitment and the spatial organization of signaling molecules in the central zone. In the case of CTLA-4–B7 engagement, these signaling molecules include the kinases phosphatidylinositol-3 kinase (PI3K)[68] and Janus kinase 2 (Jak2)[69] as well as the PP2A[70] and SHP2 phosphatases[71]; the latter is thought to attenuate TCR signaling by catalyzing the dephosphorylation of CD3[72]. AP-2 is the clathrin-associated coated pit adaptor protein complex that is involved in trafficking CTLA-4 from intracellular endosomal compartments to the cell membrane[73]. We have shown the adhesion molecule LFA-1 as oriented perpendicular to the plasma membrane and have intentionally tilted its ligand, ICAM, to emphasize that the factors controlling the molecular orientation of intrinsic membrane proteins are not well understood. As the angle between the protein and the membrane becomes more acute (see the red arrow), a smaller intermembrane distance can be accommodated.

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Signaling networks *in vivo*

A primary consideration that has not been addressed is the actual extent of such assemblies at the T cell–APC interface *in vivo*. One extreme case, which mimics the crystallographic observations, is an extensive receptor-ligand assembly that spans a significant extent of the central zone of the immunological synapse. Such a network may promote the organized recruitment of signaling molecules, as the observed ~100-Å repeat that characterizes the CTLA-4–B7 (and perhaps other receptor-ligand) complexes would impose this same distance constraint on noncovalently associated cytoplasmic kinases, phosphatases and scaffolding molecules (**Fig. 3**). This constraint would control the proximity of enzyme-substrate pairs and may thus provide a mechanism to regulate both the frequency and orientation of encounter between enzyme and substrate. In this way biologically optimal (not necessarily maximal) rates of turnover are achieved. Evidence indicates that signals are also transduced through B7 to the APC[41–43], suggesting that this type of network may play a more global role by linking T cell and APC signaling. The localized assembly of such an extended network may serve to decrease the local concentration of other signaling molecules through steric crowding and may also act as a physical barrier to diffusion that prevents entry and exit of molecules from the T cell–APC interface. Finally, the alternating CTLA-4–B7 network is highly reminiscent of the "adhesion zippers" postulated to play a role in the function of adhesion molecules such as the cadherins[44]. This suggests that costimulatory receptor-ligand engagement might provide adhesive interactions needed for a biologically optimal response at the immunological synapse.

Ultimately the types of assemblies present at the immunological synapse depend on the affinities and relative concentrations of the binding partners. For instance, a large excess of either receptor or ligand will favor the formation of "isolated" signaling complexes. In the case of limiting ligand, a cell surface complex composed of two receptor dimers, such as CTLA-4, linked by a single ligand dimer, like B7-1, will be favored. Such an assembly, although perhaps not providing the adhesive features of the extended network described above, would nonetheless still impose the ~100-Å constraint between the two adjacent receptors and any associated cytoplasmic signaling molecules. In contrast, excess ligand would favor complexes composed of a single receptor linking two independent ligand dimers. This association would not enforce any specific spatial relationship between individual receptor molecules. However, it would still direct the localization of the receptor and ligand to the immunological synapse and could result in a sufficiently high local concentration of individual CTLA-4 dimers to support signaling. Equivalent amounts of receptor and ligand at the immunological synapse would favor the formation of longer networks, as observed crystallographically. Importantly, these assembly processes are dynamic equilibria, and a distribution of species and different sized networks would be expected. These considerations are relevant to all cases in which both receptor and ligand are multivalent and may be particularly pertinent to CTLA-4 and CD28, as they compete for the same set of B7 ligands, providing the potential to form complexes containing both CD28 and CTLA-4 dimers. This analysis is likely to be overly simplistic, as it neglects potential mechanisms, such as cytoskeletal involvement, that might influence the efficiency or extent at which the costimulatory receptors and ligands can be transported to and retained at the immunological synapse[45]. Relevant to this point is the finding that only a fraction (~30%) of CD28 molecules appear to undergo lateral diffusion in the plasma membrane[46], which suggests that the majority may not be competent to bind B7 at the immunological synapse.

In addition to providing information describing the lateral organization of the costimulatory receptor–ligand pairs, the CTLA-4–B7 structures also provide constraints that are critical to understanding the relative placement of the T cell and APC plasma membranes at the immunological synapse. The maximal extent of the CTLA-4–B7 complexes are ~100–140 Å, which is consistent with the maximal dimensions of other receptor-ligand pairs that colocalize to the central zone of the immunological synapse, including MHC-TCR[35,47] and CD2-CD58[48]. In contrast, the adhesive molecules present in the peripheral zone, such as the lymphocyte function–associated antigen 1–intercellular adhesion molecule

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1 (LFA-1–ICAM-1) complex, are significantly larger, suggesting that compartmentalization is a consequence of a mechanical sorting mechanism that relies on relative molecular dimension.

Some supporting data for this sorting mechanism have been reported with the murine CD2–CD48 receptor-ligand pair as a model system. By constructing a series of chimeric molecules, in which the maximal dimension of CD48 was systematically altered, it was shown that the expression of molecules substantially larger than other molecular pairs present in the central zone were inhibitory and interfered with TCR–pMHC engagement[49]. Although these observations are consistent with the idea that the dimensions of accessory molecules are crucial for T cell signaling, the premise of a sorting mechanism based solely on molecular dimension requires careful evaluation, as there is a strong bias to view intrinsic membrane proteins as projecting perpendicular to the plane of the plasma membrane. *A priori* there is no fundamental reason for this assumption, and few data directly support this idea for the molecules in either the central or peripheral zones. In particular, it is possible to envision large molecules being accommodated within the central zone by tilting with respect to the plasma membrane. As the angle between the membrane and protein (or receptor-ligand pair) becomes more acute, larger molecular species can be accommodated by a fixed intermembrane separation (**Fig. 3**). In this context, the role of protein surface charge and carbohydrate modification as well as their interactions with the plasma membrane needs to be carefully examined with respect to molecular orientation at the cell surface[50].

Spectrum of receptor-mediated signaling mechanisms

Oligomeric cell-surface receptors use a variety of strategies to transduce signals across the plasma membrane (**Fig. 4**). One common mechanism is that of ligand-induced oligomerization, which typically involves the cross-linking of an extracellular receptor by a soluble multivalent ligand. For example, the growth hormone receptor (GHR) and the majority of the receptor tyrosine kinases (RTKs) all undergo ligand-induced dimerization[1,51] (**Fig. 4**). The GHR and RTKs are composed of monomeric extracellular ligand–binding, transmembrane and cytoplasmic domains. Upon binding of ligand to the RTK, dimerization promotes the close apposition of receptor cytoplasmic kinase domains (or associated kinases as is the case for GHR), leading to their transphosphorylation and activation, which ultimately results in the conveyance of signals to the nucleus.

The bivalent ligands that induce receptor dimerization vary in structure. For example, vascular endothelial growth factor (VEGF) is a disulfide-linked homodimer that, upon engagement of the Flt-1 RTK, results in formation of a two-fold symmetrical 2:2 receptor:ligand complex[52]. In contrast, GH is a monomeric ligand that utilizes two distinct binding surfaces to contact individual GHR monomers, which results in dimerization and formation of an asymmetric 2:1 receptor:ligand complex[51]. A different mechanism has been proposed for the fibroblast growth factor (FGF); it binds the FGF receptor and heparin in a 1:1:1 ternary complex that subsequently assembles to yield the final symmetric 2:2:2 receptor:ligand:heparin complex, which is competent to signal[53].

The erythropoetin and insulin receptors utilize a distinct signaling mechanism that involves the engagement of a monomeric ligand by preformed dimeric receptors[2,3,54]. This binding event directs a structural rearrangement of the extracellular domains that is transmitted to the cytoplasmic domains (or associated kinase domains), which results in their autophosphorylation and the initiation of signal transduction (**Fig. 4**). In contrast to the mechanism of ligand-induced dimerization, the erythropoetin and insulin receptors are preassociated in a specific oligomeric configuration that is incompatible with signaling; it is a structural rearrangement, not a change in oligomeric status, that results in signal transduction. The ligands that affect structural rearrangements in the preformed erythropoetin and insulin receptors use a similar strategy. Like GH, which is described above, they bind to their respective receptors *via* two distinct surfaces, resulting in asymmetric 2:1 receptor:ligand complexes that support signaling. It is important to stress that the erythropoetin and insulin receptors share an overall mechanistic similarity with GHR and the RTKs: individual ligand-bound receptor dimer complexes are competent to transduce signals without the need for higher order aggregation of individual receptor-ligand complexes.

In addition to the ligand-induced clustering and conformational rearrangements discussed above, other mechanisms have been proposed to regulate receptor-mediated signal transduction events. Galectin-1, a lectin specific for β-galactosides, directs the formation of a galectin-glycoprotein lattice on the T cell surface. This interferes with the recruitment of TCRs to the immunological synapse, resulting in down-regulation of T cell activity[55]. Another mechanism for regulating the clustering of cell surface receptors at the immunological synapse is mediated by the molecule agrin, an extracellular proteoglycan[56]. Agrin induces lipid raft aggregation and concomitant coclustering of CD28, CD3, MHC class I and the Lck tyrosine kinase. Interestingly, agrin is also responsible for coclustering acetylcholine receptors, sodium channels and potassium channels at the neuromuscular junction. Although the details of these agrin-associated clustering events are not well understood, the conservation of this clustering mechanism between the immune and nervous systems suggests that it may represent a general modality for regulating receptor-mediated signal transduction.

The structures of the human CTLA-4–B7-1 and CTLA-4–B7-2 complexes reveal a potential signaling mechanism that is quite distinct from those described above. Intermonomer disulfide linkage in the human CTLA-4 homodimer ensures that CTLA-4 is an obligate, preformed dimer and that its oligomeric status is therefore independent of ligand binding. In addition, structural studies have shown that, in contrast to the preassociated dimeric insulin and erythropoietin receptors, binding B7 induces no significant conformational changes in the organization of the CTLA-4 dimer interface or within the individual monomers[4,5]. These observations suggest that CTLA-4 is poised in a productive binding mode before ligand engagement and that signaling may require the cross-linking of at least two adjacent CTLA-4 dimers or perhaps the formation of an extended network of multiple CTLA-4 dimers, as observed crystallographically (**Fig. 4**). This potential mechanism differs from that used by the GH, erythropoetin, insulin and RTK receptors, in which isolated receptor dimers are competent to support signaling. These suggested mechanistic differences are directly related to the overall structural organization of the receptors and ligands. For instance, the placement of the two receptor binding surfaces on erythropoetin and insulin are such that they promote a bidentate interaction with a single receptor dimer, which results in a "closed" receptor-ligand complex that cannot participate in the formation of an extended assembly (**Fig. 4**). In contrast, placement of the respective binding sites in CTLA-4 and B7 dimers precludes the formation of bidentate interactions between individual CTLA-4 and B7 molecules; instead, it promotes the formation of "open" receptor-ligand complexes that potentially support the cross-linking and assembly of multiple CTLA-4 molecules (**Fig. 4**). Again, the relative available concentrations of receptor and ligand must be carefully considered in these situations.

The tumor necrosis factor (TNF) superfamily of receptors—including TNF receptor (TNFR), CD40 and Fas—were previously thought to be monomeric in the unbound state and to transduce signals only

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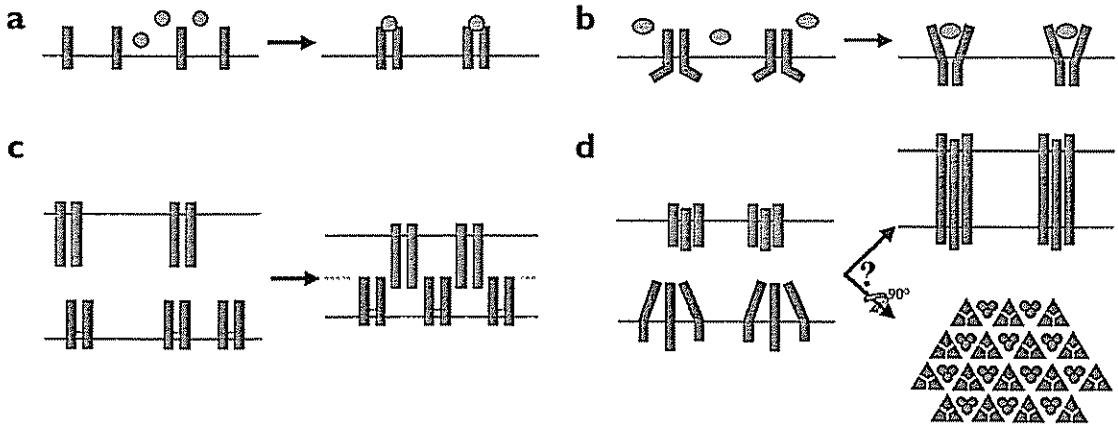


Figure 4. Mechanisms of signal transduction by cell surface receptor-ligand engagement. (a) Ligand-induced dimerization of monomeric receptors. The ligands can be either dimeric, as observed in the VEGF–Flt-1 complex[51], or monomeric and bivalent, as represented by the GH-GHR complex[52]. (b) Ligand-induced conformational change in preformed dimeric receptors is utilized in the erythropoietin–erythropoietin receptor[3] and insulin–insulin receptor complexes[3]. In these cases the ligand is monomeric and bivalent. (c) Ligand-induced ordered clustering of preassociated dimeric receptors leads to a periodic array, as observed for the CTLA-4–B7 complexes[4,5]. (d) The TNF family receptors may utilize two possible mechanisms. The first possibility involves a ligand-induced conformational change in a preformed trimeric receptor alone (top). Alternatively, the conformational change occurs in combination with trimeric ligands that cross-link multiple preformed trimeric receptors; this leads to ordered, two-dimensional receptor superclustering (bottom), which is illustrated from above, looking into the plane of the membrane[6].

upon ligand-induced trimerization by their respective trimeric ligands[57]. This view has been challenged recently by the finding that TNFR1, TNFR2, Fas, TNF-related apoptosis-inducing ligand receptor 1(TRAILR1) and CD40 exist as preassociated oligomers (trimers seem to be the favored species) on the cell surface prior to ligand engagement[6,58,59]. These observations suggest that ligand engagement may result in a conformational rearrangement in individual receptor subunits within preformed receptor complexes, which positions their associated cytoplasmic domains in an optimal orientation for the efficient recruitment of signaling molecules. Alternatively, ligand engagement may direct the formation of higher order "superclusters", in which individual trimeric ligands bridge multiple trimeric receptors, resulting in the formation of an ordered two-dimensional assembly with internal three-fold symmetry (Fig. 4)[6]. Inspection of the crystal structures of the TNF-TNFR[60] and TRAIL-DR5 complexes[61,62] suggest that the formation of these "superclusters" would also require a significant conformational rearrangement in the individual receptor subunits. Superclustering has also been proposed for the symmetric 2:2 Eph receptor (EphR)-ephrin complex[7] on the basis of crystallographic studies, which revealed a periodic two dimensional assembly that extended throughout the crystal.

Not all superclustering events result in the formation of highly symmetric, periodic assemblies. In contrast to the highly ordered networks proposed for CTLA-4–B7[4,5], TNF-TNFR[60] and the EphR-ephrin[7] complexes, the assemblies formed by IgE–IgE receptor complexes are predicted to be less regular, as individual IgE molecules will recognize diverse antigenic epitopes on a single multivalent allergen molecule[64]. This variation in epitope specificity is likely to result in the formation of heterogeneous aggregates of IgE receptors, which suggests less stringent geometric requirements for transducing signals than may be required for CTLA-4, TNFR and EphR.

Concluding remarks

A range of different mechanisms is utilized to transduce extracellular signals across the plasma membrane, including the ligand-associated conformational alteration of cell surface receptors and ligand-induced receptor oligomerization. As highlighted by the structural characterization of the CTLA-4–B7 complex[4,5], the molecular architecture of the molecules that constitute a particular receptor-ligand pair dictate the mechanistic details of a given signal transduction pathway. The complexity of the signaling assemblies that arise may range from simple receptor dimers as observed for growth factor superfamily to extensive heterogeneous clusters of IgE–IgE receptor complexes formed as the consequence of binding multivalent allergen molecules[63]. In instances where both the ligand and receptor are symmetrically multivalent, the opportunity exists for the formation of highly ordered assemblies such as the one-dimensional arrays postulated for the costimulatory molecules[4,5] and the organized two-dimensional arrays suggested for the TNF superfamily[6] and EphR[7]. The various mechanisms of ligand-induced cell surface receptor reorganization results in the assembly of cytoplasmic complexes with the structural and chemical features that support transduction of a biologically optimal signal.

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EXHIBIT 15

Introduction to Protein Structure

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THE COVER

Front: The background photograph of the cover is of a Laue x-ray diffraction pattern produced by a crystal of the plant enzyme ribulose bisphosphate carboxylase. This technique is described in Chapter 17. Information derived from such x-ray patterns, together with a knowledge of the amino acid sequence, enabled the three-dimensional arrangement of atoms in the protein to be determined. A simplified representation of this protein structure is shown in color, superimposed on the diffraction pattern. The enzyme, which is involved in the fixation of carbon dioxide, is a member of the large class of α/β barrel protein structures. This class of structures is discussed in detail in Chapter 4.

Back: Tomato bushy stunt virus is a spherical virus made from 180 protein subunits. Arms extending from sixty of these subunits contribute to an internal framework that determines the size of the correctly assembled virus particle. The interdigitated arms from three subunits meet at each of the twenty icosahedral threefold axes of the virus. One such axis is shown here with the β strands from three subunits shown in different shades of green. Virus structure is described in more detail in Chapter 11.

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ceptor for T cells one of whose principal functions is to recognize and destroy virus-infected cells (Figure 12.1b).

Antibody molecules at the surface of B cells recognize and bind intact soluble molecules or molecules on the surface of invading microorganisms. This stimulates the B cells first to multiply and to differentiate and then to synthesize and to secrete into the blood more copies of the antibody. The soluble antibody molecules bind to the cognate antigen and the complexes are recognized and removed by macrophages.

T cells, by contrast, directly destroy infected cells, and their receptors recognize antigenic determinants only when presented as part of a complex with a third important class of protein molecules, the molecules encoded by the major histocompatibility complex, or MHC, on the surface of cells. MHC molecules are so called because of the part they play in transplant rejection: each individual carries a selected set of MHC molecules on the surface of his or her cells, and the difference in the precise set of MHC molecules carried by the cells of different individuals forms the basis of discrimination between self and nonself. The physiological function of the MHC molecules, however, is to bind to degraded fragments of antigen generated inside infected cells and display them for recognition by T cells.

Thus T-cell receptors exist only on the surface of T cells, and the antigenic determinants they recognize are peptides derived from foreign proteins and complexed with MHC molecules on the surfaces of cells. Antibodies, by contrast, exist both as cell surface and as secreted soluble molecules and interact directly with intact antigens by forming very specific antigen-antibody complexes. Antibodies are the most structurally diverse of all known proteins, and any antigen presented to the immune system triggers the production of many different and specific antibodies.

In this chapter we will see that all three of the molecules directly involved in the specific recognition of antigen—immunoglobulin, the T-cell receptor, and the molecules of the MHC—belong to a family of proteins that seems to have evolved by duplication and diversification from a single ancestral domain. Because antibodies are naturally produced in very large quantities as soluble molecules, the crystal structure of immunoglobulin was solved long before either of the other two molecules could be approached, and hence the presumed ancestral domain structure is known as an immunoglobulin or immunoglobu-

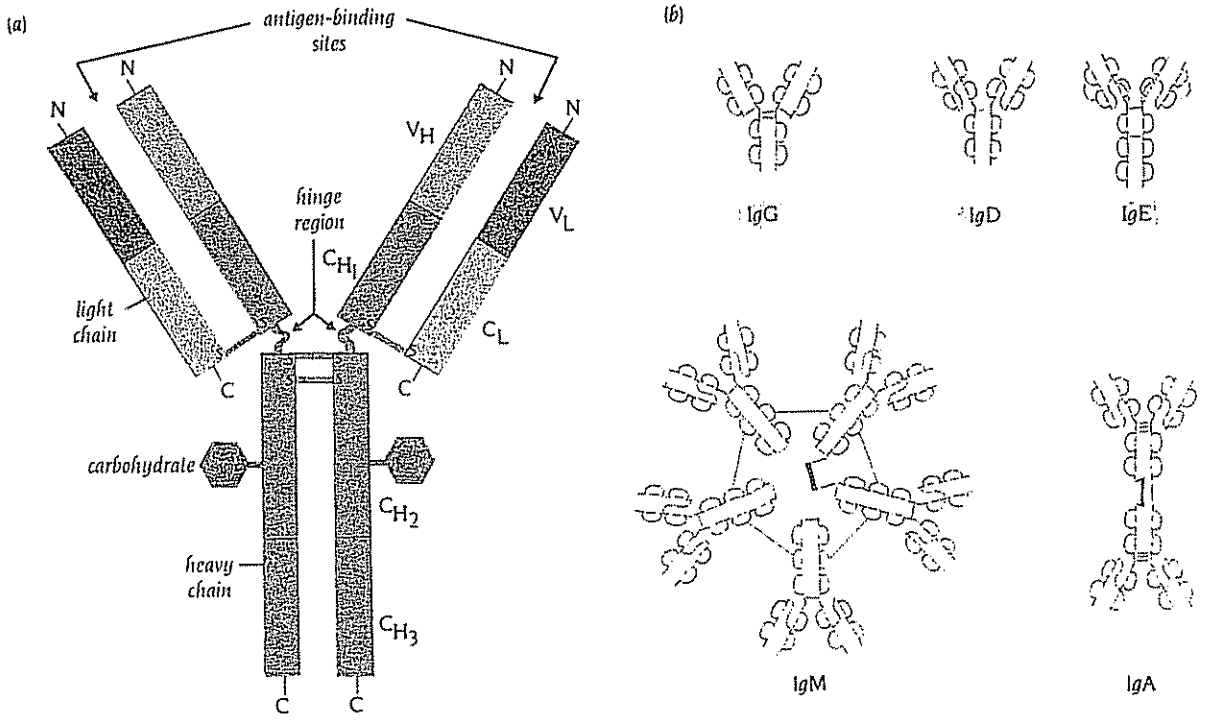


Figure 12.2 (a) The immunoglobulin molecule, IgG, is built up from two copies each of two different polypeptide chains, heavy (H) and light (L). The L chain folds into two domains: V_L with variable sequence between different IgG molecules and C_L with constant sequence. The H chain folds into four domains: one variable V_H and three constant domains, C_{H1}, C_{H2}, and C_{H3}. Disulfide bridges connect the four chains. The antigen binding sites are at the ends of the variable domains. (b) Schematic polypeptide chain structure of different immunoglobulin molecules. IgM is a pentamer where the heavy chains have one variable domain and four constant domains. An additional polypeptide chain, the J chain, (black bar) is associated with the pentameric molecule. The J chain also links two units to form the dimeric IgA. IgG, IgD and IgE are monomeric immunoglobulin molecules.

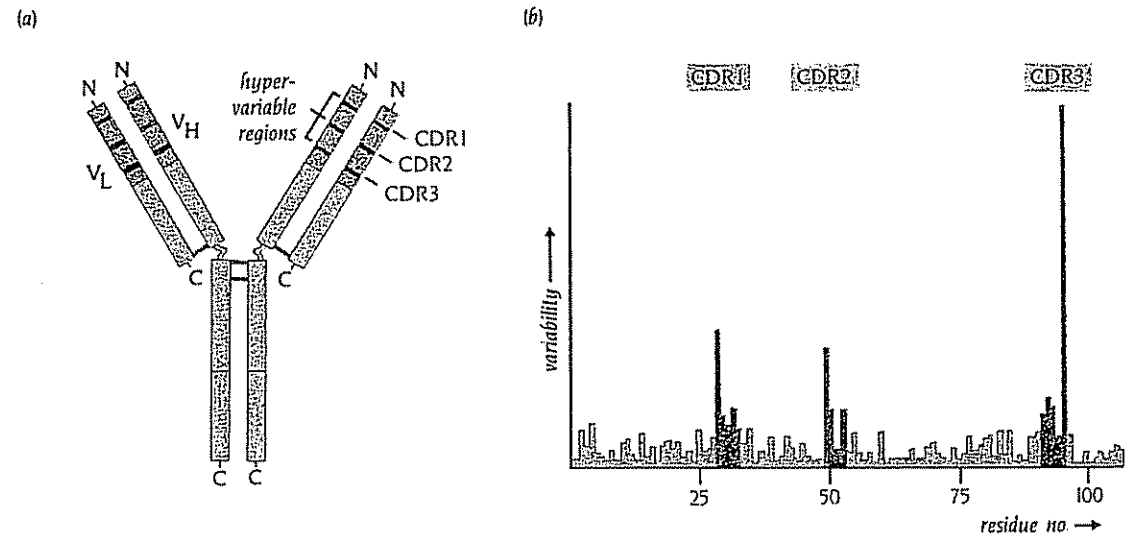


Figure 12.3 Certain regions within the 110 amino acid variable domains show a high degree of sequence variations. These regions determine the antigen specificity and are called hypervariable regions or complementarity determining regions, CDR (a). There are three such regions, CDR1–CDR3, in each variable domain. The sequences of a large number of variable domains in L chains have been compared, and the sequence variability is plotted as a function of residue number along the polypeptide chain in (b). The three large peaks in this diagram correspond to the three hypervariable regions in (a): CDR1–CDR3. [(b) Adapted from T. T. Wu and E. A. Kabat, *J. Exp Med.* 132: 211, 1970.]

lin-like domain. The T-cell receptor has only just become available in soluble form for crystallization, but the crystal structure of an MHC molecule is now known. We examine the three-dimensional structures of antibodies and their bound antigens and discuss the relationship between the genetic basis for the diversification of antibodies and the structural basis for specific recognition. We also describe strategies for the design of novel antibodies including antibodies with enzymatic function. Finally, we examine the structure of an MHC molecule and show that the structural principles for recognition and binding of antigens to MHC are quite different from those of antibodies and that they reflect the special requirement for co-recognition of MHC and antigen by the T-cell receptor.

The polypeptide chains of antibodies are divided into domains

The basic structure of all immunoglobulin (Ig) molecules comprises two identical light chains and two identical heavy chains linked together by disulfide bonds (Figure 12.2a). There are two different classes, or isotypes, of light chains, λ and κ; there is no known functional distinction between light-chain isotypes. Heavy chains, by contrast, have five different isotypes that divide the immunoglobulins into different functional classes, IgG, IgM, IgA, IgD, and IgE (Figure 12.2b), each with different effector properties in the elimination of antigen. Each class of heavy chains can combine with either of the two different classes of light chains.

Immunoglobulins of class G, IgG, which is the major type of immunoglobulin in normal human serum and which we will discuss in this chapter, are monomers of the basic unit. Each chain of an IgG molecule is divided into domains of about 110 amino acid residues. The **light chains** have two such domains, and the **heavy chains** have four.

The most remarkable feature of the antibody molecule is revealed by comparison of the amino acid sequences from many different immunoglobulin IgG molecules. This comparison shows that the amino-terminal domain of each polypeptide chain is highly variable whereas the remaining domains have constant sequences for each class. A light chain is thus built up from one amino-terminal variable domain (V_L) and one carboxy-terminal constant domain (C_L) and a heavy chain from one amino-terminal **variable domain** (V_H), followed by three **constant domains** (C_{H1}, C_{H2}, and C_{H3}).

The variable domains are not uniformly variable throughout their lengths: in particular, three small regions show much more variability than the rest of the chain; they are called hypervariable regions or **complementarity determining regions**, CDR1–CDR3 (Figure 12.3). They vary both in size and in

EXHIBIT 16

EXHIBIT 16
REDACTED IN ITS ENTIRETY

EXHIBIT 17

EXHIBIT 17
REDACTED IN ITS ENTIRETY

EXHIBIT 18

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DIFFERENTIAL REGULATORY SIGNALS DELIVERED BY ANTIBODY BINDING TO THE CD28 (Tp44) MOLECULE DURING THE ACTIVATION OF HUMAN T LYMPHOCYTES

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Molecule CD28 (Tp44) is expressed on the surface of majority of human T cells and has been implicated to play an active role in the regulation of T cell growth. The present study examines the effect of antibody binding to the CD28 molecule during T cell activation. Anti-CD28 but not isotype-matched anti-CD5 mAb consistently augmented anti-CD3-induced and IL-2-induced T cell proliferation and subsequent release of soluble CD25 molecule. When added together, mAb anti-CD28 and anti-CD5 acted synergistically to cause 2- to 7-fold enhancement of T cell activation induced by anti-CD3 mAb or IL-2 with no effect on the development of non-MHC-restricted IL-2-activated killer T cells. In contrast, alloantigen-induced T cell proliferation, soluble CD25 release, and the subsequent development of CTL were all inhibited by anti-CD28 mAb. Moreover, alloantigen-induced proliferative response of both $CD4^+$ and $CD8^+$ T cells was inhibited by anti-CD28 without affecting the cytolytic effect of CTL. Because valency of anti-CD28 binding has been implicated as an important factor in signal transduction, this was explored in the allogeneic MLR by using Fab and $F(ab')_2$ fragments of anti-CD28 mAb and anti-mouse κ mAb. The inhibitory effect of anti-CD28 mAb in the MLR was reversed by cross-linking of anti-CD28 mAb with anti-mouse κ mAb. In addition, cross-linking of the CD28 molecule on alloactivated T lymphoblasts but not that on resting T cells with anti-CD28 and anti-mouse κ induced their proliferation in the absence of the priming alloantigen. These results indicate that stimulatory or inhibitory signals delivered through the CD28 molecule are determined by the degree of cross-linking of this molecule. In addition, these results also suggest that Ag-induced CD3-TCR-mediated T cell responses are more dependent on signals delivered through the CD28 molecule than those induced with anti-CD3, and thus these results have implications for potential use of anti-CD28 in sustained propagation of Ag-specific T cells.

Human T cells utilize the CD3[2]-associated TCR complex during the specific recognition of Ag in the context of products of self MHC genes expressed on the surface of Ag-presenting cells (1–8). In addition to its cognitive function, the CD3-TCR complex also participates in the transmission of signals inside the T cells to initiate their activation (3–7). IL-2 stimulates human T lymphocytes to proliferate in the absence of external stimulus (9, 10) and induces their differentiation into potent MHC-unrestricted cytotoxic T cells with a broad spectrum antitumor cytolytic effect (11–15). The development of these IAK T cells does not appear to involve the CD3 complex and is proportional to the IL-2 concentration in culture (14, 15) (N. K. Damle, unpublished observation). Ag-independent activation of resting T cells with high concentrations of IL-2 is thought to be initiated via interaction of IL-2 with the intermediate affinity non-CD25 IL-2R (p70–75) (16–20) that are present in small numbers on resting lymphoid cells (20). In addition to the CD3-TCR complex, other Ag-nonspecific structures on the surface of T cells that transmit stimulatory signals have been identified, including molecules CD2, CD5 and CD28 (21–31). Of these the CD5 molecule has been reported to transmit only positive regulatory (stimulatory) signals (22, 23), whereas CD2, CD3 and CD28 molecules have all been reported to transmit both positive and negative regulatory (inhibitory) signals in certain experimental situations (32). In the case of molecule CD3, the distinction between positive and negative signals may depend upon the internalization of the CD3 complex (33), whereas the stimulatory vs inhibitory activity of antibody binding to the CD2 molecule is thought to be dependent upon the CD2 epitope recognized (33–36).

The CD28 molecule (Tp44), although not associated with the CD3-TCR complex (26, 27, 29), can cooperate with the CD3-TCR-directed stimulation to induce IL-2 production and cellular proliferation (26–30). In some reports, low levels of proliferation were observed after anti-CD28-induced stimulation alone (27). However, we originally reported blocking of the autologous and allogeneic MLR with anti-CD28 (24), an effect now seen by others by using Ag-specific (*Mycobacterium leprae*) class II MHC restricted T cell clones (31). Here, we report experiments designed to investigate the function of the CD28 molecule during T cell activation and functional differentiation induced with alloantigen, anti-CD3 mAb or IL-2. Our results suggest that the degree of cross-linking of the CD28 molecule controls the inhibitory vs stimulatory activity of anti-CD28 mAb, since monovalent Fab fragments of anti-CD28 mAb consistently inhibit rather than synergize with the primary stimulus,

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[2] Abbreviations used in this paper: CD, cluster designation; IAK, IL-2-activated killer; MNC, mononuclear cells; CM, culture medium.

whereas additional cross-linking of the CD28 molecule with anti-mouse κ mAb converts inhibitory activity of anti-CD28 to stimulatory activity in the allogeneic MLR. These results further emphasize the heterogeneity within the signals transmitted through the CD28 molecule during the growth of T cells dictated by the nature of the stimulus without affecting the differentiation process.

MATERIALS AND METHODS

CM. Complete CM consisted of RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with 25 mM HEPES (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 5×10^{-5} M 2-ME (Sigma), and 15% heat-inactivated pooled normal human serum (New York Blood Center, New York, NY).

mAb. Hybridomas OKT1 (IgG2a anti-CD5), OKT3 (IgG2a anti-CD3), OKT4 (IgG2a anti-CD4), OKT8 (IgG2a anti-CD8), OKM1 (IgG2b anti-CD11b), and 187.1 (rat IgG1 anti-mouse κ) were obtained from the American Type Culture Collection (Rockville, MD) and ascitic fluids from these hybridomas were generated in pristane-primed BALB/c mice. mAb 10.2 (IgG2a anti-CD5) produced in BALB/c mice and 9.3 (IgG2a anti-CD28) produced in (BALB/c × C57BL/6)F1 mice have been described before (24, 37–41). mAb L17F12 (IgG2a anti-CD5) (42) was generously provided by Dr. Edgar G. Engleman, Stanford University Medical Center, Stanford, CA. Each of the above antibodies recognizing human lymphoid antigens is an IgG2/κ molecule. Antibodies were purified on protein A-Sepharose columns, dialyzed extensively and filtered through 0.45 μm filters prior to their use in cell cultures. Rat anti-mouse κ mAb 187.1 was affinity-purified from culture supernatants of hybridoma 187.1 by using Sepharose-conjugated mouse Ig affinity column. Fluorescein-conjugated or phycoerythrin-conjugated mAb directed against various lymphoid surface molecules were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA.

Separation of peripheral blood T lymphocytes. Peripheral blood MNC from healthy normal donors (Stanford Blood Bank, Palo Alto, CA) were separated by using Ficoll-Hypaque (LSM, Litton Bionetics, Kensington, MD) density gradient centrifugation. Adherent MNC were separated from nonadherent MNC by adherence to plastic at 37°C for 45 min. T cells were purified from nonadherent MNC by a rosetting method (43) with 2-aminoethylisothiouronium bromide hydrobromide (Sigma)-treated SRBC. The rosetted T cells were freed of SRBC by hypotonic lysis of the latter. The E-rosetted T cells were further separated into low density large granular cells and small high density agranular T cells by using discontinuous Percoll gradient centrifugation with isotonic 45% Percoll as described before (15). Only high density T cells (>95% $CD2^+CD3^+CD5^+$ $CD16^-CD20^-DR^-$) were used in the experiments reported in this paper.

Isolation of subpopulations of human T cells. Subpopulations of human T cells defined by binding of mAb were isolated by a panning technique (44) that initially permitted separation of mutually exclusive subpopulations of human T cells into $CD4^+/CD4^-$ or $CD8^+/CD8^-$ subsets. The purity of both positively (bound to pans) and negatively (unbound to pans) selected subpopulations was determined by either direct or indirect immunofluorescence analysis on a FACS II equipped with Consort 40 data analysis system (Becton Dickinson). If OKT8 (anti-CD8) mAb was used as the first stage incubating antibody then >95% of the bound cells and <5% of the unbound cells were $CD8^+$. Similar subset purity was obtained when OKT4 (anti-CD4) mAb was used as the first stage reagent. T cells were also separated into $CD11b^+/CD11b^-$ subpopulations by panning with OKM1 (anti-CD11b) mAb with similar purity. Almost all of $CD11b^-$ T cells (>95%) and <3% of $CD11b^+$ T cells expressed the CD28 molecule on their surface. Similarly the vast majority of $CD28^-$ T cells expressed the CD8 and CD11b molecules on their surface. Each of the above subpopulations was examined for the presence of the T cell-specific CD3 complex with fluorescein-conjugated anti-Leu4 (CD3) mAb and was found to always include >95% $CD3^+$ cells.

Proliferative responses of T cells. Fifty thousand T cells were cultured with 1×10^4 irradiated (5000 R from a ^{137}Cs source) OKT3 hybridoma cells or HLA-DRW6$^+$ EBV-transformed B cell line, Arent, or with either 100 pM or 1 nM rIL-2 ($des\text{-}ala_1\text{-}ser_{125}$-IL-2, Cetus Corporation) in 0.2 ml CM in round-bottom microtiter wells in a humidified 5% CO_2 and 95% air atmosphere. Triplicate cultures were pulsed with 1 μCi/well of [^{3}H]thymidine (6.7 Ci/mM, New England Nuclear, Boston, MA) 16 h before harvesting of cells for measurement of radiolabel incorporated into newly synthesized DNA. The results are expressed as cpm ± SEM.

Assay for soluble IL-2R. The soluble IL-2R (CD25) molecules in the culture supernatants were quantitated by an enzyme immunoassay with "Cellfree" IL-2R test kit (T Cell Sciences, Cambridge, MA) that has been designed for the quantitative determinations of cellfree IL-2R. Undiluted culture supernatant of an IL-2-dependent T cell line was assigned a value of 1000 IL-2R U/ml and was provided as a part of the IL-2R detection kit. The details of the detection of soluble IL-2R with enzyme-immunoassay have been described before (45–47).

Generation of CTL in the allogeneic MLR. To develop alloantigen-primed CTL in the allogeneic MLR, 4×10^6 $CD3^+CD11b^-$ T cells were cultured with 2×10^6 irradiated allogeneic EBV-transformed $CD28^-$ B cells (Arent) in 4 ml of CM in 17 × 100 mm culture tubes (No. 2051, Falcon). Seven days later, viable cells from these cultures were isolated on Ficoll-Hypaque gradients, washed extensively with PBS, and resuspended at a concentration of 2.5×10^6/ml in CM to examine for their cytolytic effect on Arent and Daudi targets.

Generation of LAK T cells. To activate precursors of LAK T cells, small high density resting $CD11b^-$ ($CD28^+$) T cells were suspended at a concentration of 1×10^6/ml in CM and stimulated for 5 days in 17 × 100 mm round-bottom culture tubes (No. 2051; Falcon) (14, 15). Upon completion of the culture period, cultured T cells were washed extensively with PBS and were resuspended at a concentration of 2.5×10^6/ml in CM prior to analysis of their cytolytic activity against NK-resistant Daudi target cells. Five-day culture of $CD28^+$ T cells with IL-2 did not have any effect on the $CD28^+$ phenotype of these cells (>90% $CD3^+CD5^+CD28^+$).

Cytotoxicity assay. The cytotoxicity assay was performed in triplicate in 96-well microtiter plates (Corning) by adding effector T cells to 5×10^5 [^{51}Cr]-labeled target cells at 4 or 5 different E/T ratios ranging from 1:1 to 50:1 in a final volume of 0.2 ml of CM. For the spontaneous release control, a sample of labeled target cells was resuspended in CM alone. The cytotoxicity assays were always initiated by centrifugation of the assay plates at 50 × *g* for 4 min. After a 4-h incubation period at 37°C in a humidified 5% CO_2 atmosphere, supernatants were harvested with a Skatron-Titertek harvester. The presence of ^{51}Cr in harvested supernatants was enumerated with a gamma counter. Maximum release of ^{51}Cr from target cells was obtained by mixing 5×10^3 labeled target cells in 0.1 ml of CM with 0.1 ml of 10% Nonidet P-40 in CM. Background release was usually less than or equal to 10% of the total release.

Percent specific ^{51}Cr release was calculated using the following equation:

$$\text{Percent specific release} = \frac{\text{Experimental release} - \text{spontaneous release}}{\text{Total release} - \text{spontaneous release}} \times 100.$$

LU. Cytotoxic activity was expressed as LU per 10^6 effector cells by using a modification (48) of the original units defined by Henney (49). This system allows calculation of a single reproducible value for a large number of specimens over a wide range of E/T ratios. One LU is defined as that entity capable of causing specific lysis of one target cell over a 4-h incubation period. LU were calculated by using the following equation:

$$\text{LU} = \frac{-A \times \ln(1 - f)}{B},$$

in which A is the number of targets, B is the number of effector T cells used, and f is the fraction of target cells specifically lysed (e.g., 0.65, which is the same as 65% specific lysis). This unit of cytotoxicity is directly proportional to the number of cytotoxic T cells that induce up to 80% target cell lysis. The data were tabulated for all E/T ratios and were computed by using RPL/RS-1 language (BBN Research System, Cambridge, MA) on a VAX computer (Digital Equipment Corporation, Santa Clara, CA).

Statistical analysis was performed by paired or unpaired *t*-test.

RESULTS

Enhancement of T cell activation by anti-CD28 mAb. Stimulation of T cells with soluble anti-CD3 mAb requires the interaction of the Fc portion of anti-CD3 mAb with the FcR on monocytes (5, 8, 50, 51). To avoid the requirement for monocytes, irradiated (5000 R) OKT3 (anti-CD3) hybridoma cells were used as anti-CD3-presenting cells. Irradiated OKT3 cells exhibit minimal IgG secretion while retaining the cell surface expression of anti-CD3 mAb (C. D. Platsoucas, and N. K. Damle, unpublished observation). Such irradiated OKT3 cells by virtue of their ability

to interact through the membrane-associated antibody with the CD3 complex on T cells cause IL-1-independent polyclonal activation of T cells in the absence of monocytes with the peak proliferative response observed between day 3 and 5 of culture (52) (N. K. Damle, unpublished). Similarly, T cells can also be activated directly by culturing with IL-2 in the absence of any exogenous stimulus (mitogens or Ag) (9, 10). In the IL-2-stimulated cultures the peak response is usually observed on day 6 of culture whereas in T cell cultures stimulated with allogeneic non-T cells the peak response is usually observed on day 7 of culture.

The effect of anti-CD5 and anti-CD28 mAb on the proliferative responses of small resting CD11b$^-$ T cells (CD3$^+$CD28$^+$) (41) was examined (Table I). In these cultures anti-CD28 mAb (IgG2a) caused significant ($p < 0.01$) enhancement in the T cell response to either OKT3 cells. In contrast, anti-CD5 mAb (also IgG2a) occasionally caused modest enhancement in the T cell response that was not always statistically significant. However, when added together to cultures anti-CD5 and anti-CD28 mAb (1 μg/ml each) acted synergistically and significantly augmented ($p < 0.001$) anti-CD3-induced proliferative response of T cells. In addition to anti-CD3-stimulated T cell proliferation, the positive synergistic effects of anti-CD28 and anti-CD5 mAb were also observed on IL-2-induced T cell proliferation. Our previous studies and also those of others have shown that purified IgG2a mAb to other T cell surface structures such as CD4, CD6, CD8, and HLA-A,B,C do not augment T cell proliferation (28–30) (N. K. Damle, unpublished results).

Upon activation T cells release soluble IL-2R (CD25) molecules in the culture supernatants and thus the presence of soluble CD25 molecules in supernatants is considered to be a reliable indicator of T cell activation (45–47). Therefore, the ability of IL-2-stimulated or anti-CD3-stimulated T cells to release soluble CD25 molecules in the presence of 1 μg/ml of anti-CD28, anti-CD5, or anti-CD5 and anti-CD28 together was examined (Table II). The results showed significant increase ($p < 0.01$) in the levels of soluble CD25 molecules in the culture supernatants of T cells stimulated in the presence of both anti-CD5 and anti-CD28 mAb over that obtained in the presence of anti-CD28 alone. Addition of anti-CD5 to T cell cultures did not result in the increase in the released soluble CD25 molecules over that obtained in the absence of either mAb. Furthermore, T cells cultured with anti-CD28 and anti-CD5 mAb in the absence of any other stimulus released the CD25 molecule to the same level as that induced with culture medium alone and was usually ≤25 soluble CD25 U/ml. Thus, mAb anti-CD28 either alone or with anti-CD5 appear to exert enhancing influence on the activation of T cells only when induced by primary stimuli such as anti-CD3 or IL-2.

Inhibition of allogeneic MLR by anti-CD28 mAB. The effect of mAb anti-CD5 and anti-CD28 on the proliferative response of and subsequent release of soluble CD25 molecules by T cells activated in allogeneic MLR was examined. Table III shows that anti-CD28 caused significant inhibition of alloantigen-induced activation of T cells as reflected by reduced proliferative response in the MLR and also the reduced levels of soluble CD25 molecules released in the culture supernatants in the presence of anti-CD28. Similar presence of anti-CD5 mAb in the MLR had no significant inhibitory or enhancing effect on the activation of T cells nor did it augment the inhibitory effect of anti-CD28 mAb on the MLR response of T cells. These results indicate that the binding of mAb to the CD28 molecule delivers growth-inhibiting signals in alloactivated T cells in contrast to growth-promoting signals in anti-CD3-stimulated or IL-2-stimulated T cells.

Proliferation of T cells in the MLR results in the development of CTL. Our previous studies have shown that CD8$^+$CD28$^+$ but not CD8$^+$CD28$^-$ T cells exhibit the CTL activity upon activation in the MLR (34). Thus, the development of CTL in the MLR in the presence of anti-CD28 or anti-CD5 mAb was examined. As shown in Table IV, the presence of anti-CD28 mAb regardless of the presence of anti-CD5 mAb consistently inhibited ($p < 0.001$) the development of CTL in the allogeneic MLR. The inhibitory effect of anti-CD28 mAb on the development of CTL in the MLR could be due to the inhibition of clonal expansion (proliferation) of CD8$^+$CD28$^+$ precursors of CTL in addition to that of CD4$^+$CD28$^+$ helper/inducer cells that provide the necessary growth- and differentiation-inducing factors such as IL-2.

TABLE I

Proliferative response of T cells induced with anti-CD3 or IL-2

Stimulus[a]	[^{3}H]Thymidine Incorporation (cpm ± SEM × 10^{-3})			
	Medium	Anti-CD28	Anti-CD5	Anti-CD5 + anti-CD28
Expt. 1				
Day 3				
IL-2 100 pM	4.4 ± 0.1	10.5 ± 0.7	4.8 ± 0.4	14.1 ± 0.5
IL-2 1 nM	9.3 ± 0.2	13.4 ± 0.2	10.0 ± 1.0	19.8 ± 0.5
Anti-CD3 mAb	24.8 ± 1.8	33.8 ± 1.6	29.1 ± 2.0	46.8 ± 2.0
Day 6				
IL-2 100 pM	29.5 ± 2.8	57.3 ± 4.1	36.9 ± 2.9	98.1 ± 5.9
IL-2 1 nM	36.6 ± 3.0	49.4 ± 1.1	39.2 ± 5.0	83.9 ± 8.4
Anti-CD3 mAb	3.5 ± 0.6	11.5 ± 0.5	6.0 ± 0.9	22.5 ± 0.8
Expt. 2				
Day 3				
IL-2 100 pM	1.6 ± 0.1	6.3 ± 0.3	2.0 ± 0.1	9.4 ± 0.3
IL-2 1 nM	3.1 ± 0.2	8.4 ± 0.4	3.9 ± 0.4	14.9 ± 0.8
Anti-CD3 mAb	38.3 ± 2.1	45.3 ± 1.7	41.7 ± 0.9	58.1 ± 1.2
Day 6				
IL-2 100 pM	22.2 ± 3.4	34.2 ± 0.4	23.8 ± 1.2	39.9 ± 2.6
IL-2 1 nM	35.2 ± 2.3	47.6 ± 4.2	36.6 ± 2.0	50.2 ± 3.5
Anti-CD3 mAb	2.2 ± 0.5	4.7 ± 0.6	3.0 ± 0.2	12.4 ± 1.9

[a] Small high density CD11b$^-$ T cells (5 × 10^4) in 0.2 ml of CM were stimulated with either 100 pM IL-2 or 1 nM IL-2 or with 1 × 10^4 irradiated OKT3 (anti-CD3) hybridoma cells in the presence of 1 μg/ml of either anti-CD28 mAb, anti-CD5 mAb, or both. Proliferative responses in these cultures were measured on day 3 and day 6 of culture.

TABLE II

Release of soluble IL-2R (CD25) molecules after the activation of T cells

Stimulus[a]	Soluble IL-2R (CD25) (U/ml)			
	Expt. 1		Expt. 2	
	Day 3	Day 6	Day 3	Day 6
Anti-CD3				
No mAb	110	160	120	140
Anti-CD28	290	340	320	360
Anti-CD5	160	200	160	200
Anti-CD5 + anti-CD28	390	590	640	900
IL-2 100 pM				
No mAb	25	110	25	150
Anti-CD28	25	260	44	530
Anti-CD5	25	130	25	180
Anti-CD5 + anti-CD28	25	410	82	1200
IL-2 1 nM				
No mAb	25	110	40	220
Anti-CD28	40	220	60	570
Anti-CD5	25	140	40	300
Anti-CD5 + anti-CD28	80	400	110	1400

[a] Small high density CD11b$^-$ T cells (5×10^4) in 0.2 ml of CM were stimulated with 1×10^4 irradiated OKT3 (anti-CD3) hybridoma cells or with either 100 pM IL-2 or 1 nM IL-2 in the presence of 1 μg/ml of anti-CD28 mAb, anti-CD5 mAb, or both. Soluble IL-2R (CD25) molecules released in the supernatant were measured on day 3 and 6 of culture. T cells cultured alone for 6 days in CM or with anti-CD5 and anti-CD28 mAb in the absence of any stimulus released less than 25 soluble IL-2R U/ml.

TABLE III

Inhibition of the allogeneic MLR by anti-CD28 mAb

Stimulus[a]	[^{3}H]Thymidine incorporation (cpm ± SEM $\times 10^{-3}$)		Soluble CD25 (U/ml) Day 7
	Day 3	Day 7	
Expt. 1			
Medium alone	0.5 ± 0.02	0.8 ± 0.1	25
Alloantigen	25.1 ± 0.2	291.9 ± 12.0	1630
Alloantigen + anti-CD28 mAb	17.5 ± 1.2	145.2 ± 10.2	840
Alloantigen + anti-CD5 mAb	22.9 ± 0.2	274.9 ± 2.2	1480
Alloantigen + anti-CD5 mAb + anti-CD28 mAb	16.5 ± 1.2	150.1 ± 7.2	760
Expt. 2			
Medium alone	0.5 ± 0.1	0.6 ± 0.1	25
Alloantigen	21.1 ± 1.7	166.9 ± 12.4	1380
Alloantigen + anti-CD28 mAb	11.1 ± 0.1	85.4 ± 9.2	720
Alloantigen + anti-CD5 mAb	19.1 ± 0.8	161.6 ± 4.8	1390
Alloantigen + anti-CD5 mAb + anti-CD28 mAb	14.0 ± 0.3	81.5 ± 1.6	670

[a] Small high density CD11b$^-$ T cells (5×10^4) were cultured with 1×10^4 irradiated allogeneic CD28$^-$ B cells (EBV-transformed B cell line, Arent) in the presence of 1 μg/ml of either anti-CD28 mAb, anti-CD5 mAb, or both. Proliferative response in these cultures was measured on day 3 and day 7 of culture and soluble CD25 (IL-2R) molecules released in the supernatants were measured on day 7 of culture.

Anti-CD28 mAb does not affect development of IAK cells. Addition of anti-CD28 mAb to cultures of T cells stimulated with IL-2 or allogeneic non-T cells results in enhancement and inhibition of the T cell proliferative responses, respectively. In addition, anti-CD28 also inhibit the development of the CTL induced in the MLR. Although 100 pM IL-2 causes polyclonal proliferation of T cells such a stimulation does not usually result in the development of IL-2-activated (IAK) T cells. On the other hand, at 1 nM IL-2 T cells proliferate and further differentiate into IAK T cells (14, 15). Taking into account anti-CD28 mAb-mediated augmentation of IL-2-induced T cell activation, the effect of anti-CD28 mAb on the development of IAK T cells was examined. Thus, CD3$^+$CD28$^+$ T cells were cultured for 5 to 6 days with IL-2 in the presence of 1 μg/ml of anti-CD28 mAb either alone or with 1 μg/ml of anti-CD5 mAb after which their ability to lyse NK-resistant Daudi target cells was examined. As shown in Table V neither anti-CD28 mAb nor a combination of anti-CD28 and anti-CD5 mAb together modulated the development of IAK T cells. The above IAK activity derived from CD3$^+$CD28$^+$ T cells was not due to the outgrowth of contaminating CD28$^-$ cells and was depletable with anti-CD28 and C. Thus, anti-CD28 mAb appears to modulate the growth phase of T cells rather than their differentiation into functionally mature effector cells.

TABLE IV

Effect of anti-CD5 and anti-CD28 mAb on the development of CTL in allogeneic MLR

T Cells Cultured with[a]	LU ($\times 10^{-3}$)/10^6 Effector Cells in Expt:			
	1	2	3	4
Allogeneic B cells	98.0	39.0	54.0	62.0
Allogeneic B cells + anti-CD28 mAb	7.3	8.0	10.0	11.0
Allogeneic B cells + anti-CD5 mAb	70.0	40.0	50.0	54.0
Allogeneic B cells + anti-CD5 mAb + anti-CD28 mAb	3.3	4.1	10.0	9.0
Anti-CD5 mAb + anti-CD28 mAb	0.3	0.6	0.5	1.0

[a] CD11b$^-$ T cells were cultured for 7 days with or without irradiated allogeneic CD28$^-$ B cells (Arent) in the presence of 1 μg/ml of either anti-CD28 mAb, anti-CD5 mAb, or both mAb before the assessment of their cytotoxic activity.

TABLE V

Effect of anti-CD28 and anti-CD5 mAb on development of IAK

mAb added[a]	LU ± SEM ($\times 10^{-3}$)/10^6 Effector T Cells Activated with	
	100 pM IL-2	1 nM IL-2
Expt. 1		
No mAb	11.0 ± 3.7	36.5 ± 4.4
Anti-CD28 mAb	8.0 ± 0.4	34.2 ± 4.6
Anti-CD5 mAb	10.0 ± 2.0	31.2 ± 4.6
Anti-CD5 mAb + anti-CD28 mAb	8.0 ± 2.6	35.3 ± 4.8
Expt. 2		
No mAb	8.0 ± 2.6	27.3 ± 3.8
Anti-CD28 mAb	9.0 ± 1.0	27.8 ± 4.8
Anti-CD5 mAb	13.0 ± 5.0	26.9 ± 1.3
Anti-CD5 mAb + anti-CD28 mAb	10.0 ± 2.5	28.1 ± 4.1
Expt. 3		
No mAb	3.4 ± 0.4	46.4 ± 0.8
Anti-CD28 mAb	3.1 ± 0.5	49.9 ± 5.1
Anti-CD5 mAb	2.9 ± 0.3	51.7 ± 2.6
Anti-CD5 mAb + anti-CD28 mAb	3.9 ± 1.8	45.8 ± 5.0

[a] Small high density CD11b$^-$ T cells (3×10^5) were cultured for 5 days with either 100 pM IL-2 or 1 nM IL-2 in the presence of 1 μg/ml of either anti-CD28 mAb, anti-CD5 mAb, or both after which their ability to lyse NK-resistant Daudi target cells was examined. T cells cultured alone without IL-2 but with both anti-CD5 and anti-CD28 mAb had no cytolytic effect on Daudi target cells (1000 LU/10^6 effector cells).

Fab fragments of anti-CD28 mAb inhibit T cell activation. The role of the valency of anti-CD28 mAb in its ability to modulate the T cell activation was examined. Thus, Fab fragments, F(ab')$_2$ fragments or the intact anti-CD28 mAb were added to T cell cultures stimulated with either irradiated OKT3 hybridoma cells or allogeneic B cells and the proliferative responses in these cultures were examined. As shown in Table VI, F(ab')$_2$ fragments or intact IgG of anti-CD28 mAb augmented anti-CD3-induced proliferative response in a dose-dependent manner and inhibited alloantigen-induced proliferative re-

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TABLE VI

Effect of Fab and F(ab')$_2$ fragments of anti-CD28 mAb on activation of T cells

Anti-CD28 Added[a]	[^{3}H]Thymidine Incorporation (cpm × 10^{-3})			
	Expt. 1		Expt. 2	
	Anti-CD3	Alloantigen	Anti-CD3	Alloantigen
No mAb	15.5	12.4	50.5	19.7
Fab fragments (μg/ml)				
0.1	9.6	10.5	33.4	11.5
1.0	10.0	8.6	26.8	11.2
10.0	8.9	7.2	24.9	9.9
F(ab')$_2$ fragments (μg/ml)				
0.1	19.2	10.2	70.8	14.9
1.0	31.8	9.2	86.6	12.5
10.0	33.1	6.5	99.5	9.9
Intact IgG (μg/ml)				
0.1	17.9	9.5	75.2	16.5
1.0	31.3	8.2	90.1	12.3
10.0	34.2	6.9	95.5	10.6

[a] CD11b$^-$ T cells (5×10^4) were stimulated with 1×10^4 of either irradiated OKT3 (anti-CD3) hybridoma cells or irradiated allogeneic CD28$^-$ B cells (Arent) in the presence of anti-CD28 mAb or its Fab or F(ab')$_2$ fragments. Proliferative responses in these cultures were studied on day 3 of anti-CD3-stimulated cultures and day 7 of alloantigen-stimulated cultures. The SEM of these cultures was always <15% of the mean.

sponse of T cells. In contrast, Fab fragments of anti-CD28 mAb inhibited in a dose-dependent manner both anti-CD3-induced and alloantigen-induced proliferative responses of T cells. Similar results were obtained with IL-2-stimulated system which is purely accessory cell-independent system (data not shown). These results indicate that monovalent binding of anti-CD28 mAb always delivers growth-inhibitory signals during the activation of T cells. On the other hand, bivalent binding of anti-CD28 mAb to the CD28 delivers either growth-inhibitory or growth-augmenting signals during the activation of T cells depending on the nature of the stimulus; IL-2-induced and anti-CD3-induced responses being augmented and alloantigen-induced response being inhibited by bivalent anti-CD28.

Cross-linking of mAb bound to molecule CD28 reverses inhibition of allogeneic MLR. Anti-CD28 mAb in its monovalent and bivalent forms was consistently inhibitory to the alloantigenic stimulation of T cells. The effect of further cross-linking of the antibody bound to the CD28 molecule on the T cell response in the allogeneic MLR was then examined. Thus, CD11b$^-$ T cells were stimulated in the allogeneic MLR in the presence of 1 μg/ml of either anti-CD28 mAb or anti-CD5 mAb with or without 4 μg/ml of rat anti-mouse κ (187.1) mAb as a cross-linking agent. Table VII shows the results of three representative experiments. Anti-CD28 mAb consistently inhibited the allogeneic MLR response of T cells in the absence of the cross-linking anti-κ mAb. However, when cross-linked with anti-κ mAb the above inhibitory effect of anti-CD28 mAb on the MLR response was reversed. In contrast, anti-CD5 mAb regardless of the degree of cross-linking had no effect on the allogeneic MLR response of T cells. In addition, neither anti-CD28 mAb nor anti-CD5 mAb with or without cross-linking mAb caused the activation of resting T cells in the absence of the primary stimulus such as anti-CD3 mAb, alloantigen, or IL-2. When alloactivated T cells derived from the 10-day primary MLR were stimulated with the primary allogeneic stimulus in the presence of F(ab')$_2$ anti-CD28 mAb the secondary response of alloactivated T cells was inhibited as shown in Table VIII. However, the presence

TABLE VII

Effect of cross-linking of anti-CD28 mAb on modulation of allogeneic MLR

Treatment[a]	[^{3}H]Thymidine Incorporation (cpm ± SEM × 10^{-3}) in Expt.:		
	1	2	3
Medium alone	0.8 ± 0.1	1.1 ± 0.1	0.5 ± 0.1
Anti-CD28 mAb + anti-mouse κ mAb	0.9 ± 0.1	ND	0.7 ± 0.1
Anti-CD5 mAb + anti-mouse κ mAb	0.9 ± 0.1	ND	0.7 ± 0.1
Alloantigen	65.4 ± 2.0	51.2 ± 2.1	29.5 ± 0.8
Alloantigen + anti-mouse κ mAb	64.8 ± 1.0	52.4 ± 1.6	28.0 ± 0.6
Alloantigen + anti-CD28 mAb	36.5 ± 2.3	24.2 ± 0.9	17.4 ± 1.0
Alloantigen + anti-CD28 mAb + anti-mouse κ mAb	58.6 ± 3.6	48.9 ± 2.1	27.9 ± 1.3
Alloantigen + anti-CD5 mAb	68.1 ± 1.6	47.9 ± 1.9	30.9 ± 2.3
Alloantigen + anti-CD5 mAb + anti-mouse κ mAb	63.3 ± 0.6	55.6 ± 2.9	28.4 ± 1.4

[a] CD11b$^-$ T cells (5×10^4) were stimulated with 1×10^4 irradiated EBV-transformed allogeneic CD28$^-$ B cells (cell line, Arent) in the presence of 1 μg/ml of anti-CD5 or anti-CD28 mAb together with or without 4 μg/ml of 187.1 anti-mouse κ mAb. Proliferative responses in these cultures were measured on day 7 of culture.

TABLE VIII

Cross-linking of the CD28 molecule on alloactivated T cells supports their growth in absence of priming alloantigen

Stimulus[a]	[^{3}H]Thymidine Incorporation (cpm × 10^{-3}) in Expt.:		
	1	2	3
Medium alone	0.3	0.9	0.6
Anti-mouse κ mAb	0.5	0.2	0.6
F(ab')$_2$ anti-CD28 mAb	0.6	0.9	0.4
Alloantigen	19.8	68.2	89.8
Alloantigen + anti-mouse κ mAb	20.8	ND	90.8
Alloantigen + F(ab')$_2$ anti-CD28 mAb	10.0	40.2	60.6
Alloantigen + Fab anti-CD28 mAb	ND	33.9	ND
Alloantigen + F(ab')$_2$ anti-CD28 mAb + anti-mouse κ mAb	18.7	ND	133.6
F(ab')$_2$ anti-CD28 mAb + anti-mouse κ mAb	2.9	162.6	82.0

[a] T cells (2×10^4) isolated from a 10 day allogeneic MLR were stimulated with 5×10^3 priming allogeneic CD28$^-$ B cells (EBV-transformed B cell line T51) in the presence of F(ab')$_2$ anti-CD28 mAb or 187.1 anti-mouse κ mAb either alone or in combination. Proliferative responses in these cultures were measured on day 3. The SEM of these cultures was always <15% of the mean cpm for each data point.

of cross-linking 187.1 anti-mouse κ mAb in the secondary MLR reversed the inhibitory effect of anti-CD28 mAb. Interestingly, anti-CD28 mAb and rat 187.1 anti-mouse κ mAb together (cross-linked anti-CD28), but not alone, caused stimulation in the absence of any exogenous stimulus of activated T cells but not of the resting T cells. Thus, cross-linking of anti-CD28 mAb reverses its inhibitory effect on the allogeneic MLR response of T cells and helps maintain the state of activation of already activated T cells even in the absence of the priming Ag.

Additional experiments not shown here examining the effects of anti-CD28 and anti-CD5 mAb with or without cross-linking 187.1 anti-mouse κ mAb on the cytotoxicity of already activated CTL or IAK T cells indicated that neither anti-CD28 mAb nor anti-CD5 mAb regardless of the presence of cross-linking agent had any effect on the cytolytic effector function of already differentiated cytotoxic T cells.

Activation of cells within both CD4$^+$ and CD8$^+$ T sublineages is modulated by anti-CD28 mAb. The ability of anti-CD28 mAb to modulate the responses of CD4$^+$ and CD8$^+$ subpopulations of CD3$^+$CD28$^+$ (CD3$^+$CD11b$^-$) T cells was examined. Thus, CD4$^+$ or CD8$^+$ T cells were

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cultured with either irradiated OKT3 hybridoma cells or allogeneic B cells in the presence of anti-CD28 mAb before the measurement of their proliferative response. Table IX shows the results of this set of experiments. Anti-CD28 mAb augmented anti-CD3-induced proliferative responses and inhibited alloantigen-induced proliferative responses of both $CD4^+$ and $CD8^+$ T cells. Thus, the proliferative responses of T cells within both the $CD4^+$ and $CD8^+$ sublineages are susceptible to the modulatory effects of anti-CD28 mAb. mAb anti-CD28 and anti-CD11b recognize mutually exclusive subpopulations within the $CD3^+CD5^+$ T cells (41). When examined for the effect on the activation of $CD5^+CD11b^+$ T cells, neither anti-CD5 nor anti-CD28 mAb alone or in combination had any effect on the activation of $CD11b^+$ T cells regardless of the nature of the stimulus (data not shown). These results were not surprising in the light of the fact that $CD11b^+$ T cells do not express the CD28 molecule and thus are not expected to be susceptible to the effects of anti-CD28 mAb.

DISCUSSION

The role of T cell surface molecules during various T cell functions has been the subject of intense investigations in recent years. T cell surface molecules can be categorized into cognitive, adhesive, or growth-related categories and these include molecules CD2 (LFA-2), CD3-TCR, CD4, CD8, CD11a/CD18 (LFA-1), CD25 (Tac), LFA-3, ICAM-1, transferrin R and so on (21, 53, 54). Although the exact functions of many other T cell surface structures remain unclear at present, the role of molecule CD28 (Tp44) during T cell activation has recently begun to be appreciated. Antibody to the CD28 molecule has been observed to augment polyclonal T cell responses to agents such as PHA, Con A, or immobilized anti-CD3 mAb (25–30). Similarly, antibodies to the CD5 molecule have been shown to substitute for accessory cells in some accessory cell-dependent T cell responses (22, 23, 28). As a result, molecules CD5 and CD28 have been postulated to represent receptor molecules for "second signals" presumably delivered via accessory cell-derived growth factors or cell-cell contact (28). In the present study, we have examined modulation of both CD3-dependent and CD3-independent T cell responses by mAb directed at the CD5 and CD28 molecules. Our results point toward an interesting complexity in the modulatory effects of anti-CD28 Mab on T cell responses.

TABLE IX

Modulation of response of subpopulations of T cells by anti-CD28 mAb

Responder T Cells[a]	[^{3}H]Thymidine Incorporation (cpm ± SEM × 10^{-3}) in Cultures Stimulated with			
	Anti-CD3		Alloantigens	
	No mAb	Anti-CD28	No mAb	Anti-CD28
Expt 1				
$CD4^+$	10.9 ± 0.6	16.0 ± 1.3	17.9 ± 1.2	9.9 ± 0.5
$CD8^+$	5.6 ± 0.5	8.1 ± 0.2	7.2 ± 0.5	4.2 ± 0.1
Expt. 2				
$CD4^+$	16.5 ± 0.7	22.5 ± 1.9	46.7 ± 1.2	26.8 ± 2.9
$CD8^+$	6.7 ± 0.3	10.1 ± 1.2	12.7 ± 0.9	7.9 ± 0.2
Expt 3				
$CD4^+$	ND	ND	46.4 ± 2.1	21.6 ± 1.9
Expt 4				
$CD4^+$	ND	ND	30.6 ± 0.9	14.2 ± 0.7
Expt. 5				
$CD4^+$	ND	ND	29.5 ± 1.4	13.9 ± 1.2

[a] $CD4^+$ or $CD8^+$ T cells (5×10^4) were cultured with 1×10^4 OKT3 hybridoma cells or allogeneic $CD28^-$ B cells (Arent) in the presence or absence of 1 μg/ml of anti-CD28 mAb. Proliferative responses were measured on day 3 of anti-CD3-stimulated cultures and day 7 of alloantigen-stimulated cultures.

In agreement with previous studies (28, 29), we observed that anti-CD28 mAb upregulated anti-CD3-stimulated T cell proliferation. Although anti-CD5 mAb alone did not consistently enhance anti-CD3-induced T cell proliferation, anti-CD5 together with anti-CD28 mAb further increased the response above that observed with anti-CD28 mAb. Additive effects of anti-CD5 and anti-CD28 mAb on anti-CD3-induced T cell proliferation have been previously reported (28). The present study also shows that IL-2-activated T cells reacted to the stimulation with anti-CD28 and anti-CD5 mAb in a manner similar to anti-CD3-induced T cells, in that both the responses were upregulated with anti-CD28 mAb and further augmented when anti-CD5 mAb was included with anti-CD28 mAb. In contrast to anti-CD3-induced or IL-2-induced stimulation, alloantigen-induced stimulation was inhibited by anti-CD28 mAb but not by anti-CD5 mAb. Anti-CD28 mAb consistently exhibited 40 to 70% inhibition of alloantigen-induced proliferation and almost complete inhibition of the development of cytotoxic T cells in the allogeneic MLR. The anti-CD28 mAb-mediated inhibitory effect on the MLR was specific since anti-CD28 mAb had no effect on the IL-2-induced cytolytic activity, and anti-CD5 mAb had no inhibitory activity on the MLR. Previous studies indicate that the functional effects of anti-CD5 mAb become apparent only under conditions wherein accessory cells are either absent or suboptimally present (28, 33). In the present study, however, such suboptimal experimental conditions were not employed, and thus there was neither a growth-promoting nor an inhibitory effect of anti-CD5.

We have previously shown that valency of mAb binding to the CD28 molecule is important for the delivery of the transmembrane signal, since monovalent anti-CD28 Fab fragments did not synergize with anti-CD3 (28, 29) or with phorbol esters (J. A. Ledbetter, unpublished observation), whereas increased cross-linking of the CD28 molecule with anti-CD28 mAb and anti-mouse κ mAb (187.1) was sufficient for cytoplasmic Ca^{2+} mobilization and increased responsiveness to IL-2 (55, 56). We, therefore, investigated whether the valency of binding of anti-CD28 mAb to the CD28 molecule was related to the stimulatory versus inhibitory signals mediated through the CD28 molecule. Anti-CD28 mAb in its intact form or its $F(ab')_2$ fragments had a strong growth-promoting effect on both anti-CD3-induced and IL-2-induced proliferation and the subsequent release of soluble CD25 (IL-2R) molecule by T cells. In contrast, alloantigen-induced responses of T cells were significantly inhibited by anti-CD28 mAb (24) and this inhibitory effect of anti-CD28 mAb was neither enhanced nor reversed by anti-CD5 mAb. The above growth-promoting or inhibiting effects of anti-CD28 mAb do not appear to be Fc dependent because $F(ab')_2$ fragments of anti-CD28 mAb have biologic effects similar to those of intact anti-CD28 IgG (28, 29, 33; present study). At least bivalent binding of anti-CD28 mAb to T cells is required to elicit enhancement in the T cell growth. In contrast, monovalent Fab fragments of anti-CD28 mAb were consistently inhibitory to not only alloantigen-induced T cell activation but also that

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induced with either anti-CD3 mAb or IL-2 suggesting that cross-linking of the CD28 molecules on T cells is required for the delivery of growth-enhancing signals and that prevention of CD28 cross-linking with Fab fragments of anti-CD28 mAb may be inhibitory by blocking a normal ligand for the CD28 molecule present in the culture system. The inhibitory effect of anti-CD28 mAb on the allogeneic MLR was reversed by further cross-linking of antibody bound to the CD28 molecule with 187.1 anti-mouse κ mAb. Thus, the degree of CD28 cross-linking appears to dictate the direction of any T cell response from inhibition (Fab anti-CD28) to enhancement (cross-linked CD28).

Unlike the activation of T cells with either Ag or antibodies directed at the CD3-TCR complex, IL-2-induced T cell activation does not seem to require active participation of the CD3-TCR complex (N. K. Damle, unpublished observation). IL-2 has a direct mitogenic effect on T cells that presumably involves the participation initially of non-CD25/Tac p70–75 molecule on the surface of T cells that serves as intermediate affinity IL-2R (16–20) and upon the expression of the CD25 (Tac) molecule forms a high affinity IL-2R complex with the CD25 molecule (20). Anti-CD28 mAb significantly augmented the mitogenic effect of IL-2 on T cells in the absence of any extraneous stimulus and such augmentation in the IL-2-induced T cell stimulation requires at least bivalent binding of antibody to the CD28 molecule (data not shown). Despite the ability of anti-CD28 mAb to enhance the IL-2-induced proliferation of T cells, their ability to differentiate into IAK T cells was not affected by anti-CD28 mAb. In contrast, anti-CD28 mAb not only inhibited the proliferation of T cells in the allogeneic MLR but also their subsequent differentiation into CTL. This observation should not come as a surprise because like in the case of their IL-2-activated counterparts, proliferation of precursors of $CD8^+CD28^+$ CTL (40) is required for their differentiation in the allogeneic MLR. Since alloantigen-induced proliferation of T cells within both $CD4^+$ and $CD8^+$ sublineages is inhibited by anti-CD28 mAb the inhibition of the development of CTL activity in the allogeneic MLR by anti-CD28 mAb is consistent with the above notion. Furthermore, our recent results not described here indicate that alloantigen-induced response of cells within both $CD29^-CD45R^+$ (virgin T cells) and $CD29^+CD45R^-$ (memory T cells) subpopulations of T cells is inhibited by anti-CD28 mAb, and is restored back to normalcy when appropriately cross-linked with anti-mouse κ mAb (N. K. Damle and L. V. Doyle, unpublished observation). In addition, experiments not described here also indicate that anti-CD28 mAb regardless of the valency of CD28 cross-linking neither inhibits nor augments the cytolytic effect of activated cytotoxic T cells derived from IL-2-stimulated (IAK T cells) or alloantigen-stimulated (CTL) cultures. Moreover, anti-CD28 mAb enhances anti-CD3-induced or IL-2-induced responses of only those T cells that express the CD28 molecule on the surface. $CD11b^+$ T cells do not express the CD28 molecule and thus are not affected by anti-CD28 mAb in their proliferative responses. Taken together, these results indicate that anti-CD28 mAb modulates the proliferative events rather than differentiative events involving $CD28^+$ T cells.

The present study also reveals an interesting difference between Ag-induced T cell response and anti-CD3-induced T cell response. Despite the fact that both these T cell responses require active participation of the CD3-TCR complex, alloantigen-induced T cell activation appears to be much more dependent on the signals received through the CD28 molecule than that induced with anti-CD3. Although the biochemical basis for such a difference is not known at present, it may be related to the respective strengths (avidity) of interactions of the CD3-TCR complex with alloantigen and with anti-CD3. Conceivably, due to the high avidity of direct interaction between anti-CD3 and the CD3 molecule, such an interaction generates significant activation signal and thus, anti-CD3-induced T cell response may be less dependent on the growth-promoting signals received through the CD28 molecule. On the other hand, Ag-induced T cell response involves comparatively weak interactions between the α/β TCR (identifiable by mAb WT-31) (57) and the Ag-MHC complex to bring about CD3-mediated signal transduction and thus, may be more dependent on the growth-related signals received through the CD28 molecule. Hence, Ag-induced T cell response is more susceptible to the inhibitory effects of monovalent or bivalent anti-CD28, which when appropriately cross-linked generates required growth-supporting signals to restore the response to normalcy. Thus, in addition to the degree of cross-linking of the CD28 molecule, the extent of apparent dependence of any T cell response on the CD28-delivered signals may influence the magnitude of the response.

Although the exact role of the CD28 molecule at the biochemical level during T cell activation is yet unknown, mAb directed at this molecule has been shown to reverse the inhibitory effects of agents that increase intracellular levels of cyclic AMP such as dibutyryl cyclic AMP, forskolin, or PGE_2, and also directly increases intracellular levels of cyclic GMP (33, 55, 56). Under certain experimental conditions, anti-CD28 mAb has been shown to augment the production of IL-2 (26, 27, 29, 30) and the expression of cell-surface associated CD25 molecule (56). Increase in cyclic GMP levels is usually associated with the promotion of cytokine release by and growth of T cells, whereas similar increase in cyclic AMP is thought to mediate inhibitory effects on T cell growth (55). Similarly, crosslinking of anti-CD28 mAb on T cells results in the mobilization of intracellular Ca^{2+} (30, 33, 55, 56) which is essential for many biochemical events involved in the activation of T cells. Although not yet experimentally tested, it is likely that the intracellular Ca^{2+} mobilization by cross-linking of the CD28 molecule as reported by us (55, 56) and the functional effects of anti-CD28 observed in the present study are due to the generation of intracellular "second messengers" such as inositol 1,4,5-triphosphate and diacylglycerol which are known to initiate cellular activation (58–61).

If so, T cells stimulated with antibodies to the CD3-TCR complex may generate optimal levels of second messengers due to the avidity of their interaction, and thus may not require substantial assistance from accessory molecules like CD28. However, any assistance provided through cross-linking of the CD28 molecule would be reflected in the enhanced responsiveness as observed. Fab anti-CD28 mAB (or bivalent anti-CD28 mAb in case of allogeneic MLR) would block the multivalent cross-linking of the CD28 molecule by its natural ligand(s) and

hence, the removal of the CD28-contributed component of second messengers would reflect in the inhibition of the T cell response. Cross-linking with 187.1 anti-mouse κ mAb of the antibody bound to the CD28 molecule would recruit additional second messenger component as reflected in the reversal of the inhibitory effect on the MLR of monovalent or bivalent anti-CD28 mAb. Although the above reasoning is appealing, in reality, the activation of T cells may require assistance by other molecules in addition to the CD28 molecule. Nevertheless, the interesting modulatory effects on the T cell activation seen with the antibody directed at the CD28 molecule emphasize the crucial role played by this molecule during the growth of helper/inducer and cytolytic T cells. Moreover, the ease with which $CD2^+CD3^+CD5^+CD28^+$ helper/inducer and cytotoxic T cells can be grown in vitro and the observed inability of T cells lacking the CD28 molecule such as $CD2^+CD3^+CD5^+CD8^+CD28^-$ suppressor T cells to exhibit consistent growth (62, 63) further point toward the importance of the CD28 molecule in the propagation of T cells and have implications for the use of anti-CD28 and IL-2 in sustaining the proliferation of various Ag-specific T subpopulations before their utilization for clinical advantage.

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EXHIBIT 19

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REDACTED IN ITS ENTIRETY

EXHIBIT 20

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EXHIBIT 22

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EXHIBIT 23

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EXAMINER	
MARKS, M	
ART UNIT	PAPER NUMBER
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DATE MAILED: 03/02/90

This is a communication from the examiner in charge of your application
COMMISSIONER OF PATENTS AND TRADEMARKS

[X] This application has been examined [X] Responsive to communication filed on 29 Jan 1990 [ ] This action is made final.

A shortened statutory period for response to this action is set to expire 3 month(s), 0 days from the date of this letter. Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. [ ] Notice of References Cited by Examiner, PTO-892.
2. [ ] Notice re Patent Drawing, PTO-948.
3. [ ] Notice of Art Cited by Applicant, PTO-1449
4. [ ] Notice of Informal Patent Application, Form PTO-152
5. [ ] Information on How to Effect Drawing Changes, PTO-1474
6. [ ] _____

Part II SUMMARY OF ACTION

1. [X] Claims 1-83 are pending in the application.
   Of the above, claims 1-21 and 23-83 are withdrawn from consideration.
2. [ ] Claims _____ have been cancelled.
3. [ ] Claims _____ are allowed.
4. [X] Claims 22 ~~are~~ is rejected.
5. [X] Claims 22 ~~are~~ is objected to.
6. [ ] Claims _____ are subject to restriction or election requirement.
7. [ ] This application has been filed with informal drawings which are acceptable for examination purposes until such time as allowable subject matter is indicated.
8. [ ] Allowable subject matter having been indicated, formal drawings are required in response to this Office action.
9. [ ] The corrected or substitute drawings have been received on _____. These drawings are [ ] acceptable; [ ] not acceptable (see explanation).
10. [ ] The [ ] proposed drawing correction and/or the [ ] proposed additional or substitute sheet(s) of drawings, filed on _____ has (have) been [ ] approved by the examiner. [ ] disapproved by the examiner (see explanation).
11. [ ] The proposed drawing correction, filed _____, has been [ ] approved. [ ] disapproved (see explanation). However, the Patent and Trademark Office no longer makes drawing changes. It is now applicant's responsibility to ensure that the drawings are corrected. Corrections MUST be effected in accordance with the instructions set forth on the attached letter "INFORMATION ON HOW TO EFFECT DRAWING CHANGES", PTO-1474.
12. [X] ~~Acknowledgment is made of the~~ No claim for priority under 35 U.S.C. 119. The certified copy has [X] been received [ ] not been received [ ] been filed in parent application, serial no. _____; filed on _____
13. [ ] Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. [ ] Other

EXAMINER'S ACTION

PTOL-326 (Rev. 7-82)

Serial No. 07/347,291
Art Unit 185

Applicants' election without traverse of Group I claims 1-56 and 63-82 with the election without traverse of claim 22 as the species for examination in Paper #7 is acknowledged. Claims 1-21 and 23-83 are withdrawn from further consideration by the examiner, 37 CFR 1.142(b), as being drawn to a non-elected invention.

Applicant is advised of possible benefits under 35 USC 119, wherein an application for patent filed in the United STates may be entitled to the benefit of the filing date of a prior application filed in a foreign country. It is noted that applicants Declaration does not claim foreign priority although applicant's have filed a certified copy of EP89100787.4.

The following is a quotation of the first paragraph of 35 U.S.C. 112: The specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The specification is objected to under 35 U.S.C. 112, first paragraph, as failing to provide an adequate written description of the invention and failing to adequately teach how to make and/or use the invention (i.e. failing to provide an enabling disclosure).

The specification fails to teach how to grow any host cell in an appropriate growth medium under physiological conditions to

allow the dimerization and secretion of a biologically active peptide dimer. No physiological conditions to allow dimerization and secretion were disclosed for any bacterial cell or mammalian cell.

Claim 22 is rejected under 35 U.S.C. 112, first paragraph, for the reasons set forth in the objection to the specification.

Claim 22 is rejected under 35 U.S.C. 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention.

Claim 22 is unclear st to what is intended by a peptide in the recitation"peptide dimer". What size and identity of peptide is intended? Please clarify. Peptides which are the size of an immunoglobulin chain are usually considered by the art to be polypeptides. Claim 22 is indefinite in the recitation "growing said host cell in an appropriate growth medium under physiological conditions to allow the dimerization and secretion of said biologically active peptide dimer...". What appropriate growth medium is intended? Only methotrexate containing medium is disclosed on page 63. WHat physiological conditions are intended? Is physiological pertinent to the mammalian source of the DNA or to the cell culture? Which physiological conditions allow the dimerization and secretion of the dimer? What cell type is intended, since dimerization and physiologic conditions

vary with the host cell employed. Often reducing and oxidizing agents are required for dimerization, depending on the cell source. Please clarify each of the above indefinite points in claim 22.

The following is a quotation of the appropriate paragraphs of 35 U.S.C. 102 that form the basis for the rejections under this section made in this Office Action:

(e) the invention was described in a patent granted on an application for patent by another filed in the United States before the invention thereof by the applicant for patent, or on an international application by another who has fulfilled the requirements of paragraphs (1), (2), and (4) of section 371(c) of this title before the invention thereof by the applicant for patent.

Claim 22 is rejected under 35 U.S.C. 102(e) as being anticipated by Boss (A) or Boss (L).

Each of the Boss references teaches a method for producing a secreted biologically active peptide dimer comprising the introduction into a eukaryotic host cell :

a first DNA construct comprising a first polypeptide chain joined to a DNA sequence encoding an immunoglobulin light chain constant region - which is operably linked to both a promoter and signal sequence and

a second DNA construct comprising a second polypeptide chain joined to a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain,

growing the host cell in conditions for dimerization and secretion and isolation of the active dimer.

Boss teaches the method and eukaryotic host cell for the

production of an immunologically functional recombinant human immunoglobulin by transfection into a host cell of DNA sequences coding for both the heavy and light chain of a human immunoglobulin molecule. For example see Boss (F) figure 13, column 4 lines 52-61, column 6 line 5, and the claims. Also, please not the openness of the instant claims 2-5 which allows for the DNA sequence coding for the variable and constant regions of the Immunoglobulin to be derived from the same or different sources.

Any inquiry concerning this communication should be directed to Michelle Marks, Ph.D. whose telephone number is 703-557-0909.

RICHARD A. SCHWARTZ
PRIMARY EXAMINER
ART UNIT 181

TO SEPARATE ... D TOP AND BOTTOM EDGES, SNAP–APART ... ARD CARBON

FORM PTO-892 (REV 3-78) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

NOTICE OF REFERENCES CITED

SERIAL NO.	GROUP ART UNIT	ATTACHMENT TO PAPER NUMBER
247291	185	8

APPLICANT(S): SLEDZIEWSKI

U.S. PATENT DOCUMENTS

*		DOCUMENT NO	DATE	NAME	CLASS	SUB-CLASS	FILING DATE IF APPROPRIATE
	A	4 816 397	28 Mar 1989	Boss	435	68	23 Mar 1984
	B						
	C						
	D						
	E						
	F						
	G						
	H						
	I						
	J						
	K						

FOREIGN PATENT DOCUMENTS

*		DOCUMENT NO	DATE	COUNTRY	NAME	CLASS	SUB-CLASS	PERTINENT SHEETS DRWG	PERTINENT SPEC
	L	0 120 694	30 Oct 1984	UK	Boss	435	68		
	M								
	N								
	O								
	P								
	Q								

OTHER REFERENCES (Including Author, Title, Date, Pertinent Pages, Etc.)

R	
S	
T	
U	

EXAMINER: MS Marks DATE: 2/23/90

* A copy of this reference is not being furnished with this office action. (See Manual of Patent Examining Procedure, section 707.05 (a).)

EXHIBIT 24

United States Patent [19]

Boss et al.

[11] **Patent Number: 4,816,397**

[45] **Date of Patent: Mar. 28, 1989**

[54] **MULTICHAIN POLYPEPTIDES OR PROTEINS AND PROCESSES FOR THEIR PRODUCTION**

[75] Inventors: **Michael A. Boss, Slough; John H. Kenten; John S. Emtage,** both of High Wycombe; **Clive R. Wood,** Near Fordingbridge, all of United Kingdom

[73] Assignee: **Celltech, Limited,** Slough, United Kingdom

[21] Appl. No.: **672,265**

[22] PCT Filed: **Mar. 23, 1984**

[86] PCT No.: **PCT/GB84/00094**

§ 371 Date: **Nov. 14, 1984**

§ 102(e) Date: **Nov. 14, 1984**

[87] PCT Pub. No.: **WO84/03712**

PCT Pub. Date: **Sep. 27, 1984**

[30] **Foreign Application Priority Data**

Mar. 25, 1983 [GB] United Kingdom 8308235

[51] **Int. Cl.**[4] **C12P 21/00;** C12N 15/00; C12N 1/00; C12N 1/20

[52] **U.S. Cl.** **435/68;** 435/172.3; 435/243; 435/255; 435/320; 435/252.31; 435/252.33

[58] **Field of Search** 435/68, 172.3, 243, 435/320, 253, 255

[56] **References Cited**

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4,642,334	2/1987	Moore et al.	435/68

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088994	9/1983	European Pat. Off.
0125023	11/1984	European Pat. Off.

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Seidman et al: "Immunoglobulin light-chain structural gene sequences cloned in a bacterial plasmid," *Nature, vol. 271, pp. 582–585, 1978.*

Primary Examiner—Blondel Hazel

Attorney, Agent, or Firm—Cushman, Darby & Cushman

[57] **ABSTRACT**

Multichain polypeptides or proteins and processes for their production in cells of host organisms which have been transformed by recombinant DNA techniques. According to a first aspect of the present invention, there is provided a process for producing a heterologous multichain polypeptide or protein in a single host cell, which comprises transforming the host cell with DNA sequences coding for each of the polypeptide chains and expressing said polypeptide chains in said transformed host cell. According to another aspect of the present invention there is provided as a product of recombinant DNA technology an Ig heavy or light chain or fragment thereof having an intact variable domain. The invention also provides a process for increasing the level of protein expression in a transformed host cell and vectors and transformed host cells for use in the processes.

18 Claims, 13 Drawing Sheets

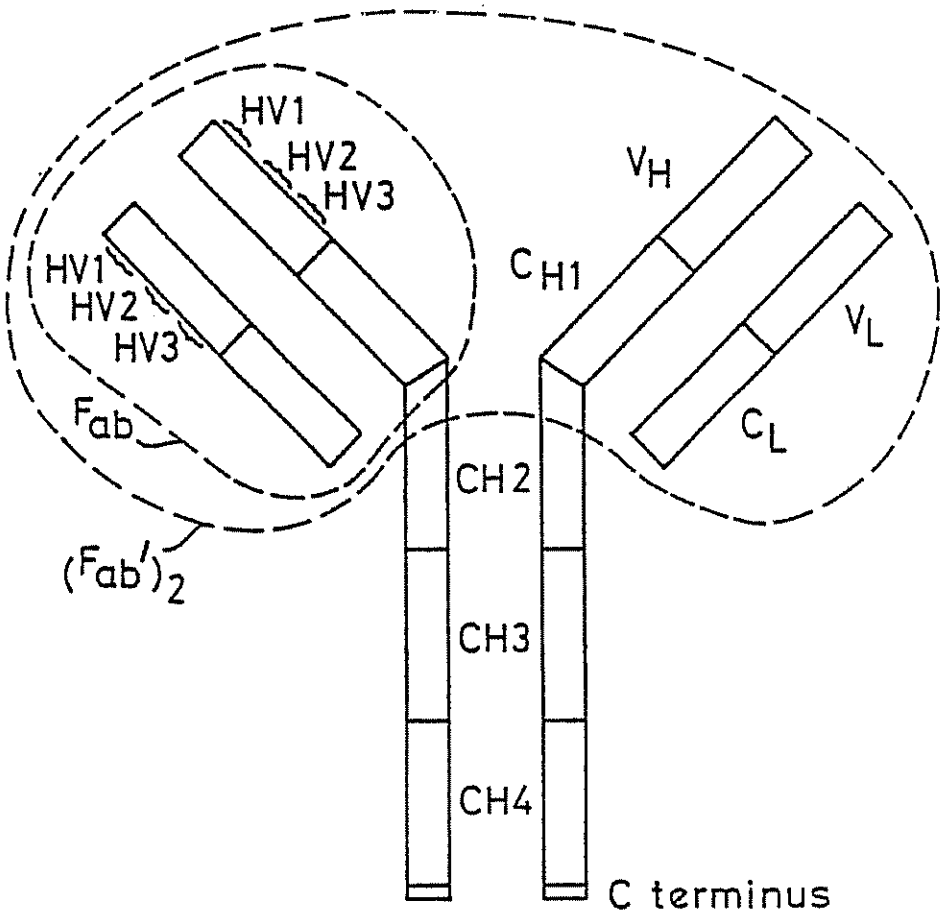
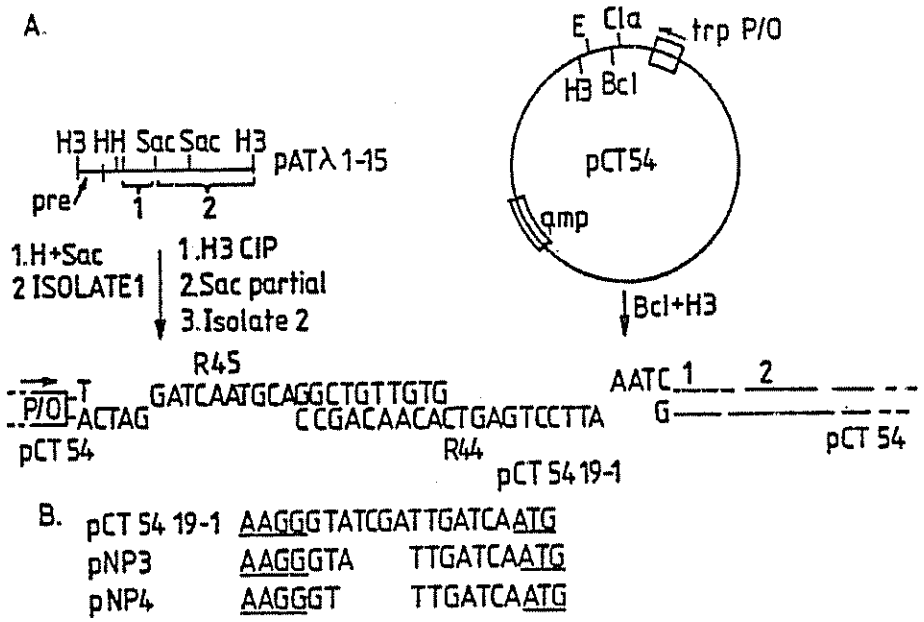

HV1
HV2
HV3
HV1
HV2
HV3
Fab
(Fab')2
VH
CH1
VL
CL
CH2
CH3
CH4
C terminus

FIG. 1



Legend. E = E corRI

H = HinfI

H3= Hind III

FIG. 2

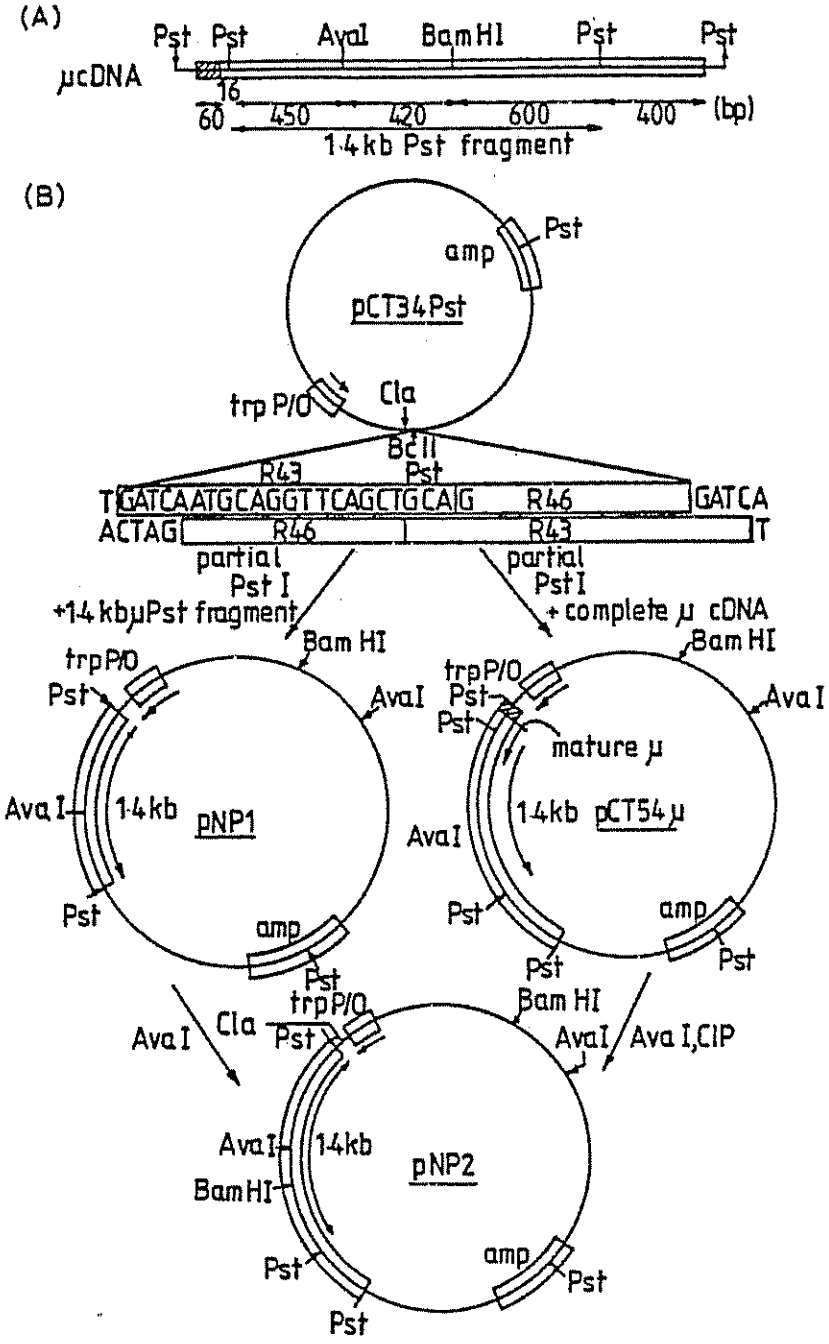

(A)
Pst Pst AvaI BamHI Pst Pst
μcDNA
16
60 450 420 600 400 (bp)
1.4kb Pst fragment
(B)
amp
Pst
pCT34Pst
trp P/O
Cla
BclI
Pst
R43
TGATCAATGCAGGTTCAGCTGCAG R46 GATCA
ACTAG R46 R43 T
partial
Pst I
+1.4kbμPst fragment
partial
Pst I
+ complete μ cDNA
Bam HI
trpP/O
Pst
AvaI
AvaI
1.4kb
pNP1
Pst
amp
Pst
Bam HI
trpP/O
Pst
Pst
AvaI
mature μ
AvaI
1.4kb
pCT54μ
Pst
amp
Pst
Pst
AvaI
Cla
trpP/O
Pst
Bam HI
AvaI
AvaI,CIP
AvaI
1.4kb
BamHI
pNP2
Pst
amp
Pst
Pst

FIG. 3

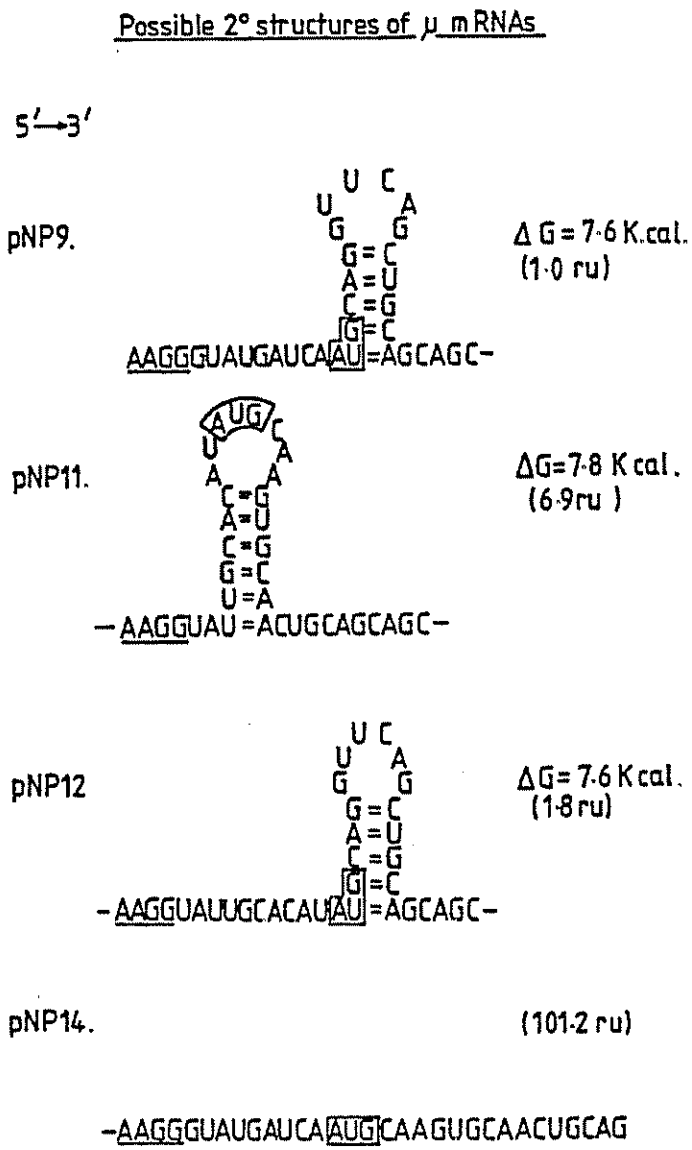

Possible 2° structures of μ mRNAs
5'→3'
pNP9.
ΔG = 7·6 K.cal.
(1·0 ru)
AAGGGUAUGAUCAAU=AGCAGC-
pNP11.
ΔG=7·8 K cal.
(6·9ru)
-AAGGUAU=ACUGCAGCAGC-
pNP12
ΔG= 7·6 K cal.
(1·8 ru)
-AAGGUAUUGCACAUAU=AGCAGC-
pNP14.
(101·2 ru)
-AAGGGUAUGAUCAAUGCAAGUGCAACUGCAG

FIG. 4

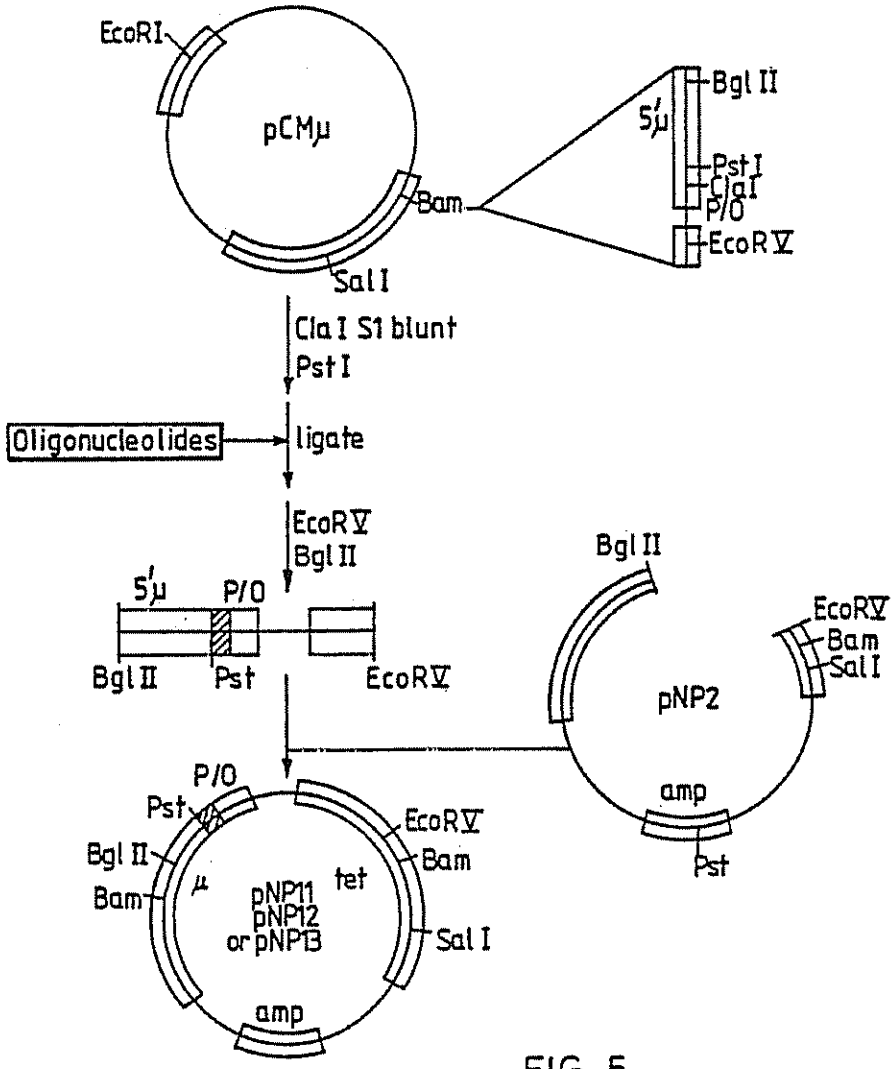

EcoRI
pCMμ
Bam
Sal I
5'μ
Bgl II
Pst I
Cla I
P/O
EcoR V
Cla I S1 blunt
Pst I
Oligonucleolides
ligate
EcoR V
Bgl II
5'μ
P/O
Bgl II
Pst
EcoR V
Bgl II
EcoR V
Bam
Sal I
pNP2
amp
Pst
P/O
Pst
Bgl II
Bam
μ
pNP11
pNP12
or pNP13
tet
EcoR V
Bam
Sal I
amp

FIG. 5

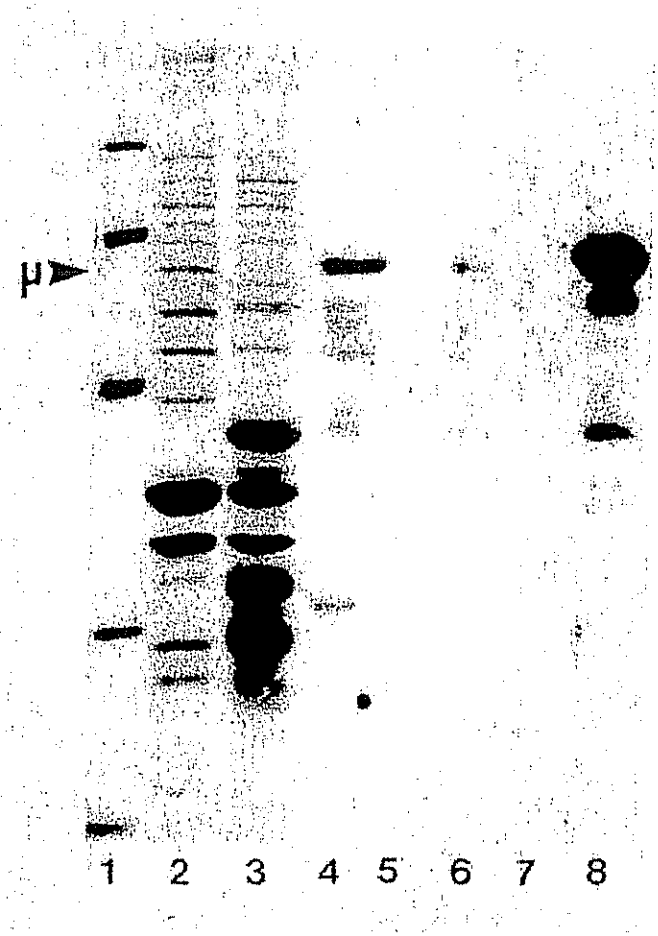


FIG. 6

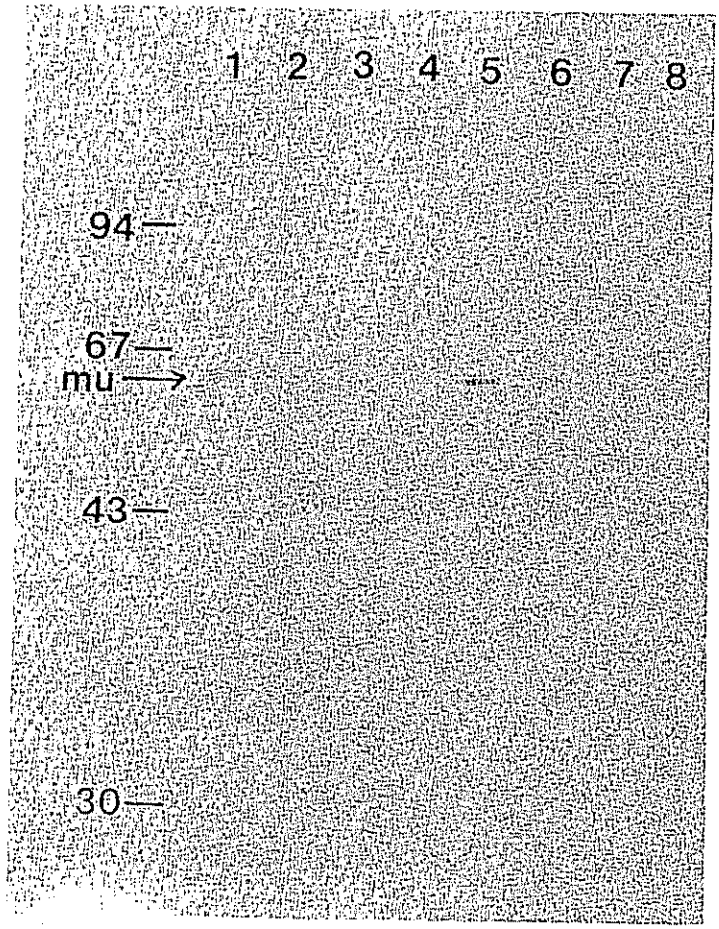


FIG. 7

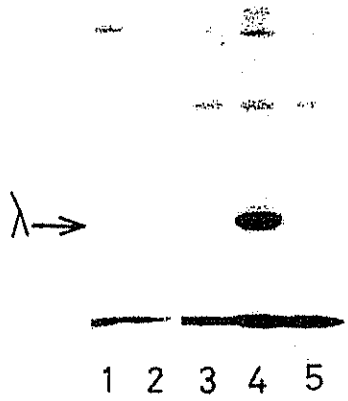


FIG. 8

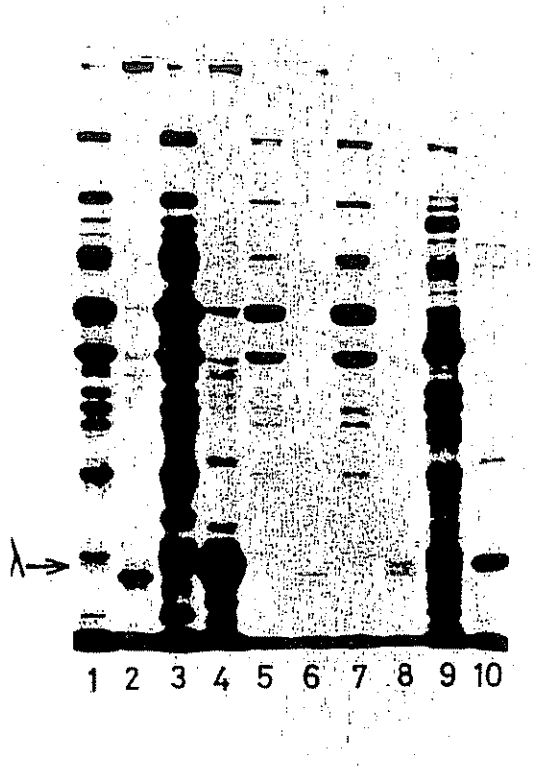


FIG. 9

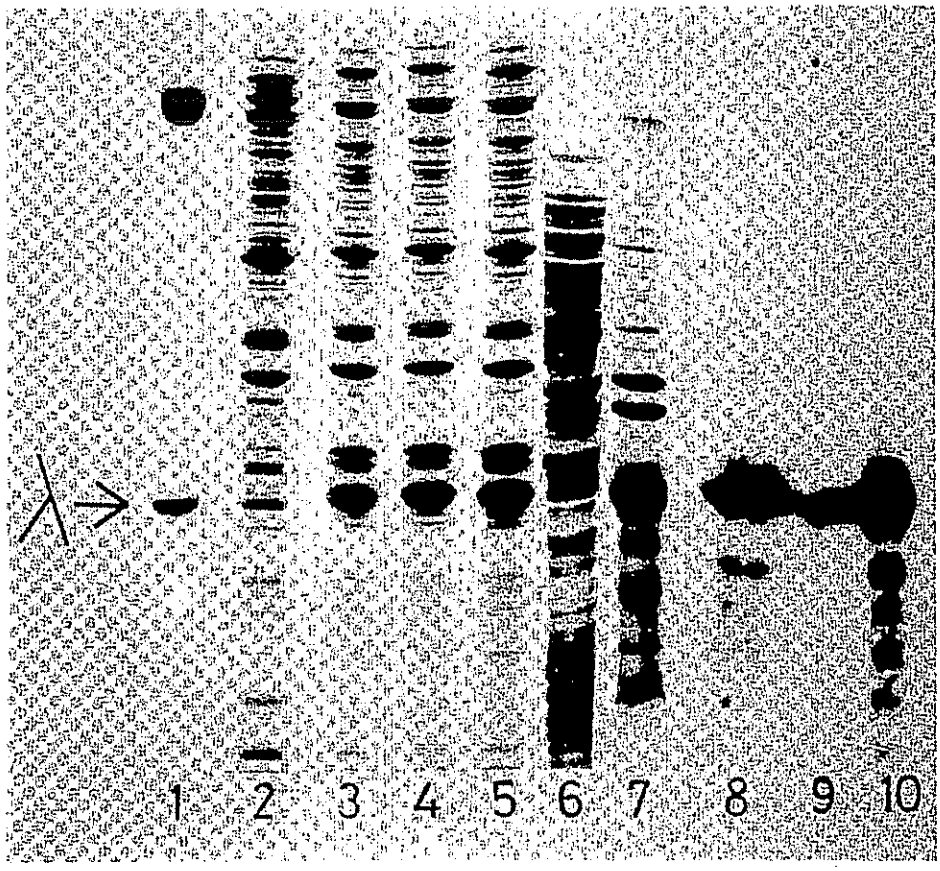


FIG. 10

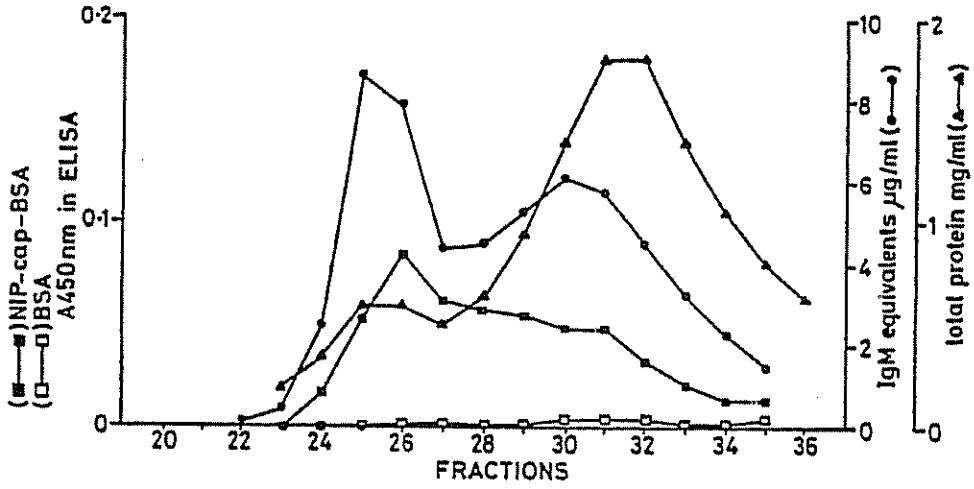

0·2
0·1
0
(■—■)NIP-cap-BSA
(□—□)BSA
A450nm in ELISA
20
22
24
26
28
30
32
34
36
FRACTIONS
10
8
6
4
2
0
IgM equivalents µg/ml (●—●)
2
1
0
total protein mg/ml (▲—▲)

FIG. 11

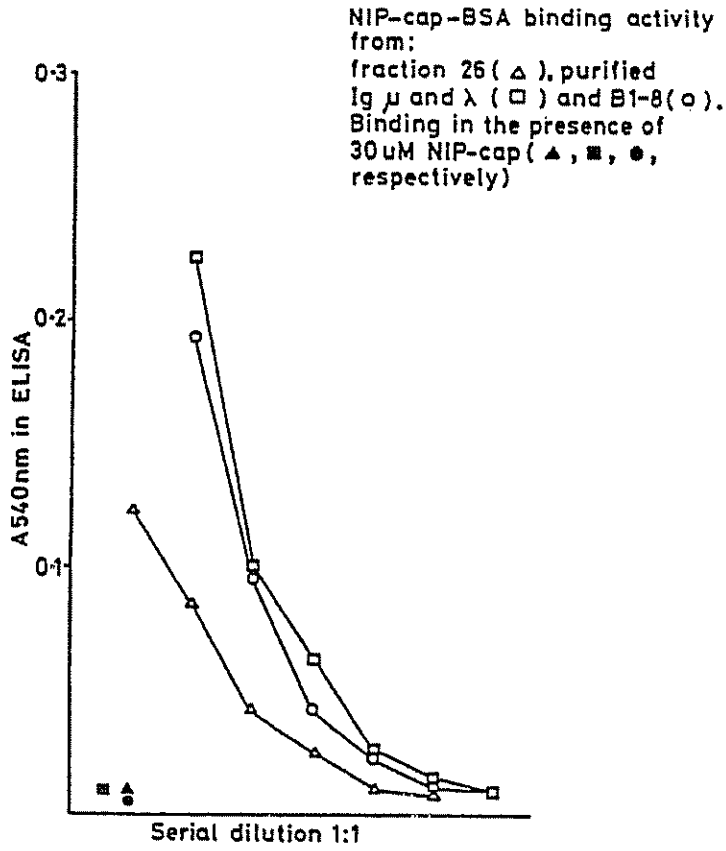
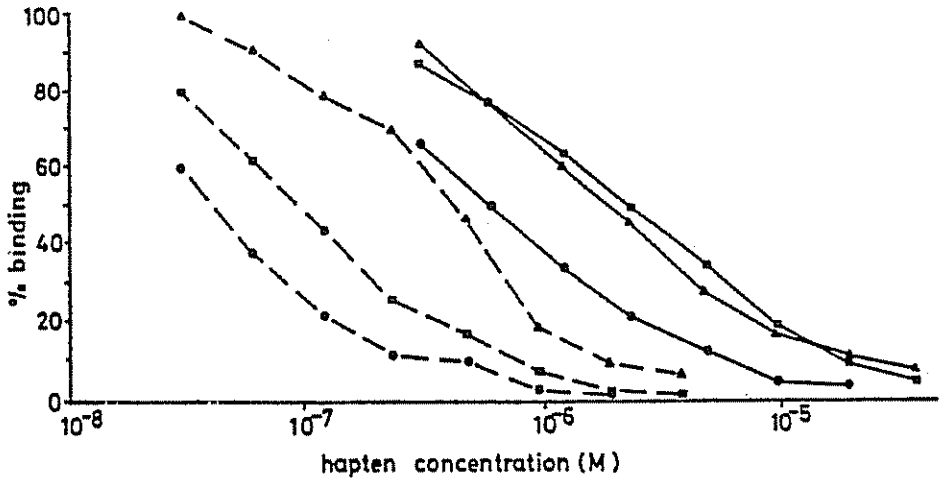

NIP-cap-BSA binding activity from:
fraction 26 (△), purified Ig μ and λ (□) and B1-8 (○).
Binding in the presence of 30 uM NIP-cap (▲ , ■ , ● , respectively)
0·3
0·2
0·1
A540nm in ELISA
Serial dilution 1:1

FIG. 12



Binding of antibodies to NIP-cap BSA B1-8 IgM (■), fraction 26 (▲), purified Ig μ or λ (●), in the presence of free NIP-cap (----) or NIP-cap (——).

FIG. 13

MULTICHAIN POLYPEPTIDES OR PROTEINS AND PROCESSES FOR THEIR PRODUCTION

This invention relates to multichain polypeptides or proteins and processes for their production in cells of host organisms which have been transformed by recombinant DNA techniques.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of heterologous (foreign) polypeptides or proteins in host cells which have been transformed with heterologous DNA sequences which code for the production of these products.

Theoretically, the recombinant DNA approach may be applied to the production of any heterologous polypeptide or protein in a suitable host cell, provided that appropriate DNA coding sequences can be identified and used to transform the host cell. In practice, when the recombinant DNA approach was first applied to the production of commercially useful products, its application for the production of any specified polypeptide or protein presented particular problems and difficulties, and the success of applying this approach to the production of any particular polypeptide or product was not readily predictable.

However, a large number of heterologous single chain polypeptides or proteins have now been produced by host cells transformed by recombinant DNA techniques. Examples of such heterologous single chain polypeptides or proteins include human interferons, the A and B chains of human insulin, human and bovine growth hormone, somatostatin, calf prochymosin and urokinase. Such transformed host cells provide a reproducible supply of authentic heterologous polypeptiede or protein which may be produced on an industrial scale using industrial fermentation technology.

It should be pointed out that some of these polypeptides, for instance urokinase, after secretion by a host cell appear as two chain molecules. However, in such cases, the molecule is synthesised by the host cell as a single chain polypeptide, coded for by a single DNA sequence, which is cleaved in the host cell subsequent to synthesis to form the two chain structure.

It is known that in both human and animal systems there are a number of polypeptides or proteins which have multichain structure in which the chains are not derived from the cleavage of a single chain polypeptide coded for by a single DNA sequence. In such cases, the gene for each of the chains may be located at different points on the same chromosome or even on different chromosomes. In these cases, the polypeptide chains are synthesised separately and then assembled into the complete molecule subsequent to synthesis. Heretofore, no such multichain polypeptide or protein has been produced by recombinant DNA techniques from a single host cell.

A particular example of a class of such multichain polypeptides or proteins is the immunoglobulins.

Immunoglobulins, commonly referred to as antibodies, are protein molecules produced in animals by B-lymphocyte cells in response to challenge with foreign antigenic agents, such as bacteria, viruses and foreign proteins. The immunoglobulins comprise a crucial part of the immune systems of humans and animals. The immunoglobulins recognise specific parts of the foreign agents and bind onto them. The specific parts are usually known as antigenic determinants or antibody binding sites. A given foreign agent is likely to have a number of different antigenic determinants.

A typical immunoglobulin (Ig) molecule is shown in FIG. 1 of the accompanying drawings, to which reference is now made. The Ig molecule comprises two identical polypeptide chains of about 600 amino acid residues (usually referred to as the heavy chains H), disulphide bonded to each other, and two identical shorter polypeptide chains of about 220 amino acid residues (usually referred to as the light chains L), each light chain being disulphide bonded to one end of each heavy chain as shown.

When the Ig molecule is correctly folded, each chain is formed into a number of distinct globular areas, usually known as domains, joined by a more linear polypeptide chain. The light chains have two such domains, one of which is of variable sequence V_L, and the other of which is of constant sequence C_L. The heavy chains have a single variable domain V_H adjacent the variable domain V_L of the light chain, and three or four constant domains $C_{H1\text{-}3 \text{ or } 4}$.

The variable domains on both the heavy and the light chains each contain three hypervariable regions (HV1–3) which, when the Ig molecule is correctly folded, are located adjacent one another and form the antigen binding site. It is this area which recognises and binds to the antigenic determinant for which the Ig molecule is specific.

The constant domains of the Ig molecule do not take part in the binding to the antigenic determinant, but mediate the actions triggered by the binding of the Ig molecule to the antigenic determinant. It is believed that this triggering is caused by an allosteric effect induced by the binding of the Ig molecule to the antigenic determinant. The constant domain may enable the Ig molecule to fix complement or may cause mast cells to release histamine.

Ig's may be categorised by class or subclass, depending on which of a number of possible heavy chain constant domains they contain, there being eight such possible heavy chains in mice. Thus, for instance, and Ig molecule with a μ heavy chain belongs to the class IgM, and one with a γ_1 heavy chain to the class IgG_1.

Ig's may also contain one of two light chains, designated as κ and λ light chains, which have different constant domains and different sets of variable domains.

The structure of the Ig molecule and the location of the genes coding for the various domains thereof are discussed more fully by Early and Hood, in Genetic Engineering, Principles and Methods, Vol. 3, pages 153–158 (edited by Setlow and Hollaender, Plenum Press).

It is known that Ig molecules on digestion with selected enzymes can produce a number of immunologically functional fragments. Two such fragments are known as the F_{ab} and $(F_{ab'})_2$ fragments. The F_{ab} fragment comprises one light chain linked to the V_H and C_{H1} domains of a heavy chain as shown in FIG. 1. The $(F_{ab'})_2$ fragment consists essentially of two F_{ab} fragments linked together by a small additional portion of the heavy chains as shown in FIG. 1. These fragments and other similar fragments can be of use in various tests and diagnostic and medical methods.

The principle method employed for the production of Ig's involves the immunisation of susceptible animals with the antigenic agent to provide an immune reaction. Generally the animal is immunised a second time to

improve the yield of Ig. The animal is then bled and the Ig is recovered from the serum.

However, the product of this method is not a homogeneous protein. The animal will produce Ig of different classes and also Ig specific for each of the different antigenic determinants on the antigenic agent, and its blood will therefore contain a heterogeneous mixture of Ig's. Obtaining a specific Ig of particular class and desired specificity from such a mixture requires very difficult and tedious purification procedures.

Recently, it has become possible to produce a homogeneous Ig of a single class and a single specificity by a technique first described by Kohler and Milstein (Nature, 256, 495–479, 1975). The technique involves the fusion of single Ig-producing parent cells with cancer cells to produce a monoclonal hybridoma cell which produces the Ig. Ig produced by this technique is usually known as monoclonal antibody. The nature of the monoclonal Ig is determined by the class and specificity of the Ig produced by the parent cell.

Recently attempts have been made to use recombinant DNA techniques to produce fragments of Ig molecules. For instance, Amster et al. (Nucleic Acid Research, 8, No. 9, 1980, pp 2055 to 2065) disclose the cloning of double stranded cDNA sequences encoding for a mouse Ig light chain into a plasmid. An E. Coli strain transformed by the plasmid synthesised a protein thought to comprise the complete constant domain of the light chain and about 40 amino acid residues of its variable region.

Kemp and Cowman (Proc. Natl. Acad. Sci. USA, 78, 1981 , pp 4520 to 4524) disclose the cloning of cDNA sequences encoding for mouse heavy chain fragments and the transforming of an E. Coli strain which then synthesised heavy chain polypeptide fragments.

In both these cases, the polypeptides were produced as fusion proteins, in which the fragments of the Ig polypeptides were fused with additional non-Ig polypeptide sequences, and the incomplete variable domains. Thus, the polypeptide chains produced in these studies were not immunologically functional polypeptides as they were incapable of combining with complementary heavy or light chains to provide Ig molecules having intact antigen binding sites and immunological function.

Research studies have also been carried out in mammalian systems. For instance, Falkner and Zachau (Nature, 298, 1982, pp 286 to 288) report the cloning of cDNA sequences encoding for mouse light chains into a plasmid which was used to transfect genomic eukaryotic cells which could then transiently synthesise light chains.

Rice and Baltimore (Proc. Natl. Acad. Sci. USA, 79, 1982, pp 7862 to 7865) report on the transfection of a functionally rearranged K light chain Ig gene into a murine leukemia virus-transformed lymphoid cell line. The cell line is then able to express the gene continuously. In both these cases, the K genes used to transfect the mammalian cells were obtained from myeloma cells and the K polypeptides produced were of indeterminate immunological function.

A further approach is exemplified in a series of papers by Valle et al. (Nature, 291, 1981, 338–340; Nature, 300, 1982, 71–74and J. Mol. Biol., 160, 1982, 459–474), which describe the microinjection of mRNAs encoding for heavy or light chains of Ig isolated from a mouse myeloma line into oocytes of Xenopus laevis. Under certain conditions complete Ig molecules were formed. However, the mRNAs were obtained from myeloma cells and the Ig molecules were of indeterminate immunological function.

It can thus be seen that hitherto it has not been possible to produce functional Ig by recombinant DNA technology.

According to a first aspect of the present invention, there is provided a process for producing a heterologous multichain polypeptide or protein in a single host cell, which comprises transforming the host cell with DNA sequences coding for each of the polypeptide chains and expressing said polypeptide chains in said transformed host cell.

According to a second aspect of the present invention, there is provided a heterologous multichain polypeptide or protein produced by recombinant DNA technology from a single host cell.

The present invention is of particular, but not exclusive, application in the production of Ig molecules and immunologically functional Ig fragments of the type referred to above. However, it will be appreciated that the present invention can be applied to the production of other multichain polypeptides or proteins.

In relation to the product of Ig molecules according to the invention it will be appreciated that, in order to produce a functional molecule, the DNA sequences used to transform the host cell will need to encode for at least the V_L and V_H domains of an Ig molecule. Moreover, these domains will need to be complementary so that when the two polypeptide chains fold together they form an antigen binding site of predetermined specificity.

Preferably, the Ig molecule or fragment include a complete light chain and at least the C_{H1} domain in addition to the V_H domain of the heavy chain. Most preferably the Ig molecule is intact.

It has also been shown by the present applicants that it is now possible to produce individual heavy and light chains having intact variable domains. This has not previously been possible. Therefore, according to a third aspect of the present invention there is provided as a product of recombinant DNA technology an Ig heavy or light chain or fragment thereof having an intact variable domain.

Advantageously, the Ig molecule or functional fragment thereof according to the present invention has a variable region (formed by the V_L and V_H domains) which defines a binding site for an antigenic determinant of clinical or industrial importance. The DNA coding sequences necessary to produce such a molecule may be derived from naturally occuring or hybridoma (monoclonal)Ig-producing cells with the desired specificity.

The constant domains of the Ig molecule or fragment, if present, may be derived from the same cell line as the variable region. However, the constant domains may be specifically altered, partially or completely omitted, or derived from a cell line producing a different class of Ig to provide Ig molecules or fragments having desired properties.

For example, an Ig molecule may be produced having variable domains (V_H and V_L) idential with those from a monoclonal antibody having a desired specificity, and constant domain(s) from a different monoclonal antibody having desired properties, for instance to provide human compatibility or to provide a complement binding site.

Such alterations in the amino acid sequence of the constant domains may be achieved by suitable mutation or partial synthesis and replacement or partial or complete substitution of appropriate regions of the corresponding DNA coding sequences. Substitute constant domain portions may be obtained from compatible recombinant DNA sequences.

The invention may be utilised for the production of Ig molecules or fragments useful for immunopurification, immunoassays, cytochemical labelling and targetting methods, and methods of diagnosis or therapy. For example, the Ig molecule or fragment may bind to a therapeutically active protein such as interferon or a blood clotting factor, for example Factor VIII, and may therefore be used to produce an affinity chromatorgraphy medium for use in the immunopurification or assay of the protein.

It is also envisaged that the Ig molecule may be synthesised by a host cell with another peptide moiety attached to one of its constant domains. Such a further peptide moiety may be cytotoxic or enzymatic. Alternatively, the moiety may be useful in attaching the Ig molecule to a biological substrate, such a cell or tissue, or to a non-biological substrate, such as a chromatography medium. Such a peptide moiety is herein referred to as a structural peptide moiety.

It is further envisaged that cytotoxic, enzymic or structural peptide moieties could be attached to the Ig molecule by normal peptide chemical methods, as are already known in the art, rather than by being synthesised with the Ig molecule.

The Ig molecule or fragment may also comprise a therapeutic agent in its own right. For instance, an Ig molecule or fragment specific for D blood group antigen may be useful for the prevention of haemolytic disease of the new born.

Any suitable recombinant DNA technique may be used in the production of the multichain polypeptides or proteins of the present invention. Typical expression vectors such as plasmids are constructed comprising DNA sequences coding for each of the chains of the polypeptide or protein.

It will be appreciated that a single vector may be constructed which contains the DNA sequences coding for more than one of the chains. For instance, the DNA sequences coding for Ig heavy and light chains may be inserted at different positions on the same plasmid.

Alternatively, the DNA sequence coding for each chain may be inserted individually into a plasmid, thus producing a number of constructed plasmids, each coding for a particular chain. Preferably the plasmids into which the sequences are inserted are compatible.

The or each constructed plasmid is used to transform a host cell so that each host cell contains DNA sequences coding for each of the chains in the polypeptide or protein.

Suitable expression vectors which may be use for cloning in bacterial systems include plasmids, such as Col E1, pcR1, pBR322, pACYC 184 and RP4, phage DNA or derivatives of any of these.

For use in cloning in yeast systems, suitable expression vectors include plasmids based on a 2 micron origin.

Any plasmid containing an appropriate mammalian gene promoter sequence may be used for cloning in mammalian systems. Such vectors include plasmids derived from, for instance, pBR322, bovine papilloma virus, retroviruses, DNA viruses and vaccinia viruses.

Suitable host cells which may be used for expression of the heterologous multichain polypeptide or protein include bacteria, such as *E. coli* and *B. subtilis*, Streptomyces, yeasts, such as *S. cervisiae*, and eukaryotic cells, such as insect or mammalian cell lines. Examples of suitable bacterial host cells include *E. Coli* HB 101, *E. Coli* X1776, *E. Coli* X2882, *E. Coli* PS 410, *E. Coli* MRC 1, *E. Coli* RV308, *E. Coli* E103S and *E. Coli* B.

The present invention also includes constructed expression vectors and transformed host cells for use in producing the multichain polypeptides or proteins of the present invention.

After expression of the individual chains in the same host cell, they may be recovered to provide the complete multichain polypeptide or protein in active form, for instance to provide an Ig molecule of predetermined immunological function.

It is envisaged that in preferred forms of the invention, the individual chains will be processed by the host cell to form the complete polypeptide or protein which advantageously is secreted therefrom.

However, it may be that the individual chains may be produced in insoluble or membrane-bound form. It may therefore be necessary to solubilise the individual chains and allow the chains to refold in solution to form the active multichain polypeptide or protein. A suitable procedure for solubilising polypeptide chains expressed in insoluble or membrane-bound form is disclosed in our copending application No. (Protein Recovery, Agent's Ref. GF 402120 and 402121).

It will be appreciated that the present application shows for the first time that it is possible to transform a host cell so that it can express two or more separate polypeptides which may be assembled to form a complete multichain polypeptide or protein. There is no disclosure or suggestion of the present invention in the prior art, which relates solely to the production of a single chain heterologous polypeptide or protein from each host cell.

The present invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

FIG. **1** shows a diagrammatic representation of a typical intact Ig molecule;

FIG. **2** shows the construction of plasmids for the direct synthesis of a λ light chain in *E. Coli;*

FIG. **3** shows the construction of plasmids for the direct synthesis of a μ heavy chain in *E. Coli;*

FIG. **4** is a diagrammatic representation of μ*m*RNA sequences around the initiation codon;

FIG. **5** shows the construction of plasmids having altered secondary structure around the initiation codon;

FIG. **6** is a polyacrylamide gel showing expression and distribution of μ protein from *E. Coli* B;

FIG. **7** is a polyacrylamide gel showing pulse chase autoradiograms of μ protein in *E. Coli* B and in *E. Coli* HB101;

FIG. **8** is a polyacrylamide gel showing the results of λ gene expression in *E. Coli;*

FIG. **9** is a polyacrylamide gel showing the distribution of recombinant λ light chain polypeptide between the soluble and insoluble cell fractions;

FIG. **10** is a polyacrylamide gel showing expression and distribution of λ protein from *E. Coli* E103S;

FIG. **11** shows the results of the fracitonation of μ and λ protein expressed by *E. Coli* B on DEAE Sephacel;

FIG. 12 shows the specific hapten binding of reconstituted Ig molecules; and

FIG. 13 shows the heteroclitic nature of the hapten binding of the reconstituted Ig molecules.

In the following examples, there is described the production of Ig light and heavy chain polypeptides derived from monoclonal antibodies which recognise and bind to the antigenic determinant 4-hydroxy-3-nitrophenyl acetyl (NP), using *E. coli* and *S. cerevisiae* as the host cells Recombinant DNA techniques were used to enable the host cells to express both the polypeptide chains.

It will be appreciated that the invention is not limited to the specific methods and construction described hereafter.

Construction of Lambda Light Chain Expression Plasmid

FIG. 2, to which reference is now made, shows schematically the method used to construct a λ light chain expression plasmid.

It was decided to express the lambda gene in *E. coli* by direct expression of the gene lacking the eucaryotic signal peptide but containing a methionine initiator residue at the amino-terminus (met-lambda). The approach used for bacterial synthesis of met-lambda was to reconstruct the gene in vitro from restriction fragments of a cDNA clone and to utilise synthetic DNA fragments for insertion into the bacterial plasmid pCT54 (Emtage et al., proc. Natl. Acad. Sci. USA., 80, 3671 to 3675, 1983). This vector contains the *E. coli* trp promoter, operator and leader ribosome binding site; in addition 14 nucleotides downstream of the ribosome binding site is an initiator ATG followed immediately by $EcoR_1$ and HindIII sites and the terminator for *E. coli* RNA polymerase from bacteriophage T7.

As a source of light chain we used a plasmid pABλ-1-15 which contains a full-length λ_1 light chain cDNA cloned into the PstI site of pBR322. This λ_1 light chain is derived from a monoclonal antibody, designated S43, which binds to 4-hydroxy-3-nitrophenylacetyl (NP) haptens.

In order to create a HindIII site 3' to the end of the lambda gene for insertion into the HindIII site of pCT54, the cDNA was excised from pAB λ 1-15 using PstI. The cohesive ends were blunt ended using the Klenow fragment of DNA polymerase and synthetic HindIII linker molecules of sequence 5'-CCAAGCTTGG-3' ligated. The DNA was digested with HindIII and the 850bp lambda gene isolated by gel electrophoresis and cloned into HindIII cut pAT153 to yield plasmid pAT λ 1-15. The 3' end of the lambda gene was isolated from pAT λ 1-15 by HindIII plus partial SacI digestion as a 630bp SacI-HindIII fragment (2 in FIG. 2). The HindIII cohesive end was dephosphorylated by calf intestinal alkaline phosphatase during isolation of the fragment to prevent unwanted ligations at this end in subsequent reactions.

A HinfI restriction site is located between codons 7 and 8 and the lambda sequence. The 5' end of the lambda gene was isolated as a 148bp HinfI to sacI fragment (1 in FIG. 2).

Two oligodeoxyribonucleotides were designed to restore condons 1-8, and to provide an initiator ATG as well as BclI and HinfI sticky ends. The two chemically synthesised oligonucleotides made to facilitate assembly of the gene had the sequences:

R45 5'-pGATCAATGCAGGCTGTTGTG 3'

R44 3' CCGACAACACTGAGTCCTTAp- 5'

pCT54 was cut with both Bcl1 and HindIII and the resulting linear molecules isolated, mixed together with the two oligodeoxyribonucleotide linkers R44 and R45 and both fragments 1 and 2, and ligated using T4 ligase (FIG. 2). The mixture was used to transform *E. coli* DH1 to ampicillin resistance. Recombinant clones in pCT54 were identified by hybridisation of DNA from replica plated colonies on nitrocellulose to a nick-translated probe derived from the pAT λ 1-15 insert.

A clone was identified which hybridised to lambda cDNA and also showed the predicted restriction fragment pattern. This plasmid (designated pCT54 19-1) was sequenced from the ClaI site and shown to have the anticipated sequence except that there was a mutation of the fourth codon from CTG to ATG, changing the amino acid at this point from valine the methionine.

The sequence in this area was:

```
. . . GATTGATCA ATG CAG GCT GTT ATG ACT CAG GAA TCT GCA CTC ACC ACA TCA
        met  gln  ala  val  met  thr  gln  glu  ser  ala  leu  thr  thr  ser
```

The restriction enzyme sites in pCT54 between the Shine and Dalgarno sequence (AAGG), which is important for ribosome binding, and the ATG allow for the adjustment of the SD-ATG distance, an important parameter in determining expression rates. The SD-ATG distance was reduced by cutting the plasmid with ClaI or BclI and creating blunt ended species by digestion with S_1 nuclease. 2 μg of ClaI cut DNA was digested with 200 units of S_1 nuclease for 30 minutes at 30° using standard buffer conditions. The solution was deproteinised with phenol and the DNA recovered by ethanol precipitation. This DNA on religation with T4 DNA ligase and transformation into *E. coli* strain HB101 gave rise to a number of plasmids which had lost the ClaI or BclI site.

The plasmids which had lost their ClaI site were sequenced in the region surrounding the initiator ATG.

```
. . AAGGGTATTGATCAATG CAG . . .   plasmid pNP3
     SD                met  glu

. . AAGGGTTTGATCAATG CAG          plasmid pNP4
     SD                met  glu
```

In order to achieve high level expression a number of other approaches were followed. Firstly, a series of constructs were obtained which had increasing amounts of the 3' untranslated region of the cDNA removed by Bal 31 exonuclease. Secondly, a high copy number plasmid containing λcDNA was constructed. This plasmid contained a par function (Meacock, P. A. and Cohen, S. N., Cell, 20, 529-542, 1980) as well as being present in high copy number. Thirdly, the pNP3 plasmid was transformed into a number of protease-deficient strains or into HB101 in conjunction with a protease deficient dominant acting plasmid (Grossman, A. D. et al, Cell, 32, 151-159, 1983).

Construction of μ Heavy Chain Expression Plasmid

The full-length μ heavy chain cDNA derived from the NP binding monoclonal antibody B1-8 had been cloned into the PstI site of pBR322 yielding a plasmid designated pABμ-11 (Bothwell et al, Cell, 24, 625-637, 1981). In order to achieve high level expression, the μ cDNA minus the eukaryotic leader was reconstructed into pCT54. The construction of the μ heavy chain expression plasmid is shown diagramatically in FIG. 3. Two chemically synthesised oligonucleotides were made to facilitate this. These have the following sequences:

R43 5' GATCAATGCAGGTTCAGCTGCA 3'

R46 3' TTACGTCCAAGTCG 5'

These were ligated into BclI cut pCT54 using T4 DNA ligase and the resulting plasmid designated pCT54 Pst. The linkers were designed to replace the sequence in pCT54 between the BclI site and the ATG, to provide an internal PstI site and to recreate the sequence from pABμ-11 5' to the PstI site up to codon +1. pCT54 Pst was cut briefly was PstI, treated with alkaline phosphatase and full length DNA glass isolated from a 1% agarose gel. Similarly pABμ-11 was briefly cut with PstI and the full length μ insert isolated following agarose gel electrophoresis. The μ cDNA was ligated with the full length pCT54Pst fragment using T4 DNA ligase under standard conditions and a plasmid designated pCT54μ was identified which by restriction enzyme analysis was shown in contain a full-length μ insert. The plasmid was sequenced around the 5' linker region and was found to have the anticipated sequence:

5' . . .
TGATCAATGCAGGTTCAGCT-
GCAGGGGGGGGATGGATGGAG . . . 3',

demonstrating that it was, indeed, a full length clone. A complete PstI digest of pCT54μ liberated a 1.4 Kb fragment which was purified by 0.8% agrose gel electrophoresis and glass powder isolation. This was ligated with PstI cut pCT54 Pst (see above) using T4 DNA ligase under standard conditions, followed by transformation into HB101. A plasmid designated pNP1 was isolated which was shown by restriction endonuclease pattern analysis to contain the 1.4 Kb μcDNA fragment in an appropriate orientation (FIG. 3). pNP1 was a plasmid which consisted of an appropriate 5' end for expression, whilst pCT54 contained an appropriate 3' end. The full length gene was reconstructed into pCT54 by cutting both pNP1 and PCT54μ with AvaI which cuts once in the plasmid and once in the μ gene. Both digests were run on a 1% agrose gel and the 1.9 kb fragment from pNP1 and the 3.65 kb fragment from pCT54μ isolated. Following alkaline phosphatase treatment of the 3.65 pCT54μ fragment, the two pieces of DNA were ligated to each other and transformed into HB101. A plasmid designated pNP2 was identified which demonstrated the correct restriction endonuclease pattern. It was sequenced in the area surrounding the initiator ATG and found to have the anticipated sequence:

5' . . .
TTGATCAATGCAGGTTCAGCTGCAG-
CAGCCTGGGGCTGAGCTTGTGAAG . . . 3'

The vector pCT54 had been constructed to include two restriction sites (BclI and ClaI) between the S-D sequence (AAGG) and the initiation codon so that the distance between these sequence elements could be varied. As most *E. coli* mRNAs have 6-11 nucleotides between the S-D sequence and the AUG, the distance in pNP2 was reduced by modification at the ClaI site. pNP2 was cut with ClaI and incubated with S1. The amount of S1 nuclease was adjusted so that some DNA molecules would lose 1-2 extra base pairs as a result of 'nibbling' by the enzyme. This DNA on religation with T4 DNA ligase and transformation into *E. coli* strain HB101 gave rise to a number of colonies harbouring plasmids which had lost the ClaI site. The sequences around the initiation codon of four plasmids pNP223, pNP261, pNP9 and pNP282 were determined and are given in Table 1, part A below.

TABLE 1

		S—D to ATG distance (no. of residues)	Sequence (5' - 3') Clot	Expression:(mu)(ru) expts. A	B	C
(A)	pNP2	14	—AAAAAGGGTATCGATTGATCA[ATG]CAGGTTCAG—	0.7	0.6	0.8
S1 derivatives of pNP2	pNP223	12	—AAAAAGGGTATATTGATCA[ATG]CAGGTTCAG—	—	0.7	0.6
	pNP261	10	—AAAAAGGGTATTGATCA[ATG]CAGGTTCAG—	—	1.1	1.1
	pNP282	7	—AAAAAGGGTGATCA[ATG]CAGGTTCAG—	—	0.7	0.6
	pNP9	9	—AAAAAGGGTATGATCA[ATG]CAGGTTCAG—	1.0	1.0	1.0
(B)	pNP11	11	—AAAAAGGGTATTGCACAT[ATG]CAAGTGCAA—	5.3	6.3	9.1
oligo constructs	pNP12	11	—AAAAAGGGTATTGCACA[ATG]CAGGTTCAG—	1.8	2.3	1.3

TABLE 1-continued

	S···D to ATG distance (no. of residues)	Sequence (5' - 3') Clot	Expression:(mu)(ru) expts. A	B	C
pNP14	9	—AAAAAGGGTATGATCA[ATG]CAAGTGCAA—	72.2	106.7	124.6
pNP8	—	—	77.0	53.4	145 5
pCT70	—	—	0	0	0

The expression of these constructs in *E. coli* B was examined in three experiments, and concentrations of μ protein were determined by ELISA as relative units (ru), a measure of μ concentration normalised to pNP9. The results are shown in Table 1.

Inductions were carried out by resuspending cells at 1:50 dilution from overnight cultures into induction medium, consisting of: KH_2PO_4 (3 g/l), Na_2HPO_4 (6 g/l), NaCl (0.5 g/l), Difco vitamin assay casamino acids (30 g/l), NH_4Cl (4 g/l), glycerol (16 g/l), proline (0.5 g/l), Difco yeast extract (1 g/l), $CaCl_2.6H_2O$ (0.022 g/l), $MgSO_4.7H_2O$ (0.025 g/l), thiamine (0.01 g/l) and carbenicillin (0.1 g/l). Cultures were shaken at 37° C. and cells harvested by centrifugation.

ELISA assays were performed essentially as described by Patel et al. (Nucleic Acid Research, 10, 5605 to 5620, 1982), using affinity purified goat anti-mouse IgM (Tago) and affinity purified goat anti-mouse IgM (Tago) conjugated peroxidase, with 3,3',5,5' tetramethylbenzidine (Miles) as a substrate. Bacterial cell pellets were boiled for 2 minutes in 9M urea (BRL) and loaded directly into the ELISA, such that all wells contained 2.25M urea, 10 mM Tris-HCl pH 7.8 150 mM NaCl, 0.05% NP40 and 0.5% casein (Hammarsten, B. D. H.).

Altering the S-D to ATG distance with nuclease S1 was found to increase the level of μ expression, relative, to the parental plasmid pNP2, but only to a small extent (compare pNP9). The optimal S-G to AUG distance was found to be 9-10 nucleotides, as found in pNP9 and pNP261 respectively.

Secondary Structure Analysis

Hairpin loops were identified by use of the computer programme HAIRGU developed by Dr. Roger Staden. The ΔG values were calculated as described by Trioco et al. (Nature NB, 246, 40-41, 1973).

Analysis of the potential secondary structure of μ mRNA encoded by pNP2 and pNP9 (one of the S1 derivatives of pNP2) revealed an extensive array of possible hairpin loops. Attention was focused on the region of this mRNA containing the ribosome binding site since the sequestering of S-D sequences and initiation codons into secondary structure may inhibit the initiation of translation. It was found that a hairpin loop including the U and G of the initiation codon could be formed (see FIG. 4). This hairpin loop is formed entirely within coding sequences and has a $\Delta G = -7.6$ kcal. No secondary structures were found which buried the S-D sequence, and none which mutually excluded the hairpin loop described above, and which had a lower ΔG.

To test whether expression of μ protein could be influenced by changes designed to alter potential secondary structure in the ribosome binding region, we used synthetic oligonucleotides to mutagenise this region of μ mRNA. The plasmids were constructed by insertion of a pair of oligonucleotides between the blunted Cla I site and the 5' Pst site of μ in pNP2 (see FIG. 5). The cloning strategy used to accomplish this, however, was multi-staged due to the presence of four Pst sites in pNP2. The vector pACYC184CM, which lacks Cla I and Pst I sites, was first constructed by cutting pACYC184 at its unique Cla I site, 'filling-in' the cohesive termini using T4 DNA polymerase and religating. Next, PACYC184CM was cut with BamHI, and the BamHI fragment from pNP2, bearing the 5'μ sequence, was inserted into it to form pCMμ (see FIG. 4). The vector pCMμ contained unique Cla I and Pst sites which were required for the oligonucleotide cloning (see FIG. 5).

pcMμ was cut with Cla I to completion, blunted with nuclease S1, and then cut with Pst I. The cut plasmid was purified by agarose gel electrophoresis and ligated separately with pairs of oligonucleotides, with the latter in a 100-fold molar excess. The pairs of oligonucleotides used were: R131 and R132 (for pNP11); R196 and R197 (for pNP12); and R202 and R203 (for pNP14). These have the sequences given below.

TGCACATATGCAAGTGCAACTGCA (R131),

GTTGCACTTGCATATGTGCA (R132),

GCTGAACCTGCATATGTGCA (R196),

TGCACATATGCAGGTTCAGCTGCA (R197),

GTTGCACTTGCATTGATC (R202),

GATCAATGCAAGTGCAACTGCA (R203).

The ligation mixture was then used to transform HB101. Recombinant clones were detected by colony hybridisation, on nitrocellulose filters, using one of each pair of oligonucleotides as a probe. Positive clones were sequenced and shown to have the correct nucleotide sequence. The μ sequences containing the cloned oligonucleotides were excised on Eco RV-Bgl II fragments. These were ligated to Eco RV-Bgl II cut pNP2 to reconstruct the full length μ genes (see FIG. 5). Three different plasmids were created by these procedures; pNP11, pNP12 and pNP14, having the nucleotide sequences shown in Table 1 above.

In the construct pNP11 the potentially deleterious hairpin loop of pNP2 was abolished by changing the residues in the degenerate position of three codons and by introducing a different S-D to AUG sequence. These changes allow the region between the S-D and AUG to base pair with the 5' end of μ coding sequence to form a hairpin loop of $\Delta G = -7.8$ kcal, approximately equal to that of pNP2, but leaving the AUG and S-D sequences exposed (see FIG. 4). The only other mutually exclusive hairpin loops are of $\Delta G = -2.3$, -3.6 and $+0.4$ kcal.

The construct pNP12 retains the S-D to AUG sequence of pNP11, but has the coding sequence of pNP2, such that the hairpin loop of pNP2 can form (see FIG. 4).

The final μ construct was pNP14, which had the S-D to AUG sequence of pNP9, but the 5' coding sequence of pNP11, so that the hairpin loop of pNP12 could not form. The construct pNB14 had no secondary structure burying the S-D or μ AUG.

μ Protein Expression from Constructs with Differing mRNA Secondary Structures

E. coli B strains containing plasmids pNP9, pNP11, pNP12 and pNP14 were grown in induction medium and samples removed for ELISA assay. The concentration of μ was increased 6–7 fold in *E. coli* B cells containing pNP11 relative to pNP9 (see Table 1, Part b). Plasmid pNP12, which has the S-D to AUG sequence of pNP11, but the coding sequence of pNP9, showed only a two-fold increase in level of μ expression compared to pNP9.

Plasmid pNP14, which has the S-D to AUG sequence of pNP9 but the 5'μcoding sequence of pNP11, and thus differed from pNP9 only in three residues, was found to express μ protein over 90 times that found in pNP9.

A plasmid, pNP8, has also been constructed, from which μ is expressed, also from the trp promoter, as part of a trpE-μ fusion protein containing the amino terminal 53 amino acids of trpE and the carboxyl terminal 503 amino acids of μ. The rationale for making this fusion gene was that μ would be translated from an effective bacterial ribosome binding site rather than from one of unknown efficiency. Then, by comparing the amounts of trpE-μ produced from pNP8 with those of μ produced from the different constructs, an estimate of relative ribosome binding efficiency may be made. When the trpE-μ gene in pNP8 was induced and the products quantitated by ELISA, it was found that the μ expression in strains containing pNP8 and pNP14 was very similar (see Table 1, part B). The ribosome binding site (RBS) sequences of pNP11, pNP12 and pNP14 were designed in order to test the hypothesis that sequestering of the initiation codon into secondary structure inhibits translation initiation, and to increase the rate of initiation by releasing the initiation codon. In pNP11, the RBS hairpin loop of pNP2 and pNP9 was abolished by changing the 5'μcoding sequence in the degenerate position of three codons. In addition, the S-D to AUG sequence was changed to allow the base-pairing of this region with the 5' coding sequence. The result of these changes is that the initiation codon is exposed in the loop of a hairpin with the S-D sequence at the base of the stem (see FIG. 4). This hairpin loop had a ΔG approximately equivalent to the ΔG of the pNP9 hairpin loop. Strains containing pNP11 produced 6–7 times as much μ as did those with pNP9. To test whether this was due to the change in S-D to ATG sequence characteristics, rather than to the liberation of the initiation codon, pNP12 was constructed as a control. The latter has the S-D to AUG sequence of pNP11, but retains the 5'μ coding sequence of pNP9, so that the RBS hairpin loop of pNP11 cannot form, but the potentially deleterious one of pNP9 can form. The level of μ expression from pNP12 was found to be increased by only two-fold relative to pNP9. This increase is probably due to an altered S-D to AUG distance, a U immediately 5' to the initiation codon, and the S-D to AUG sequence being G-poor each of which has been shown to be advantageous. It is therefore likely that the increased expression from pNP11 is due to a preferable mRNA secondary structure. The RBS hairpin loop of pNP9 probably acts to inhibit translation while creation of another hairpin loop which exposes the initiation codon is responsible for the increased expression.

The plasmid pNP14 was constructed, such that it had the S-D to AUG sequence of pNP9, but the coding sequence of pNP11. The expression plasmid pNP14, therefore, differed from pNP9 in only three residues, those in the degenerate position of three codons, yet strains containing pNP14 expressed over ninety times more μ than those containing pNP9. This increase in expression cannot be explained on the basis of the three residue changes optimizing codon usage, for they introduce less favoured codons, as judged by the frequency of their occurrence in strongly expressed *E. coli* genes.

The increased expression is best explained by the abolition of the RBS hairpin loop in pNP9. The magnitude of the increases in expression observed, and the few residue changes introduced, makes it difficult for these results to be interpreted in terms of changes in mRNA stability.

The expression of pNP8, which encodes the trpE-μ fusion protein from a native trpE RBS, was also examined, and found to express at approximately the same level as pNP14. This may indicate that the RBS of pNP14 is equivalent to that of trpE in terms of its efficiency in directing translation initiation. The native trpE RBS of pNP8 and that of pNP14 were found to have no secondary structures hindering the use of their RBS sequences.

Expression of μ Protein in *E. Coli*

E. Coli B cells containing the μ expression plasmid pNP11 were grown under inducing conditions and soluble and insoluble extracts prepared, and analysed by SDS-PAGE. A novel band was seen after staining the gel with Coomassie blue in the lane containing proteins from the insoluble fraction (see FIG. 6, lane 2). This band was not seen in the negative control lane which contained proteins from the same fraction from cells harbouring pCT70 (see FIG. 6, lane 3). The novel band was found to migrate to a position corresponding to a protein of a molecular weight within less than 5% of the actual molecular weight of non-glycosylated μ of 62.5 Kd. A duplicate set of lanes were transferred to nitrocellulose, and Western blotted. Alignment of the stained gel and the blot autoradiogram confirms that this novel band is antigenically related to IgM (see FIG. 6, lanes 4 and 8). No band was found in extracts from cells containing pCT70 (see FIG. 6, lanes 5 and 7). Only a low amount of μ was found in the soluble fraction (see FIG. 6, lane 6).

A greatly increased level of expression of μ was found in *E. Coli* B compared to HB101. Pulse chase analysis demonstrated that in *E. Coli* B, a similar level of μ protein was detected after a 60 minute chase (FIG. 7, lane 3) as was seen after the initial labelling period (FIG. 7, lane 1). In HB101, however, very little μ protein could be seen after a 10 minute chase (FIG. 7, lane 7), and none after 30 minutes (FIG. 7, lane 8), compared to the amount detected after the initial labelling period (FIG. 7, lane 5).

For pulse chase analysis, inductions were set up as described above, except that the medium used consisted of: proline (0.3 g/L), leucine (0.1 g/L), Difco methio-

nine assay medium (5 g/L), glucose (60 mg/L), thiamine (10 mg/L), $CaCl_2$ (22 mg/L), $MgSO_4$ (0.25 g/L)m and carbenicillin (0.1 g/L). During exponential growth cells were pulse labelled with 30 μCi/ml L-[^{35}S]methionine for 2 minutes, after which unlabelled methione (100 μg/ml) was added and the incubation continued for the times indicated.

Induced *E. Coli* B cells harbouring pNP14 when examined by phase contrast microscopy were found to contain inclusion bodies.

Expression of λ Light Chain in *E. Coli*

A fresh 1 ml overnight culture of *E. Coli* HB101 or RV308 containing the plasmid under study was grown in L Broth (Maniatis et al,, Molecular Cloning, Cold Spring Harbor Laboratory, 1982), supplemented with carbenicillin to 100 μg/ml.

A 1:100 dilution of this culture was made into M9 medium (Maniatis, 1982, op.cit.) supplemented with glucose, vitamin B_1, carbenicillin, leucine and proline and the cultures shaken at 37° for 5–6 hours. At this time ^{35}S-methionine was added for 5–20 minutes. The cells were then harvested by centrifugation, lysed by boiling in 1% SDS for 2 minutes and diluted by addition of a buffer consisting of 2% Triton X-100, 50mM Tris pH 8, 0.15M NaCl and 0.1 mM EDTA. Immunoprecipitations were carried out by addition of antisera to aliquots of labelled *E. Coli* extracts and, after incubation at 4° overnight, immune complexes were isolated by binding to Staphylococcus aureus fixed cells. The complexes were dissociated by boiling in 60 mM Tris pH 6.8 buffer containing 10% glycerol, 3% SDS and 5% mercaptoethanol and the liberated proteins analysed on 10% or 12.5% acrylamide/SDS gels (Laemmli, UK. Nature, 227, 680–685, 1970). Gels were stained with Coomassie Brilliant blue to visualise the protein bands while labelled proteins were detected on Fuji-RX film by fluorography using 1M sodium salicylate.

HB101 cells containing pNP3 and other plasmids were grown under inducing conditions to an OD_{600}=0.6 and the proteins present examined by specific immunoprecipitation substantially as described above. The results obtained are given in FIG. 8, in which lanes 1 and 2 respectively were extracts from pCT54-19 and pNP3, immunoprecipitated with normal rabbit serum; lanes 3, 4 and 5 were extracts immunoprecipitated with rabbit anti-lambda serum and represent pNP4 (lane 3), pNP3 (lane 4) and pCT54-19 (lane 5). The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of FIG. 8. All of the plasmids produced a protein which reacted with rabbit anti-mouse λ light chain serum and comigrated with authentic λ_1 light chain from the mouse myeloma MOPC104E. pNP3 however produced the most of this protein (compare lane 4 with lanes 3 and 5 in FIG. 8). No such band was detected with control immunoprecipitations using normal rabbit serum (FIG. 8, lanes 1 and 2). Studies using brief pulses with ^{35}S-methionine followed by chasing with excess cold methionine indicated that the recombinant λ light chain had a ½ life of about 20 minutes.

In vitro transcription/translation (Pratt et al, Nucleic Acids Research, 9, 4459–447, 1981) confirmed that pNP3 coded for a protein which comigrated with authentic λ light chain. This 25 Kd product was synthesized in vitro at a rate comparable to that of β-lactamase indicating that it is synthesised in vivo at a level of 0.5% of total *E. Coli* proteins. This figure is in good agreement with the percentage of recombinant λ product synthesised in vivo (0.4–2%) as determined from the following equation:

$$\% \text{ specific } CPM = \frac{100 \times (CPM \text{ with anti-}\lambda) - (CPM \text{ with normal serum})}{\text{total } CPM}$$

The procedure used to disrupt cells was as follows (Emtage J S et al, Proc. Natl. Acad. Sci., 80, 3671–3715, 1983). *E. Coli* HB101/pNP3 grown under inducing conditions were harvested and resuspended in 0.05M Tris pH 8, 0.233M NaCl, 5% glycerol containing 130 g/ml of lysozyme and incubated at 4° C. or room temperature for 20 minutes. Sodium deoxycholate was then added to a final concentration of 0.05% and 10 μg of DNAase 1 (from bovine pancreas) was added per g wet wt of *E. Coli*. The solution was incubated at 15° C. for 30 minutes by which time the viscosity of the solution had decreased markedly. The extract was centrifuged (at 10,000×g for 15 minutes for small volumes (1 ml) or 1 hour for larger volumes) to produce a soluble and an insoluble fraction.

For immunoprecipitations, the soluble fraction was diluted in the Triton-containing buffer described above and the insoluble fraction solubilised by boiling in 1% SDS followed by dilution in Triton-containing buffer. HB101 cells containing pNP3 or pNP4 were grown under inducing conditions, pulse labelled with [^{35}S]methionine, separated into soluble and insoluble fractions and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results obtained are shown in FIG. 9 in which lanes 1 and 3 correspond to the soluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lanes 2 and 4 correspond to the insoluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively, lanes 5 and 7 correspond to the soluble fraction from HB101-pNP4 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lane 9 corresponds to the soluble fraction from HB101-pNP3, and lane 10 corresponds to the insoluble fraction from HB101-pNP3. The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of FIG. 9. When such a procedure was carried out using pNP3, the recombinant λ light chain protein was present in the insoluble fraction (FIG. 9, lane 4) rather than the soluble fraction (FIG. 9, lane 3). No 25 Kd band comigrating with authentic λ_1 light chain was present when either the soluble or insoluble fraction were immunoprecipitated using normal rabbit serum (FIG. 9, lanes 1 and 2). By inference it is likely that all Ig light chains will fractionate as in this specific example.

In the absence of specific immunoprecipitation a novel protein band was not visible from extracts of HB101 containing pNP3 nor was the λ protein found to accumulate. However, there was a dramatic difference when pNP3 was induced in the K12 strain E103s. In this strain λ protein was found to accumulate during induction until the cells reaches stationary phase (FIG. 10, lanes 3–5) to a level of about 150 times that found in HB101 as determined by an ELISA (enzyme linked immunosorbent assay). These cells were found to contain inclusion bodies which appeared refractile under light microscopy, a phenomenon characteristic of high

level expression of foreign proteins. An estimate of the pecentage of total *E. coli* protein represented by recombinant λ protein was obtained by separating the proteins by gel electrophoresis, staining them, with Coomassie blue and scanning the stained gel with a Joyce-Loebl chromoscan 3. This method showed that λ was the major protein present (FIG. 10, lane 5) and represented 13% or total *E. coli* protein. The λ protein had a half-life of 20 minute in HB101 but accumulated to very high levels in E103s, suggesting that lambda protein was much more stable in the latter strain. After cell lysis and centrifugation of HB101 or E103s containing pNP3, λ light chain was detected in the insoluble (FIG. 10, lanes 7 and 10) but not in the soluble fractions (FIG. 10, lanes 6), as determined by Coomassie blue staining. The identity of the major Coomassie blue stained band as λ protein was confirmed by Western blot analysis (FIG. 10, lanes 8–10). The presence of such immunoreactive bands was specific to pNP3 containing cells. When extracts from cells containing pCT70, a prochymosin expressing plasmid, were subjected to the same analysis no bands were detected. This more sensitive technique showed that a small amount of the λ protein was in the soluble fraction (FIG. 10, lane 9). The presence of a number of distinct immunoreactive proteins all smaller than full-length protein were also detected. These may result from proteolytic degradation of of λ protein, from premature termination of transcription or from internal initiation of translation.

Expression of μ and Lambda Polypeptides in the same Bacterial Cell

Each of the Ig μ and λ genes in expression plasmids were transformed into the same *E. Coli* cell to direct the synthesis of both Ig μ and λ polypeptides. In order to overcome plasmid incompatibility and provide a second antibiotic resistance marker, the trp promoter and λ sequences were excised from pNP3 on a Hind III-Bam HI fragment and inserted into the Hind III-Bam HI fragment of pACYC 184. The resultant plasmid pACYC λ caused the *E. coli* to grow very poorly. This weak growth was thought to result from read-through of RNA polymerase into the origin of replication. However, the inhibition of growth was virtually eliminated by the cloning in of the baceteriophage T7 transcriptional terminator at the Hind III site of plasmid pACYC λ.

This terminator functions in both orientations. The resultant plasmid pAC λ T7-1 has a chloroamphenicol resistance gene and an origin compatible with the pBR 322-derived origin on pNP14, the Ig μ expressing plasmid. Transformation of both plasmids in the same *E. Coli B* was achieved in two steps. Firstly pNP14 was introduced followed by pAC λ T7- I in two sequential transformations to give ampicillin and chloramphenicol resistant clones.

E. Coli B cells derived form this double-transformant clone showed the presence of inclusion bodies and two novel polypeptide bands on stained gels of the insoluble fraction after lysis. These two bands correspond both with immunological activity by Western blotting for Ig μ and Ig λ and in their expected molecular weights. It has thus been shown that the double-transformant clone expresses both the heterologous genes. This had not hitherto been shown.

The presence of λ light chain in the insoluble fraction was a useful purification step since it both concentrated the protein and separated if from the bulk of *E. Coli* soluble proteins.

For further purification of the λ light chain, the cell debris were dissolved in 10 mM Tris-HCl pH8.0, 25% formamide, 7M urea, 1 mM EDTA and 2 mM dithiothreitol. This material was loaded onto a DEAE Sephacel column (Pharmacia) (1×25 cm at a flow rate of 5 ml/hr) which had been equilibrated in 9M urea, 10 mM Tris-HCl pH 8.0, 1 mM EDTA and 2 mM DTT. The DEAE Sephacel column was developed using a 0–150 mM NaCl gradient in loading buffer. The eluted peak λ light chain immunoreactivity, corresponding to the major peak of protein, was diluted to a final concentration of 2.25M urea, 10 mM Tris-Hcl pH 8.0, 1 mM EDTA, 2 mM DTT and loaded onto an octylSepharose column (Pharmacia) (2.5×10 cm). Material was eluted by use of a urea gradient of 2.25–9M urea. The peak material was pooled, dialysed into ammonium bicarbonate and lyophilised. Following this step, only a single band of Coomassie blue stainable material corresponding to recombinant λ protein was visualised by SDS-PAGE.

The μ heavy chain was purified from 9M urea solubilised pellets by anion exchange chromatogrpahy and chromatofocussing (Pharmacia).

It was of great interest to determine whether the concomitant expression of μ and λ would lead to the formation of functional IgM. In order to determine this, extracts were made from *E. coli* containing both Ig μ and λ polypeptides and these tested for antigen binding. We used a two-site sandwich ELISA which detects μ chain binding to haptenalated bovine serum albumin (NIP-caproate-BSA).

In the NIP binding assay, bovine serum albumin (BSA) is reacted with an equimolar amount of NIP-Cap-N-hydroxy-succinamide at pH 7.5 in 10 mM phosphate buffer. The resulting NIP-Cap-BSA is separated from free NIP-Cap on a G-50 Sephadex column.

Microtitre plates (96 well Nunc Immuno Plate 1) are coated with 100 μl of a solution of 10 μg/ml NIP-BSA in sodium carbonate/sodium bicarbonate 0.1M, pH 9.6 buffer (coating buffer) overnight at 4° C.

The coated plates are then blocked for non-specific binding by addition of 100 μl of 0.5% casein in coating buffer (blocking buffer) and incubating at 37° C. for 1 hour.

The coated and blocked plates are then washed three times in 150 mM NaCl, 0.05% NP40, 20 mM Tris-HCl, pH 7.8 buffer (washing buffer).

The washed plates are shaken free of excess washing buffer and samples are added in their buffer or typically in 0.5% casein in washing buffer (sample buffer) to a 100 μl final volume.

In order to demonstrate the nature of the binding to NIP-BSA, samples for testing were supplemented with either NIP or NP is free solution at various concentrations (from 60 to 0.3 μM for NIP or from 600 to 3 μM for NP).

The plates with samples are then incubated at 37° C. for one hour and thereafter washed three times with washing buffer.

The washed plates are then innoculated with 100 μl of anti μ-peroxidase conjugate (1:1000 dilution; TAGO Inc) in sample buffer, and incubated for one hour at 37° C. The plates are then washed three times in washing buffer.

The washed plates are innoculated with 100 μl of 0.1M Na acetate-citrate, pH 6.0, 0.1 mg/ml tetramethyl-

benzidine, 13 mM H_2O_2 (peroxidase substrate) and incubated at room temperature for one hour. The reaction is terminated by the addition of 25 μl of 2.5M H_2SO_4.

The microtitre plates are then read in a plate reader (Dynatech) at 450 λm with a reference of 630 λm. The A_{450} was related to the level of a standard protein, Bl-8 anti-NIP IgM.

This assay demonstrates sensitivity to 60 pg of Bl-8 IgM. The extracts were prepared as soluble and insoluble material. The insoluble material was solubilized in the same buffer used in lysis but containing 8M urea followed by its dilution for assay.

In order to obtain activity for the Ig μ and λ, extracts were made of the insoluble fraction and these dialysed into buffer conditions in which disulphide interchange will occur at a higher frequency.

Production of functional antibodies from *E. Coli* expressing both heavy and light chains was achieved by lysing the cells and clarifying the supernatant by centrifugation. The insoluble material was washed, followed by sonication (3 times for 3 minutes), and finally dissolved in 9M urea, 50 mM glycine-NaOH pH 10.8, 1 mM EDTA, and 20 mM 2-mercaptoethanol. This extract was dialysed for 40 hours against 3 changes of 20 vols. of 100 mM KCl, 50 mM glycine-NaOH pH 10.8, 5% glycerol, 0.05 mM EDTA, 0.5 mM reduced glutathione and 0.1 mM oxidised glutathione. The dialysate was cleared by centrifugation at 30,000 g for 15 minutes and loaded directly onto DEAE Sephacel, followed by development with a 0–0.5M KCl linear gradient in 10 mM Tris-HCl, 0.5 mM EDTA, pH 8.0.

The purified Ig μ and λ were treated as above, except that no anion exchange chromatography was carried out. The preparation was finally dialysed into phosphate buffered saline, 5% glycerol, 0.01% sodium azide and 0.5 mM EDTA pH 7.4.

The results from assays of material processed in this way indicated that some activity was obtained. The level of activity obtained in this way was too low to do any detailed studies on, so the resultant dialysate was purified by anion exchange chromatography (FIG. **11**). This process resulted in the isolation of significant NIP-cap-BSA binding activity over that of background binding to BSA (FIG. **11**). The assay of the fractions for the level of Ig μ, expressed as Bl-8 IgM equivalents demonstrated two peaks of activity. This was not found to correlate with full length Ig μ by Western blotting. The first peak observed may represent a fragment of Ig μ. The separation of NIP-CAP-BSA binding activity from the majority of full length Ig μ and protein indicates that the hapten binding activity is contained within a particular molecular species formed at low efficiency.

The processing of insoluble material obtained from Ig μ expression in *E. Coli* produced a similar IgM protein profile but without NIP-cap-BSA binding activity. This demonstrates that the activity recovered was a property of the combined immunoglobulin expression, not of some *E. Coli* factor, or of the Ig μ heavy chain alone.

Further studies of the characteristics of the hapten (NIP-cap-BSA) binding were carried out. As samples were diluted they showed less binding to hapten in a very similar way to the original antibody (FIG. **12**). Free hapten was found to inhibit most of the binding activity in both indiluted and diluted samples. Using Bl-8 antibody as a standard for both IgM and hapten binding, the specific activity of the assembled antibody was calculated to be 1.4×10^4 gm/gm of IgM equivalents. This value demonstrates the inefficient recovery of activity, but possibly represents an underestimate of the specific activity due to an overestimate of full-length Ig μ in these fractions, as described above.

Heteroclitic Nature of Recombined Antibody

Detailed specificity of binding to NIP-cap-BSA was investigated by comparing the assembled antibodies with Bl-8 IgM in the presence of free NIP-cap and NP-cap (FIG. **13**). Both Bl-8 IgM and the assembled antibodies showed that higher NP-cap than NIP-cap concentrations were required to inhibit NIP-cap-BSA binding.

The heteroclitic nature is demonstrated by the molar ratio of NIP to NP at 50% inhibition. The concentrations of NIP and NP at 50% inhibition (I50) were found to be similar for both Bl-8 and the assembled antibodies as shown in Table 2. Also the NP I50/NIP I50 ratios were similar (Table 2).

TABLE 2

Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA solid phase. SD = Deviation.

	NIP I50 uM	NP I50 uM	NP I50/ NIP I50
Bl-8 IgM	0.13 (SD, 0.05)	3.7 (SD, 2.9)	29
Fraction 26	0.24 (SD, 0.09)	1.9 (SD, 0.4)	6
Fraction 27 and 28	0.11 (SD, 0.02)	1.1 (SD, 0.3)	10
Purified μ and λ	0.4	0.84	22

It has thus been shown that not only is it possible to express μ and λ proteins having intact variable domains, but also that it is possible to express both μ and λ proteins on compatible plasmids in the same cell. This latter has not hitherto been disclosed or even suggested. Moreover, it proved possible to derive functional Ig molecules from *E. Coli* cells which expressed both μ and λ proteins.

It has thus been shown that the process of the present invention produces a functional Ig molecule by recombinant DNA techniques.

Construction of Expression Plasmids for Yeast

The plasmids used for expression in yeast (*Saccharomyces cerevisiae*) are based on pBR322 and the yeast 2 micron plasmid. These plasmids are the subject of co-pending European Patent Application EP 0 073 635, the disclosure of which is incorporated hereby reference. The yeast phosphoglycerate kinase gene (PGK) provides the 5′ sequence necessary for initiation of transcription and insertion of genes can be made at the unique BglII site in the plasmid pMA 3013 described in the above mentioned European patent application. This plasmid has now been renamed pMA91 as is so referred to hereinafter.

(a) met-mu (μ)

BglII and BclI produce compatible 5′-GATC ends. The met-μ gene from pNP2 was excised on a partial BclI fragment and ligated into the unique BglII site of pMA91.

(b) Pre-mu (μ)

The plasmid pCT54 u containing the full lengths pre-μ cDNA was digested with Hind III. This cuts at the 3′ side of the μ cDNA. This Hind III site was changed to a BclI site by incubation with T_4 DNA polymerase in the presence of all 4 nucleotides. The linker R107 of sequence TTTTGATCAAAA, which contains an internal BclI was ligated to the DNA obtained above. After ligation one aliquot of the resultant

DFNA was digested with BclI and AccI and the μ AccI-BclI fragment was isolated from the resultant mixture by gel electrophoresis. A separate aliquot of the resultant DNA was digested with MboII and ligated with chemically synthesised oligonucleotide linkers R121 and R112 which have cohesive BclI and MboII ends.

R121 5' GATCAATGGGATGGAGCTGT 3'

R112 5' TTACCCTACCTCGAC 3'

The ligation reaction was terminated and the resultant DNA digested with AccI to give a μ MboII-AccI fragment which was isolated from the resultant mixture by gel electrophoresis on a 5% polyacrylamide gel.

The two μ fragments obtained above were ligated together and then digested with BclI to eliminate unwanted ligation products after which the resultant pre-μ DNA was ligated to BglII cut plasmid pMA91.

The resultant ligation mix was used to transform *E. Coli* strain HB101 to ampicillin resistance. A colony of the transformed bacteria was isolated and was found to contain a plasmid exhibiting the predicted enzyme digestion pattern. The 5' end of the inserted DNA from this plasmid was sequenced and shown to have the anticipated sequence for a pre-μ cDNA.

(c) met-lambda (λ)

The met-λ DNA was cloned into the BglII site of plasmid pMA91. Plasmid pCT54 Clone 1 was cut with Hind III to the 3' side of the met-λ cDNA and this cleavage site was altered to a BclI site in a similar manner as previously described for pNP2. Following this the plasmid DNA was cut 5' of the λ gene at the BclI site and the resultant λ DNA was cloned into the BglII site of pMA91.

(d) Pre-lambda (λ)

Pre-λ cDNA was reconstructed into pMA 91 as follows. The plasmid pCT54 was digested with BclI and Hind III and the resultant vector DNA was isolated by gel electrophoresis and ligated together with two synthetic oligonucleotides, R162 and R163, and the two fragments from plasmid pAT λ 1-15.

R162 5' GATCAATGGCCTGGATI 3'

R163 5' GTGAAATCCAGGCCATT 3'

These fragments were produced by digesting pAT λ 1-15 with FokI and Hind III and isolating the 5'300 base pair FokI fragment and the 3' 600 base pair FokI-Hind III fragment by gel electrophoresis. The resultant ligation mixture was used to transform *E. Coli* strain HB101 to ampicillin resistance and a colony of bacteria containing a plasmid having the predicted correct restriction enzyme digestion pattern was isolated. The 5' end of the inserted DNA was sequenced and found to have the anticipated sequence of a pre-λ cDNA. This plasmid was digested with Hind III and the resultant Hind III cleaved ends converted to a BclI site by blunting with T_4 polymerase and ligating with the linker R107. The resultant plasmid was then digested with BclI and the pre-λ cDNA isolated on a BclI fragment by gel electrophoresis. This BclI fragment was then ligated to BglI cut pMA91 to give a pMA 91 plasmid containing the full length pre-λ cDNA.

Expression of Immunoglobulin Genes in Yeast

The pMA91 derivative plasmids containing the pre-λ and pre-μ genes as prepared above were used to transform *Saccharomyces cerevisiae* yeast host organisms and the pre-λ and pre-μ genes were expressed by the transformed cells.

Saccharomyces cerevisiae strain MD46 when transformed with pMA91 pre-λ or pMA91 pre-μ gave rise to colonies which expressed immunoreactive proteins as revealed on Western blots. Yeast cells containing plasmid pMA91 pre-λ produced a protein which reacted with anti-λ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ. Similarly yeast cells containing pMA 91 pre-μ produced a protein which reacted with anti-μ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ protein. These observations indicate that both pre-μ and pre-μ are being processed to the corresponding mature proteins within the yeast host cell environment.

Additionally the λ protein product (but not the λ protein) was shown to be glycosylated. Cells were incubated in the presence or absence of tunicamycin at a concentration of 15 μg/ml. This compound specifically arrests the N-linked glycosylation of proteins. Cell extracts derived from cells incubated in the absence of tunicamycin showed higher molecular weight bands (as revealed by Western blotting) whilst extracts from cells incubated in the presence of tunicamycin showed no such higher molecular weight bands.

When cells were lysed with glass beads and the soluble and insoluble fractions examined it was found that the μ and λ proteins were exclusively in the insoluble fractions, as determined by Western blotting.

Secretion

After incubation transformed yeast cells were spun down and 1 ml volumes of supernatant were removed and passed through BSA-coated 0.2 μM Millipore filters to remove any remaining cellular material. ELISA assays for μ and λ protein were then carried out on the filtered supernatants. Only supernatants from pMA91 pre-λ harbouring cells showed detectable levels of immunoglobulin protein. Increased amounts of λ protein was detected in the supernatant when cells were grown in minimal medium to OD_{660}=0.2 and then spun down and resuspended in YPAB and harvested at OD_{660}=1-.0-1.5. Thus the λ protein but not the μ protein is secreted from yeast cells.

Intracellular Location

After incubation cells containing pMA91 pre-μ were converted to spheroplasts and fixed in 5% acetic acid/95% ethanol (v/v) and incubated with fluorescein conjugated goat anti-mouse μ. The fixed cells were examined by fluorescent microscopy and the μ protein was found to be localised in the periphery of the spheroplasts and especially in vacuoles.

Expression of both μ and λ Proteins in the Same Cells

In order to express both genes in the same host cells it was necessary to provide compatible plasmids for use in transformation. The pre-λ gene was excised from pMA91 pre-λ on a Hind III fragment and inserted into the Hind III site of plasmid pLG89 (Griti L, and Davies J, Gene, 25 179-188, 1983). This plasmid contains a ura3 marker which can be used as a positive selection for

transformed host cells. A convenient ura3 host organism is *S. cerevisiae* strain X4003-5B. Both plasmids, pMA91 pre-μ and pLG89 pre-λ, were transformed into this strain, and colonies were grown which contained together both the ura3 and leu2 markers. After incubation of the transformed cells both λ and μ proteins together were detected in the same cultures of X4003-5B using ELISA techniques. Levels of expression were comparable to those obtained for the individual genes in MD46 cells and for the λ gene alone in X4000-5B.

In addition, to check for assembly of the λ and μ proteins in vivo the following procedure was followd.

After the growth to an OD_{660}=1, the transformed X4003-5B cell were spun down and resuspended in buffer, either 5 mM borate buffer at pH 8.0 or phosphate buffer at pH 5.8 either with or without detergent (e.g. 0.5% Triton X). The suspended cells were then lysed by vortexing with glass heads in buffer as above and the insoluble material was spun down. After centrifugation aliquots of the supernatant were assayed in a NIP binding assay (as described previously for assembled *E. Coli* μ and λ proteins). Specific antigen binding activity was detected in the supernatant and this activity was shown to be specifically competed out by free NIP (the specific antigen). These results indicated that μ and λ protein were expressed within the X4003-5 B cells and assembled into functional immunoglobulin molecules in vivo.

In the above experiments the culture medium used for incubating the transformed cells was yeast minimal medium (containing, per liter, 6.7 g DIFCO yeast nitrogen base without amino acids, 10 g glucose, and 200 mg each of histidine, tryptophan, methionine and adenine). When the λ protein alone was being expressed the medium contained in addition 200 mg/l of leucine, and when the μ protein alone was being expressed the medium contained in addition 200 mg/l of uracil.

We claim:

1. A process for producing an Ig molecule or an immunologially functional Ig fragment comprising at least the variable domains of the Ig heavy and light chains, in a single host cell, comprising the steps of:

(i) transforming said single host cell with a first DNA sequence encoding at least the variable domain of the Ig heavy chain and a second DNA sequence encoding at least the variable domain of the Ig light chain, and

(ii) independently expessing said first DNA sequence and said second DNA sequence so that said Ig heavy and light chains are produced as separate molecules in said transformed single host cell.

2. The process according to claim **1** wherein said first and second DNA sequences are present in different vectors.

3. The process according to claim **1** wherein said first and second DNA sequences are present in a single vector.

4. A process according to claim **2** wherein the or each vector is a plasmid.

5. A process according to claim **4**, wherein the plasmid is col E1, pcR1, pBR322, RP4 or phage DNA.

6. A process according to claim **1** wherein the host cell is a baceterium or a yeast.

7. A process according to claim **6**, wherein the host cell is *E. Coli, B. subtilis* or *S. cerevisiae*.

8. A process according to claim **7**, wherein the host cell is *E. Coli* strain HB101, X1776, X2887, PS410, MRC 1 RV308, B or E1035.

9. A process according to claim **1** wherein the Ig heavy and light chains are expressed in the host cell and secreted therefrom as an immunologically functional Ig molecule or Ig fragment.

10. A process according to claim **1** wherein the Ig heavy and light chains are produced in insoluble or membrane bound form and are solubilised and allowed to refold in solution to form an immunologically functional Ig molecule or Ig fragment.

11. A process according to claim **1**, wherein the DNA sequences code for the complete Ig heavy and light chains.

12. A process according to claim **1**, wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from the same source as the variable domain to which it is attached.

13. A process according to claim **1**, wherein said first or said second DNA sequence further encodes at least one constant domain, wherein the constant domain is derived from a source different from that from which the variable domain to which it is attached is derived.

14. A process according to claim **1**, wherein said first and second DNA sequences are derived from one or more monoclonal antibody producing hybridomas.

15. A vector comprising a first DNA sequence encoding at last a variable domain of an Ig heavy chain and a second DNA sequence encoding at least a variable domain of an Ig light chain wherein said first DNA sequence and said second DNA sequence are located in said vector at different insertion sites.

16. A vector according to claim **15**, which is a plasmid.

17. A host cell transformed with a vector according to claims **15**.

18. A transformed host cell comprising at least two vectors, at least one of said vectors comprising a DNA sequence encoding at least a variable domain of an Ig heavy chain and at least another one of said vectors comprising a DNA sequence encoding at least the variable domain of an Ig light chain.

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EXHIBIT 25

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EP 0 120 694 B1

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Description

This invention relates to processes for the production of immunoglobulin molecules or immunologically functional Ig fragments in cells of host organisms which have been transformed by recombinant DNA techniques.

In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of heterologous (foreign) polypeptides or proteins in host cells which have been transformed with heterologous DNA sequences which code for the production of these products.

Theoretically, the recombinant DNA approach may be applied to the production of any heterologous polypeptide or protein in a suitable host cell, provided that appropriate DNA coding sequences can be identified and used to transform the host cell. In practice, when the recombinant DNA approach was first applied to the production of commercially useful products, its application for the production of any specified polypeptide or protein presented particular problems and difficulties, and the success of applying this approach to the production of any particular polypeptide or product was not readily predictable.

However, a large number of heterologous single chain polypeptides or proteins have now been produced by host cells transformed by recombinant DNA techniques. Examples of such heterologous single chain polypeptides or proteins include human interferons, the A and B chains of human insulin, human and bovine growth hormone, somatostatin, calf prochymosin and urokinase. Such transformed host cells provide a reproducible supply of authentic heterologous polypeptide or protein which may be produced on an industrial scale using industrial fermentation technology.

It should be pointed out that some of these polypeptides, for instance urokinase, after secretion by a host cell appear as two chain molecules. However, in such cases, the molecule is synthesised by the host cell as a single chain polypeptide, coded for by a single DNA sequence, which is cleaved in the host cell subsequent to synthesis to form the two chain structure.

It is known that in both human and animal systems there are a number of polypeptides or proteins which have multichain structure in which the chains are not derived from the cleavage of a single chain polypeptide coded for by a single DNA sequence. In such cases, the gene for each of the chains may be located at different points on the same chromosome or even on different chromosomes. In these cases, the polypeptide chains are synthesised separately and then assembled into the complete molecule subsequent to synthesis. Heretofore, no such multichain polypeptide or protein has been produced by recombinant DNA techniques from a single host cell.

A particular example of a class of such multichain polypeptides or proteins is the immunoglobulins.

Immunoglobulins, commonly referred to as antibodies, are protein molecules produced in animals by B-lymphocyte cells in response to challenge with foreign antigenic agents such as bacteria, viruses and foreign proteins. The immunoglobulins comprise a crucial part of the immune systems of humans and animals. The immunoglobulins recognise specific parts of the foreign agents and bind onto them. The specific parts are usually known as antigenic determinants or antibody binding sites. A given foreign agent is likely to have a number of different antigenic determinants.

A typical immunoglobulin (Ig) molecule is shown in Figure 1 of the accompanying drawings, to which reference is now made. The Ig molecule comprises two identical polypeptide chains of about 600 amino acid residues (usually referred to as the heavy chains, H), disulphide bonded to each other, and two identical shorter polypeptide chains of about 220 amino acid residues (usually referred to as the light chains, L), each light chain being disulphide bonded to one end of each heavy chain as shown.

When the Ig molecule is correctly folded, each chain is formed into a number of distinct globular areas, usually known as domains, joined by a more linear polypeptide chain. The light chains have two such domains, one of which is of variable sequence V_L, and the other of which is of constant sequence C_L. The heavy chains have a single variable domain V_H, adjacent the variable domain V_L of the light chain, and three or four constant domains C_H1-3 or 4.

The variable domains on both the heavy and the light chains each contain three hypervariable regions (HV1-3) which, when the Ig molecule is correctly folded, are located adjacent one another and form the antigen binding site. It is this area which recognises and binds to the antigenic determinant for which the Ig molecule is specific.

The constant domains of the Ig molecule do not take part in the binding to the antigenic determinant, but mediate the actions triggered by the binding of the Ig molecule to the antigenic determinant. It is believed that this triggering is caused by an allosteric effect induced by the binding of the Ig molecule to the antigenic determinant. The constant domain may enable the Ig molecule to fix complement or may cause mast cells to release histamine.

Igs may be categorised by class or subclass, depending on which of a number of possible heavy chain constant domains they contain, there being eight such possible heavy chains in mice. Thus, for instance, an Ig molecule with a μ heavy chain belongs to the class IgM, and one with a γ_1 heavy chain to the class IgG_1.

Igs may also contain one of two light chains, designated as κ and λ light chains, which have different constant domains and different sets of variable domains.

The structure of the Ig molecule and the location of the genes coding for the various domains thereof are discussed more fully by Early and Hood, in Genetic Engineering, Principles and Methods, Vol 3, pages 153-158 (edited by Setlow and Hollaender, Plenum Press).

It is known that Ig molecules on digestion with selected enzymes can produce a number of immunologically functional fragments. Two such fragments are known as the Fab and $(Fab')_2$ fragments. The Fab fragment comprises one light chain linked to the V_H and C_H1 domains of a heavy chain as shown in Figure 1. The $(Fab')_2$ fragment consists essentially of two Fab fragments linked together by a small additional portion of the heavy chains as shown in Figure 1. These fragments and other similar fragments can be of use in various tests and diagnostic and medical methods.

The principle method employed for the production of Igs involves the immunisation of susceptible animals with the antigenic agent to provide an immune reaction. Generally the animal is immunised a second time to improve the yield of Ig. The animal is then bled and the Ig is recovered from the serum.

However, the product of this method is not a homogeneous protein. The animal will produce Igs of different classes and also Igs specific for each of the different antigenic determinants on the antigenic agent, and its blood will therefore contain a heterogenous mixture of Igs. Obtaining a specific Ig of particular class and desired specificity from such a mixture requires very difficult and tedious purification procedures.

Recently, it has become possible to produce a homogeneous Ig of a single class and a single specificity by a technique first described by Kohler and Milstein (Nature, 256, 495-479, 1975). The technique involves the fusion of single Ig-producing parent cells with cancer cells to produce a monoclonal hybridoma cell which produces the Ig. Ig produced by this technique is usually known as monoclonal antibody. The nature of the monoclonal Ig is determined by the class and specificity of the Ig produced by the parent cell.

Recently, attempts have been made to use recombinant DNA techniques to produce fragments of Ig molecules. For instance, Amster *et al.* (Nucleic Acids Research, 8, 9, 1980, pp 2055 to 2065) disclose the cloning of double stranded cDNA sequences encoding a mouse Ig light chain into a plasmid. An *E. Coli* strain transformed by the plasmid synthesised a protein thought to comprise the complete constant domain of the light chain and about 40 amino acid residues of its variable region

Kemp and Cowman (Proc. Natl. Acad. Sci. USA, 78, 1981, pp 4520 to 4524) disclose the cloning of cDNA sequences encoding mouse heavy chain fragments and the transforming of an *E. Coli* strain which then synthesised heavy chain polypeptide fragments.

In both these cases, the polypeptides were produced as fusion proteins, in which the fragments of the Ig polypeptides were fused with additional non-Ig polypeptide sequences, and with incomplete variable domains. Thus, the polypeptide chains produced in these studies were not immunologically functional polypeptides as they were incapable of combining with complementary heavy or light chains to provide Ig molecules having intact antigen binding sites and immunological function.

Research studies have also been carried out in mammalian systems. For instance, Falkner and Zachau (Nature, 298, 1982, pp 286 to 288) report the cloning of cDNA sequences encoding mouse light chains into a plasmid which was used to transfect genomic eukaryotic cells which could then transiently synthesise light chains.

Rice and Baltimore (Proc. Natl. Acad. Sci. USA, 79 1982, pp 7862 to 7865) report on she transfection of a functionally rearranged K light chain Ig gene into a murine leukaemia virus-transformed lymphoid cell line. The cell line is then able to express the gene continuously. In both these cases, the K genes used to transfect the mammalian cells were obtained from myeloma cells and the K polypeptides produced were of indeterminate immunological function.

A further approach is exemplified in a series of papers by Valle *et al.* (Nature, 291, 1981, 338-340; Nature, 300, 1982, 71-74; and J. Mol. Biol., 160, 1982, 459-474), which describe the microinjection of mRNAs encoding heavy or light chains of Ig isolated from a mouse myeloma line into oocytes of *Xenopus laevis*. Under certain conditions complete Ig molecules were formed. However, the mRNAs were obtained from myeloma cells and the Ig molecules were of indeterminate immunological function.

It can thus be seen that hitherto it has not been possible to produce functional Ig by recombinant DNA technology.

According to a first aspect of the present invention, there is provided a process for producing a heterologous Ig molecule or an immunologically functional Ig fragment in a single host cell, which comprises transforming the host cell with separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains and expressing each of said polypeptide chains separately in said transformed single host cell.

According to a second aspect of the present invention, there is provided a vector for use in the above process, comprising separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains.

Preferably, the vector is a plasmid.

The present invention also provides a host cell transformed with a vector as defined above.

The present invention further provides a host cell transformed with two separate vectors, the first vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig heavy chain, and the second vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig light chain.

In the production of Ig molecules according to the invention it will be appreciated that, in order to produce a functional molecule, the DNA sequences used to transform the host cell will need to encode for at least the V_L and V_H domains of an Ig molecule. Moreover, these domains will need to be complementary so that when the two polypeptide chains fold together they form an antigen binding site of predetermined specificity.

Preferably, the Ig molecule or fragment produced by the process includes a complete light chain and at least the C_{H1} domain in addition to the V_H domain of the heavy chain. Most preferably the process produces a complete Ig molecule.

Advantageously, the Ig molecule or functional fragment thereof produced according to the present invention has a variable region (formed by the V_L and V_H domains) which defines a binding site for an antigenic determinant of clinical or industrial importance. The DNA coding sequences necessary to produce such a molecule may be derived from naturally occurring or hybridoma (monoclonal) Ig-producing cells with the desired specificity.

The constant domains of the Ig molecule or fragment, if present, may be derived from the same cell line as the variable region. However, the constant domains may be specifically altered, partially or completely omitted, or derived from a cell line producing a different class of Ig to provide Ig molecules or fragments having desired properties.

For example, an Ig molecule may be produced having variable domains (V_H and V_L) identical with those from a monoclonal antibody having a desired specificity, and constant domain(s) from a different monoclonal antibody having desired properties, for instance to provide human compatibility or to provide a complement binding site.

Such alterations in the amino acid sequence of the constant domains may be achieved by suitable mutation or partial synthesis and replacement or partial or complete substitution of appropriate regions of the corresponding DNA coding sequences. Substitute constant domain portions may be obtained from compatible recombinant DNA sequences.

The invention may be utilised for the production of Ig molecules or fragments useful for immunopurification, immunoassays, cytochemical labelling and targetting methods, and methods of diagnosis or therapy. For example, the Ig molecule or fragment may bind to a therapeutically active protein such as interferon or a blood clotting factor, for example Factor VIII, and may therefore be used to produce an affinity chromatography medium for use in the immunopurification or assay of the protein.

It is also envisaged that the Ig molecule may be synthesised by a host cell with another peptide moiety attached to one of its constant domains. Such a further peptide moiety may be cytotoxic or enzymatic. Alternatively, the moiety may be useful in attaching the Ig molecule to a biological substrate, such as a cell or tissue, or to a non-biological substrate, such as a chromatography medium. Such a peptide moiety is herein referred to as a structural peptide moiety.

It is further envisaged that cytotoxic, enzymic or structural peptide moieties could be attached to the Ig molecule by normal peptide chemical methods, as are already known in the art, rather than by being synthesised with the Ig molecule.

The Ig molecule or fragment may also comprise a therapeutic agent in its own right. For instance, an Ig molecule or fragment specific for D blood group antigen may be useful for the prevention of haemolytic disease of the new born.

Any suitable recombinant DNA technique may be used in the process of the invention for the production of Ig molecules or fragments. Typical expression vectors such as plasmids are constructed comprising DNA sequences coding for each of the chains of the Ig molecule or fragment.

It will be appreciated that a single vector may be constructed which contains the DNA sequences coding for more than one of the chains. For instance, the DNA sequences coding for Ig heavy and light chains may be inserted at different positions on the same plasmid.

Alternatively, the DNA sequence coding for each chain may be inserted individually into a plasmid, thus producing a number of constructed plasmids, each coding for a particular chain. Preferably the plasmids into which the sequences are inserted are compatible.

The or each constructed plasmid is used to transform a host cell so that each host cell contains DNA sequences coding for each of the chains in the Ig molecule or fragment.

For use in cloning in yeast systems, suitable expression vectors include plasmids based on a 2 micron origin.

Any plasmid containing an appropriate mammalian gene promoter sequence may be used for cloning in mammalian systems. Such vectors include plasmids derived from, for instance, pBR322, bovine papilloma virus, retroviruses, DNA viruses and vaccinia viruses.

Suitable host cells which may be used for expression of the Ig molecule or fragment include yeasts, such as *S. cervisiae*, and eukaryotic cells, such as insect or mammalian cell lines.

The present invention also includes constructed expression vectors and transformed host cells for use in producing the Ig molecules or fragments.

After expression of the individual chains in the same host cell, they may be recovered to provide the Ig molecule or fragment in active form, for instance to provide an Ig molecule of predetermined immunological function.

It is envisaged that in preferred forms of the invention, the individual chains will be processed by the host cell to form the complete polypeptide or protein which advantageously is secreted therefrom.

It will be appreciated that the present application shows for the first time that it is possible to transform a host cell so that it can express two or more separate polypeptides which may be assembled to form a complete Ig molecule or fragment. There is no disclosure or suggestion of the present invention in the prior art, which relates solely to the production of a single chain heterologous polypeptide or protein from each host cell.

The present invention will now be described, by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 shows a diagrammatic representation of a typical intact Ig molecule;

Figure 2 shows the construction of plasmids for the direct synthesis of a λ light chain in E. Coli;

Figure 3 shows the construction of plasmids for the direct synthesis of a μ heavy chain in E. Coli;

Figure 4 is a diagrammatic representation of μ mRNA sequences around the initiation codon;

Figure 5 shows the construction of plasmids having altered secondary structure around the initiation codon;

Figure 6 is a polyacrylamide gel showing expression and distribution of μ protein from E. Coli B;

Figure 7 is a polyacrylamide gel showing pulse chase autoradiograms of μ protein in E Coli B and in E Coli HB101;

Figure 8 is a polyacrylamide gel showing the results of λ gene expression in E. Coli;

Figure 9 is a polyacrylamide gel showing the distribution of recombinant λ light chain polypeptide between the soluble and insoluble cell fractions;

Figure 10 is a polyacrylamide gel showing expression and distribution of λ protein from E. Coli E103S;

Figure 11 shows the results of the fracitonation of μ and λ protein expressed by E. Coli B on DEAE Sephacel;

Figure 12 shows the specific hapten binding of reconstituted Ig molecules; and

Figure 13 shows the heteroclitic nature of the hapten binding of the reconstituted Ig molecules.

In the following examples, there is described the production of Ig light and heavy chain polypeptides derived from monoclonal antibodies which recognise and bind to the antigenic determinant 4-hydroxy-3-nitrophenyl acetyl (NP), using E.coli and S. cerevisiae as the host cells Recombinant DNA techniques were used to enable the host cells to express both the polypeptide chains.

It will be appreciated that the invention is not limited to the specific methods and construction described hereafter.

Construction of Lambda Light Chain Expression Plasmid

Figure 2, to which reference is now made, shows schematically the method used to construct a λ light chain expression plasmid.

It was decided to express the lambda gene in E. coli by direct expression of the gene lacking the eucaryotic signal peptide but containing a methionine initiator residue at the amino-terminus (met-lambda). The approach used for bacterial synthesis of met-lambda was to reconstruct the gene in vitro from restriction fragments of a cDNA clone and to utilise synthetic DNA fragments for insertion into the bacterial plasmid pCT54 (Emtage et al, Proc. Natl. Acad. Sci. USA., 80, 3671 to 3675, 1983). This vector contains the E. coli trp promoter, operator and leader ribosome binding site; in addition 14 nucleotides downstream of the ribosome binding site is an initiator ATG followed immediately by EcoR1 and HindIII sites and the terminator for E. coli RNA polymerase from bacteriophage T7.

As a source of light chain we used a plasmid pABλ1-15 which contains a full-length λ_1 light chain cDNA cloned into the PstI site of pBR322. This λ_1 light chain is derived from a monoclonal antibody, designated S43, which binds to 4-hydroxy-3-nitrophenylacetyl (NP) haptens.

In order to create a HindIII site 3' to the end of the lambda gene for insertion into the HindIII site of pCT54, the cDNA was excised from pABλ1-15 using Pst1. The cohesive ends were blunt ended using the Klenow fragment of DNA polymerase and synthetic HindIII linker molecules of sequence 5'-CCAAGCTTGG-3' ligated. The DNA was digested with HindIII and the 850bp lambda gene isolated by gel electrophoresis and cloned into HindIII cut pAT153 to yield plasmid pATλ1-15. The 3' end of the lambda gene was isolated from pATλ1-15 by HindIII plus partial Sac1 digestion as a 630bp SacI-HindIII fragment (2 in Figure 2). The HindIII cohesive end was dephosphorylated by calf intestinal alkaline phosphatase during isolation of the fragment to prevent unwanted ligations at this end in subsequent reactions.

A HinfI restriction site is located between codons 7 and 8 and the lambda sequence. The 5' end of the lambda gene was isolated as a 148bp HinfI to SacI fragment (1 in Figure 2)

Two oligodeoxyribonucleotides were designed to restore codons 1-8, and to provide an initiator ATG as well as Bc1I and HinfI sticky ends. The two chemically synthesised oligonucleotides made to facilitate assembly of the gene had the sequences:

```
R45   5' -pGATCAATGCAGGCTGTTGTG    3'

R44          3'       CCGACAACACTGAGTCCTTAp-    5'
```

pCT54 was cut with both Bcl1 and HindIII and the resulting linear molecules isolated, mixed together with the two oligodeoxyribonucleotide linkers R44 and R45 and both fragments 1 and 2, and ligated using T4 ligase (Figure 2). The mixture was used to transform E. coli DH1 to ampicillin resistance. Recombinant clones in pCT54 were identified by hybridisation of DNA from replica plated colonies on nitrocellulose to a nick-translated probe derived from the pATλ1-15 insert.

A clone was identified which hybridised to lambda cDNA and also showed the predicted restriction fragment pattern. This plasmid (designated pCT54 19-1) was sequenced from the ClaI site and shown to have the anticipated sequence except that there was a mutation of the fourth codon from CTG to ATG, changing the amino acid at this point from valine to methionine.

The sequence in this area was:

```
...GATTGATCA.ATG.CAG.GCT.GTT.ATG.ACT.CAG.GAA.TCT.GCA.CTC.ACC.ACA.TCA.

                met gln ala val met thr gln glu ser ala leu thr thr ser
```

The restriction enzyme sites in pCT54 between the Shine and Dalgarno sequence (AAGG), which is important for ribosome binding, and the ATG allow for the adjustment of the SD-ATG distance, an important parameter in determining expression rates. The SD-ATG distance was reduced by cutting the plasmid with ClaI or BclI and creating blunt ended species by digestion with S_1 nuclease. 2 µg of ClaI cut DNA was digested with 200 units of S_1 nuclease for 30 minutes at 30° using standard buffer conditions. The solution was deproteinised with phenol and the DNA recovered by ethanol precipitation. This DNA on religation with T4 DNA ligase and transformation into E.coli strain HB101 gave rise to a number of plasmids which had lost the ClaI or BclI site.

The plasmids which had lost their ClaI site were sequenced in the region surrounding the initiator ATG.

```
....AAGGGTATTGATCAATG CAG....    plasmid pNP3
    SD            met glu

....AAGGGTTTGATCAATG CAG         plasmid pNP4
    SD            met glu
```

In order to achieve high level expression a number of other approaches were followed. Firstly, a series of constructs were obtained which had increasing amounts of the 3' untranslated region of the cDNA removed by Bal 31 exonuclease. Secondly, a high copy number plasmid containing λ cDNA was constructed. This plasmid contained a par function (Meacock, P. A.and Cohen, S.N.Cell, 20, 529-542, 1980) as well as being present in high copy number. Thirdly, the pNP3 plasmid was transformed into a number of protease-deficient strains or into HB101 in conjunction with a protease deficient dominant acting plasmid (Grossman, A.D.et al Cell, 32, 151-159, 1983).

Construction of μ Heavy Chain Expression Plasmid

The full-length μ heavy chain cDNA derived from the NP binding monoclonal antibody B1-8 had been cloned into the PstI site of pBR322 yielding a plasmid designated pABμ-11 (Bothwell et al, Cell, 24, 625-637, 1981). In order to achieve high level expression, the μ cDNA minus the eukaryotic leader was reconstructed into pCT54. The construction of the μ heavy chain expression plasmid is shown diagramatically in Figure 3. Two chemically synthesised oligonucleotides were made to facilitate this. These have the following sequences:

```
R43    5'  GATCAATGCAGGTTCAGCTGCA   3'
R46    3'      TTACGTCCAAGTCG       5'
```

These were ligated into BclI cut pCT54 using T4 DNA ligase and the resulting plasmid designated pCT54 Pst. The linkers were designed to replace the sequence in pCT54 between the BclI site and the ATG, to provide an internal PstI site and to recreate the sequence from pABμ-11 5' to the PstI site up to codon +1. pCT54 Pst was cut briefly with PstI, treated with alkaline phosphatase and full length DNA glass isolated from a 1% agarose gel. Similarly pABμ-11 was briefly cut with PstI and the full length μ insert isolated following agarose gel electrophoresis. The μ cDNA was ligated with the full length pCT54Pst fragment using T4 DNA ligase under standard conditions and a plasmid designated pCT54μ was identified which by restriction enzyme analysis was shown to contain a full-length μ insert. The plasmid was sequenced around the 5' linker region and was found to have the anticipated sequence:
5' ...TGATCAATGCAGGTTCAGCTGCAGGGGGGGGGATGGGATGGAG... 3', demonstrating that it was, indeed, a full length clone. A complete PstI digest of pCT54μ liberated a 1.4 Kb fragment which was purified by 0.8% agrose gel electrophoresis and glass powder isolation. This was ligated with PstI cut pCT54 Pst (see above) using T4 DNA ligase under standard conditons, followed by transformation into HB101. A plasmid designated pNP1 was isolated which was shown by restriction endonuclease pattern analysis to contain the 1.4 Kb μcDNA fragment in an appropriate orientation (Figure 3). pNP1 was a plasmid which consisted of an appropriate 5' end for expression, whilst pCT54 contained an appropriate 3' end. The full length gene was reconstructed into pCT54 by cutting both pNP1 and PCT54μ with AvaI which cuts once in the plasmid and once in the μ gene. Both digests were run on a 1% agrose gel and the 1.9 Kb fragment from pNP1 and the 3.65 Kb fragment from pCT54μ isolated. Following alkaline phosphatase treatment of the 3.65 Kb pCT54μ fragment, the two pieces of DNA were ligated to each other and transformed into HB101. A plasmid designated pNP2 was identified which demonstrated the correct restriction endonuclease pattern. It was sequenced in the area surrounding the initiator ATG and found to have the anticipated sequence:

5' ....TTGATCAATGCAGGTTCAGCTGCAGCAGCCTGGGGCTGAGCTTGTGAAG.... 3'

The vector pCT54 had been constructed to include two restriction sites (Bcl1 and Cla1) between the S-D sequence (AAGG) and the initiation codon so that the distance between these sequence elements could be varied. As most E.coli mRNAs have 6-11 nucleotides between the S-D sequence and the AUG the distance in pNP2 was reduced by modification at the Cla1 site. pNP2 was cut with Cla1 and incubated with S1. The amount of S1 nuclease was adjusted so that some DNA molecules would lose 1-2 extra base pairs as a result of 'nibbling' by the enzyme. This DNA on religation with T4 DNA ligase and transformation into E.coli strain HB101 gave rise to a number of colonies harbouring plasmids which had lost the Cla1 site. The sequences around the initiation codon of four plasmids pNP223, pNP261, pNP9 and pNP282 were determined and are given in Table 1, part A below.

TABLE 1

		S-D to ATG distance (no. of residues)	Sequence (5' - 3') (ClaI)	Expression (mu) expts. A	B	C (ru)
(A)	pNP2	14	-AAAAAGGGTATCGATTGATCAATGCAGGTTCAG-	0.7	0.6	0.8
S1 derivatives of pNP2	pNP223	12	-AAAAAGGGTATATTGATCAATGCAGGTTCAG-	-	0.7	0.6
	pNP261	10	-AAAAAGGGTATTGATCAATGCAGGTTCAG-	-	1.1	1.1
	pNP282	7	-AAAAAGGGTGATCAATGCAGGTTCAG-	-	0.7	0.6
	pNP9	9	-AAAAAGGGTATGATCAATGCAGGTTCAG-	1.0	1.0	1.0
(B)	pNP11	11	-AAAAAGGGTATTGCACATATGCAAGTGCAA-	5.3	6.3	9.1
oligo constructs	pNP12	11	-AAAAAGGGTATTGCACATATGCAGGTTCAG-	1.8	2.3	1.3
	pNP14	9	-AAAAAGGGTATGATCAATGCAAGTGCAA-	72.2	106.7	124.6
	pNP8	-	-	77.0	53.4	145.5
	pCT70	-	-	0	0	0

The expression of these constructs in E. coli B was examined in three experiments, and concentrations of μ protein were determined by ELISA as relative units (ru), a measure of μ concentration normalised to pNP9. The results are shown in Table 1.

Inductions were carried out by resuspending cells at 1:50 dilution from overnight cultures into induction medium, consisting of: KH_2PO_4 (3g/L), Na_2HPO_4 (6g/L), NaCl (0.5g/L), Difco vitamin assay casamino acids (30g/L), NH_4Cl (4g/L) glycerol (16g/L), proline (0.5g/L), Difco yeast extract (1g/L), $CaCl_2.6H_2O$ (0.022g/L), $MgSO_4.7H_2O$ (0.025g/L), thiamine (0.01g/L) and carbenicillin (0.1g/L). Cultures were shaken at 37°C and cells harvested by centrifugation.

ELISA assays were performed essentially as described by Patel et al. (Nucleic Acid Research, 10, 5605 to 5620, 1982), using affinity purified goat anti-mouse IgM (Tago) and affinity purified goat anti-mouse IgM (Tago) conjugated toperoxidase, with 3, 3', 5, 5' tetramethylbenzidine (Miles) as a substrate. Bacterial cell pellets were boiled for 2 minutes in 9M urea (BRL) and loaded directly into the ELISA, such that all wells contained 2.25M urea, 10mM Tris-HCl pH7.8, 150mM NaCl, 0.05% NP40 and 0.5% casein (Hammarsten, B.D.H.).

Altering the S-D to ATG distance with nuclease S1 was found to increase the level of μ expression, relative to the parental plasmid pNP2, but only to a small extent (compare pNP9). The optimal S-D to AUG distance was found to be 9-10 nucleotides, as found in pNP9 and pNP261 respectively.

Secondary Structure Analysis

Hairpin loops were identified by use of the computer programme HAIRGU developed by Dr. Roger Staden. The ΔG values were calculated as described by Trioco et al. (Nature NB, 246, 40-41, 1973).

Analysis of the potential secondary structure of μ mRNA encoded by pNP2 and pNP9 (one of the S1 derivatives or pNP2) revealed an extensive array of possible hairpin loops. Attention was focused on the region of this mRNA containing the ribosome binding site since the sequestering of S-D sequences and initiation codons into secondary structure may inhibit the initiation of translation. It was found that a hairpin loop including the U and G of the initiation codon could be formed (see Figure 4). This hairpin loop is formed entirely within coding sequences and has a ΔG = -7.6 kcal. No secondary structures were found whichburied the S-D sequence, and none which mutually excluded the hairpin loop described above, and which had a lower ΔG.

To test whether expression of μ protein could be influenced by changes designed to alter potential secondary structure in the ribosome binding region we used synthetic oligonucleotides to mutagenise this region of μ mRNA. The plasmids were constructed by insertion of a pair of oligonucleotides between the blunted Cla I site and the 5' Pst site of μ in pNP2 (see Figure 5). The cloning strategy used to accomplish this, however, was multi-staged due to the presence of four Pst sites in pNP2. The vector pACYC184CM, which lacks Cla I and Pst I sites, was first constructed by cutting pACYC184 at its unique Cla I site, 'filling-in' the cohesive termini using T4 DNA polymerase and religating. Next, PACYC184CM was cut with BamHI, and the BamHI fragment from pNP2, bearing the 5' μ sequence, was inserted into it to form pCMμ (see Figure 4). The vector pCMμ contained unique Cla I and Pst sites which were required for the oligonucleotide cloning (see Figure 5).

pCMμ was cut with Cla I to completion, blunted via nuclease S1, and then cut with Pst I. The cut plasmid was purified by agarose gel electrophoresis and ligated separately with pairs of oligonucleotides, with the latter in a 100-fold molar excess. The pairs of oligonucleotides used were: R131 and R132 (for pNP11); R196 and R197 (for pNP12); and R202 and R203 (for pNP14). These have the sequences given below.

TGCACATATGCAAGTGCAACTGCA (R131), GTTGCACTTGCATATGTGCA (R132),
GCTGAACCTGCATATGTGCA (R196), TGCACATATGCAGGTTCAGCTGCA (R197),
GTTGCACTTGCATTGATC (R202), GATCAATGCAAGTGCAACTGCA (R203).

The ligation mixture was then used to transform HB101. Recombinant clones were detected by colony hybridisation, on nitrocellulose filters, using one of each pair of oligonucleotides as a probe. Positive clones were sequenced and shown to have the correct nucleotide sequence. The μ sequences containing the cloned oligonucleotides were excised on Eco RV-3gl II fragments. These were ligated to Eco RV-Bgl II cut pNP2 to reconstruct the full length μ genes (see Figure 5). Three different plasmids were created by these procedures; pNP11, pNP12 and pNP14, having the nucleotide sequences shown in Table 1 above.

In the construct pNP11 the potentially deleterious hairpin loop of pNP2 was abolished by changing the residues in the degenerate position of three codons and by introducing a different A-D to AUD sequence. These changes allow the region between the S-D and AUG to base pair with the 5' end of μ coding sequence to form a hairpin loop of ΔG = -7.8 kcal, approximately equal to that of pNP2, but leaving the AUG and S-D sequences exposed (see Figure 4). The only other mutually exclusive hairpin loops are of ΔG = -2.3, -3.6 and +0.4 kcal.

The construct pNP12 retains the S-D to AUG sequence of pNP11, but has the coding sequence of pNP2, such that the hairpin loop of pNP2 can form (see Figure 4).

The final μ construct was pNP11, which had the S-D to AUG sequence of pNP9, but the 5' coding sequence of pNP11, so that the hairpin loop of pNP12 could not form. The construct pNP14 had no secondary structure burying the S-D or μ AUG.

μ Protein Expression from Constructs with Differing mRNA Secondary Structures

E.coli B strains containing plasmids pNP9, pNP11, pNP12 and pNP14 were grown in induction medium and samples removed for ELISA assay. The concentration of μ was increased 6-7 fold in E.coli B cells containing pNp11 relative to pNP9 (see Table 1, Part b). Plasmid pNP12, which has the S-D to AUG

sequence of pNP11, but the coding sequence of pNP9, showed only a two-fold increase in level of μ expression compared to pNP9.

Plasmid pNP14, which has the S-D to AUG sequence of pNP9 but the 5' μ coding sequence of pNP11, and thus differed from pNP9 only in three residues, was found to express μ protein over 90 times that found in pNP9.

A plasmid, pNP8, has also been constructed, from which μ is expressed, also from the trp promoter, as part of a trpE-μ fusion protein containing the amino terminal 53 amino acids of trpE and the carboxyl terminal 503 amino acids of μ. The rationale for making this fusion gene was that μ would be translated from an efficient bacterial ribosome binding site rather than from one of unknown efficiency. Then, by comparing the amounts of trpE-μ produced from pNP8 with those of μ produced from the different constructs, an estimate of relative ribosome binding efficiency may be made. When the trpE-μ gene in pNP8 was induced and the products quantitated by ELISA, it was found that the μ expression in strains containing pNP8 and pNP14 were very similar (see Table 1, part B).

The ribosome binding site (RBS) sequences of pNP11, pNP12 and nPnp14 were designed in order to test the hypothesis that sequestering of the initiation codon into secondary structure inhibits translation initiation, and to increase the rate of initiation by releasing the initiation codon. In pNP11, the RBS hairpin loop of pNP2 and pNP9 was abolished by changing the 5' μ coding sequence in the degenerate position of three codons In addition, the S-D to AUG sequence was changed to allow the base-pairing of this region with the 5' coding sequence. The result of these changes is that the initiation codon is exposed in the loop of a hairpin with the S-D sequence at the base of the stem (see Figure 4). This hairpin loop had a ΔG approximately equivalent to the ΔG of the pNP9 hairpin loop. Strains containing pNP11 produced 6-7 times as much μ as did those with pNP9. To test whether this was due to the change in S-D to ATG sequence characteristics, rather than to the liberation of the initiation codon, pNP12 was constructed as a control. The latter has the S-D to AUG sequence of pNP11, but retains the 5' μ coding sequence of pNP9, so that the RBS hairpin loop of pNP11 cannot form, but the potentially deleterious one of pNP9 can form. The level of μ expression from pNP12 was found to be increased by only two-fold relative to pNP9. This increase is probably due to an altered S-D to AUG distance, a U immediately 5' to the initiation codon , and the S-D to AUG sequence being G-poor each of which has been shown to be advantageous. It is therefore likely that the increased expression from pNP11 is due to a preferable mRNA secondary structure. The RBS hairpin loop of pNP9 probably acts to inhibit translation while creation of another hairpin loop which exposes the initiation codon is responsible for the increased expression.

The plasmid pNP14 was constructed, such that it had the S-D to AUG sequence of pNP9, but the coding sequence Of pNP11. The expression plasmid pNP14, therefore, differed from pNP9 in only three residues, those in the degenerate position of three codons, yet strains containing pNP14 expressed over ninety times more μ than those containing pNP9. This increase in expression cannot be explained on the basis of the three residue changes optimizing codon usage, for they introduce less favoured codons, as judged by the frequency of their occurrence in strongly expressed E.coli genes.

The increased expression is best explained by the abolition of the RBS hairpin loop in pNP9. The magnitude of the increases in expression observed, and the few residue changes introduced, makes it difficult for these results to be interpreted in terms of changes in mRNA stability.

The expression or pNP8, which encodes the trpE-μ fusion protein from a native trpE RBS, was also examined, and found to express at approximately the same level as pNP14. This may indicate that the RBS of pNP14 is equivalent to that of trpE in terms of its efficiency in directing translation initiation. The native trpE RBS of pNP8 and that of pNP14 were found to have no secondary structures hindering the use of their RBS sequences.

Expression of μ Protein in E. Coli

E. Coli B cells containing the μ expression plasmid pNP11 were grown under inducing conditions and soluble and insoluble extracts prepared, and analysed by SDS-PAGE. A novel band was seen after staining the gel with Coomassie blue in the lane containing proteins from the insoluble fraction (see Figure 6, lane 2). This band was not seen in the negative control lane which contained proteins from the same fraction from cells harbouring pCT70 (see Figure 6,lane 3). The novel band was found to migrate to a position corresponding to a protein of a molecular weight within less than 5% of the actual molecular weight of non-glycosylated μ of 62.5Kd. A duplicate set of lanes were transferred to nitrocellulose, and Western blotted. Alignment of the stained gel and the blot autoradiogram confirms that this novel band is antigenically related to IgM (see Figure 6, lanes 4 and 8). No band was found in extracts from cells containing pCT70 (see Figure 6, lanes 5 and 7). Only a low amount of μ was found in the soluble fraction (see Figure 6, lane

6).

A greatly increased level of expression of μ was found in E. Coli B compared to HB101. Pulse chase analysis demonstrated that in E. Coli B,a similar level of μ protein was detected after a 60 minute chase (figure 7, lane 3) as was seen after the initial labelling period (Figure 7, lane 1). In HB101, however, very little μ protein could be seen after a 10 minute chase (Figure 7, lane 7), and none after 30 minutes (Figure 7, lane 8), compared to the amount detected after the initial labelling period (Figure 7, lane 5).

For pulse chase analysis, inductions were set up as described above,except that the medium used consisted of: proline (0.3g/L), leucine (0.1g/L), Difco methionine assay medium (5g/L), glucose (60mg/L), thiamine (10mg/L), $CaCl_2$ (22mg/L), $MgSO_4$ (0.25g/L)m and carbenicillin (0,1g/L). During exponential growth cells were pulse labelled with 30 μCi/ml L-[^{35}S] methionine for 2 minutes, after which unlabelled methione (100 μg/ml) was added and the incubation continued for the times indicated.

Induced E. Coli B cells harbouring pNP14 when examined by phase contrast microscopy were found to contain inclusion bodies.

Expression of λ Light Chain in E.Coli

A fresh 1 ml overnight culture of E.Coli HB101 or RV308 containing the plasmid under study was grown in L Broth (Maniatis et al,, Molecular Cloning, Cold Spring Harbor Laboratory, 1982), supplemented with carbenicillin to 100 μg/ml.

A 1:100 dilution of this culture was made into M9 medium (Maniatis, 1982, op.cit.) supplemented with glucose, vitamin B_1, carbenicillin, leucine and proline and the cultures shaken at 37° for 5-6 hours. At this time ^{35}S-methionine was added for 5-20 minutes. The cells were then harvested by centrifugation, lysed by boiling in 1% SDS for 2 minutes and diluted by addition of a buffer consisting of 2% Triton X-100, 50 mM Tris pH 8, 0.15 M NaCl and 0.1 mM EDTA. Immunoprecipitations were carried out by addition of antisera to aliquots of labelled E.Coli extracts and, after incubation at 4° overnight, immune complexes were isolated by binding to Staphylococcus aureus fixed cells. The complexes were dissociated by boiling in 60 mM Tris pH 6.8 buffer containing 10% glycerol, 3% SDS and 5% mercaptoethanol and the liberated proteins analysed on 10% or 12.5% acrylamide/SDS gels (Laemmli, UK. Nature, 227, 680-685, 1970). Gels were stained with Coomassie Brilliant blue to visualise the protein bands while labelled proteins were detected on Fuji-RX film by fluorography using 1 M sodium salicylate.

HB101 cells containing pNP3 and other plasmids were grown under inducing conditions to an OD_{600} = 0.6 and the proteins present examined by specific immunoprecipitation substantially as described above. The results obtained are given in Figure 8, in which lanes 1 and 2 respectively were extracts from pCT54-19 and pNP3, immunoprecipitated with normal rabbit serum; lanes 3, 4 and 5 were extracts immunoprecipitated with rabbit anti-lambda serum and represent pNP4 (lane 3), pNP3 (lane 4) and pCT54-19 (lane 5). The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of Figure 8. All of the plasmids produced a protein which reacted with rabbit anti-mouse λ light chain serum and comigrated with authentic λ_1 light chain from the mouse myeloma MOPC104E. pNP3 however produced the most of this protein (compare lane 4 with lanes 3 and 5 in Figure 8). No such band was detected with control immunoprecipitations using normal rabbit serum (Figure 8, lanes 1 and 2). Studies using brief pulses with ^{35}S-methionine followed by chasing with excess cold methionine indicated that the recombinant λ light chain had a 1/2 life of about 20 minutes.

In vitro transcription/translation (Pratt et al, Nucleic Acids Research, 9, 4459-447, 1981) confirmed that pNP3 coded for a protein which comigrated with authentic λ light chain. This 25 Kd product was synthesised in vitro at a rate comparable to that of β-lactamase indicating that it is synthesised in vivo at a level of 0.5% of total E.Coli proteins. This figure is in good agreement with the percentage of recombinant λ product synthesised in vivo (0.4-2%) as determined from the following equation:

$$\% \text{ specific CPM} = \frac{100 \times (\text{CPM with anti-}\lambda) - (\text{CPM with normal serum})}{\text{total CPM}}$$

The procedure used to disrupt cells was as follows (Emtage J S et al, Proc. Natl. Acad. Sci., 80, 3671-3715, 1983). E.Coli HB101/pNP3 grown under inducing conditions were harvested and resuspended in 0.05 M Tris pH 8, 0.233 M NaCl, 5% glycerol containing 130 g/ml of lysozyme and incubated at 4°C or room temperature for 20 minutes. Sodium deoxycholate was then added to a final concentration of 0.05% and 10 μg of DNAase 1 (from bovine pancreas) was added per g wet wt of E.Coli. The solution was incubated at

15 °C for 30 minutes by which time the viscosity of the solution had decreased markedly. The extract was centrifuged (at 10,000 x g for 15 minutes for small volumes (1 ml) or 1 hour for larger volumes) to produce a soluble and an insoluble fraction.

For immunoprecipitations, the soluble fraction was diluted in the Triton-containing buffer described above and the insoluble fraction solubilised by boiling in 1% SDS followed by dilution in Triton-containing buffer. HB101 cells containing pNP3 or pNP4 were grown under inducing conditions, pulse labelled with [^{35}S] methionine, separated into soluble and insoluble fractions and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results obtained are shown in Figure 9 in which lanes 1 and 3 correspond to the soluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lanes 2 and 4 correspond to the insoluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively, lanes 5 and 7 correspond to the soluble fraction from HB101-pNP4 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lane 9 corresponds to the soluble fraction from HB101-pNP3, and lane 10 corresponds to the insoluble fraction from HB101-pNP3. The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of Figure 9. When such a procedure was carried out using pNP3, the recombinant λ light chain protein was present in the insoluble fraction (Figure 9, lane 4) rather than the soluble fraction (Figure 9, lane 3). No 25 Kd band comigrating with authentic λ_1 light chain was present when either the soluble or insoluble fraction were immunoprecipitated using normal rabbit serum (Figure 9, lanes 1 and 2). By inference it is likely that all Ig light chains will fractionate as in this specific example.

In the absence of specific immunoprecipitation a novel protein band was not visible from extracts of HB101 containing pNP3 nor was the λ protein found to accumulate. However, there was a dramatic difference when pNP3 was induced in the K12 strain E103s. In this strain λ protein was found to accumulate during induction until the cells reaches stationary phase (figure 10, lanes 3-5) to a level of about 150 times that found in HB101 as determined by an ELISA (enzyme linked immunosorbent assay). These cells were found to contain inclusion bodies which appeared refractile under light microscopy, a phenomenon characteristic of high level expression of foreign proteins. An estimate of the percentage of total E. Coli protein represented by recombinant λ protein was obtained by separating the proteins by gel electrophoresis, staining them with Coomassie blue and scanning the stained gel with a Joyce-Loebl chromoscan 3. This method showed that λ was the major protein present (Figure 10, lane 5) and represented 13% of total E. Coli protein. The λ protein had a half-life of 20 minute in HB101 but accumulated to very high levels in E103s, suggesting that Lambda protein was much more stable in the latter strain. After cell lysis and centrifugation of HB101 or E103s containing pNP3, λ light chain was detected in the insoluble (Figure 10, lanes 7 and 10) but not in the soluble fractions (Figure 10, lanes 6), as determined by Coomassie blue staining. The identity of the major Coomassie blue stained band as λ protein was confirmed by Western blot analysis (Figure 10, lanes 8-10). The presence of such immunoreactive bands was specific to pNP3 containing cells. When extracts from cells containing pCT70, a prochymosin expressing plasmid, were subjected to the same analysis no bands were detected. This more sensitive technique showed that a small amount of the λ protein was in the soluble fraction (Figure 10, lane 9). The presence of a number of distinct immunoreactive proteins all smaller than full-length protein were also detected. These may result from proteolytic degradation of of λ protein, from premature termination of transcription or from internal initiation of translation.

Expression of μ and Lambda Polypeptides in the same Bacterial Cell

Each of the Ig μ and λ genes in expression plasmids were transformed into the same E.Coli cell to direct the synthesis of both Ig μ and λ polypeptides. In order to overcome plasmid incompatibility and provide a second antibiotic resistance marker, the trp promoter and λ sequences were excised from pNP3 on a Hind III - Bam HI fragment and inserted into the Hind III - Bam HI fragment of pACYC 184. The resultant plasmid pACYC λ caused the E.Coli to grow very poorly. This weak growth was thought to result from read-through of RNA polymerase into the origin of replication. However, the inhibition of growth was virtually eliminated by the cloning in of the bacteriophage T7 transcriptional terminator at the Hind III site of plasmid pACYC λ .

This terminator functions in both orientations. The resultant plasmid pAC λ T7-1 has a chloramphenicol resistance gene and an origin compatible with the pBR 322-derived origin on pNP14, the Ig μ expressing plasmid. Transformation of both plasmids in the same E.Coli B was achieved in two steps. Firstly pNP14 was introduced followed by pAC λ T7 - I in two sequential transformations to give ampicillin and chloramphenicol resistant clones

E.Coli B cells derived form this double-transfomant clone showed the presence of inclusion bodies and two novel polypeptide bands on stained gels of the insoluble fraction after lysis. These two bands correspond both with immunological activity by Western blotting for Ig μ and Ig λ and in their expected molecular weights. It has thus been shown that the double-transformant clone expresses both the heterologous genes. This had not hitherto been shown.

The presence of λ light chain in the insoluble fraction was a useful purification step since it both concentrated the protein and separated it from the bulk of E. Coli soluble proteins.

For further purification of the λ light chain, the cell debris were dissolved in 10mM Tris-HCl pH8.0, 25% formamide, 7M urea, 1mM EDTA and 2mM dithiothreitol. This material was loaded onto a DEAE Sephacel column (Pharmacia) (1x 25cm at a flow rate of 5ml/hr) which had been equilibrated in 9M urea, 10mM Tris-HCl pH8.0, 1mM EDTA and 2mM DTT. The DEAE Sephacel column was developed using a 0-150mM NaCl gradient in loading buffer. The eluted peak λ light chain immunoreactivity, corresponding to the major peak of protein, was diluted to a final concentration of 2.25M urea, 10mM Tris-Hcl pH8.0, 1mM EDTA, 2mM DTT and loaded onto an octyl-Sepharose column (Pharmacia) (2.5 x 10cm). Material was eluted by use of a urea gradient of 2.25-9M urea. The peak material was pooled, dialysed into ammonium bicarbonate and lyophilised. Following this step, only a single band of Coomassie blue stainable material corresponding to recombinant λ protein was visualised by SDS-PAGE.

The μ heavy chain was purified from 9M urea solubilised pellets by anion exchange chromatogrpahy and chromatofocussing (Pharmacia)

It was of great interest to determine whether the concomitant expression of μ and λ would lead to the formation of functional IgM. In order to determine this, extracts were made from E. Coli containing both Ig μ and λ polypeptides and these tested for antigen binding. We used a two-site sandwich ELISA which detects μ chain binding to haptenalated bovine serum albumin (NIP-caproate-BSA)

In the NIP binding assay, bovine serum albumin (BSA) is reacted with an equimolar amount of NIP-Cap-N-hydroxy-succinamide at pH 7.5 in 10mM phosphate buffer. The resulting NIP-Cap-BSA is separated from free NIP-Cap on a G-50 Sephadex column.

Microtitre plates (96 well Nunc Immuno Plate 1) are coated with 100 μl of a solution of 10 μg/ml NIP-BSA in-sodium carbonate/sodium bicarbonate 0.1M, pH 9.6 buffer (coating buffer) overnight at 4 • C.

The coated plates are then blocked for non-specific binding by addition of 100 μl of 0.5% casein in coating buffer (blocking buffer) and incubating at 37 • C for 1 hour.

The coated and blocked plates are then washed three times in 150 mM NaCl, 0.05% NP40, 20mM Tris-HCl, pH 7.8 buffer (washing buffer).

The washed plates are shaken free of excess washing buffer and samples are added in their buffer or typically in 0.5% casein in washing buffer (sample buffer) to a 100 μl final volume.

In order to demonstrate the nature of the binding to NIP-BSA, samples for testing were supplemented with either NIP or NP is free solution at various concentrations (from 60 to 0.3 μM for NIP or from 600 to 3 μM for NP).

The plates with samples are then incubated at 37 • C for one hour and thereafter washed three times with washing buffer.

The washed plates are then innoculated with 100 μl of anti μ-peroxidase conjugate (1:1000 dilution; TAGO Inc) in sample buffer, and incubated for one hour at 37 • C. The plates are then washed three times in washing buffer.

The washed plates are innoculated with 100 μl of 0.1M Na acetate-citrate, pH 6.0, 0.1 mg/ml tetramethylbenzidine, 13mM H_2O_2 (peroxidase substrate) and incubated at room temperature for one hour. The reaction is terminated by the addition of 25μl of 2.5M H_2SO_4.

The microtitre plates are then read in a plate reader (Dynatech) at 450 nm with a reference of 630 nm. The A_{450} was related to the level of a standard protein, B1-8 anti-NIP IgM.

This assay demonstrates sensitivty to 60pg of B1-8 IgM. The extracts were prepared as soluble and insoluble material. The insoluble material was solubilized in the same buffer used in lysis but containing 8M urea followed by its dilution for assay.

In order to obtain activity for the Ig μ and λ, extracts were made of the insoluble fraction and these dialysed into buffer conditions in which disulphide interchange will occur at a higher frequency.

Production of functional antibodies from E. Coli expressing both heavy and light chains was achieved by lysing the cells and clarifying the supernatant by centrifugation. The insoluble material was washed, followed by sonication (3 times for 3 minutes), and finally dissolved in 9M urea, 50mM glycine-NaOH pH10.8, 1mM EDTA, and 20mM 2-mercaptoethanol. This extract was dialysed for 40 hours against 3 changes of 20 vols. of 100mM KCl, 50mM glycine-NaOH pH10.8, 5% glycerol, 0.05mM EDTA, 0.5mM reduced glutathione and 0.1mM oxidised glutathione. The dialysate was cleared by centrifugation at 30,000g

for 15 minutes and loaded directly onto DEAE Sephacel, followed by development with a 0-0.5M KCl linear gradient in 10mM Tris-HCl, 0.5mM EDTA, pH8.0.

The purified Ig μ and λ were treated as above, except that no anion exchange chromatography was carried out. The preparation was finally dialysed into phosphate buffered saline, 5% glycerol, 0.01% sodium azide and 0.5mM EDTA pH7.4.

The results from assays of material processed in this way indicated that some activity was obtained. The level of activity obtained in this way was too low to do any detailed studies on, so the resultant dialysate was purified by anion exchange chromatography (Figure 11). This process resulted in the isolation of significant NIP-cap-BSA binding activity over that of background binding to BSA (Figure 11). The assay of the fractions for the level of Ig μ, expressed as B1-8 IgM equivalents demonstrated two peaks of activity. This was not found to correlate with full length Ig μ by Western blotting. The first peak observed may represent a fragment of Ig μ. The separation of NIP-CAP-BSA binding activity from the majority of full length Ig μ and protein indicates that the hapten binding activity is contained within a particular molecular species formed at low efficiency.

The processing of insoluble material obtained from Ig μ expression in E. Coli produced a similar IgM protein profile but without NIP-cap-BSA binding activity. This demonstrates that the activity recovered was a property of the combined immunoglobulin expression, not of some E. Coli factor, or of the Ig μ heavy chain alone.

Further studies of the characteristics of the hapten (NIP-cap-BSA) binding were carried out. As samples were diluted they showed less binding to hapten in a very similar way to the original antibody (Figure 12). Free hapten was found to inhibit most of the binding activity in both indiluted and diluted samples. Using B1-8 antibody as a standard for both IgM and hapten binding, the specific activity of the assembled antibody was calculated to be 1.4×10^4 gm/gm of IgM equivalents. This value demonstrates the inefficient recovery of activity, but possibly represents an underestimate of the specific activity due to an overestimate of full-length Ig μ in these fractions, as described above.

Heteroclitic Nature of Recombined Antibody

Detailed specificity of binding to NIP-cap-BSA was investigated by comparing the assembled antibodies with B1-8 IgM in the presence of free NIP-cap and NP-cap (Figure 13). Both B1-8 IgM and the assembled antibodies showed that higher NP-cap than NIP-cap concentrations were required to inhibit NIP-cap-BSA binding.

The heteroclitic nature is demonstrated by the molar ratio of NIP to NP at 50% inhibition. The concentrations of NIP and NP at 50% inhibition (I50) were found to be similar for both B1-8 and the assembled antibodies as shown in Table 2. Also the NP I50/NIP I50 ratios were similar (Table 2).

Table 2. Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA solid phase.

SD = Standard Deviation.

	NIP I50 uM	NP I50 uM	NP I50 / NIP I50
B1-8 IgM	0.13 (SD, 0.05)	3.7 (SD,2.9)	29
Fraction 26	0.34 (SD, 0.09)	1.9 (SD,0.4)	6
Fraction 27 and 28	0.11 (SD,0.02)	1.1 (SD,0.3)	10
Purified μ and λ	0.4	0.84	22

It has thus been shown that not only is it possible to express μ and λ proteins having intact variable domains, but also that it is possible to express both μ and λ proteins on compatible plasmids in the same cell. This latter has not hitherto been disclosed or even suggested. Moreover, it proved possible to derive functional Ig molecules from E. Coli cells which expressed both μ and λ proteins.

It has thus been shown that the process of the present invention produces a functional Ig molecule by recombinant DNA techniques.

Construction of Expression Plasmids for Yeast

The plasmids used for expression in yeast (Saccharomyces cerevisiae) are based on pBR322 and the yeast 2 micron plasmid. These plasmids are the subject of copending European Patent Application EP 0 073 635, the disclosure of which is incorporated hereby reference. The yeast phosphoglycerate kinase gene (PGK) provides the 5' sequence necessary for initiation of transcription and insertion of genes can be made at the unique BglII site in the plasmid pMA 3013 described in the above mentioned European patent application. This plasmid has now been renamed pMA91 as is so referred to hereinafter.

a) met-mu (μ)

BglII and BclI Produce compatible 5'-GATC ends. The met-μ gene from pNP2 was excised on a partial BclI fragment and ligated into the unique BglII site of pMA91.

b) Pre-mu (μ)

The plasmid pCT54 u containing the full length pre-μ cDNA was digested with Hind III. This cuts at the 3' side of the μ cDNA. This Hind III site was changed to a BclI site by incubation with T_4 DNA polymerase in the presence of all 4 nucleotides. The linker R107 of sequence TTTTGATCAAAA, which contains an internal BclI was ligated to the DNA obtained above. After ligation one aliquot of the resultant DNA was digested with BclI and AccI and the μ AccI-BclI fragment was isolated from the resultant mixture by gel electrophoresis. A separate aliquot of the resultant DNA was digested with MboII and ligated with chemically synthesised oligonucleotide linkers R121 and R112 which have cohesive BclI and MboII ends.

```
R121  5'  GATCAATGGGATGGAGCTGT  3'

R112  5'  TTACCCTACCTCGAC      3'
```

The ligation reaction was terminated and the resultant DNA digested with AccI to give a μ MboII-AccI fragment which was isolated from the resultant mixture by gel electrophoresis on a 5% polyacrylamide gel.

The two μ fragments obtained above were ligated together and then digested with BclI to eliminate unwanted ligation products after which the resultant pre-μ DNA was ligated to BglII cut plasmid pMA91.

The resultant ligation mix was used to transform E.Coli strain HB101 to ampicillin resistance. A colony of the transformed bacteria was isolated and was found to contain a plasmid exhibiting the predicted enzyme digestion pattern. The 5' end of the inserted DNA from this plasmid was sequenced and shown to have the anticipated sequence for a pre-μ cDNA.

c) met-lambda (λ)

The met-λ DNA was cloned into the BglII site of plasmid pMA91. Plasmid pCT54 Clone 1 was cut with Hind III to the 3' side of the met-λ cDNA and this cleavage site was altered to a BclI site in a similar manner as previously described for pNP2. Following this the plasmid DNA was cut 5' of the λ gene at the BclI site and the resultant λ DNA was cloned into the BglII site of pMA91.

d) Pre-lambda (λ)

Pre-λ cDNA was reconstructed into pMA 91 as follows. The plasmid pCT54 was digested with BclI and Hind III and the resultant vector DNA was isolated by gel electrophoresis and ligated together with two synthetic oligonucleotides, R162 and R163, and the two fragments from plasmid pATλ1-15.

```
R162  5'  GATCAATGGCCTGGATT    3'

R163  5'  GTGAAATCCAGGCCATT    3'
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These fragments were produced by digesting pATλ1-15 with FokI and Hind III and isolating the 5' 300 base pair FokI fragment and the 3' 600 base pair FokI-Hind III fragment by gel electrophoresis. The resultant ligation mixture was used to transform E.Coli strain HB101 to ampicillin resistance and a colony

of bacteria containing a plasmid having the predicted correct restriction enzyme digestion pattern was isolated. The 5' end of the inserted DNA was sequenced and found to have the anticipated sequence of a pre-λ cDNA. This plasmid was digested with Hind III and the resultant Hind III cleaved ends converted to a BclI site by blunting with T_4 polymerase and ligating with the linker R107. The resultant plasmid was then digested with BclI and the pre-λ cDNA isolated on a BclI fragment by gel electrophoresis. This BclI fragment was then ligated to BglI cut pMA91 to give a pMA 91 plasmid containing the full length pre-λ cDNA.

Expression of Immunogloublin Genes in Yeast

The pMA91 derivative plasmids containing the pre-λ and pre-μ genes as prepared above were used to transform Saccharomyces cerevisiae yeast host organisms and the pre-λ and pre-μ genes were expressed by the transformed cells.

Saccharomyces cerevisiae strain MD46 when transformed with pMA91 pre-λ or pMA91 pre-μ gave rise to colonies which expressed immunoreactive proteins as revealed on Western blots. Yeast cells containing plasmid pMA91 pre-λ produced a protein which reacted with anti-λ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ . Similarly yeast cells containing pMA 91 pre-μ produced a protein which reacted with anti-μ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature μ protein. These observations indicate that both pre-λ and pre-μ are being processed to the corresponding mature proteins within the yeast host cell environment.

Additionally the μ protein product (but not the λ protein) was shown to be glycosylated. Cells were incubated in the presence or absence of tunicamycin at a concentration of 15 μg/ml. This compound specifically arrests the N-linked glycosylation of proteins. Cell extracts derived from cells incubated in the absence of tunicamycin showed higher molecular weight bands (as revealed by Western blotting) whilst extracts from cells incubated in the presence of tunicamycin showed no such higher molecular weight bands.

When cells were lysed with glass beads and the soluble and insoluble fractions examined it was found that the μ and λ proteins were exclusively in the insoluble fractions, as determined by Western blotting.

Secretion

After incubation transformed yeast cells were spun down and 1 ml volumes of supernatant were removed and passed through BSA-coated 0.2 μM Millipore filters to remove any remaining cellular material. ELISA assays for μ and λ protein were then carried out on the filtered supernatants. Only supernatants from pMA91 pre-λ harbouring cells showed detectable levels of immunoglobulin protein. Increased amounts of λ protein was detected in the supernatant when cells were grown in minimal medium to OD_{650} = 0.2 and then spun down and resuspended in YPAB and harvested at OD_{650} = 1.0-1.5. Thus the λ protein but not the μ protein is secreted from yeast cells.

Intracellular Location

After incubation cells containing pMA91 pre-μ were converted to spheroplasts and fixed in 5% acetic acid/95% ethanl (v/v) and incubated with fluorescein conjugated goat anti-mouse μ. The fixed cells were examined by fluorescent microscopy and the μ protein was found to be localised in the periphery of the spheroplasts and especially in vacuoles.

Expression of both μ and λ Proteins in the Same Cells

In order to express both genes in the same host cells it was necessary to provide compatible plasmids for use in transformation. The pre-λ gene was excised from pMA91 pre-λ on a Hind III fragment and inserted into the Hind III site of plasmid pLG89 (Griti L, and Davies J, Gene, 25 179-188, 1983). This plasmid contains a ura3 marker which can be used as a positive selection for transformed host cells. A convenient ura3$^-$ host organism is S. cerevisiae strain X4003-5B. Both plasmids, pMA91 pre-μ and pLG89 pre-λ , were transformed into this strain, and colonies were grown which contained together both the ura3 and leu2 markers. After incubation of the transformed cells both λ and μ proteins together were detected in the same cultures of X4003-5B using ELISA techniques. Levels of expression were comparable to those obtained for the individual genes in MD46 cells and for the λ gene alone in X4003-5B.

In addition, to check for assembly of the λ and μ proteins in vivo the following procedure was followd.

After the growth to an $OD_{550} \cong 1$, the transformed X4003-5B cells were spun down and resuspended in buffer, either 5 mM borate buffer at pH 8.0 or phosphate buffer at pH 5.8 either with or without detergent (e.g. 0.5% Triton X). The suspended cells were then lysed by vortexing with glass beads in buffer as above and the insoluble material was spun down. After centrifugation aliquots of the supernatant were assayed in a NIP binding assay (as described previously for assembled E.Coli μ and λ proteins). Specific antigen binding activity was detected in the supernatant and this activity was shown to be specifically competed out by free NIP (the specific antigen). These results indicated that μ and λ protein were expressed within the X4003-5B cells and assembled into functional immunogloublin molecules in vivo.

In the above experiments the culture medium used for incubating the transformed cells was yeast minimal medium (containing, per litre, 6.7 g DIFCO yeast nitrogen base without amino acids, 10 g glucose, and 200 mg each of histidine, tryptophan, methionine and adenine). When the λ protein alone was being expressed the medium contained in additon 200 mg/l of leucine, and when the μ protein alone was being expressed the medium contained in addition 200 mg/l of uracil.

Claims

1. A process for producing a heterologous Ig molecule or an immunologically functional Ig fragment in a single host cell, which comprises transforming the host cell with separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains and expressing each of said polypeptide chains separately in said transformed single host cell.

2. The process of claim 1, wherein each DNA sequence is inserted individually into a vector and the individual vectors are used to transform the single host cell.

3. The process of claim 1, wherein the two DNA sequences are inserted into separate parts of a vector which is used to transform the single host cell.

4. The process of claim 2 or claim 3, wherein the or each vector is a plasmid.

5. The process of any one of claims 1 to 4, wherein the host cell is a eukaryotic host cell.

6. The process of claim 5, wherein the host cell is a yeast.

7. The process of claim 6, wherein the host cell is S. cerevisiae.

8. The process of claim 5, wherein the host cell is a mammalian cell.

9. The process of any one of claims 1 to 8, wherein the polypeptide chains are secreted by the host cell.

10. The process of any one of claims 1 to 9, for producing an Ig molecule or fragment having at least one constant domain, wherein the or each constant domain is derived from the same source as the variable domain to which it is attached.

11. The process of any one of claims 1 to 9, for producing an Ig molecule or fragment having at least one constant domain, wherein the or each constant domain is derived from a source different from that from which the variable domain to which it is attached is derived.

12. The process of any one of claims 1 to 11, wherein the DNA sequences code for complete Ig heavy and light chains.

13. The process of any one of claims 1 to 12, wherein the DNA sequences code for only one constant domain.

14. The process of any one of claims 1 to 13, wherein at least one of the DNA sequences encodes at least the variable region of an Ig heavy or light chain and also a cytotoxic, enzymic or structural polypeptide moiety whereby the host cell is transformed so as to produce an Ig molecule or fragment having attached thereto said cytotoxic, enzymic or structural polypeptide moiety.

15. The process of any one of claims 1 to 14, wherein the DNA coding sequences for the Ig molecule or fragment are derived from one or more monoclonal antibody-producing hybridomas

16. A vector for use in the process of any one of claims 1 to 15, comprising separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains.

17. A vector according to claim 16, which is a plasmid.

18. A host cell transformed with the vector of claim 16 or claim 17.

19. A host cell transformed with two separate vectors, the first vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig heavy chain, and the second vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig light chain.

Patentansprüche

1. Verfahren zur Herstellung eines heterologen Ig-Moleküls oder eines immunologisch funktionellen Ig-Fragments in einer einzigen Wirtszelle umfassend das Transformieren der Wirtszelle mit getrennten DNA-Sequenzen, die für die entsprechenden Polypeptidketten kodieren, welche zumindest die variablen Domänen der schweren und leichten Ig-Ketten umfassen, und das getrennte Exprimieren der Polypeptidketten in der transformierten einzigen Wirtszelle.

2. Verfahren nach Anspruch 1, bei dem jede DNA-Sequenz individuell in einen Vektor insertiert wird und die individuellen Vektoren zur Transformation der einzigen Wirtszelle verwendet werden.

3. Verfahren nach Anspruch 1, bei dem die beiden DNA-Sequenzen in getrennte Bereiche eines Vektors insertiert werden, welcher zur Transformation der einzigen Wirtszelle verwendet wird.

4. Verfahren nach Anspruch 2 oder 3, bei dem der oder jeder Vektor ein Plasmid ist.

5. Verfahren nach einem der Ansprüche 1 bis 4, bei dem die Wirtszelle eine eukaryontische Wirtszelle ist.

6. Verfahren nach Anspruch 5, bei dem die Wirtszelle eine Hefe ist.

7. Verfahren nach Anspruch 6, bei dem die Wirtszelle S. cerevisiae ist.

8. Verfahren nach Anspruch 5, bei dem die Wirtszelle eine Säugerzelle ist.

9. Verfahren nach einem der Ansprüche 1 bis 8, bei dem die Polypeptidketten von der Wirtszelle exprimiert und sekretiert werden.

10. Verfahren nach einem der Ansprüche 1 bis 9 zur Herstellung eines Ig-Moleküls oder -Fragments mit zumindest einer konstanten Domäne, wobei sich die oder jede konstante Domäne von derselben Quelle wie die variable Domäne ableitet, an die sie angeheftet ist.

11. Verfahren nach einem der Ansprüche 1 bis 10 zur Herstellung eines Ig-Moleküls oder -Fragments mit zumindest einer konstanten Domäne, wobei die oder jede konstante Domäne aus einer anderen Quelle als derjenigen stammt, aus der sich die variable Domäne ableitet, an welche sie angeheftet ist.

12. Verfahren nach einem der Ansprüche 1 bis 11, bei dem die DNA-Sequenzen für vollständige schwere und leichte Ig-Ketten kodieren.

13. Verfahren nach einem der Ansprüche 1 bis 12, bei dem die DNA-Sequenzen für lediglich eine konstante Domäne kodieren.

14. Verfahren nach einem der Ansprüche 1 bis 10, bei dem die DNA-Sequenzen für eine schwere oder leichte Ig-Kette oder ein Fragment davon mit einer vollständigen variablen Domäne kodieren.

15. Verfahren nach einem der Ansprüche 1 bis 14, bei dem zumindest eine der DNA-Sequenzen zumindest für die variable Region einer schweren oder leichten Ig-Kette und ferner für eine cytotoxische, enzymatische oder strukturelle Polypeptidkomponente kodiert, wodurch die Wirtszelle dahingehend transformiert wird, daß sie ein Ig-Molekül oder -Fragment bildet, an welches die cytotoxische, enzymatische oder strukturelle Polypeptidkomponente angeheftet ist.

16. Verfahren nach einem der Ansprüche 1 bis 15, bei dem die für das Ig-Molekül oder -Fragment kodierenden DNA-Sequenzen aus einem oder mehreren monoklonale Antikörper bildenden Hybridomzellen stammen.

17. Vektor zur Verwendung in dem Verfahren gemäß einem der Ansprüche 1 bis 16, welcher die für die entsprechenden Polypeptidketten, welche zumindest die variablen Domänen der schweren und leichten Ig-Ketten umfassen, kodierenden DNA-Sequenzen getrennt umfaßt.

18. Vektor nach Anspruch 17, welcher ein Plasmid ist.

19. Eine mit dem Vektor gemäß Anspruch 17 oder Anspruch 18 transformierte Wirtszelle.

20. Eine mit zwei getrennten Vektoren transformierte Wirtszelle, wobei der erste Vektor eine DNA-Sequenz umfaßt, die für eine Polypeptidkette kodiert, welche zumindest die variable Domäne der schweren Ig-Kette umfaßt, und der zweite Vektor eine DNA-Sequenz umfaßt, die für eine Polypeptidkette kodiert, welche zumindest die variable Domäne der leichten Ig-Kette umfaßt.

Revendications

1. Procédé pour la production d'une molécule d'Ig hétérologue ou d'un fragment d'Ig immunologiquement fonctionnel dans une cellule hôte unique, qui comprend la transformation de la cellule hôte avec des séquences d'ADN séparées codant respectivement pour des chaînes polypeptidiques comprenant au moins les domaines variables des chaînes lourdes et légères d'Ig et exprimant chacune des dites chaînes polypeptidiques séparément dans ladite cellule hôte unique transformée.

2. Procédé selon la revendication 1, dans lequel chaque séquence d'ADN est insérée individuellement dans un vecteur et les vecteurs individuels sont utilisés pour transformer la cellule hôte unique.

3. Procédé selon la revendication 1, dans lequel les deux séquences d'ADN sont insérées dans des parties séparées d'un vecteur qui est utilisé pour transformer la cellule hôte unique.

4. Procédé selon l'une des revendications 2 ou 3, dans lequel le ou chaque vecteur est un plasmide.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel la cellule hôte est une cellule hôte eucaryote.

6. Procédé selon la revendication 5, dans lequel la cellule hôte est une levure.

7. Procédé selon la revendication 6, dans lequel la cellule hôte est S. cerevisiae.

8. Procédé selon la revendication 5, dans lequel la cellule hôte est une cellule de mammifère.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel les chaînes polypeptidiques sont exprimées et sécrétées par la cellule hôte.

10. Procédé selon l'une quelconque des revendications 1 à 9, pour la production d'une molécule ou d'un fragment d'Ig ayant au moins un domaine constant, dans lequel le ou chaque domaine constant est dérivé de la même source que le domaine variable auquel il est fixé.

11. Procédé selon l'une quelconque des revendications 1 à 10, pour la production d'une molécule ou d'un fragment d'Ig ayant au moins un domaine constant, dans lequel le ou chaque domaine constant est dérivé d'une source différente de celle de laquelle le domaine variable auquel il est attaché est dérivé.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel les séquences d'ADN codent pour des chaînes lourdes et légères d'Ig complètes.

13. Procédé selon l'une quelconque des revendications 1 à 12, dans lequel les séquences d'ADN codent pour seulement un domaine constant.

14. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel les séquences d'ADN codent pour une chaîne lourde ou légère d'Ig ou un fragment de celle-ci ayant un domaine variable complet.

15. Procédé selon l'une quelconque des revendications 1 à 14, dans lequel au moins une des séquences d'ADN code pour au moins la région variable d'une chaîne lourde ou légère d'Ig et également une unité polypeptidique cytotoxique, enzymatique ou structurale, la cellule hôte étant ainsi transformée de façon à produire une molécule ou un fragment d'Ig auquel est fixée ladite unité polypeptidique cytotoxique, enzymatique ou structurale.

16. Procédé selon l'une quelconque des revendications 1 à 15, dans lequel les séquences codantes d'ADN pour la molécule ou le fragment d'Ig sont dérivées d'un ou plusieurs hybridomes monoclonaux produisant des anticorps.

17. Vecteur pour utilisation dans le procédé selon l'une quelconque des revendications 1 à 16, comprenant des séquences d'ADN séparées codant respectivement pour des chaînes polypeptidiques comprenant au moins les domaines variables des chaînes lourdes et légères d'Ig.

18. Vecteur selon la revendication 17, qui est un plasmide.

19. Cellule hôte transformée avec le vecteur selon l'une des revendications 17 ou 18.

20. Cellule hôte transformée avec deux vecteurs séparés, le premier vecteur comprenant une séquence d'ADN codant pour une chaîne polypeptidique comprenant au moins le domaine variable de la chaîne lourde d'Ig, et le second vecteur comprenant une séquence d'ADN codant pour une chaîne polypeptidique comprenant au moins le domaine variable de la chaîne légère d'Ig.

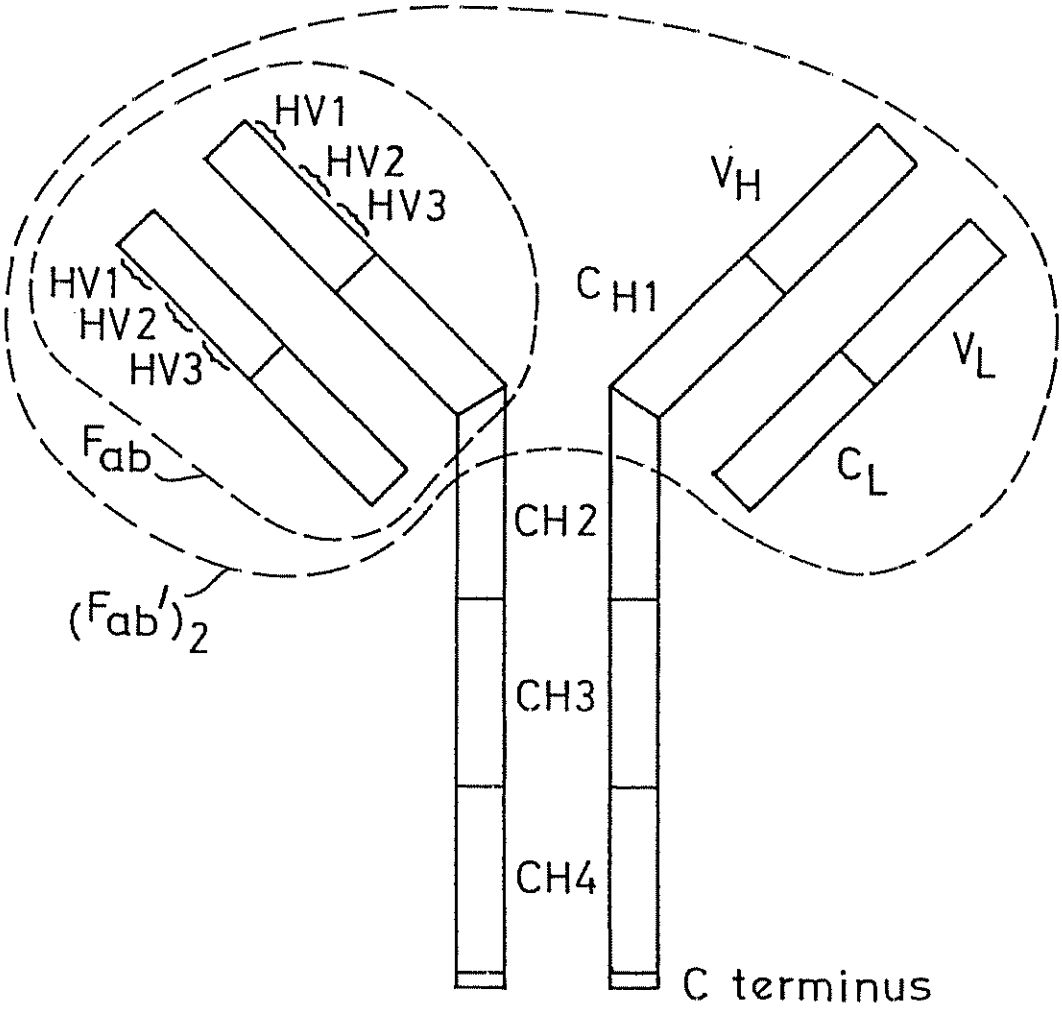
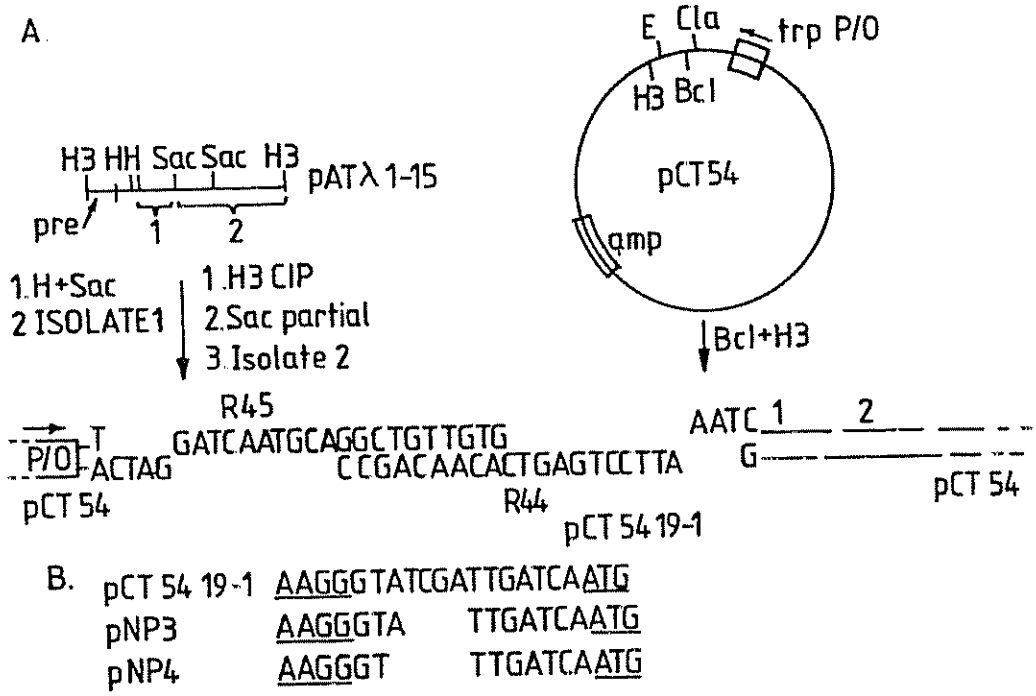

HV1
HV2
HV3
HV1
HV2
HV3
Fab
$(Fab')_2$
V_H
C_{H1}
V_L
C_L
CH2
CH3
CH4
C terminus

FIG. 1



B.

pCT 54 19-1	AAGGGTATCGATTGATCAATG	
pNP3	AAGGGTA	TTGATCAATG
pNP4	AAGGGT	TTGATCAATG

Legend. E = E corRI

H = HinfI

H3= Hind III

FIG. 2

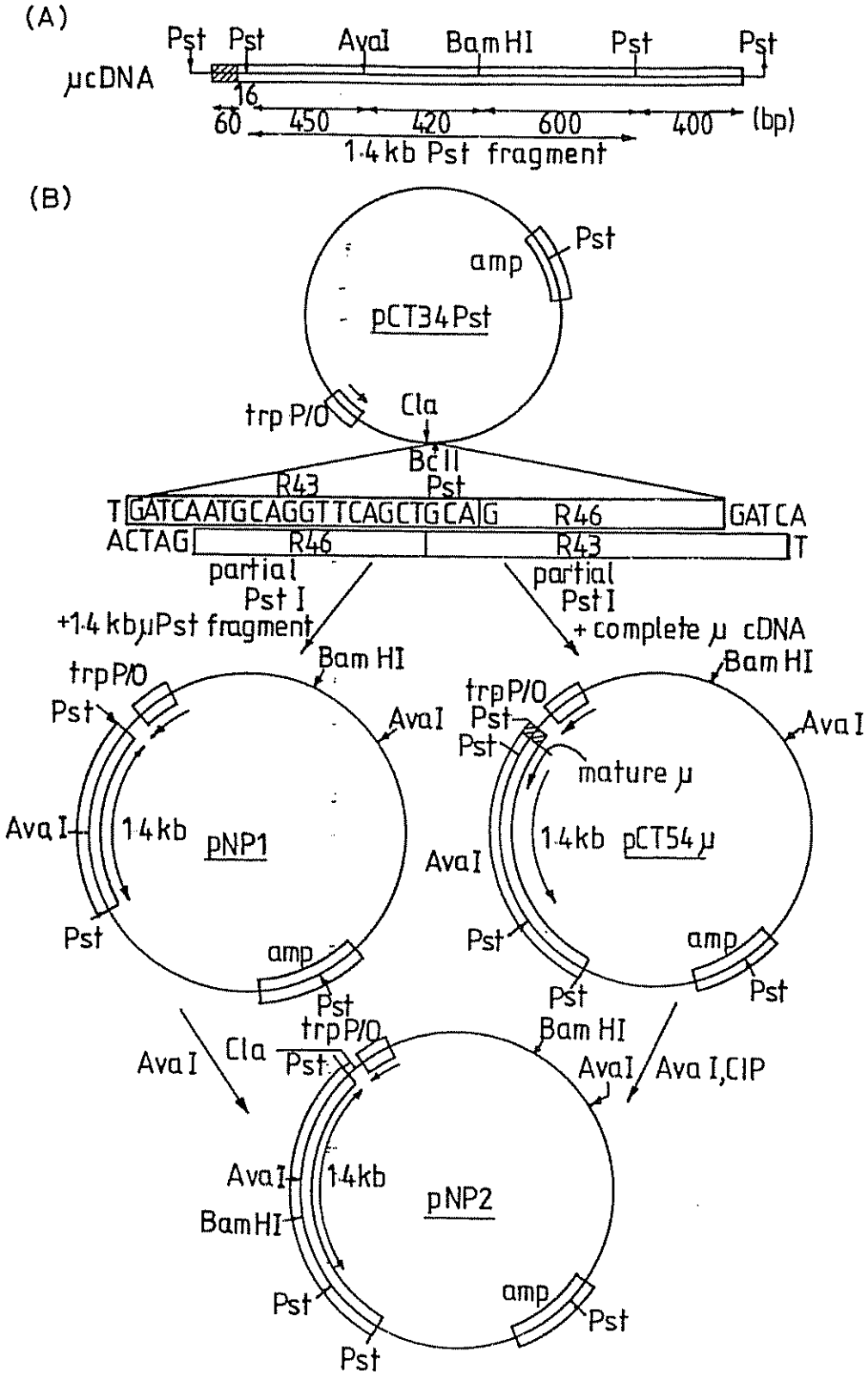

(A)
μcDNA
Pst Pst AvaI BamHI Pst Pst
16
60 450 420 600 400 (bp)
1.4kb Pst fragment
(B)
amp
Pst
pCT34Pst
trp P/O
Cla
BclI
R43
Pst
T GATCAATGCAGGTTCAGCTGCA G R46 GATCA
ACTAG R46 R43 T
partial
Pst I
+1.4 kbμPst fragment
partial
Pst I
+ complete μ cDNA
Bam HI
trpP/O
Pst
AvaI
Ava I
1.4kb
pNP1
amp
mature μ
1.4kb pCT54μ
Cla
AvaI
Ava I,CIP
BamHI
pNP2

FIG. 3

Possible 2° structures of μ mRNAs

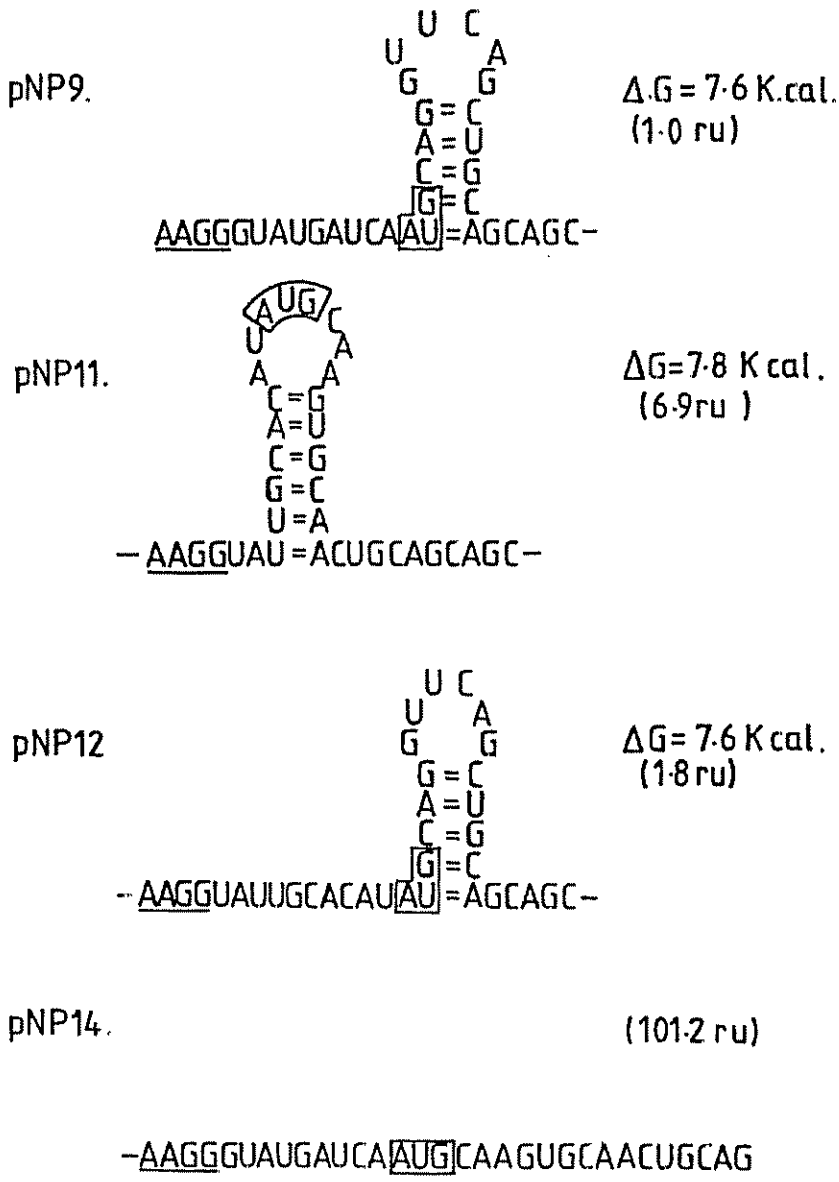


FIG. 4

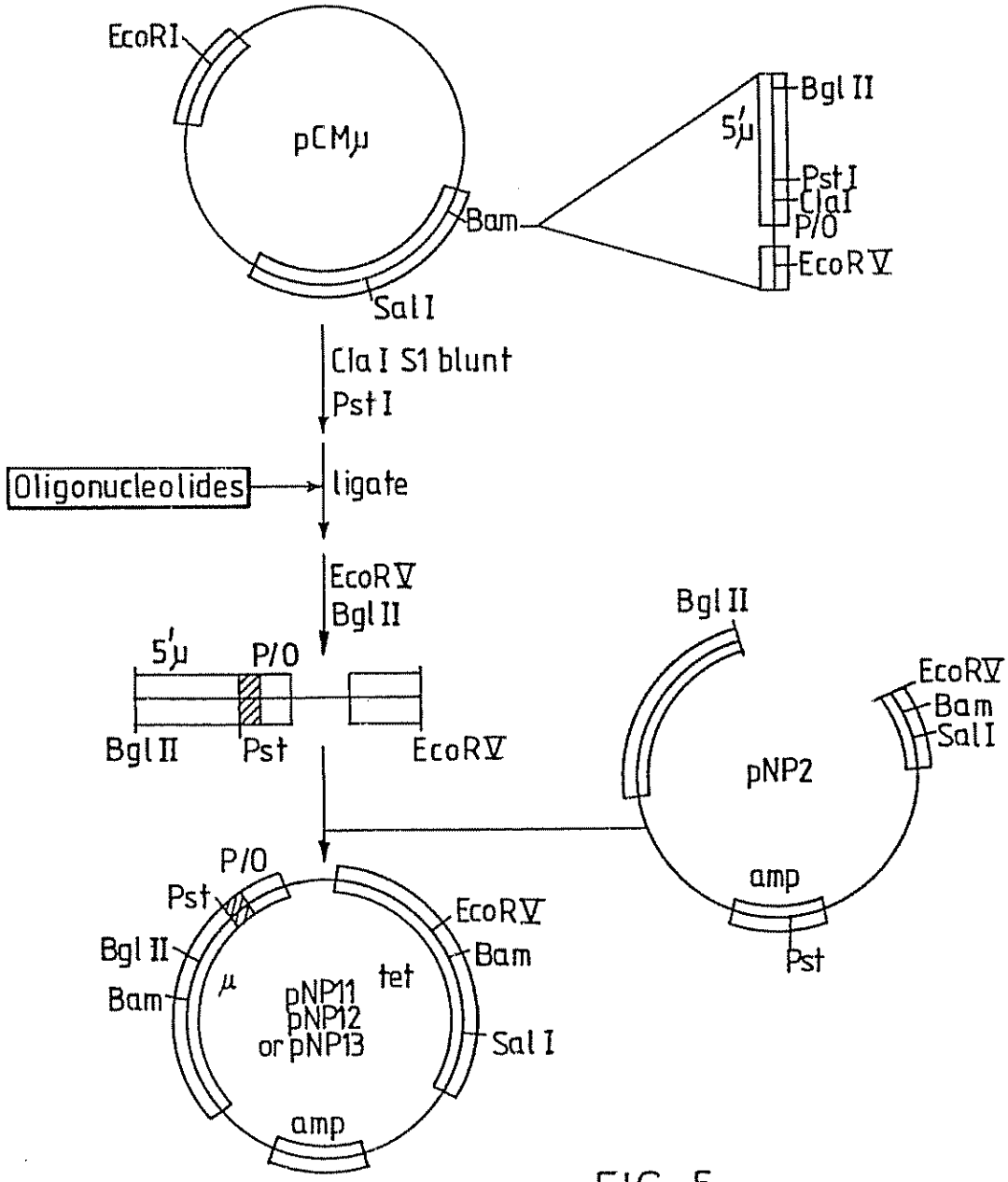

EcoRI
pCMμ
Bam
Sal I
5'μ
Bgl II
Pst I
Cla I
P/O
EcoR V
Cla I S1 blunt
Pst I
Oligonucleolides
ligate
EcoR V
Bgl II
5'μ
P/O
Bgl II
Pst
EcoR V
Bgl II
EcoR V
Bam
Sal I
pNP2
amp
Pst
P/O
Pst
Bgl II
Bam
μ
tet
pNP11
pNP12
or pNP13
EcoR V
Bam
Sal I
amp

FIG. 5



FIG. 6

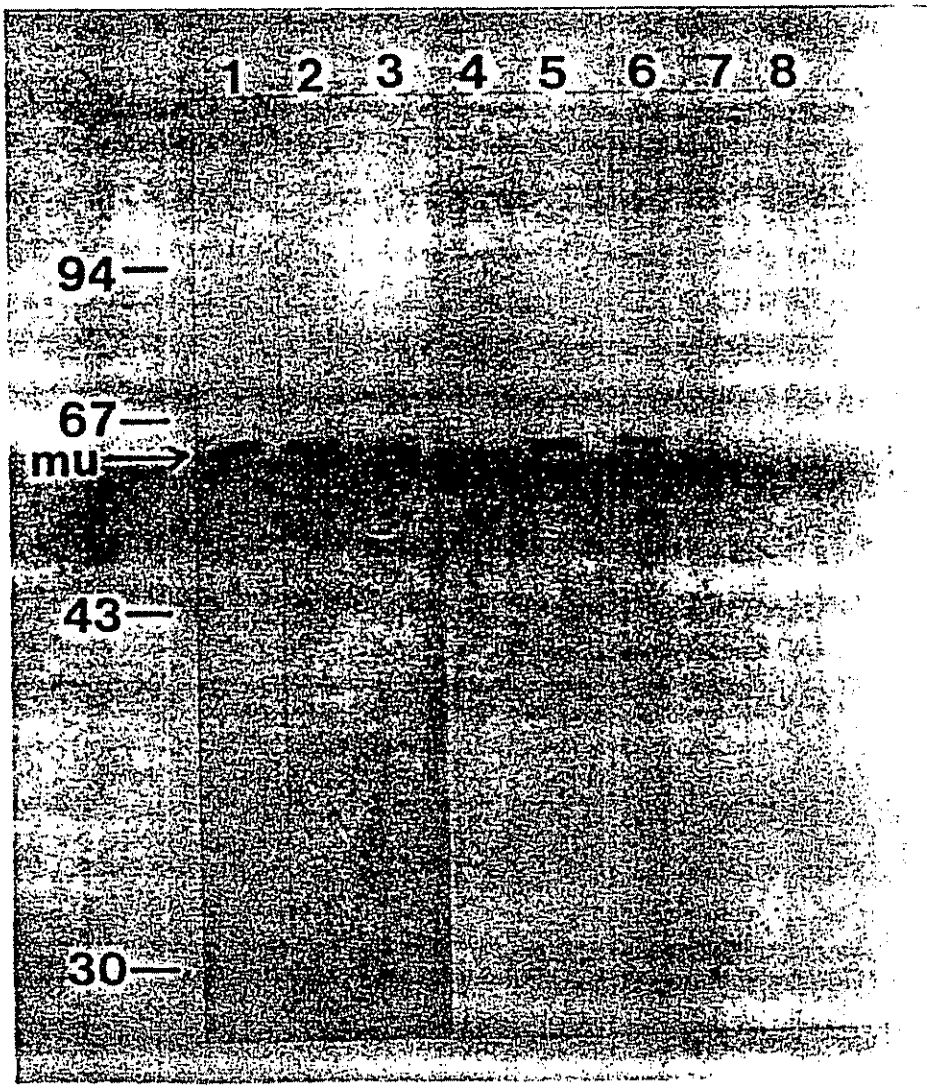


FIG. 7

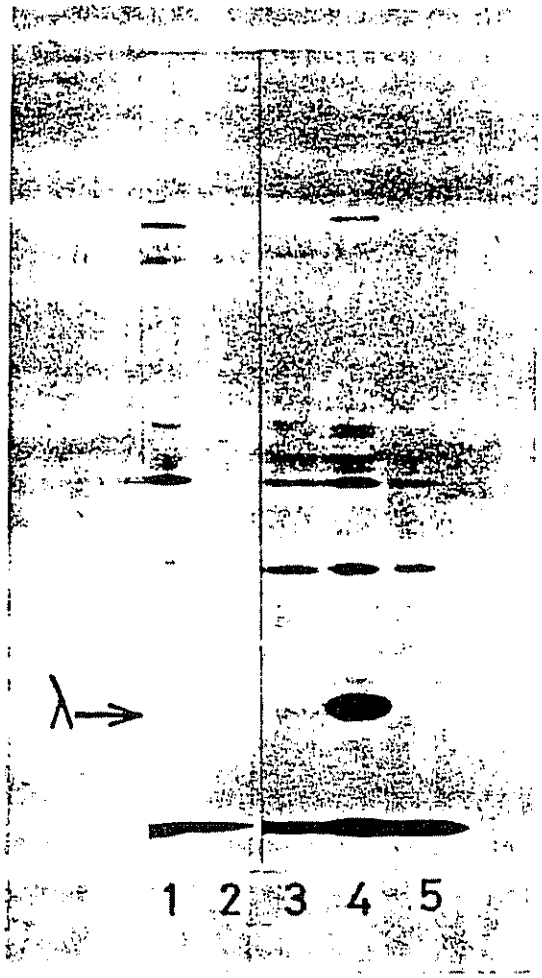


FIG. 8

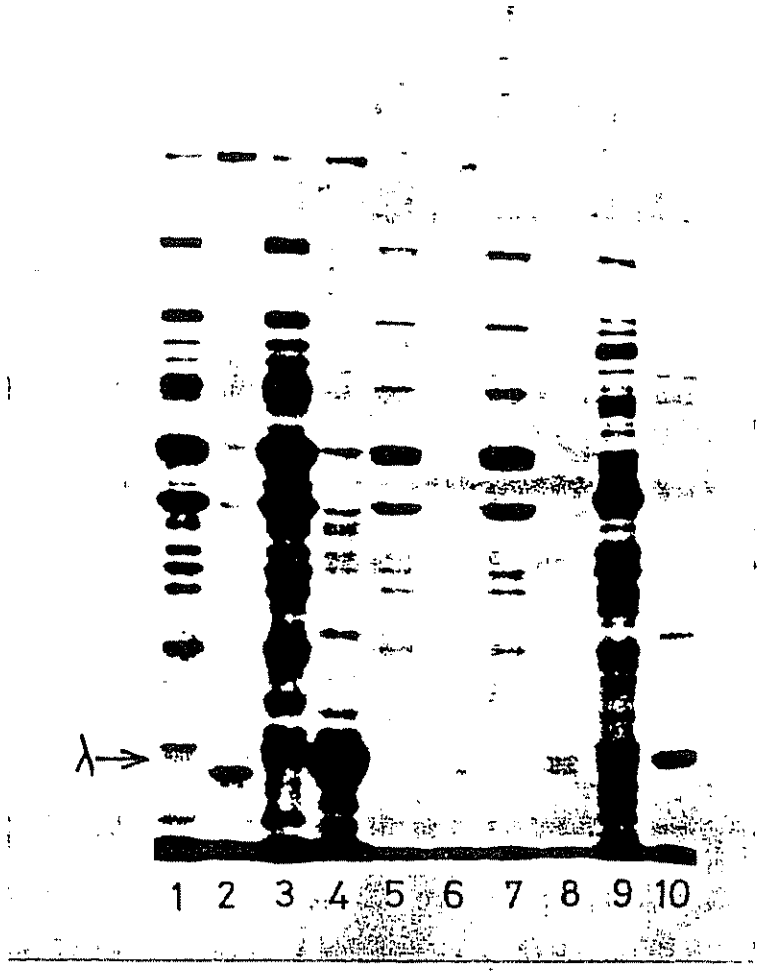


FIG. 9

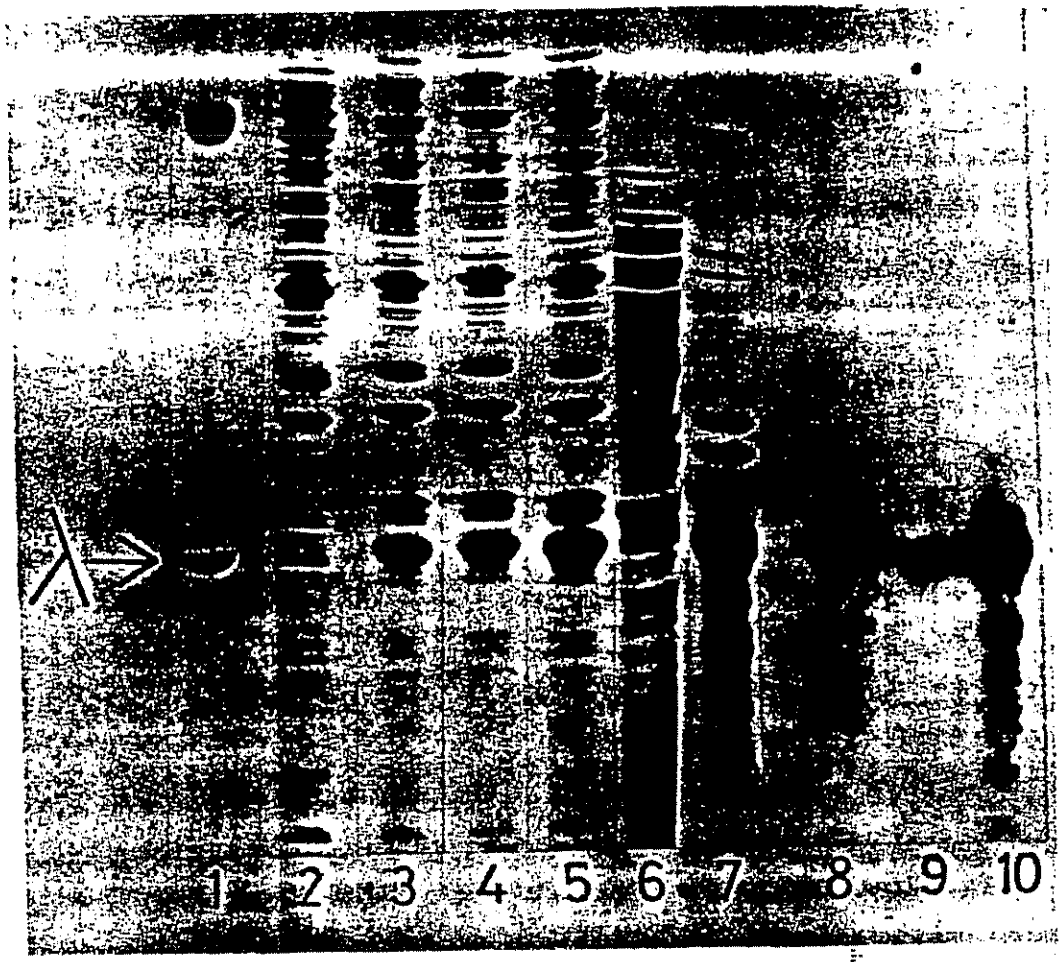


FIG. 10

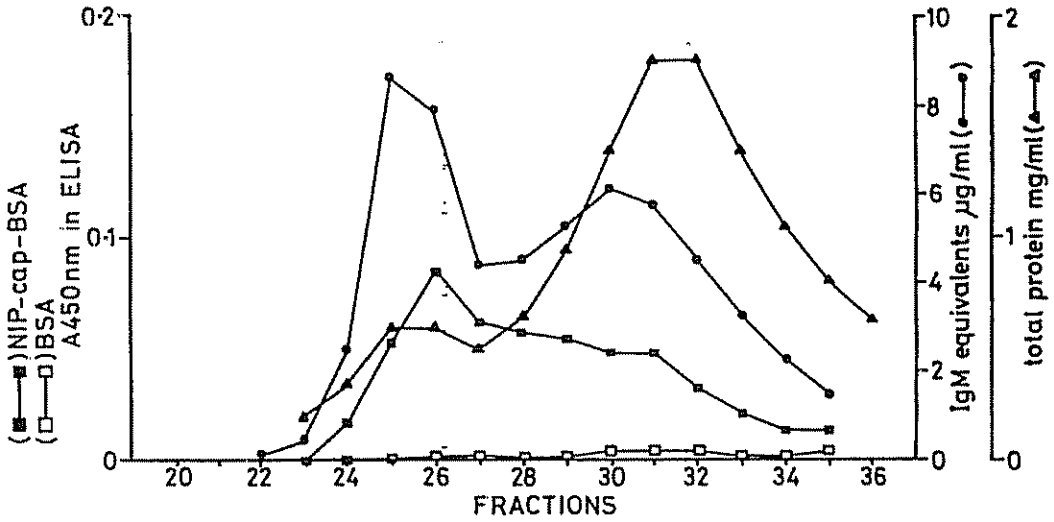

(■—■)NIP-cap-BSA
(□—□)BSA
A450nm in ELISA
0·2
0·1
0
20
22
24
26
28
30
32
34
36
FRACTIONS
10
8
6
4
2
0
IgM equivalents µg/ml (●—●)
2
1
0
total protein mg/ml (▲—▲)

FIG. 11

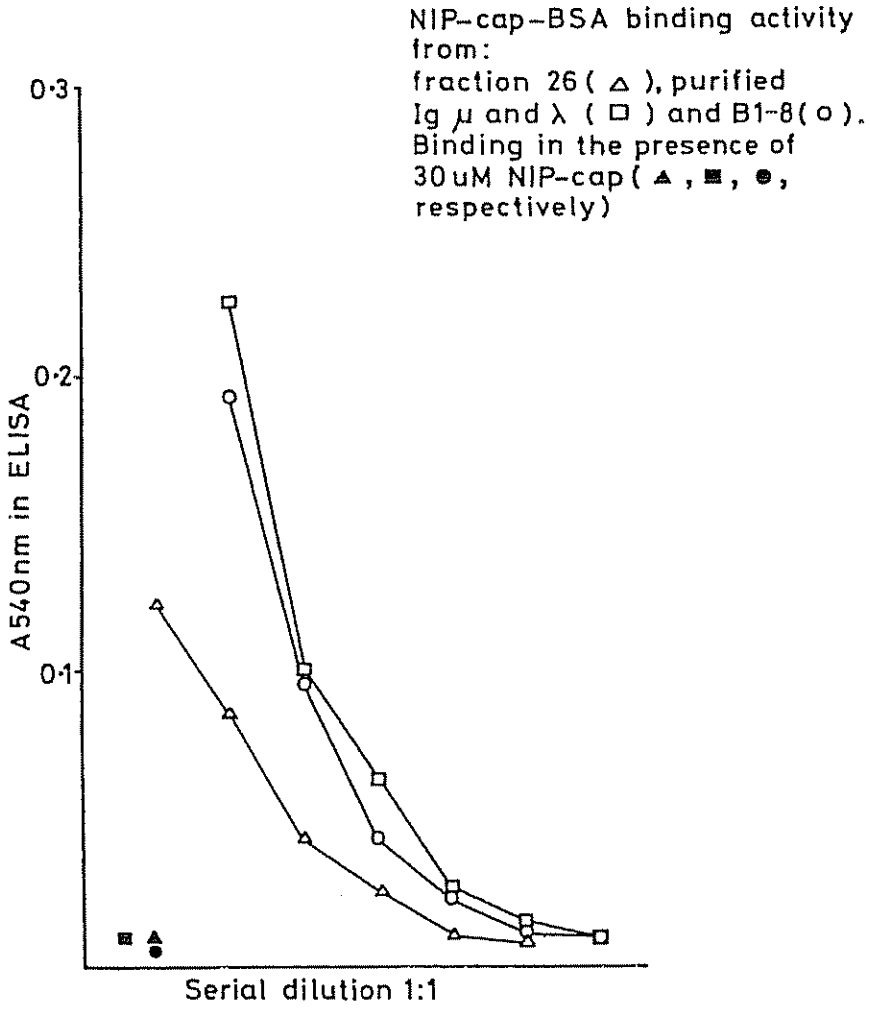
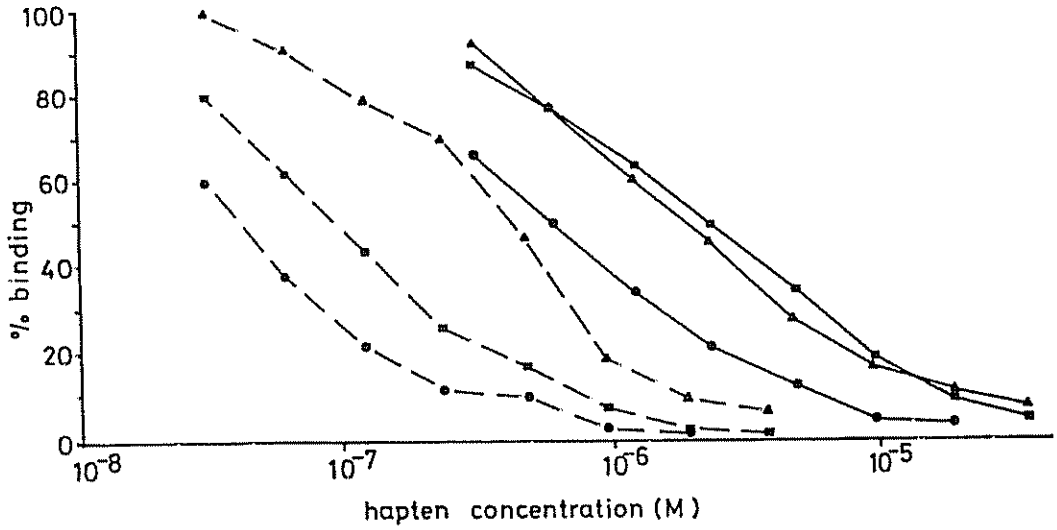

NIP-cap-BSA binding activity from:
fraction 26 (△), purified Ig μ and λ (□) and B1-8(o).
Binding in the presence of 30 uM NIP-cap (▲ , ■ , ● , respectively)
0·3
0·2
0·1
A540nm in ELISA
Serial dilution 1:1

FIG. 12



Binding of antibodies to NIP-cap BSA B1-8 IgM (■), fraction 26 (▲), purified Ig μ or λ (●), in the presence of free NIP-cap (-----) or NIP-cap (——).

FIG. 13

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Office européen des brevets

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(54) **Processes for the production of Ig molecules**

Verfahren zur Herstellung von Immunglobulinen

Procédés pour la production des immunoglobulines

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- **DNA, vol 1, no.2, August 24, 1982, Mary Ann Liebert, Inc, Publishers. J. Haley et al "Porcine Relaxin: Molecular Cloning and cDNA structure", pages 155-161**

Remarks:

The file contains technical information submitted after the application was filed and not included in this specification

EP 0 120 694 B2

Description

[0001] This invention relates to processes for the production of immunoglobulin molecules or immunologically functional Ig fragments in yeast cells which have been transformed by recombinant DNA techniques.

[0002] In recent years advances in molecular biology based on recombinant DNA techniques have provided processes for the production of heterologous (foreign) polypeptides or proteins in host cells which have been transformed with heterologous DNA sequences which code for the production of these products.

[0003] Theoretically, the recombinant DNA approach may be applied to the production of any heterologous polypeptide or protein in a suitable host cell, provided that appropriate DNA coding sequences can be identified and used to transform the host cell. In practice, when the recombinant DNA approach was first applied to the production of commercially useful products, its application for the production of any specified polypeptide or protein presented particular problems and difficulties, and the success of applying this approach to the production of any particular polypeptide or product was not readily predictable.

[0004] However, a large number of heterologous single chain polypeptides or proteins have now been produced by host cells transformed by recombinant DNA techniques. Examples of such heterologous single chain polypeptides or proteins include human interferons, the A and B chains of human insulin, human and bovine growth hormone, somatostatin, calf prochymosin and urokinase. Such transformed host cells provide a reproducible supply of authentic heterologous polypeptide or protein which may be produced on an industrial scale using industrial fermentation technology.

[0005] It should be pointed out that some of these polypeptides, for instance urokinase, after secretion by a host cell, appear as two chain molecules. However, in such cases, the molecule is synthesised by the host cell as a single chain polypeptide, coded for by a single DNA sequence, which is cleaved in the host cell subsequent to synthesis to form the two chain structure.

[0006] It is known that in both human and animal systems there are a number of polypeptides or proteins which have multichain structure in which the chains are not derived from the cleavage of a single chain polypeptide coded for by a single DNA sequence. In such cases, the gene for each of the chains may be located at different points on the same chromosome or even on different chromosomes. In these cases, the polypeptide chains are synthesised separately and then assembled into the complete molecule subsequent to synthesis. Heretofore, no such multichain polypeptide or protein has been produced by recombinant DNA techniques from a single host cell.

[0007] A particular example of a class of such multichain polypeptides or proteins is the immunoglobulins.

[0008] Immunoglobulins, commonly referred to as antibodies, are protein molecules produced in animals by B-lymphocyte cells in response to challenge with foreign antigenic agents such as bacteria, viruses and foreign proteins. The immunoglobulins comprise a crucial part of the immune systems of humans and animals. The immunoglobulins recognise specific parts of the foreign agents and bind onto them. The specific parts are usually known as antigenic determinants or antibody binding sites. A given foreign agent is likely to have a number of different antigenic determinants.

[0009] A typical immunoglobulin (Ig) molecule is shown in Figure 1 of the accompanying drawings, to which reference is now made. The Ig molecule comprises two identical polypeptide chains of about 600 amino acid residues (usually referred to as the heavy chains, H), disulphide bonded to each other, and two identical shorter polypeptide chains of about 220 amino acid residues (usually referred to as the light chains, L), each light chain being disulphide bonded to one end of each heavy chain as shown.

[0010] When the Ig molecule is correctly folded, each chain is formed into a number of distinct globular areas, usually known as domains, joined by a more linear polypeptide chain. The light chains have two such domains, one of which is of variable sequence V_L, and the other of which is of constant sequence C_L. The heavy chains have a single variable domain V_H, adjacent the variable domain V_L of the light chain, and three or four constant domains C_H1-3 or 4.

[0011] The variable domains on both the heavy and the light chains each contain three hypervariable regions (HV1-3) which, when the Ig molecule is correctly folded, are located adjacent one another and form the antigen binding site. It is this area which recognises and binds to the antigenic determinant for which the Ig molecule is specific.

[0012] The constant domains of the Ig molecule do not take part in the binding to the antigenic determinant, but mediate the actions triggered by the binding of the Ig molecule to the antigenic determinant. It is believed that this triggering is caused by an allosteric effect induced by the binding of the Ig molecule to the antigenic determinant. The constant domain may enable the Ig molecule to fix complement or may cause mast cells to release histamine.

[0013] Igs may be categorised by class or subclass, depending on which of a number of possible heavy chain constant domains they contain, there being eight such possible heavy chains in mice. Thus, for instance, an Ig molecule with a μ heavy chain belongs to the class IgM, and one with a γ1 heavy chain to the class IgG_1.

[0014] Igs may also contain one of two light chains, designated as κ and λ light chains, which have different constant domains and different sets of variable domains.

[0015] The structure of the Ig molecule and the location of the genes coding for the various domains thereof are discussed more fully by Early and Hood, in Genetic Engineering, Principles and Methods, Vol. 3, pages 153-158 (edited

by Setlow and Hollaender, Plenum Press).

[0016] It is known that Ig molecules on digestion with selected enzymes can produce a number of immunologically functional fragments. Two such fragments are known as the Fab and $(Fab')_2$ fragments. The Fab fragment comprises one light chain linked to the V_H and C_H1 domains of a heavy chain as shown in Figure 1. The $(Fab')_2$ fragment consists essentially of two Fab fragments linked together by a small additional portion of the heavy chains as shown in Figure 1. These fragments and other similar fragments can be of use in various tests and diagnostic and medical methods.

[0017] The principle method employed for the production of Igs involves the immunisation of susceptible animals with the antigenic agent to provide an immune reaction. Generally the animal is immunised a second time to improve the yield of Ig. The animal is then bled and the Ig is recovered from the serum.

[0018] However, the product of this method is not a homogeneous protein. The animal will produce Igs of different classes and also Igs specific for each of the different antigenic determinants on the antigenic agent, and its blood will therefore contain a heterogenous mixture of Igs. Obtaining a specific Ig of particular class and desired specificity from such a mixture requires very difficult and tedious purification procedures.

[0019] Recently, it has become possible to produce a homogeneous Ig of a single class and a single specificity by a technique first described by Kohler and Milstein (Nature, 256, 495-479, 1975). The technique involves the fusion of single Ig-producing parent cells with cancer cells to produce a monoclonal hybridoma cell which produces the Ig. Ig produced by this technique is usually known as monoclonal antibody. The nature of the monoclonal Ig is determined by the class and specificity of the Ig produced by the parent cell.

[0020] Recently, attempts have been made to use recombinant DNA techniques to produce fragments of Ig molecules. For instance, Amster *et al.* (Nucleic Acids Research, 8, 9, 1980, pp 2055 to 2065) disclose the cloning of double stranded cDNA sequences encoding a mouse Ig light chain into a plasmid. An *E. Coli* strain transformed by the plasmid synthesised a protein thought to comprise the complete constant domain of the light chain and about 40 amino acid residues of its variable region.

[0021] Kemp and Cowman (Proc. Natl. Acad. Sci. USA, 78, 1981, pp 4520 to 4524) disclose the cloning of cDNA sequences encoding mouse heavy chain fragments and the transforming of an *E. Coli* strain which then synthesised heavy chain polypeptide fragments.

[0022] In both these cases, the polypeptides were produced as fusion proteins, in which the fragments of the Ig polypeptides were fused with additional non-Ig polypeptide sequences, and with incomplete variable domains. Thus, the polypeptide chains produced in these studies were not immunologically functional polypeptides as they were incapable of combining with complementary heavy or light chains to provide Ig molecules having intact antigen binding sites and immunological function.

[0023] Research studies have also been carried out in mammalian systems. For instance, Falkner and Zachau (Nature, 298, 1982, pp 286 to 288) report the cloning of cDNA sequences encoding mouse light chains into a plasmid which was used to transfect genomic eukaryotic cells which could then transiently synthesise light chains.

[0024] Rice and Baltimore (Proc. Natl. Acad. Sci. USA, 79 1982, pp 7862 to 7865) report on the transfection of a functionally rearranged K light chain Ig gene into a murine leukaemia virus-transformed lymphoid cell line. The cell line is then able to express the gene continuously. In both these cases, the K genes used to transfect the mammalian cells were obtained from myeloma cells and the K polypeptides produced were of indeterminate immunological function.

[0025] A further approach is exemplified in a series of papers by Valle *et al.* (Nature, 291, 1981, 338-340; Nature, 300, 1982, 71-74; and J. Mol. Biol., 160, 1982, 459-474), which describe the microinjection of mRNAs encoding heavy or light chains of Ig isolated from a mouse myeloma line into oocytes *of Xenopus laevis*. Under certain conditions complete Ig molecules were formed. However, the mRNAs were obtained from myeloma cells and the Ig molecules were of indeterminate immunological function.

[0026] It can thus be seen that hitherto it has not been possible to produce functional Ig by recombinant DNA technology.

[0027] According to a first aspect of the present invention, there is provided a process for producing a heterologous Ig molecule or an immunologically functional Ig fragment in a single yeast host cell, which comprises transforming the host cell with separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains and expressing each of said polypeptide chains separately in said transformed single yeast host cell.

[0028] The present invention also provides a yeast host cell transformed with a vector for use in the process of the first aspect of the invention, as defined in claim 13.

[0029] The present invention further provides a yeast host cell transformed with two separate vectors, the first vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig heavy chain, and the second vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig light chain.

[0030] In the production of Ig molecules according to the invention it will be appreciated that, in order to produce a functional molecule, the DNA sequences used to transform the host cell will need to encode for at least the V_L and V_H

domains of an Ig molecule. Moreover, these domains will need to be complementary so that when the two polypeptide chains fold together they form an antigen binding site of predetermined specificity.

[0031] Preferably, the Ig molecule or fragment produced by the process includes a complete light chain and at least the C_{H1} domain in addition to the V_H domain of the heavy chain. Most preferably the process produces a complete Ig molecule.

[0032] Advantageously, the Ig molecule or functional fragment thereof produced according to the present invention has a variable region (formed by the V_L and V_H domains) which defines a binding site for an antigenic determinant of clinical or industrial importance. The DNA coding sequences necessary to produce such a molecule may be derived from naturally occurring or hybridoma (monoclonal) Ig-producing cells with the desired specificity.

[0033] The constant domains of the Ig molecule or fragment, if present, may be derived from the same cell line as the variable region. However, the constant domains may be specifically altered, partially or completely omitted, or derived from a cell line producing a different class of Ig to provide Ig molecules or fragments having desired properties.

[0034] For example, an Ig molecule may be produced having variable domains (V_H and V_L) identical with those from a monoclonal antibody having a desired specificity, and constant domain(s) from a different monoclonal antibody having desired properties, for instance to provide human compatibility or to provide a complement binding site.

[0035] Such alterations in the amino acid sequence of the constant domains may be achieved by suitable mutation or partial synthesis and replacement or partial or complete substitution of appropriate regions of the corresponding DNA coding sequences. Substitute constant domain portions may be obtained from compatible recombinant DNA sequences.

[0036] The invention may be utilised for the production of Ig molecules or fragments useful for immunopurification, immunoassays, cytochemical labelling and targetting methods, and methods of diagnosis or therapy. For example, the Ig molecule or fragment may bind to a therapeutically active protein such as interferon or a blood clotting factor, for example Factor VIII, and may therefore be used to produce an affinity chromatography medium for use in the immunopurification or assay of the protein.

[0037] It is also envisaged that the Ig molecule may be synthesised by a yeast host cell with another peptide moiety attached to one of its constant domains. Such a further peptide moiety may be cytotoxic or enzymatic. Alternatively, the moiety may be useful in attaching the Ig molecule to a biological substrate, such as a cell or tissue, or to a non-biological substrate, such as a chromatography medium. Such a peptide moiety is herein referred to as a structural peptide moiety.

[0038] It is further envisaged that cytotoxic, enzymic or structural peptide moieties could be attached to the Ig molecule by normal peptide chemical methods, as are already known in the art, rather than by being synthesised with the Ig molecule.

[0039] The Ig molecule or fragment may also comprise a therapeutic agent in its own right. For instance, an Ig molecule or fragment specific for D blood group antigen may be useful for the prevention of haemolytic disease of the new born.

[0040] Any suitable recombinant DNA technique may be used in the process of the invention for the production of Ig molecules or fragments. Typical expression vectors such as plasmids are constructed comprising DNA sequences coding for each of the chains of the Ig molecule or fragment.

[0041] It will be appreciated that a single vector may be constructed which contains the DNA sequences coding for more than one of the chains. For instance, the DNA sequences coding for Ig heavy and light chains may be inserted at different positions on the same plasmid.

[0042] Alternatively, the DNA sequence coding for each chain may be inserted individually into a plasmid, thus producing a number of constructed plasmids, each coding for a particular chain. Preferably the plasmids into which the sequences are inserted are compatible.

[0043] The or each constructed plasmid is used to transform a yeast host cell so that each host cell contains DNA sequences coding for each of the chains in the Ig molecule or fragment.

[0044] For use in cloning in yeast systems, suitable expression vectors include plasmids based on a 2 micron origin.

[0045] Suitable yeast host cells which may be used for expression of the Ig molecule or fragment include *S. cervisiae*.

[0046] It is envisaged that the individual chains will be processed by the host cell to form the complete polypeptide or protein which advantageously is secreted therefrom.

[0047] It will be appreciated that the present application shows for the first time that it is possible to transform a yeast host cell so that it can express two or more separate polypeptides which may be assembled to form a complete Ig molecule or fragment. There is no disclosure or suggestion of the present invention in the prior art, which relates solely to the production of a single chain heterologous polypeptide or protein from each host cell.

[0048] The present invention will now be described, by way of example only, with reference to the accompanying drawings, in which:-

Figure 1 shows a diagrammatic representation of a typical intact Ig molecule;

Figure 2 shows the construction of plasmids for the direct synthesis of a λ light chain in E. Coli;
Figure 3 shows the construction of plasmids for the direct synthesis of a μ heavy chain in E. Coli;
Figure 4 is a diagrammatic representation of μ mRNA sequences around the initiation codon;
Figure 5 shows the construction of plasmids having altered secondary structure around the initiation codon;
Figure 6 is a polyacrylamide gel showing expression and distribution of μ protein from E. Coli B;
Figure 7 is a polyacrylamide gel showing pulse chase autoradiograms of μ protein in E. Coli B and in E. Coli HB101;
Figure 8 is a polyacrylamide gel showing the results of λ gene expression in E. Coli;
Figure 9 is a polyacrylamide gel showing the distribution of recombinant λ light chain polypeptide between the soluble and insoluble cell fractions;
Figure 10 is a polyacrylamide gel showing expression and distribution of λ protein from E. Coli E103S;
Figure 11 shows the results of the fracitonation of μ and λ protein expressed by E. Coli B on DEAE Sephacel;
Figure 12 shows the specific hapten binding of reconstituted Ig molecules; and
Figure 13 shows the heteroclitic nature of the hapten binding of the reconstituted Ig molecules.

[0049] In the following examples, there is described the production of Ig light and heavy chain polypeptides derived from monoclonal antibodies which recognise and bind to the antigenic determinant 4-hydroxy-3-nitrophenyl acetyl (NP), using E.coli and S. cerevisiae as the host cells. Recombinant DNA techniques were used to enable the host cells to express both the polypeptide chains.

[0050] It will be appreciated that the invention is not limited to the specific methods and construction described hereafter.

Construction of Lambda Light Chain Expression Plasmid

[0051] Figure 2, to which reference is now made, shows schematically the method used to construct a λ light chain expression plasmid.

[0052] It was decided to express the lambda gene in E. coli by direct expression of the gene lacking the eucaryotic signal peptide but containing a methionine initiator residue at the amino-terminus (met-lambda). The approach used for bacterial synthesis of met-lambda was to reconstruct the gene in vitro from restriction fragments of a cDNA clone and to utilise synthetic DNA fragments for insertion into the bacterial plasmid pCT54 (Emtage et al., Proc. Natl. Acad. Sci. USA., 80, 3671 to 3675, 1983). This vector contains the E. coli trp promoter, operator and leader ribosome binding site; in addition 14 nucleotides downstream of the ribosome binding site is an initiator ATG followed immediately by EcoR1 and HindIII sites and the terminator for E. coli RNA polymerase from bacteriophage T7.

[0053] As a source of light chain we used a plasmid pABλ1-15 which contains a full-length λ_1 light chain cDNA cloned into the PstI site of pBR322. This λ_1 light chain is derived from a monoclonal antibody, designated S43, which binds to 4-hydroxy-3-nitrophenylacetyl (NP) haptens.

[0054] In order to create a HindIII site 3' to the end of the lambda gene for insertion into the HindIII site of pCT54, the cDNA was excised from pABλ1-15 using Pst1. The cohesive ends were blunt ended using the Klenow fragment of DNA polymerase and synthetic HindIII linker molecules of sequence 5'-CCAAGCTTGG-3' ligated. The DNA was digested with HindIII and the 850bp lambda gene isolated by gel electrophoresis and cloned into HindIII cut pAT153 to yield plasmid pATλ1-15. The 3' end of the lambda gene was isolated from pATλ1-15 by HindIII plus partial Sac1 digestion as a 630bp SacI-HindIII fragment (2 in Figure 2). The HindIII cohesive end was dephosphorylated by calf intestinal alkaline phosphatase during isolation of the fragment to prevent unwanted ligations at this end in subsequent reactions.

[0055] A HinfI restriction site is located between codons 7 and 8 and the lambda sequence. The 5' end of the lambda gene was isolated as a 148bp HinfI to SacI fragment (1 in Figure 2).

[0056] Two oligodeoxyribonucleotides were designed to restore codons 1-8, and to provide an initiator ATG as well as Bc1I and HinfI sticky ends. The two chemically synthesised oligonucleotides made to facilitate assembly of the gene had the sequences:

```
R45   5' -pGATCAATGCAGGCTGTTGTG    3'

R44          3'      CCGACAACACTGAGTCCTTAp-    5'
```

[0057] pCT54 was cut with both Bc1I and HindIII and the resulting linear molecules isolated, mixed together with the

two oligodeoxyribonucleotide linkers R44 and R45 and both fragments 1 and 2, and ligated using T4 ligase (Figure 2). The mixture was used to transform E. coli DH1 to ampicillin resistance. Recombinant clones in pCT54 were identified by hybridisation of DNA from replica plated colonies on nitrocellulose to a nick-translated probe derived from the pATλ1-15 insert.

[0058] A clone was identified which hybridised to lambda cDNA and also showed the predicted restriction fragment pattern. This plasmid (designated pCT54 19-1) was sequenced from the ClaI site and shown to have the anticipated sequence except that there was a mutation of the fourth codon from CTG to ATG, changing the amino acid at this point from valine to methionine.

[0059] The sequence in this area was:

```
...GATTGATCA.ATG.CAG.GCT.GTT.ATG.ACT.CAG.GAA.TCT.GCA.CTC.ACC.ACA.TCA.

                met gln ala val met thr gln glu ser ala leu thr thr ser
```

The restriction enzyme sites in pCT54 between the Shine and Dalgarno sequence (AAGG), which is important for ribosome binding, and the ATG allow for the adjustment of the SD-ATG distance, an important parameter in determining expression rates. The SD-ATG distance was reduced by cutting the plasmid with ClaI or BclI and creating blunt ended species by digestion with S_1 nuclease. 2 µg of ClaI cut DNA was digested with 200 units of S_1 nuclease for 30 minutes at 30° using standard buffer conditions. The solution was deproteinised with phenol and the DNA recovered by ethanol precipitation. This DNA on religation with T4 DNA ligase and transformation into E.coli strain HB101 gave rise to a number of plasmids which had lost the ClaI or BclI site.

[0060] The plasmids which had lost their ClaI site were sequenced in the region surrounding the initiator ATG.

```
....AAGGGTATTGATCAATG CAG....    plasmid pNP3
    SD            met glu
```

```
....AAGGGTTTGATCAATG CAG         plasmid pNP4
    SD            met glu
```

In order to achieve high level expression a number of other approaches were followed. Firstly, a series of constructs were obtained which had increasing amounts of the 3' untranslated region of the cDNA removed by Bal 31 exonuclease. Secondly, a high copy number plasmid containing λ cDNA was constructed. This plasmid contained a par function (Meacock, P. A and Cohen, S.N. Cell, 20, 529-542, 1980) as well as being present in high copy number. Thirdly, the pNP3 plasmid was transformed into a number of protease-deficient strains or into HB101 in conjunction with a protease deficient dominant acting plasmid (Grossman, A.D et al Cell, 32, 151-159, 1983).

Construction of µ Heavy Chain Expression Plasmid

[0061] The full-length µ heavy chain cDNA derived from the NP binding monoclonal antibody B1-8 had been cloned into the PstI site of pBR322 yielding a plasmid designated pABµ-11 (Bothwell et al, Cell, 24, 625-637, 1981). In order to achieve high level expression, the µ cDNA minus the eukaryotic leader was reconstructed into pCT54. The construction of the µ heavy chain expression plasmid is shown diagramatically in Figure 3. Two chemically synthesised oligonucleotides were made to facilitate this. These have the following sequences:

```
R43    5'  GATCAATGCAGGTTCAGCTGCA   3'
```

R46 3' TTACGTCCAAGTCG 5'

These were ligated into BclI cut pCT54 using T4 DNA ligase and the resulting plasmid designated pCT54 Pst. The linkers were designed to replace the sequence in pCT54 between the BclI site and the ATG, to provide an internal PstI site and to recreate the sequence from pABμ-11 5' to the PstI site up to codon +1. PCT54 Pst was cut briefly with PstI, treated with alkaline phosphatase and full length DNA glass isolated from a 1% agarose gel. Similarly pABμ-11 was briefly cut with PstI and the full length μ insert isolated following agarose gel electrophoresis. The μ cDNA was ligated with the full length pCT54Pst fragment using T4 DNA ligase under standard conditions and a plasmid designated pCT54μ was identified which by restriction enzyme analysis was shown to contain a full-length μ insert. The plasmid was sequenced around the 5' linker region and was found to have the anticipated sequence:
5' .....TGATCAATGCAGGTTCAGCTGCAGGGGGGGGATGGGATGGAG..... 3', demonstrating that it was, indeed, a full length clone. A complete PstI digest of pCT54μ liberated a 1.4 Kb fragment which was purified by 0.8% agrose gel electrophoresis and glass powder isolation. This was ligated with PstI cut pCT54 Pst (see above) using T4 DNA ligase under standard conditons, followed by transformation into HB101. A plasmid designated pNP1 was isolated which was shown by restriction endonuclease pattern analysis to contain the 1.4 Kb μcDNA fragment in an appropriate orientation (Figure 3). pNP1 was a plasmid which consisted of an appropriate 5' end for expression, whilst pCT54 contained an appropriate 3' end. The full length gene was reconstructed into pCT54 by cutting both pNP1 and PCT54μ with AvaI which cuts once in the plasmid and once in the μ gene. Both digests were run on a 1% agrose gel and the 1.9 Kb fragment from pNP1 and the 3.65 Kb fragment from pCT54μ isolated. Following alkaline phosphatase treatment of the 3.65 Kb pCT54μ fragment, the two pieces of DNA were ligated to each other and transformed into HB101. A plasmid designated pNP2 was identified which demonstrated the correct restriction endonuclease pattern. It was sequenced in the area surrounding the initiator ATG and found to have the anticipated sequence:

5' ....TTGATCAATGCAGGTTCAGCTGCAGCAGCCTGGGGCTGAGCTTGTGAAG.... 3'

[0062] The vector pCT54 had been constructed to include two restriction sites (BclI and ClaI) between the S-D sequence (AAGG) and the initiation codon so that the distance between these sequence elements could be varied. As most E.coli mRNAs have 6-11 nucleotides between the S-D sequence and the AUG the distance in pNP2 was reduced by modification at the ClaI site. pNP2 was cut with ClaI and incubated with S1. The amount of S1 nuclease was adjusted so that some DNA molecules would lose 1-2 extra base pairs as a result of 'nibbling' by the enzyme. This DNA on religation with T4 DNA ligase and transformation into E.coli strain HB101 gave rise to a number of colonies harbouring plasmids which had lost the ClaI site. The sequences around the initiation codon of four plasmids pNP223, pNP261, pNP9 and pNP282 were determined and are given in Table 1, part A below.

TABLE 1

		S-D to ATG distance (no. of residues)	Sequence (5' - 3') ClaI	Expression (ru) expts. A	B	C
(A)	pNP2	14	-AAAAAGGGTATCGATTGATCAATGCAGGTTCAG-	0.7	0.6	0.8
S1 derivatives of pNP2	pNP223	12	-AAAAAGGGTATATTGATCAATGCAGGTTCAG-	-	0.7	0.6
	pNP261	10	-AAAAAGGGTATTGATCAATGCAGGTTCAG-	-	1.1	1.1
	pNP282	7	-AAAAAGGGTGATCAATGCAGGTTCAG-	-	0.7	0.6
	pNP9	9	-AAAAAGGGTATGATCAATGCAGGTTCAG-	1.0	1.0	1.0
(B)	pNP11	11	-AAAAAGGGTATTGCACATATGCAAGTGCAA-	5.3	6.3	9.1
oligo constructs	pNP12	11	-AAAAAGGGTATTGCACATATGCAGGTTCAG-	1.8	2.3	1.3
	pNP14	9	-AAAAAGGGTATGATCAATGCAAGTGCAA-	72.2	106.7	124.6
	pNP8	-	-	77.0	53.4	145.5
	pCT70	-	-	0	0	0

[0063] The expression of these constructs in E. coli B was examined in three experiments, and concentrations of μ protein were determined by ELISA as relative units (ru), a measure of μ concentration normalised to pNP9. The results are shown in Table 1.

[0064] Inductions were carried out by resuspending cells at 1:50 dilution from overnight cultures into induction medium, consisting of: KH_2PO_4 (3g/L), Na_2HPO_4 (6g/L), NaCl (0.5g/L), Difco vitamin assay casamino acids (30g/L), NH_4Cl (4g/L),glycerol (16g/L), proline (0.5g/L), Difco yeast extract (1g/L), $CaCl_2.6H_2O$ (0.022g/L), $MgSO_4.7H_2O$ (0.025g/L), thiamine (0.01g/L) and carbenicillin (0.1g/L). Cultures were shaken at 37°C and cells harvested by centrifugation.

[0065] ELISA assays were performed essentially as described by Patel et al. (Nucleic Acid Research, 10, 5605 to 5620, 1982), using affinity purified goat anti-mouse IgM (Tago) and affinity purified goat anti-mouse IgM (Tago) conjugated to peroxidase, with 3, 3', 5, 5' tetramethylbenzidine (Miles) as a substrate. Bacterial cell pellets were boiled for 2 minutes in 9M urea (BRL) and loaded directly into the ELISA, such that all wells contained 2.25M urea, 10mM Tris-HCl pH7.8, 150mM NaCl, 0.05% NP40 and 0.5% casein (Hammarsten, B.D.H.).

[0066] Altering the S-D to ATG distance with nuclease S1 was found to increase the level of μ expression, relative to the parental plasmid pNP2, but only to a small extent (compare pNP9). The optimal S-D to AUG distance was found to be 9-10 nucleotides, as found in pNP9 and pNP261 respectively.

Secondary Structure Analysis

[0067] Hairpin loops were identified by use of the computer programme HAIRGU developed by Dr. Roger Staden. The ΔG values were calculated as described by Trioco et al. (Nature NB, 246, 40-41, 1973).

[0068] Analysis of the potential secondary structure of μ mRNA encoded by pNP2 and pNP9 (one of the S1 derivatives or pNP2) revealed an extensive array of possible hairpin loops. Attention was focused on the region of this mRNA containing the ribosome binding site since the sequestering of S-D sequences and initiation codons into secondary structure may inhibit the initiation of translation. It was found that a hairpin loop including the U and G of the initiation codon could be formed (see Figure 4). This hairpin loop is formed entirely within coding sequences and has a ΔG = -7.6 kcal. No secondary structures were found which buried the S-D sequence, and none which mutually excluded the hairpin loop described above, and which had a lower ΔG.

[0069] To test whether expression of μ protein could be influenced by changes designed to alter potential secondary structure in the ribosome binding region we used synthetic oligonucleotides to mutagenise this region of μ mRNA. The

plasmids were constructed by insertion of a pair of oligonucleotides between the blunted Cla It site and the 5' Pst site of μ in pNP2 (see Figure 5). The cloning strategy used to accomplish this, however, was multi-staged due to the presence of four Pst sites in pNP2. The vector pACYC184CM, which lacks Cla I and Pst I sites, was first constructed by cutting pACYC184 at its unique Cla I site, 'filling-in' the cohesive termini using T4 DNA polymerase and religating. Next, PACYC184CM was cut with BamHI, and the BamHI fragment from pNP2, bearing the 5' μ sequence, was inserted into it to form pCMμ (see Figure 4). The vector pCMμ contained unique Cla I and Pst sites which were required for the oligonucleotide cloning (see Figure 5).

[0070] pCMμ was cut with Cla I to completion, blunted via nuclease S1, and then cut with Pst I. The cut plasmid was purified by agarose gel electrophoresis and ligated separately with pairs of oligonucleotides, with the latter in a 100-fold molar excess. The pairs of oligonucleotides used were: R131 and R132 (for pNP11); R196 and R197 (for pNP12); and R202 and R203 (for pNP14). These have the sequences given below.

TGCACATATGCAAGTGCAACTGCA (R131), GTTGCACTTGCATATGTGCA (R132),
GCTGAACCTGCATATGTGCA (R196), TGCACATATGCAGGTTCAGCTGCA (R197),
GTTGCACTTGCATTGATC (R202), GATCAATGCAAGTGCAACTGCA (R203).

[0071] The ligation mixture was then used to transform HB101. Recombinant clones were detected by colony hybridisation, on nitrocellulose filters, using one of each pair of oligonucleotides as a probe. Positive clones were sequenced and shown to have the correct nucleotide sequence. The μ sequences containing the cloned oligonucleotides were excised on Eco RV-3gl II fragments. These were ligated to Eco RV-Bgl II cut pNP2 to reconstruct the full length μ genes (see Figure 5). Three different plasmids were created by these procedures; pNP11, pNP12 and pNP14, having the nucleotide sequences shown in Table 1 above.

[0072] In the construct pNP11 the potentially deleterious hairpin loop of pNP2 was abolished by changing the residues in the degenerate position of three codons and by introducing a different A-D to AUD sequence. These changes allow the region between the S-D and AUG to base pair with the 5' end of μ coding sequence to form a hairpin loop of ΔG = -7.8 kcal, approximately equal to that of pNP2, but leaving the AUG and S-D sequences exposed (see Figure 4). The only other mutually exclusive hairpin loops are of ΔG = -2.3, -3.6 and + 0.4 kcal.

[0073] The construct pNP12 retains the S-D to AUG sequence of pNP11, but has the coding sequence of pNP2, such that the hairpin loop of pNP2 can form (see Figure 4).

[0074] The final μ construct was pNP11, which had the S-D to AUG sequence of pNP9, but the 5' coding sequence of pNP11, so that the hairpin loop of pNP12 could not form. The construct pNP14 had no secondary structure burying the S-D or μ AUG.

μ Protein Expression from Constructs with Differing mRNA Secondary Structures

[0075] E.coli B strains containing plasmids pNP9, pNP11, pNP12 and pNP14 were grown in induction medium and samples removed for ELISA assay. The concentration of μ was increased 6-7 fold in E.coli B cells containing pNp11 relative to pNP9 (see Table 1, Part b). Plasmid pNP12, which has the S-D to AUG sequence of pNP11, but the coding sequence of pNP9, showed only a two-fold increase in level of μ expression compared to pNP9.

[0076] Plasmid pNP14, which has the S-D to AUG sequence of pNP9 but the 5' μ coding sequence of pNP11, and thus differed from pNP9 only in three residues, was found to express μ protein over 90 times that found in pNP9.

[0077] A plasmid, pNP8, has also been constructed, from which μ is expressed, also from the trp promoter, as part of a trpE-μ fusion protein containing the amino terminal 53 amino acids of trpE and the carboxyl terminal 503 amino acids of μ. The rationale for making this fusion gene was that μ would be translated from an efficient bacterial ribosome binding site rather than from one of unknown efficiency. Then, by comparing the amounts of trpE-μ produced from pNP8 with those of μ produced from the different constructs, an estimate of relative ribosome binding efficiency may be made. When the trpE-μ gene in pNP8 was induced and the products quantitated by ELISA, it was found that the μ expression in strains containing pNP8 and pNP14 were very similar (see Table 1, part B).

The ribosome binding site (RBS) sequences of pNP11, pNP12 and nPnp14 were designed in order to test , the hypothesis that sequestering of the initiation codon into secondary structure inhibits translation initiation, and to increase the rate of initiation by releasing the initiation codon. In pNP11, the RBS hairpin loop of pNP2 and pNP9 was abolished by changing the 5' μ coding sequence in the degenerate position of three codons. In addition, the S-D to AUG sequence was changed to allow the base-pairing of this region with the 5' coding sequence. The result of these changes is that the initiation codon is exposed in the loop of a hairpin with the S-D sequence at the base of the stem (see Figure 4). This hairpin loop had a ΔG approximately equivalent to the ΔG of the pNP9 hairpin loop. Strains containing pNP11

produced 6-7 times as much μ as did those with pNP9. To test whether this was due to the change in S-D to ATG sequence characteristics, rather than to the liberation of the initiation codon, pNP12 was constructed as a control. The latter has the S-D to AUG sequence of pNP11, but retains the 5' μ coding sequence of pNP9, so that the RBS hairpin loop of pNP11 cannot form, but the potentially deleterious one of pNP9 can form. The level of μ expression from pNP12 was found to be increased by only two-fold relative to pNP9. This increase is probably due to an altered S-D to AUG distance, a U immediately 5' to the initiation codon , and the S-D to AUG sequence being G-poor each of which has been shown to be advantageous. It is therefore likely that the increased expression from pNP11 is due to a preferable mRNA secondary structure. The RBS hairpin loop of pNP9 probably acts to inhibit translation while creation of another hairpin loop which exposes the initiation codon is responsible for the increased expression.

[0078] The plasmid pNP14 was constructed, such that it had the S-D to AUG sequence of pNP9, but the coding sequence of pNP11. The expression plasmid pNP14, therefore, differed from pNP9 in only three residues, those in the degenerate position of three codons, yet strains containing pNP14 expressed over ninety times more μ than those containing pNP9. This increase in expression cannot be explained on the basis of the three residue changes optimizing codon usage, for they introduce less favoured codons, as judged by the frequency of their occurrence in strongly expressed E.coli genes.

[0079] The increased expression is best explained by the abolition of the RBS hairpin loop in pNP9. The magnitude of the increases in expression observed, and the few residue changes introduced, makes it difficult for these results to be interpreted in terms of changes in mRNA stability.

[0080] The expression of pNP8, which encodes the trpE-μ fusion protein from a native trpE RBS, was also examined, and found to express at approximately the same level as pNP14. This may indicate that the RBS of pNP14 is equivalent to that of trpE in terms of its efficiency in directing translation initiation. The native trpE RBS of pNP8 and that of pNP14 were found to have no secondary structures hindering the use of their RBS sequences.

Expression of μ Protein in E. Coli

[0081] E. Coli B cells containing the μ expression plasmid pNP11 were grown under inducing conditions and soluble and insoluble extracts prepared, and analysed by SDS-PAGE. A novel band was seen after staining the gel with Coomassie blue in the lane containing proteins from the insoluble fraction (see Figure 6, lane 2). This band was not seen in the negative control lane which contained proteins from the same fraction from cells harbouring pCT70 (see Figure 6, lane 3). The novel band was found to migrate to a position corresponding to a protein of a molecular weight within less than 5% of the actual molecular weight of non-glycosylated μ of 62.5Kd. A duplicate set of lanes were transferred to nitrocellulose, and Western blotted. Alignment of the stained gel and the blot autoradiogram confirms that this novel band is antigenically related to IgM (see Figure 6, lanes 4 and 8). No band was found in extracts from cells containing pCT70 (see Figure 6, lanes 5 and 7). Only a low amount of μ was found in the soluble fraction (see Figure 6, lane 6).

[0082] A greatly increased level of expression of μ was found in E. Coli B compared to HB101. Pulse chase analysis demonstrated that in E. Coli B, a similar level of μ protein was detected after a 60 minute chase (Figure 7, lane 3) as was seen after the initial labelling period (Figure 7, lane 1). In HB101, however, very little μ protein could be seen after a 10 minute chase (Figure 7, lane 7), and none after 30 minutes (Figure 7, lane 8), compared to the amount detected after the initial labelling period (Figure 7, lane 5).

[0083] For pulse chase analysis, inductions were set up as described above, except that the medium used consisted of: proline (0.3g/L), leucine (0.1g/L), Difco methionine assay medium (5g/L), glucose (60mg/L), thiamine (10mg/L), $CaCl_2$ (22mg/L), $MgSO_4$ (0.25g/L) and carbenicillin (0.1g/L). During exponential growth cells were pulse labelled with 30 μCi/ml L-[^{35}S] methionine for 2 minutes, after which unlabelled methione (100 μg/ml) was added and the incubation continued for the times indicated.

[0084] Induced E. Coli B cells harbouring pNP14 when examined by phase contrast microscopy were found to contain inclusion bodies.

Expression of λ Light Chain in E.Coli

[0085] A fresh 1 ml overnight culture of E.Coli HB101 or RV308 containing the plasmid under study was grown in L Broth (Maniatis et al, Molecular Cloning, Cold Spring Harbor Laboratory, 1982), supplemented with carbenicillin to 100 μg/ml.

[0086] A 1:100 dilution of this culture was made into M9 medium (Maniatis, 1982, op.cit.) supplemented with glucose, vitamin B_1, carbenicillin, leucine and proline and the cultures shaken at 37° for 5-6 hours. At this time ^{35}S-methionine was added for 5-20 minutes. The cells were then harvested by centrifugation, lysed by boiling in 1% SDS for 2 minutes and diluted by addition of a buffer consisting of 2% Triton X-100, 50 mM Tris pH 8, 0.15 M NaCl and 0.1 mM EDTA. Immunoprecipitations were carried out by addition of antisera to aliquots of labelled E.Coli extracts and, after incubation

at 4° overnight, immune complexes were isolated by binding to Staphylococcus aureus fixed cells. The complexes were dissociated by boiling in 60 mM Tris pH 6.8 buffer containing 10% glycerol, 3% SDS and 5% mercaptoethanol and the liberated proteins analysed on 10% or 12.5% acrylamide/SDS gels (Laemmli, UK. Nature, 227, 680-685, 1970). Gels were stained with Coomassie Brilliant blue to visualise the protein bands while labelled proteins were detected on Fuji-RX film by fluorography using 1 M sodium salicylate.

[0087] HB101 cells containing pNP3 and other plasmids were grown under inducing conditions to an OD_{600} = 0.6 and the proteins present examined by specific immunoprecipitation substantially as described above. The results obtained are given in Figure 8, in which lanes 1 and 2 respectively were extracts from pCT54-19 and pNP3, immunoprecipitated with normal rabbit serum; lanes 3, 4 and 5 were extracts immunoprecipitated with rabbit anti-lambda serum and represent pNP4 (lane 3), pNP3 (lane 4) and pCT54-19 (lane 5). The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of Figure 8. All of the plasmids produced a protein which reacted with rabbit anti-mouse λ light chain serum and comigrated with authentic λ_1 light chain from the mouse myeloma MOPC104E. pNP3 however produced the most of this protein (compare lane 4 with lanes 3 and 5 in Figure 8). No such band was detected with control immunoprecipitations using normal rabbit serum (Figure 8, lanes 1 and 2). Studies using brief pulses with ^{35}S-methionine followed by chasing with excess cold methionine indicated that the recombinant λ light chain had a 1/2 life of about 20 minutes.

[0088] In vitro transcription/translation (Pratt et al, Nucleic Acids Research, 9, 4459-447, 1981) confirmed that pNP3 coded for a protein which comigrated with authentic λ light chain. This 25 Kd product was synthesised in vitro at a rate comparable to that of β-lactamase indicating that it is synthesised in vivo at a level of 0.5%of total E.Coli proteins. This figure is in good agreement with the percentage of recombinant λ product synthesised in vivo (0.4-2%) as determined from the following equation:

$$\% \text{ specific CPM} = \frac{100 \times (\text{CPM with anti-}\lambda) - (\text{CF}^{M} \text{ with normal serum})}{\text{total CFM}}$$

The procedure used to disrupt cells was as follows (Emtage J S et al, Proc. Natl. Acad. Sci., 80, 3671-3715, 1983). E. Coli HB101/pNP3 grown under inducing conditions were harvested and resuspended in 0.05 M Tris pH 8, 0.233 M NaCl, 5% glycerol containing 130 g/ml of lysozyme and incubated at 4°C or room temperature for 20 minutes. Sodium deoxycholate was then added to a final concentration of 0.05% and 10 µg of DNAase 1 (from bovine pancreas) was added per g wet wt of E.Coli. The solution was incubated at 15°C for 30 minutes by which time the viscosity of the solution had decreased markedly. The extract was centrifuged (at 10,000 x g for 15 minutes for small volumes (1 ml) or 1 hour for larger volumes) to produce a soluble and an insoluble fraction.

[0089] For immunoprecipitations, the soluble fraction was diluted in the Triton-containing buffer described above and the insoluble fraction solubilised by boiling in 1% SDS followed by dilution in Triton-containing buffer. HB101 cells containing pNP3 or pNP4 were grown under inducing conditions, pulse labelled with [^{35}S] methionine, separated into soluble and insoluble fractions and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The results obtained are shown in Figure 9 in which lanes 1 and 3 correspond to the soluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lanes 2 and 4 correspond to the insoluble fraction from HB101-pNP3 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively, lanes 5 and 7 correspond to the soluble fraction from HB101-pNP4 immunoprecipitated with normal rabbit serum and rabbit anti-lambda serum respectively; lane 9 corresponds to the soluble fraction from HB101-pNP3, and lane 10 corresponds to the insoluble fraction from HB101-pNP3. The position of unlabelled lambda protein from MOPC104E is indicated on the left hand side of Figure 9. When such a procedure was carried out using pNP3, the recombinant λ light chain protein was present in the insoluble fraction (Figure 9, lane 4) rather than the soluble fraction (Figure 9, lane 3). No 25 Kd band comigrating with authentic λ_1 light chain was present when either the soluble or insoluble fraction were immunoprecipitated using normal rabbit serum (Figure 9, lanes 1 and 2). By inference it is likely that all Ig light chains will fractionate as in this specific example.

[0090] In the absence of specific immunoprecipitation a novel protein band was not visible from extracts of HB101 containing pNP3 nor was the λ protein found to accumulate. However, there was a dramatic difference when pNP3 was induced in the K12 strain E103s. In this strain λ protein was found to accumulate during induction until the cells reach stationary phase (Figure 10, lanes 3-5) to a level of about 150 times that found in HB101 as determined by an ELISA (enzyme linked immunosorbent assay). These cells were found to contain inclusion bodies which appeared refractile under light microscopy, a phenomenon characteristic of high level expression of foreign proteins. An estimate of the percentage of total E. Coli protein represented by recombinant λ protein was obtained by separating the proteins by gel electrophoresis, staining them with Coomassie blue and scanning the stained gel with a Joyce-Loebl chromoscan 3. This method showed that λ was the major protein present (Figure 10, lane 5) and represented 13% of total E. Coli protein. The λ protein had a half-life of 20 minute in HB101 but accumulated to very high levels in E103s, suggesting

that Lambda protein was much more stable in the latter strain. After cell lysis and centrifugation of HB101 or E103s containing pNP3, λ light chain was detected in the insoluble (Figure 10, lanes 7 and 10) but not in the soluble fractions (Figure 10, lanes 6), as determined by Coomassie blue staining. The identity of the major Coomassie blue stained band as λ protein was confirmed by Western blot analysis (Figure 10, lanes 8-10). The presence of such immunoreactive bands was specific to pNP3 containing cells. When extracts from cells containing pCT70, a prochymosin expressing plasmid, were subjected to the same analysis no bands were detected. This more sensitive technique showed that a small amount of the λ protein was in the soluble fraction (Figure 10, lane 9). The presence of a number of distinct immunoreactive proteins all smaller than full-length protein were also detected. These may result from proteolytic degradation of of λ protein, from premature termination of transcription or from internal initiation of translation.

Expression of μ and Lambda Polypeptides in the same Bacterial Cell

[0091] Each of the Ig u and λ genes in expression plasmids were transformed into the same E.Coli cell to direct the synthesis of both Ig μ and λ polypeptides. In order to overcome plasmid incompatibility and provide a second antibiotic resistance marker, the trp promoter and λ sequences were excised from pNP3 on a Hind III - Bam HI fragment and inserted into the Hind III - Bam HI fragment of pACYC 184. The resultant plasmid pACYC λ caused the E.Coli to grow very poorly. This weak growth was thought to result from read-through of RNA polymerase into the origin of replication. However, the inhibition of growth was virtually eliminated by the cloning in of the bacteriophage T7 transcriptional terminator at the Hind III site of plasmid pACYC λ.

[0092] This terminator functions in both orientations. The resultant plasmid pAC λ T7-1 has a chloramphenicol esistance gene and an origin compatible with the pBR 322-derived origin on pNP14, the Ig μ expressing plasmid. Transformation of both plasmids in the same E.Coli B was achieved in two steps. Firstly pNP14 was introduced followed by pAC λ T7 - I in two sequential transformations to give ampicillin and chloramphenicol resistant clones.

[0093] E.Coli B cells derived from this double-transfomant clone showed the presence of inclusion bodies and two novel polypeptide bands on stained gels of the insoluble fraction after lysis. These two bands; correspond both with immunological activity by Western blotting for Ig μ and Ig λ and in their expected molecular weights. It has thus been shown that the double-transformant clone expresses both the heterologous genes. This had not hitherto been shown.

[0094] The presence of λ light chain in the insoluble fraction was a useful purification step since it both concentrated the protein and separated it from the bulk of E. Coli soluble proteins.

[0095] For further purification of the λ light chain, the cell debris were dissolved in 10mM Tris-HCl pH8.0, 25% formamide, 7M urea, 1mM EDTA and 2mM dithiothreitol. This material was loaded onto a DEAE Sephacel column (Pharmacia) (1x 25cm at a flow rate of 5ml/hr) which had been equilibrated in 9M urea, 10mM Tris-HCl pH8.0, 1mM EDTA and 2mM DTT. The DEAE Sephacel column was developed using a 0-150mM NaCl gradient in loading buffer. The eluted peak λ light chain immunoreactivity, corresponding to the major peak of protein, was diluted to a final concentration of 2.25M urea, 10mM Tris-Hcl pH8.0, 1mM EDTA, 2mM DTT and loaded onto an octyl-Sepharose column (Pharmacia) (2.5 x 10cm). Material was eluted by use of a urea gradient of 2.25-9M urea. The peak material was pooled, dialysed into ammonium bicarbonate and lyophilised. Following this step, only a single band of Coomassie blue stainable material corresponding to recombinant λ protein was visualised by SDS-PAGE.

[0096] The μ heavy chain was purified from 9M urea solubilised pellets by anion exchange chromatogrpahy and chromatofocussing (Pharmacia).

[0097] It was of great interest to determine whether the concomitant expression of μ and λ would lead to the formation of functional IgM. In order to determine this, extracts were made from E. Coli containing both Ig μ and λ polypeptides and these tested for antigen binding. We used a two-site sandwich ELISA which detects μ chain binding to haptenalated bovine serum albumin (NIP-caproate-BSA).

[0098] In the NIP binding assay, bovine serum albumin (BSA) is reacted with an equimolar amount of NIP-Cap-N-hydroxy-succinamide at pH 7.5 in 10mM phosphate buffer. The resulting NIP-Cap-BSA is separated from free NIP-Cap on a G-50 Sephadex column.

[0099] Microtitre plates (96 well Nunc Immuno Plate 1) are coated with 100 μl of a solution of 10 μg/ml NIP-BSA in sodium carbonate/sodium bicarbonate 0.1M, pH 9.6 buffer (coating buffer) overnight at 4°C.

[0100] The coated plates are then blocked for non-specific binding by addition of 100 μl of 0.5% casein in coating buffer (blocking buffer) and incubating at 37°C for 1 hour.

[0101] The coated and blocked plates are then washed three times in 150 mM NaCl, 0.05% NP40, 20mM Tris-HCl, pH 7.8 buffer (washing buffer).

[0102] The washed plates are shaken free of excess washing buffer and samples are added in their buffer or typically in 0.5% casein in washing buffer (sample buffer) to a 100 μl final volume.

[0103] In order to demonstrate the nature of the binding to NIP-BSA, samples for testing were supplemented with either NIP or NP in free solution at various concentrations (from 60 to 0.3 μM for NIP or from 600 to 3 μM for NP).

[0104] The plates with samples are then incubated at 37°C for one hour and thereafter washed three times with

washing buffer.

[0105] The washed plates are then innoculated with 100 µl of anti µ-peroxidase conjugate (1:1000 dilution; TAGO Inc) in sample buffer, and incubated for one hour at 37°C. The plates are then washed three times in washing buffer.

[0106] The washed plates are innoculated with 100 µl of 0.1M Na acetate-citrate, pH 6.0, 0.1 mg/ml tetramethylbenzidine, 13mM H_2O_2 (peroxidase substrate) and incubated at room temperature for one hour. The reaction is terminated by the addition of 25µl of 2.5M H_2SO_4.

[0107] The microtitre plates are then read in a plate reader (Dynatech) at 450 nm with a reference of 630 nm. The 450 was related to the level of a standard protein, B1-8 anti-NIP IgM.

[0108] This assay demonstrates sensitivty to 60pg of B1-8 IgM. The extracts were prepared as soluble and insoluble material. The insoluble material was solubilized in the same buffer used in lysis but containing 8M urea followed by its dilution for assay.

[0109] In order to obtain activity for the Ig µ and λ, extracts were made of the insoluble fraction and these dialysed into buffer conditions in which disulphide interchange will occur at a higher frequency.

[0110] Production of functional antibodies from E. Coli expressing both heavy and light chains was achieved by lysing the cells and clarifying the supernatant by centrifugation. The insoluble material was washed, followed by sonication (3 times for 3 minutes), and finally dissolved in 9M urea, 50mM glycine-NaOH pH10.8, 1mM EDTA, and 20mM 2-mercaptoethanol. This extract was dialysed for 40 hours against 3 changes of 20 vols. of 100mM KCl, 50mM glycine-NaOH pH10.8, 5% glycerol, 0.05mM EDTA, 0.5mM educed glutathione and 0.1mM oxidised glutathione. The dialysate was cleared by centrifugation at 30,000g for 15 minutes and loaded directly onto DEAE Sephacel, followed by development with a 0-0.5M KCl linear gradient in 10mM Tris-HCl, 0.5mM EDTA, pH8.0.

[0111] The purified Ig µ and λ were treated as above, except that no anion exchange chromatography was carried out. The preparation was finally dialysed into phosphate buffered saline, 5% glycerol, 0.01% sodium azide and 0.5mM EDTA pH7.4.

[0112] The results from assays of material processed in this way indicated that some activity was obtained. The level of activity obtained in this way was too low to do any detailed studies on, so the resultant dialysate was purified by anion exchange chromatography (Figure 11). This process resulted in the isolation of significant NIP-cap-BSA binding activity over that of background binding to BSA (Figure 11). The assay of the fractions for the level of Ig µ, expressed as B1-8 IgM equivalents, demonstrated two peaks of activity. This was not found to correlate with full length Ig µ by Western blotting. The first peak observed may represent a fragment of Ig µ. The separation of NIP-CAP-BSA binding activity from the majority of full length Ig µ. and protein indicates that the hapten binding activity is contained within a particular molecular species formed at low efficiency.

[0113] The processing of insoluble material obtained from Ig µ expression in E. Coli produced a similar IgM protein profile but without NIP-cap-BSA binding activity. This demonstrates that the activity recovered was a property of the combined immunoglobulin expression, not of some E. Coli factor, or of the Ig µ heavy chain alone.

[0114] Further studies of the characteristics of the hapten (NIP-cap-BSA) binding were carried out. As samples were diluted they showed less binding to hapten in a very similar way to the original antibody (Figure 12). Free hapten was found to inhibit most of the binding activity in both indiluted and diluted samples. Using B1-8 antibody as a standard for both IgM and hapten binding, the specific activity of the assembled antibody was calculated to be 1.4×10^4 gm/gm of IgM equivalents. This value demonstrates the inefficient recovery of activity, but possibly represents an underestimate of the specific activity due to an overestimate of full-length Ig µ in these fractions, as described above.

Heteroclitic Nature of Recombined Antibody

[0115] Detailed specificity of binding to NIP-cap-BSA was investigated by comparing the assembled antibodies with B1-8 IgM in the presence of free NIP-cap and NP-cap (Figure 13). Both B1-8 IgM and the assembled antibodies showed that higher NP-cap than NIP-cap concentrations were required to inhibit NIP-cap-BSA binding.

[0116] The heteroclitic nature is demonstrated by the molar ratio of NIP to NP at 50% inhibition. The concentrations of NIP and NP at 50% inhibition (I50) were found to be similar for both B1-8 and the assembled antibodies as shown in Table 2. Also the NP I50/NIP I50 ratios were similar (Table 2).

Table 2.

Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA solid phase.
SD = Standard Deviation.

	NIP I50 uM	NP I50 uM	$\frac{\text{NP I50}}{\text{NIP I50}}$
B1-8 IgM	0.13 (SD, 0.05)	3.7 (SD,2.9)	29
Fraction 26	0.34 (SD, 0.09)	1.9 (SD,0.4)	6

Table 2. (continued)

Hapten concentration at 50% inhibition (I50) of binding of antibodies to NIP-cap-BSA spiked			
	NIP I50 uM	NP I50 uM	NIP I50
Fraction 27 and 28	0.11 (SD,0.02)	1.1 (SD,0.3)	10
Purified μ and λ	0.4	0.84	22

[0117] It has thus been shown that not only is it possible to express μ and λ proteins having intact variable domains, but also that it is possible to express both μ and λ proteins on compatible plasmids in the same cell. This latter has not hitherto been disclosed or even suggested. Moreover, it proved possible to derive functional Ig molecules from E. Coli cells which expressed both μ and λ proteins.

Construction of Expression Plasmids for Yeast

[0118] The plasmids used for expression in yeast (Saccharomyces cerevisiae) are based on pBR322 and the yeast 2 micron plasmid. These plasmids are the subject of copending European Patent Application EP 0 073 635, the disclosure of which is incorporated herein by reference. The yeast phosphoglycerate kinase gene (PGK) provides the 5' sequence necessary for initiation of transcription and insertion of genes can be made at the unique BglII site in the plasmid pMA 3013 described in the above mentioned European patent application. This plasmid has now been renamed pMA91 as is so referred to hereinafter.

a) met-mu (μ)

[0119] BglII and BclI produce compatible 5'-GATC ends. The met-μ gene from pNP2 was excised on a partial BclI fragment and ligated into the unique BglII site of pMA91.

b) Pre-mu (μ)

[0120] The plasmid pCT54 μ containing the full length pre-μ cDNA was digested with Hind III. This cuts at the 3' side of the μ cDNA. This Hind III site was changed to a BclI site by incubation with T_4 DNA polymerase in the presence of all 4 nucleotides. The linker R107 of sequence TTTTGATCAAAA, which contains an internal BclI was ligated to the DNA obtained above. After ligation one aliquot of the resultant DNA was digested with BclI and AccI and the μ AccI-BclI fragment was isolated from the resultant mixture by gel electrophoresis. A separate aliquot of the resultant DNA was digested with MboII and ligated with chemically synthesised oligonucleotide linkers R121 and R112 which have cohesive BclI and MboII ends.

R121 5' GATCAATGGGATGGAGCTGT 3'

R112 5' TTACCCTACCTCGAC 3'

The ligation reaction was terminated and the resultant DNA digested with AccI to give a μ MboII-AccI fragment which was isolated from the resultant mixture by gel electrophoresis on a 5% polyacrylamide gel.

[0121] The two μ fragments obtained above were ligated together and then digested with BclI to eliminate unwanted ligation products after which the resultant pre-μ DNA was ligated to BglII cut plasmid pMA91.

[0122] The resultant ligation mix was used to transform E.Coli strain HB101 to ampicillin resistance. A colony of the transformed bacteria was isolated and was found to contain a plasmid exhibiting the predicted enzyme digestion pattern. The 5' end of the inserted DNA from this plasmid was sequenced and shown to have the anticipated sequence for a pre-μ cDNA.

c) met-lambda (λ)

[0123] The met-λ DNA was cloned into the BglII site of plasmid pMA91. Plasmid pCT54 Clone 1 was cut with Hind III to the 3' side of the met-λ cDNA and this cleavage site was altered to a BclI site in a similar manner as previously

described for pNP2. Following this the plasmid DNA was cut 5' of the λ gene at the BclI site and the resultant λ DNA was cloned into the BglII site of pMA91.

d) Pre-lambda (λ)

[0124] Pre-λ cDNA was reconstructed into pMA 91 as follows. The plasmid pCT54 was digested with BclI and Hind III and the resultant vector DNA was isolated by gel electrophoresis and ligated together with two synthetic oligonucleotides, R162 and R163, and the two fragments from plasmid pATλ1-15.

R162 5' GATCAATGGCCTGGATT 3'

R163 5' GTGAAATCCAGGCCATT 3'

These fragments were produced by digesting pATλ1-15 with FokI and Hind III and isolating the 5' 300 base pair FokI fragment and the 3' 600 base pair FokI-Hind III fragment by gel electrophoresis. The resultant ligation mixture was used to transform E.Coli strain HB101 to ampicillin resistance and a colony of bacteria containing a plasmid having the predicted correct restriction enzyme digestion pattern was isolated. The 5' end of the inserted DNA was sequenced and found to have the anticipated sequence of a pre-λ cDNA. This plasmid was digested with Hind III and the resultant Hind III cleaved ends converted to a BclI site by blunting with T_4 polymerase and ligating with the linker R107. The resultant plasmid was then digested with BclI and the pre-λ cDNA isolated on a BclI fragment by gel electrophoresis. This BclI fragment was then ligated to BglI cut pMA91 to give a pMA 91 plasmid containing the full length pre-λ cDNA.

Expression of Immunogloublin Genes in Yeast

[0125] The pMA91 derivative plasmids containing the pre-λ and pre-μ genes as prepared above were used to transform Saccharomyces cerevisiae yeast host organisms and the pre-λ and pre-μ genes were expressed by the transformed cells.

[0126] Saccharomyces cerevisiae strain MD46 when transformed with pMA91 pre-λ or pMA91 pre-μ gave rise to colonies which expressed immunoreactive proteins as revealed on Western blots. Yeast cells containing plasmid pMA91 pre-λ produced a protein which reacted with anti-λ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature λ. Similarly yeast cells containing pMA 91 pre-μ produced a protein which reacted with anti-μ antiserum and co-migrated on polyacrylamide gels with bacterially synthesised mature μ protein. These observations indicate that both pre-λ and pre-μ are being processed to the corresponding mature proteins within the yeast host cell environment.

[0127] Additionally the μ protein product (but not the λ protein) was shown to be glycosylated. Cells were incubated in the presence or absence of tunicamycin at a concentration of 15 μg/ml. This compound specifically arrests the N-linked glycosylation of proteins. Cell extracts derived from cells incubated in the absence of tunicamycin showed higher molecular weight bands (as revealed by Western blotting) whilst extracts from cells incubated in the presence of tunicamycin showed no such higher molecular weight bands.

[0128] When cells were lysed with glass beads and the soluble and insoluble fractions examined it was found that the μ and λ proteins were exclusively in the insoluble fractions, as determined by Western blotting.

Secretion

[0129] After incubation transformed yeast cells were spun down and 1 ml volumes of supernatant were removed and passed through BSA-coated 0.2 μM Millipore filters to remove any remaining cellular material. ELISA assays for μ and λ protein were then carried out on the filtered supernatants. Only supernatants from pMA91 pre-λ harbouring cells showed detectable levels of immunoglobulin protein. Increased amounts of λ protein was detected in the supernatant when cells were grown in minimal medium to OD_{660} = 0.2 and then spun down and resuspended in YPAB and harvested at OD_{660} = 1.0-1.5. Thus the λ protein but not the μ protein is secreted from yeast cells.

Intracellular Location

[0130] After incubation cells containing pMA91 pre-μ were converted to spheroplasts and fixed in 5% acetic acid/

95% ethanol (v/v) and incubated with fluorescein conjugated goat anti-mouse μ. The fixed cells were examined by fluorescent microscopy and the μ protein was found to be localised in the periphery of the spheroplasts and especially in vacuoles.

Expression of both μ and λ Proteins in the Same Cells

[0131] In order to express both genes in the same host cells it was necessary to provide compatible plasmids for use in transformation. The pre-λ gene was excised from pMA91 pre-λ on a Hind III fragment and inserted into the Hind III site of plasmid pLG89 (Griti L, and Davies J, Gene, 25 179-188, 1983). This plasmid contains a ura3 marker which can be used as a positive selection for transformed host cells. A convenient ura3⁻ host organism is S. cerevisiae strain X4003-5B. Both plasmids, pMA91 pre-μ and pLG89 pre-λ , were transformed into this strain, and colonies were grown which contained together both the ura3 and leu2 markers. After incubation of the transformed cells both λ and μ proteins together were detected in the same cultures of X4003-5B using ELISA techniques. Levels of expression were comparable to those obtained for the individual genes in MD46 cells and for the λ gene alone in X4003-5B.

[0132] In addition, to check for assembly of the λ and μ proteins in vivo the following procedure was followed.

[0133] After the growth to an $OD_{660} \equiv 1$, the transformed X4003-5B cells were spun down and resuspended in buffer, either 5 mM borate buffer at pH 8.0 or phosphate buffer at pH 5.8 either with or without detergent (e.g. 0.5% Triton X). The suspended cells were then lysed by vortexing with glass beads in buffer as above and the insoluble material was spun down. After centrifugation aliquots of the supernatant were assayed in a NIP binding assay (as described previously for assembled E.Coli μ and λ proteins). Specific antigen binding activity was detected in the supernatant and this activity was shown to be specifically competed out by free NIP (the specific antigen). These results indicated that μ and λ protein were expressed within the X4003-5B cells and assembled into functional immunogloublin molecules in vivo.

[0134] In the above experiments the culture medium used for incubating the transformed cells was yeast minimal medium (containing, per litre, 6.7 g DIFCO yeast nitrogen base without amino acids, 10 g glucose, and 200 mg each of histidine, tryptophan, methionine and adenine). When the λ protein alone was being expressed the medium contained in additon 200 mg/l of leucine, and when the μ protein alone was being expressed the medium contained in addition 200 mg/l of uracil.

Claims

1. A process for producing a heterologous Ig molecule or an immunologically functional Ig fragment in a single yeast host cell, which comprises transforming the host cell with separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains and expressing each of said polypeptide chains separately in said transformed single yeast host cell.

2. The process of claim 1, wherein each DNA sequence is inserted individually into a vector and the individual vectors are used to transform the single host cell.

3. The process of claim 1, wherein the two DNA sequences are inserted into separate parts of a vector which is used to transform the single host cell.

4. The process of claim 2 or claim 3, wherein the or each vector is a plasmid.

5. The process of any one of claims 1 to 4, wherein the host cell is S. Cerevisiae.

6. The process of any one of claims 1 to 5, wherein the polypeptide chains are secreted by the host cell.

7. The process of any one of claims 1 to 6, for producing an Ig molecule or fragment having at least one constant domain, wherein the or each constant domain is derived from the same source as the variable domain to which it is attached.

8. The process of any one of claims 1 to 6, for producing an Ig molecule or fragment having at least one constant domain, wherein the or each constant domain is derived from source different from that from which the variable domain to which it is attached is derived.

9. The process of any one of claims 1 to 8, wherein the DNA sequences code for complete Ig heavy and light chains.

10. The process of any one of claims 1 to 8, wherein the DNA sequences code for only one constant domain.

11. The process of any one of claims 1 to 10, wherein at least one of the DNA sequences encodes at least the variable region of an Ig heavy or light chain and also a cytotoxic, enzymic or structural polypeptide moiety whereby the host cell is transformed so as to produce an Ig molecule or fragment having attached thereto said cytotoxic, enzymic or structural polypeptide moiety.

12. The process of any one of claims 1 to 11, wherein the DNA coding sequences for the Ig molecule or fragment are derived from one or more monoclonal antibody-producing hybridomas.

13. A vector for use in the process of any one of claims 1 to 12, comprising separate DNA sequences respectively encoding polypeptide chains comprising at least the variable domains of the Ig heavy and light chains.

14. A vector according to claim 13, which is a plasmid.

15. A yeast host cell transformed with the vector of claim 13 or claim 14.

16. A yeast host cell transformed with two separate vectors, the first vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig heavy chain, and the second vector comprising a DNA sequence encoding a polypeptide chain comprising at least the variable domain of the Ig light chain.

Patentansprüche

1. Verfahren zur Herstellung eines heterologen Ig-Moleküls oder eines immunologisch funktionellen Ig-Fragments in einer einzelnen Hefewirtszelle, welches das Transformieren der Wirtszelle mit getrennten DNA-Sequenzen mit entsprechend kodierenden Polypeptidketten, umfassend mindestens die variablen Domänen der schweren und leichten Ig-Ketten, und die getrennte Expression jeder der Polypeptidketten in der transformierten einzelnen Hefewirtszelle umfaßt.

2. Verfahren nach Anspruch 1, wobei jede DNA-Sequenz individuell in einen Vektor insertiert wird und der individuelle Vektor zur Transformation der einzelnen Wirtszelle verwendet wird.

3. Verfahren nach Anspruch 1, wobei die zwei DNA-Sequenzen in getrennte Abschnitte eines Vektors insertiert werden, welcher zur Transformation der einzelnen Wirtszelle verwendet wird.

4. Verfahren nach Anspruch 2 oder Anspruch 3, wobei der oder jeder Vektor ein Plasmid ist.

5. Verfahren nach einem der Ansprüche 1 bis 4, wobei die Wirtszelle S.cerevisiae ist.

6. Verfahren nach einem der Ansprüche 1 bis 5, wobei die Polypeptidketten von der Wirtszelle sekretiert werden.

7. Verfahren nach einem der Ansprüche 1 bis 6 zur Herstellung eines Ig-Moleküls oder -Fragments mit mindestens einer konstanten Domäne, wobei die oder jede konstante Domäne aus der gleichen Quelle wie die variable Domäne, an die sie gebunden ist, stammt.

8. Verfahren nach einem der Ansprüche 1 bis 6 zur Herstellung eines Ig-Moleküls oder -Fragments mit mindestens einer konstanten Domäne, wobei die oder jede konstante Domäne aus einer Quelle stammt, welche unterschiedlich von der ist, aus welcher die variable Domäne, an die sie gebunden ist, stammt.

9. Verfahren nach einem der Ansprüche 1 bis 8, wobei die DNA-Sequenzen vollständige schwere und leichte Ig-Ketten kodieren.

10. Verfahren nach einem der Ansprüche 1 bis 8, wobei die DNA-Sequenzen nur eine konstante Domäne kodieren.

11. Verfahren nach einem der Ansprüche 1 bis 10, wobei mindestens eine der DNA-Sequenzen mindestens die variable Region einer schweren oder leichten Ig-Kette und auch einen cytotoxischen, enzymatischen oder strukturellen Polypeptidteil kodiert, wodurch die Wirtszelle transformiert wird, um ein Ig-Molekül oder -Fragment herzu-

stellen, welches daran gebunden den cytotoxischen, enzymatischen oder strukturellen Polypeptidanteil aufweist.

12. Verfahren nach einem der Ansprüche 1 bis 11, wobei die DNA-kodierenden Sequenzen für das Ig-Molekül oder -Fragment aus einem oder mehreren monoklonalen Antikörper-erzeugenden Hybridomas stammen.

13. Vektor zur Verwendung in einem Verfahren aus einem der Ansprüche 1 bis 12, umfassend getrennte DNA-Sequenzen mit entsprechend kodierenden Polypeptidketten, umfassend mindestens die variablen Domänen der schweren und leichten Ig-Ketten.

14. Vektor nach Anspruch 13, welcher ein Plasmid ist.

15. Hefewirtszelle, transformiert mit dem Vektor nach Anspruch 13 oder Anspruch 14.

16. Hefewirtszelle, transformiert mit zwei getrennten Vektoren, wobei der erste Vektor eine DNA-Sequenz umfaßt, welche eine Polypeptidkette kodiert, welche mindestens die variable Domäne der schweren Ig-Kette umfaßt, und der zweite Vektor eine DNA-Sequenz umfaßt, welche eine Polypeptidkette kodiert, welche mindestens die variable Domäne der leichten Ig-Kette umfaßt.

Revendications

1. Procédé pour la production d'une molécule d'Ig hétérologue ou d'un fragment d'Ig immunologiquement fonctionnel dans une cellule hôte unique de levure, qui comprend la transformation de la cellule hôte avec des séquences d'ADN séparées codant respectivement pour des chaînes polypeptidiques comprenant au moins les domaines variables des chaînes lourdes et légères d'Ig et exprimant chacune desdites chaînes polypeptidiques séparément dans ladite cellule hôte unique transformée de levure.

2. Procédé selon la revendication 1, dans lequel chaque séquence d'ADN est insérée individuellement dans un vecteur, et les vecteurs individuels sont utilisés pour transformer la cellule hôte unique.

3. Procédé selon la revendication 1, dans lequel les deux séquences d'ADN sont insérées dans des parties séparées d'un vecteur qui est utilisé pour transformer la cellule hôte unique.

4. Procédé selon la revendication 2 ou 3, dans lequel le ou chaque vecteur est un plasmide.

5. Procédé selon l'une quelconque des revendications 1 à 4, dans lequel la cellule hôte est S. cerevisiae.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel les chaînes polypeptidiques sont sécrétées par la cellule hôte.

7. Procédé selon l'une quelconque des revendications 1 à 6, pour la production d'une molécule ou d'un fragment d'Ig ayant au moins un domaine constant, où le ou chaque domaine constant dérive de la même source que le domaine variable auquel il est fixé.

8. Procédé selon l'une quelconque des revendications 1 à 6, pour la production d'une molécule ou d'un fragment d'Ig ayant au moins un domaine constant, où le ou chaque domaine constant dérive d'une source différente de celle dont dérive le domaine variable auquel il est fixé.

9. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel les séquences d'ADN codent pour des chaînes lourdes et légères d'Ig complètes.

10. Procédé selon l'une quelconque des revendications 1 à 8, dans lequel les séquences d'ADN codent pour seulement un domaine constant.

11. Procédé selon l'une quelconque des revendications 1 à 10, dans lequel au moins l'une des séquences d'ADN code pour au moins la région variable d'une chaîne lourde ou légère d'Ig et également une unité polypeptidique cytotoxique, enzymatique ou structurale, la cellule hôte étant ainsi transformée de façon à produire une molécule ou un fragment d'Ig auquel est fixée ladite unité polypeptidique cytotoxique, enzymatique ou structurale.

12. Procédé selon l'une quelconque des revendications 1 à 11, dans lequel les séquences codantes d'ADN pour la molécule ou le fragment d'Ig dérivent d'un ou plusieurs hybridomes produisant des anticorps monoclonaux.

13. Vecteur pour utilisation dans le procédé selon l'une quelconque des revendications 1 à 12, comprenant des séquences d'ADN séparées codant respectivement pour des chaînes polypeptidiques comprenant au moins les domaines variables des chaînes lourdes et légères d'Ig.

14. Vecteur selon la revendication 13, qui est un plasmide.

15. Cellule hôte de levure transformée avec le vecteur selon la revendication 13 ou 14.

16. Cellule hôte de levure transformée par deux vecteurs séparés, le premier vecteur comprenant une séquence d'ADN codant pour une chaîne polypeptidique comprenant au moins le domaine variable de la chaîne lourde d'Ig, et le deuxième vecteur comprenant une séquence d'ADN codant pour une chaîne polypeptidique comprenant au moins le domaine variable de la chaîne légère d'Ig.

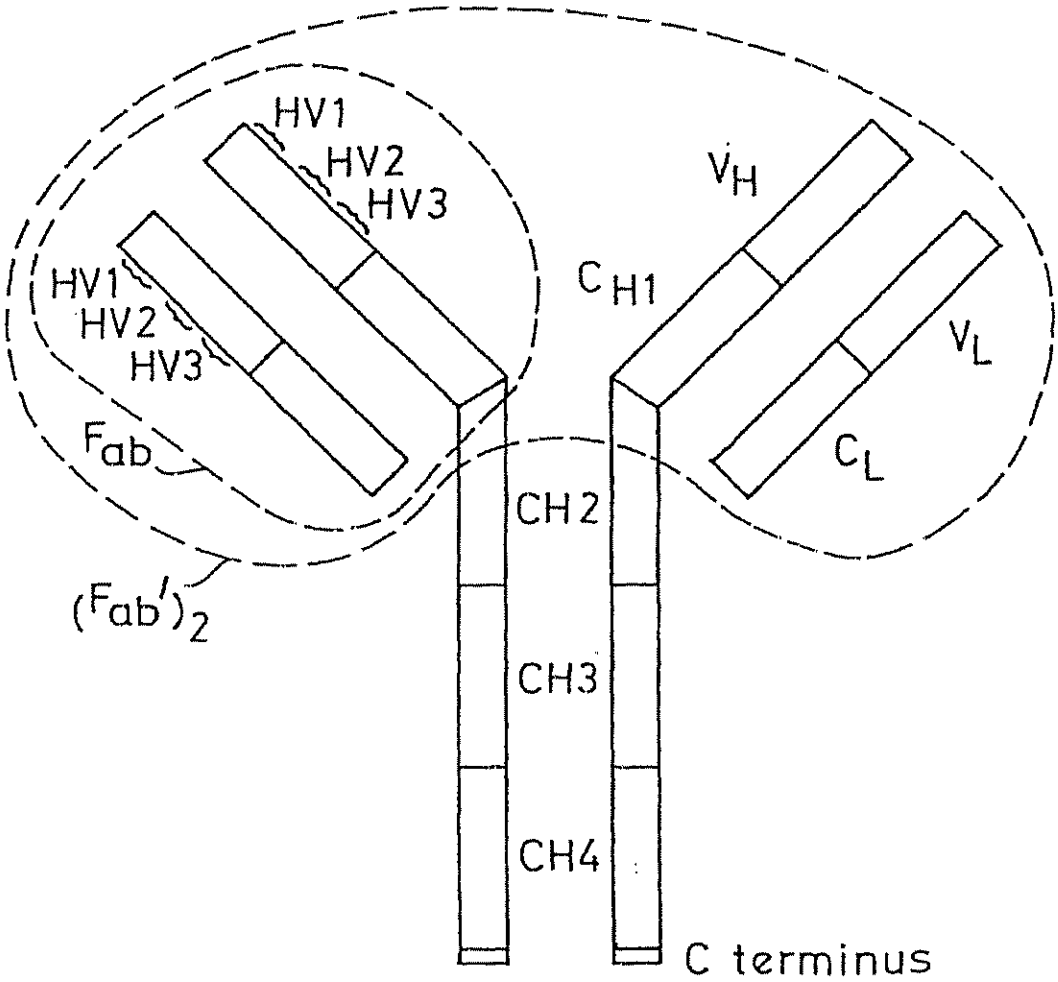
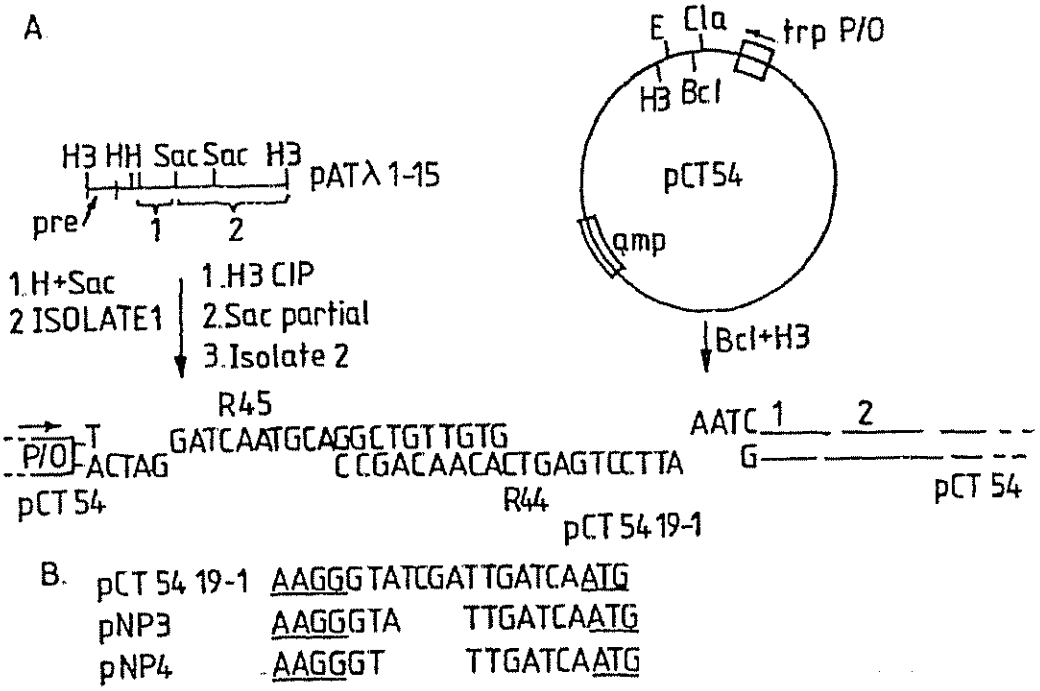

HV1
HV2
HV3
HV1
HV2
HV3
Fab
(Fab')2
VH
CH1
VL
CL
CH2
CH3
CH4
C terminus

FIG. 1



B.

pCT 54 19-1	AAGGGTATCGATTGATCAATG	
pNP3	AAGGGTA	TTGATCAATG
pNP4	AAGGGT	TTGATCAATG

Legend. E = E corRI

H = HinfI

H3= Hind III

FIG. 2

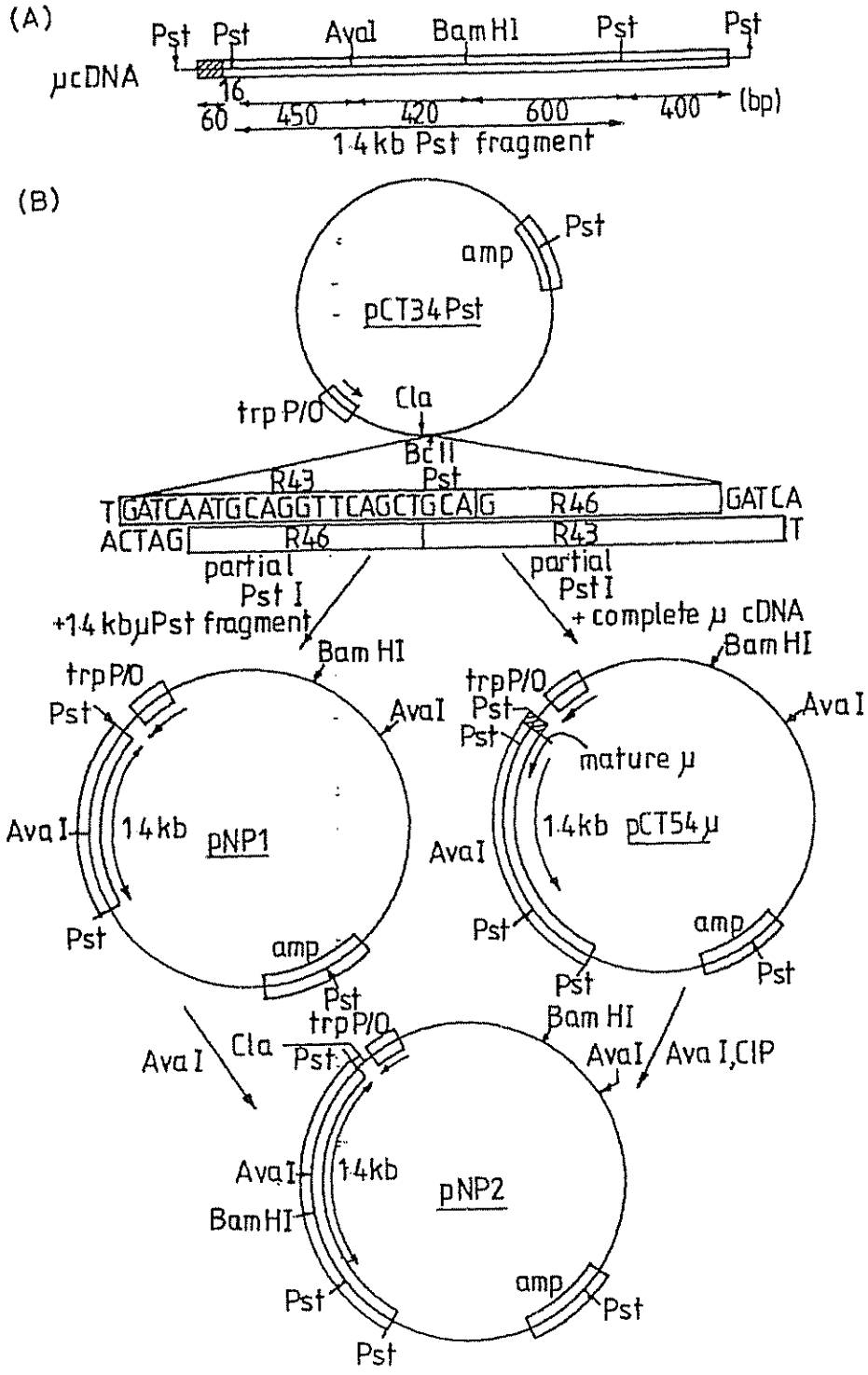

(A)
Pst Pst AvaI BamHI Pst Pst
μcDNA
16
60 450 420 600 400 (bp)
1.4kb Pst fragment
(B)
amp
Pst
pCT34Pst
trp P/O
Cla
BclI
Pst
R43
T GATCAATGCAGGTTCAGCTGCA G R46 GATCA
ACTAG R46 R43 T
partial
Pst I
+1.4kbμPst fragment
partial
Pst I
+ complete μ cDNA
Bam HI
trpP/O
Pst
AvaI
AvaI
14kb
pNP1
Pst
amp
Pst
Bam HI
trpP/O
Pst
Pst
AvaI
mature μ
1.4kb
pCT54μ
AvaI
Pst
amp
Pst
Pst
AvaI
Cla
trpP/O
Pst
Bam HI
AvaI
AvaI,ClP
AvaI
14kb
pNP2
BamHI
Pst
amp
Pst
Pst

FIG. 3

Possible 2° structures of μ mRNAs

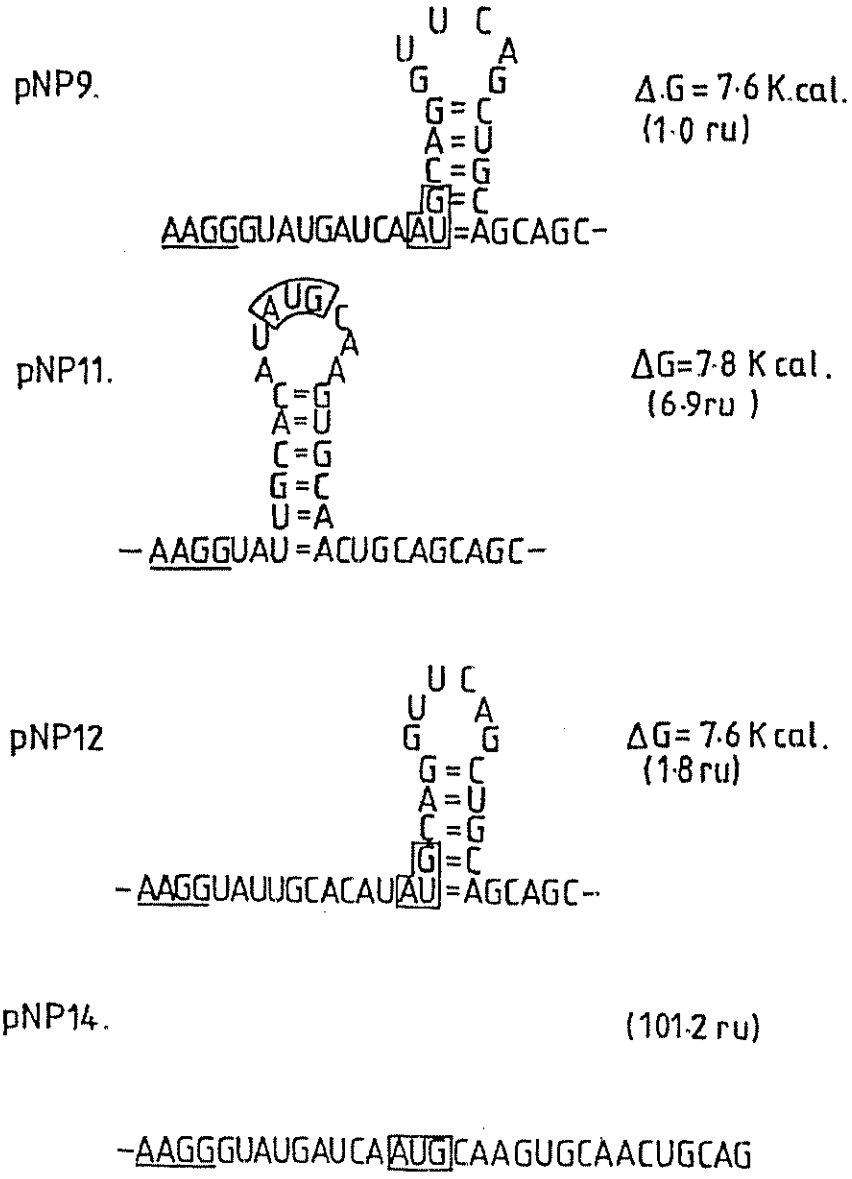


FIG. 4

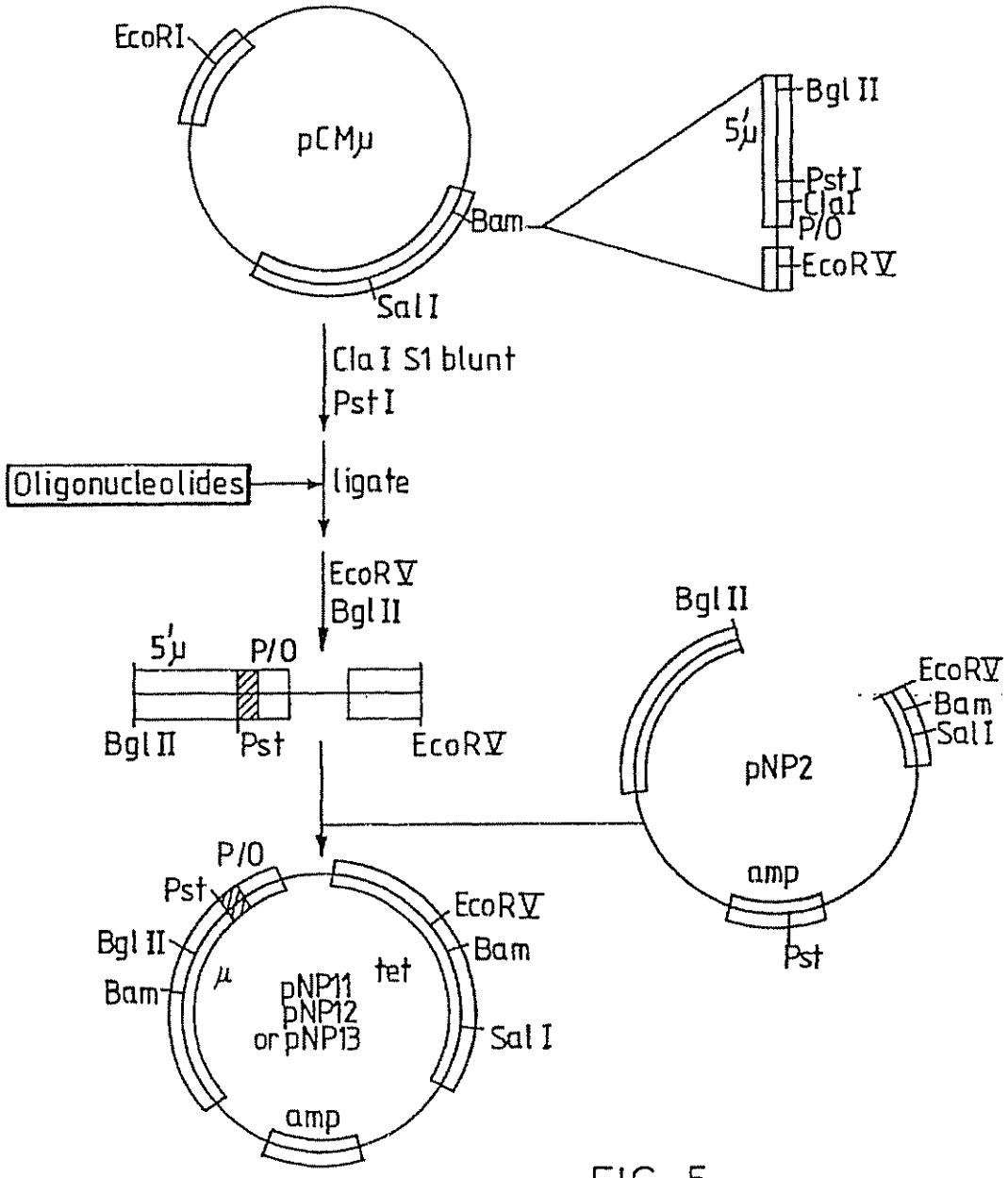

EcoRI
pCMμ
Bam
Sal I
5'μ
Bgl II
Pst I
Cla I
P/O
EcoR V
Cla I S1 blunt
Pst I
Oligonucleolides
ligate
EcoR V
Bgl II
5'μ
P/O
Bgl II
Pst
EcoR V
Bgl II
EcoR V
Bam
Sal I
pNP2
amp
Pst
P/O
Pst
Bgl II
Bam
μ
tet
pNP11
pNP12
or pNP13
EcoR V
Bam
Sal I
amp

FIG. 5



FIG. 6

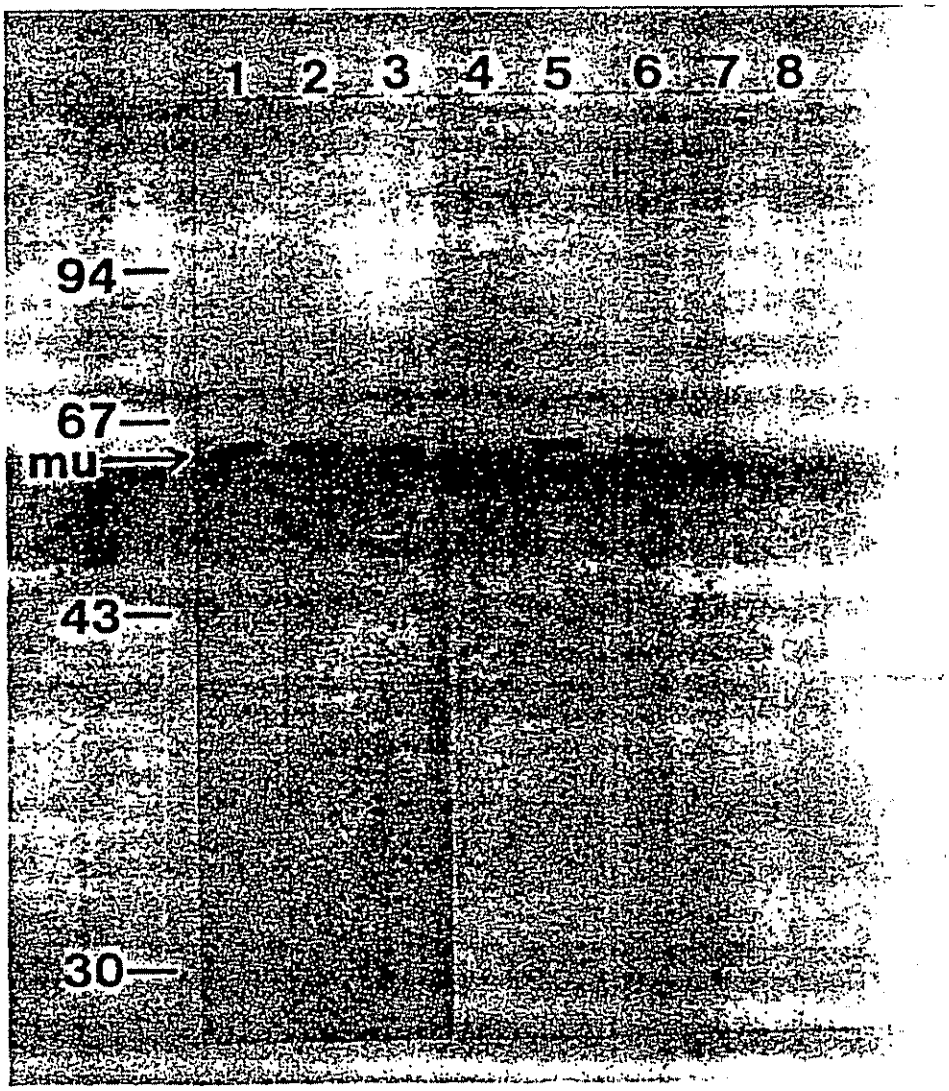


FIG. 7

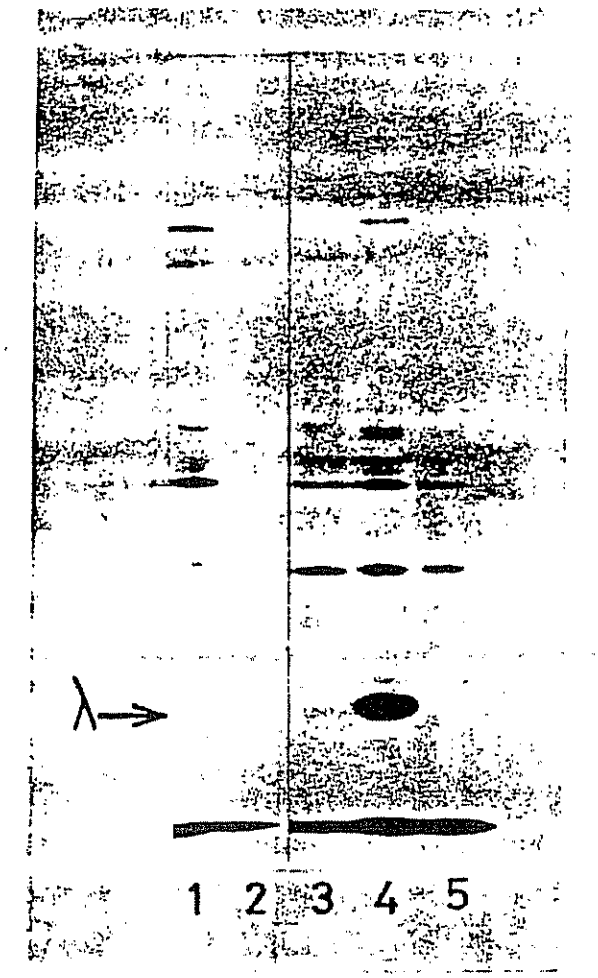


FIG. 8

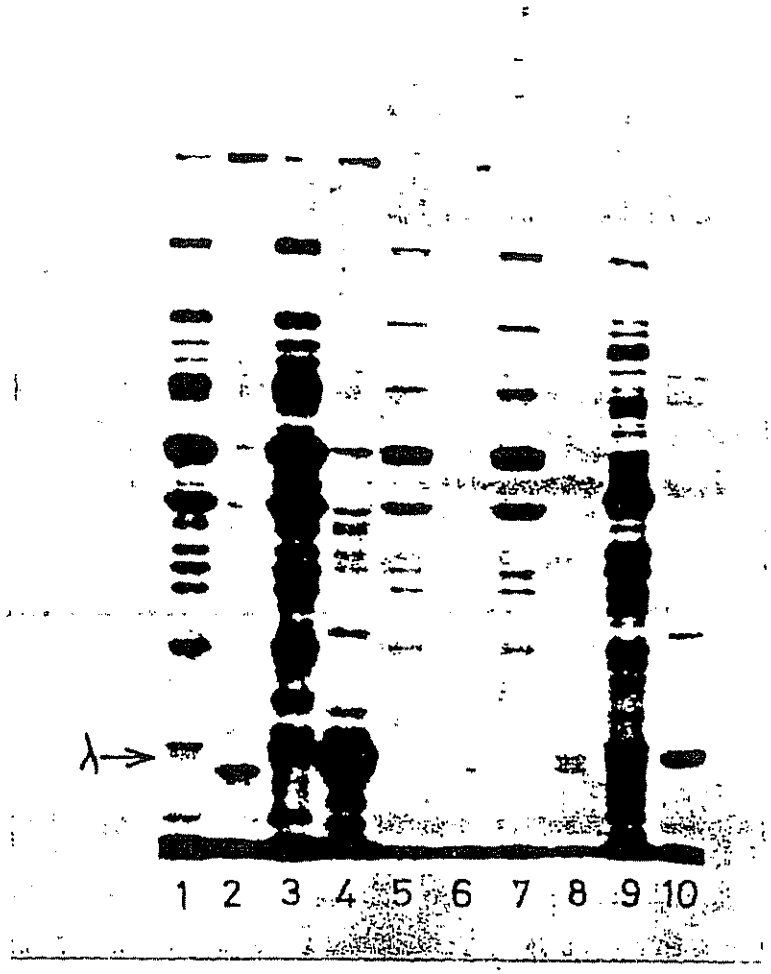


FIG. 9

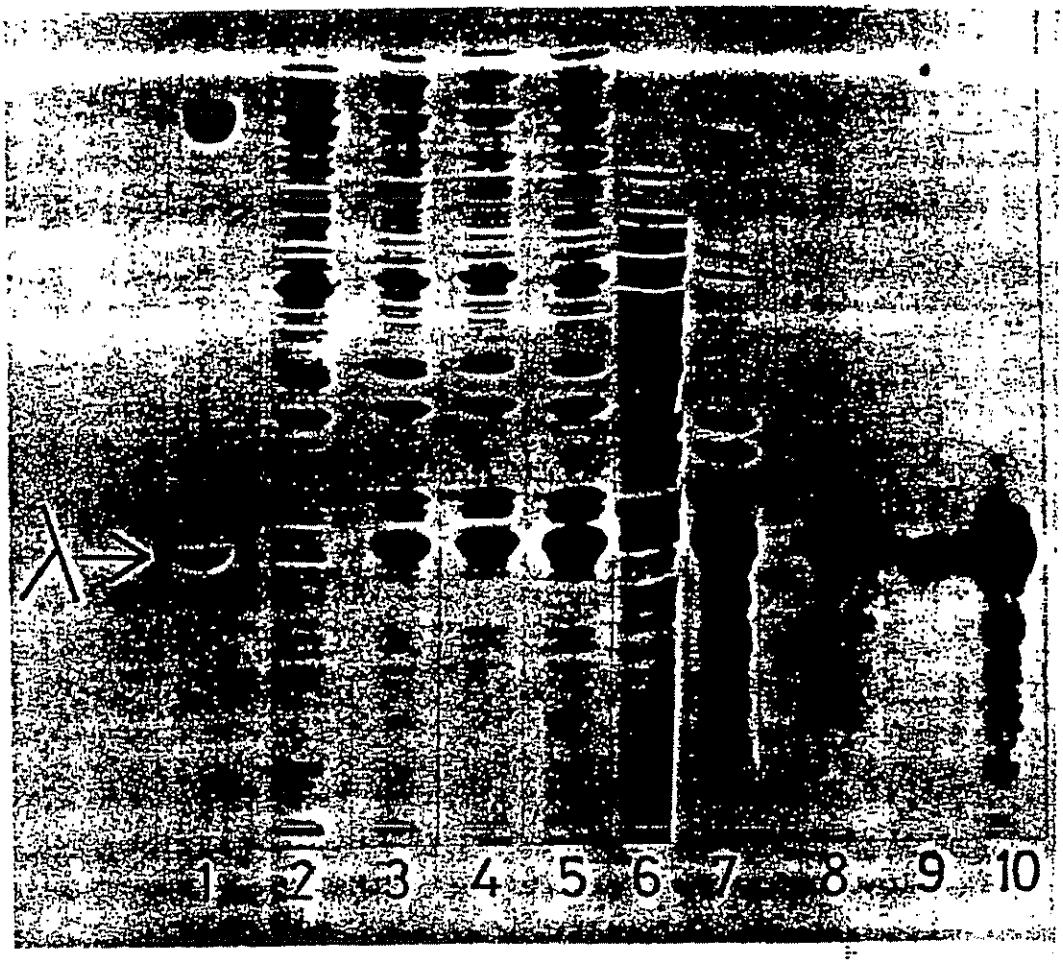


FIG. 10

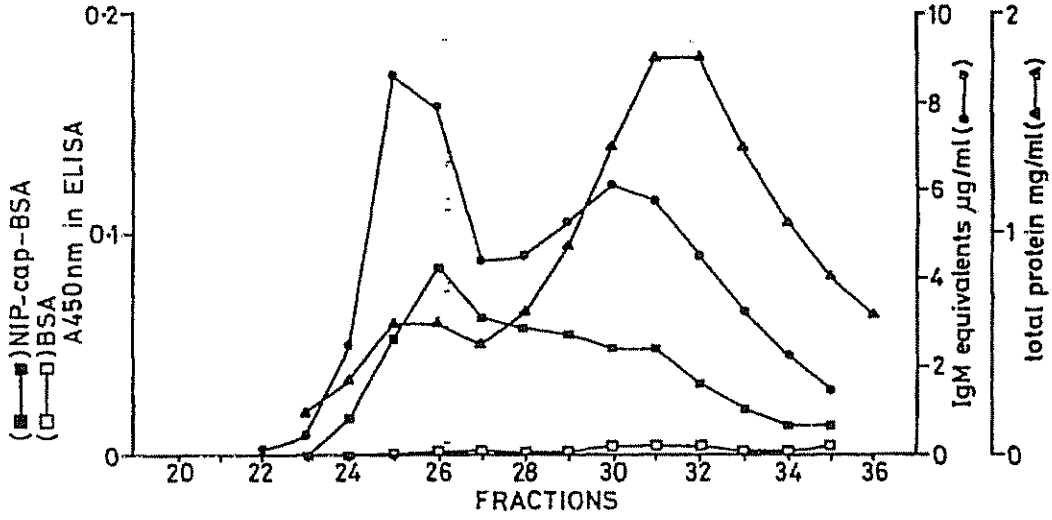

(■—■)NIP-cap-BSA
(□—□)BSA
A450nm in ELISA
0·2
0·1
0
20
22
24
26
28
30
32
34
36
FRACTIONS
10
8
6
4
2
0
IgM equivalents µg/ml (●—●)
2
1
0
total protein mg/ml (▲—▲)

FIG. 11

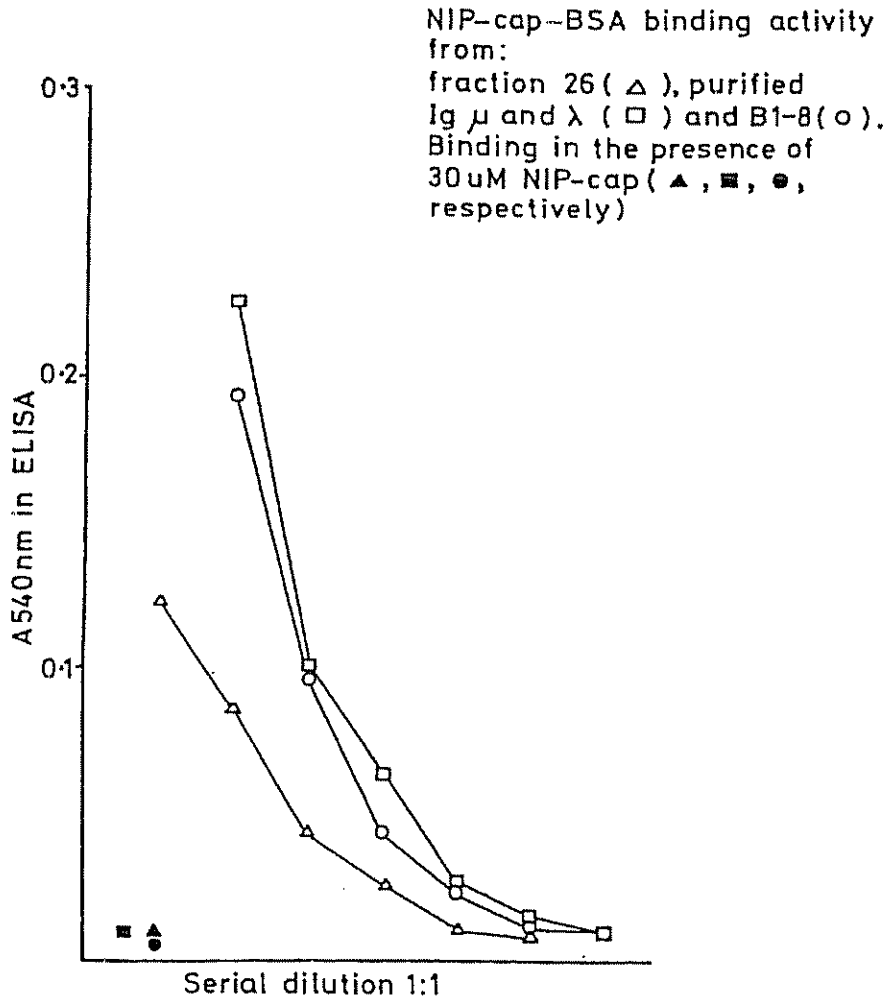
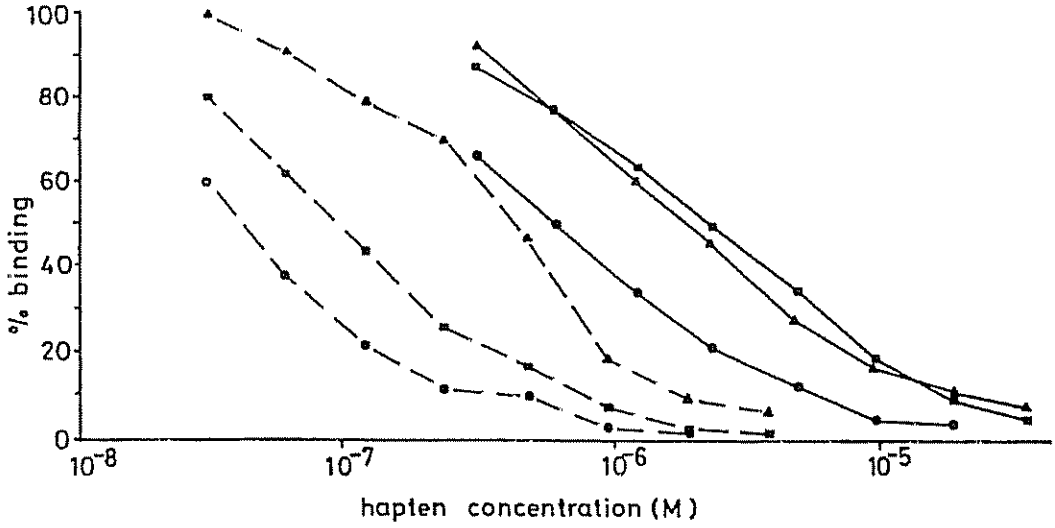

NIP-cap-BSA binding activity from:
fraction 26 (△), purified Ig μ and λ (□) and B1-8 (o).
Binding in the presence of 30 uM NIP-cap (▲ , ■ , ● , respectively)
0·3
0·2
0·1
A540nm in ELISA
Serial dilution 1:1

FIG. 12



Binding of antibodies to NIP-cap BSA B1-8 IgM (■), fraction 26 (▲), purified Ig μ or λ (●), in the presence of free NIP-cap(----) or NIP-cap (——).

FIG. 13

EXHIBIT 26

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AUG 10 1990

PATENT

I hereby certify that on the date specified below this correspondence is being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, D.C. 20231.

July 2, 1990
Date

David J. Maki

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants	: Andrzej Z. Sledziewski et al.
Serial No.	: 07/347,291
Filed	: May 2, 1989
For	: METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE PEPTIDE DIMERS
Examiner	: M. Marks
Art Unit	: 185
Docket No.	: 990008.446C2
Date	: June 29, 1990

Commissioner of Patents and Trademarks
Washington, D.C. 20231

AMENDMENT

Sir:

In response to the Office Action dated March 2, 1990, please extend the period of time for response one month, to expire on July 2, 1990. Enclosed are a Request for Extension of Time and the requisite fee.

In response to the Office Action dated March 2, 1990, please amend the above-identified patent application as follows:

In the Specification:

On page 17, line 11, please change "Claesson-Walsh" to "Claesson-Welsh".

On page 17, line 24, please change "prediceted" to "predicted".

On page 17, line 35, please delete the second occurrence of "followed".

On page 18, line 18, please change "receptorsm" to "receptors,".

On page 19, line 12, please change "seuqences" to "sequences".

On page 20, line 33, after "99-105", please insert -- 1985 --.

N/E On page 22, line 20, please change "or" to "of".

On page 23, line 12, please delete ".".

On page 24, line 35, please change "9424,494" to "946,873".

On page 26, line 26, please delete ".".

N/E On page 35, line 13, please change "kgt11" to "λgt11".

On page 64, line 23, please change "µl10%" to "µl 10%"

On page 65, line 6, please delete "(10)".

On page 67, line 34, please change "is" to "was".

On page 74, line 34, please change "11" to "µl".

In the Claims:

Please amend claim 22 as follows:

22. (Amended) A method for producing a secreted, biologically active, dimerized polypeptide fusion [peptide dimer], comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a first polypeptide chain of a non-immunoglobulin polypeptide [peptide] dimer joined to a DNA sequence encoding an immunoglobulin light chain constant region;

introducing into said host cell a second DNA construct comprising a transcriptional promoter operatively linked to a second secretory signal sequence followed

downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of a non-immunoglobulin polypeptide [peptide] dimer joined to a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3 and C_H4;

growing said host cell in an appropriate growth medium under physiological conditions to allow the [dimerization and] secretion of a dimerized polypeptide fusion comprising a first polypeptide chain of a non-immunoglobulin polypeptide dimer joined to an immunoglobulin light chain constant region and a second polypeptide chain of a non-immunoglobulin polypeptide dimer joined to at least one immunoglobulin heavy chain constant region domain [peptide dimer]; and

isolating said biologically active, dimerized polypeptide fusion [peptide dimer] from said host cell.

REMARKS

Reconsideration of the application in view of the above amendments and the following remarks is requested. Claims 22 is in this case. Claim 22 has been amended.

Applicants have previously claimed priority from European Application Serial No. 89100787.4 through a communication filed September 18, 1989. Within this communication, an appropriate declaration referring to the European application was enclosed.

Claim 22 has been rejected under 35 U.S.C. § 112, first paragraph. The Examiner does not believe that the specification teaches how to grow any host cell in an appropriate growth medium under physiological conditions to allow dimerization and secretion of a biologically active peptide dimer.

Applicants respectfully traverse this ground of rejection. Applicants have amended claim 22 to recite "growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of said

biologically active, dimerized polypeptide fusion comprising a first polypeptide chain of a non-immunoglobulin polypeptide dimer joined to an immunoglobulin light chain constant region and a second polypeptide chain of a non-immunoglobulin polypeptide dimer joined to at least one immunoglobulin heavy chain constant region domain". Applicants submit that this amendment has been made to more particularly point out and distinctly claim certain aspects of applicants' invention. This amendment is fully supported, for example, on pages 28-29 of the specification. Applicants believe that the claim, as amended, more clearly points out that the dimerization of the polypeptide fusions occurs as the polypeptide progresses through the secretory pathway within the host cell and that biologically active, dimerized polypeptide fusions are secreted into the medium. Thus, the term "physiological conditions" means those conditions within the host cell. Pages 20 and 23-28 of the specification teach suitable host cells, which include mammalian and fungal cells, for use in the present invention. Suitable vectors, selectable markers, promoters, terminators, and secretory signals for use in the expression of the claimed dimerized polypeptide fusions in fungal and mammalian cells are also disclosed. The specification teaches methods for introducing DNA constructs capable of directing the expression of the claimed dimerized polypeptide fusions into host cells, for example, at pages 25 and 27 of the specification. Applicants submit that the specification provides sufficient teaching for one of ordinary skill in the art to grow any host cell in appropriate growth medium under physiological conditions to allow the secretion of a biologically active, dimerized polypeptide fusion. Pages 28 and 29 of the specification, for example, teach nutrients required in appropriate growth medium for mammalian and fungal host cells of the present invention and the general requirements for a growth medium for yeast, a representative host cell. Mammalian cells require a medium that emulates serum and should be grown under conditions wherein the temperature is maintained at approximately 37°C and the pH and

the level of CO_2 are constantly maintained. It would be evident to one of ordinary skill in the art, given the guidance in the specification, to procure an appropriate mammalian cell growth medium from a commercial source or to prepare such a medium given the formulations supplied by the manufacturers or supplied by, for example, the American Type Culture Collection (Rockville, Md.). Applicants submit that the appropriate growth medium and physiological conditions suitable for the growth of fungal cells is well known in the art. Pontecorvo et al. (Adv. Genet. 5:141-238, 1953; a copy of which is enclosed) describe media and conditions for the growth of Aspergillus spp. Applicants submit that on the basis of the above amendment and remarks, the Examiner's rejection under Section 112 has been obviated.

Claim 22 was also rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner believes that it is unclear what is intended by the recitation of "peptide dimer."

Applicants respectfully traverse this ground of rejection. Applicants have amended claim 22 to more particularly point out and distinctly claim certain aspects of applicants' invention. This amendment is fully supported, for example, on page 21 of the specification. Applicants submit that "polypeptide dimer" refers to polypeptide dimers requiring dimerization for biological activity or enhancement of biological activity as recited, for example, on page 18 of the specification. Examples of such dimers are listed therein. A polypeptide is defined as "a peptide formed by the union of an indefinite (usually large) number of amino acids" (Illustrated Stedman's Medical Dictionary, 24th ed., Williams & Wilkins, Baltimore, Md., 1982). Applicants submit that "non-immunoglobulin polypeptide dimers" extend in size to that of immunoglobulin chains and are limited only by the requirement that such polypeptides require dimerization for biological activity. Applicants respectfully submit that on the basis of

the above remarks, the Examiner's rejection under Section 112, second paragraph, has been obviated.

Claim 22 was also rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as the invention. The Examiner believes that claim 22 is indefinite in the recitation "growing said host cell in an appropriate growth medium under physiological conditions to allow the dimerization and secretion of said biologically active peptide dimer."

Applicants respectfully traverse this ground of rejection. Applicants have amended claim 22 to recite "growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of said biologically active polypeptide dimer." Applicants submit that such an amendment more particularly points out and distinctly claims certain aspects of applicants' invention. As noted previously, the term "physiological conditions" refers to those conditions required for the growth of the host cell. As discussed above, applicants submit that the dimerization of the polypeptide fusions of the present invention is carried out during passage of the polypeptide through the secretion pathway of the host cell, thus the conditions for dimerization are determined by the host cell. The specification, on pages 28 and 29, for example, provides ample guidance for one of ordinary skill in the art to grow host cells. As discussed above, it would be evident to one skilled in the art to purchase an appropriate growth medium for a particular mammalian cell line from a commercial source or to prepare the medium using the formulations supplied by the commercial manufacturers or using formulations supplied by, for example, the American Type Culture Collection. As noted previously, the term "physiological conditions" refer to those conditions required for the growth of the host cell. Applicants submit that the dimerization of the polypeptide fusions of the present invention is carried out during passage of the polypeptide through the secretion pathway of the host cell. Thus, the

conditions for dimerization are determined by the host cell. Applicants respectfully submit that on the basis of the above amendment and remarks, the Exmaminer's rejection under Section 112, second paragraph, has been obviated.

Claim 22 was rejected under 35 U.S.C. § 102(e) as being anticipated by Boss et al. (U.S. Patent No. 4,816,397) and Boss et al. (EP 120,694).

Applicants respectfully traverse this ground of rejection. Boss et al. do not teach or suggest the dimerized polypeptide fusions or methods of producing such fusions as claimed in the subject application. The claimed dimerized polypeptide fusions are non-immunoglobulin polypeptides fused to at least an immunoglobulin constant region wherein the dimerization is driven by the immunoglobulin portion of the molecule. Boss et al. do not teach or suggest the use of the immunoglobulin constant region in the dimerization of non-immunoglobulin polypeptides. Applicants respectfully submit that on the basis of the above remarks, the Examiner's rejection under 35 U.S.C. § 102(e) has been overcome.

On the basis of the above amendments and remarks, reconsideration of the subject application and its allowance are requested.

Respectfully submitted
Andrzej Z. Sledziewski et al.
SEED and BERRY

David J. Maki
Registration No. 31,392

DJM:ag

Enclosures:
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WDAM16/8719C2P1/V2

EXHIBIT 27

EXHIBIT 27
REDACTED IN ITS ENTIRETY

EXHIBIT 28

EXHIBIT 28
REDACTED IN ITS ENTIRETY

EXHIBIT 29

EXHIBIT 29
REDACTED IN ITS ENTIRETY

EXHIBIT 30

Research ... Immunology 146 (9) 130-140 1995

CD28/CTLA-4 receptor structure, binding stoichiometry and aggregation during T-cell activation

P.S. Linsley, J. Ledbetter, R. Peach and I. Bajorath

Bristol-Myers Squibb Pharmaceutical Research Institute, 3005 First Avenue, Seattle, WA 98121 (USA)

Introduction

The interactions of T lymphocytes with antigen-presenting cells (APC) initiates T-cell activation required for immunity to foreign pathogens, transplanted organs, or to self tissue during autoimmune disease. Activation of T lymphocytes by APC interaction can be explained by a two-signal model (Schwartz, 1992). The specificity (signal one) of a T-cell immune response is generated by recognition of antigenic peptide/MHC complexes by T-cell antigen receptors (TCR). Improper TCR engagement (Sloan-Lancaster *et al.*, 1994), or engagement without additional T cell/APC interactions leads to T-cell anergy rather than activation. A successful immune response thus requires additional costimulatory interactions (second signals) between the T cell and the APC.

Work from many laboratories has established that a key T-cell costimulatory signal is provided by the interaction of CD28 and CTLA-4 receptors on T cells with B7 counterreceptors on APC (Linsley *et al.*, 1993). Two B7 molecules are currently known, B7-1 (CD80) and B7-2 (CD86) (June *et al.*, 1994). CTLA4Ig, a soluble form of CTLA-4 which binds B7 molecules with high avidity, blocks T-lymphocyte activation *in vitro* (Linsley *et al.*, 1991), *in vivo* immune responses to model antigens (Linsley *et al.*, 1992b), organ graft rejection (Pearson *et al.*, 1994; Turka *et al.*, 1992) and development of spontaneous lupus-like autoimmune disease (Finck *et al.*, 1994). These findings demonstrate the importance of CD28/CTLA-4:B7 costimulatory signals.

The initiation of CD28/CTLA-4:B7 signals requires interactions of the extracellular domains of these molecules, but little is known of these interactions at the molecular level of detail, or of how they lead to generation of costimulatory signals. The extracellular regions of each of these molecules share some sequence similarity with other members of the immunoglobulin superfamily (IgSF). However, these molecules and their interactions have unique characteristics, which determine the generation of costimulatory signals. In this chapter, we will focus on interactions of the extracellular domains of CD28, CTLA-4, and B7 molecules. We summarize current knowledge of their structures, and their interactions, in relation to other IgSF members; and discuss implications of these interactions for signal transduction.

Structure and binding properties of IgSF receptors

Many cell surface receptor molecules and their ligands are members of the IgSF (Williams *et al.*, 1988). These molecules typically contain one or more immunoglobulin (Ig)-like domains in their extracellular regions. Ig domains may display only limited sequence similarity, often reduced to the presence of a few conserved residues characteristic of the Ig fold (Williams *et al.*, 1988; Harpaz *et al.*, 1994). Single Ig domains consist of two closely packed antiparallel β-sheets, and their β-strands are connected by loops following conserved topology (Williams *et al.*, 1988). The two β-sheets in variable (V-) domains include four (A, B, E, D,) and five (G, F, C, C' C") β-strands, respectively. The β-sheets in constant (C-) domains usually include four (A, B, E, D) and three (G, F, C) strands, respectively. The two β-sheet faces and three or four loops at each end of the sheets are exposed to solvent and thus provide a structural scaffold for a variety of intermolecular interactions. Despite sharing a common structural scaffold, members of the IgSF show distinct structural properties, and engage in intermolecular interactions in many different ways.

Three-dimensional (3D) structures of many IgSF molecules have been determined by X-ray crystallography and NMR techniques. Antibodies were the first IgSF members whose 3D structures were elucidated (Padlan, 1994; Davies *et al.*, 1993; Alzari *et al.*, 1988). Structures determined for non-antibody cell surface molecules which contain Ig domains include: MHC class I (Bjorkman *et al.*, 1987) and II (Stern *et al.*, 1994) molecules; CD2 (Driscoll *et al.*, 1991; Jones *et al.*, 1992); CD4 (Wang *et al.*, 1990; Ryu *et al.*, 1990; Brady *et al.*, 1993); CD8 (Leahy *et al.*, 1992); human growth hormone receptor (GHR) (De Vos *et al.*, 1992); prolactin receptor (Somers *et*

al., 1994); tissue factor (Harlos *et al.*, 1994); E-cadherin (Overduin *et al.*, 1995); and VCAM-1 (Jones *et al.*, 1995).

Despite the number of IgSF structures known, only a limited number of structures for receptor/ligand complexes have been directly determined. At present, besides antibody/antigen complexes, only structures of the human growth hormone (hGH)/GHR (De Vos *et al.*, 1992) and the homologous hGH/prolactin receptor (Somers *et al.*, 1994) are available. Thus, for most non-antibody IgSF molecules, it is not (yet) possible to "see" their binding sites and to understand the stoichiometry of their interactions directly. Instead, receptor-ligand interactions have been examined indirectly by mutagenesis and binding studies (e.g., CD2 (Arulanandam *et al.*, 1993), CD4 (Mœbius *et al.*, 1991), and CD8 (Giblin *et al.*, 1994)). Examples of the variety of binding stoichiometries of IgSF receptor/ligand complexes are schematically shown in figure 1.

Antibodies (or immunoglobulin, Ig) represent the paradigm of molecular recognition by IgSF molecules. Heavy and light chains, each consisting of a V-domain followed by three or one C-domains, respectively, form a well-defined heterodimer. The V-domain dimer (Fv) contains the antigen-binding site which is formed by a combination of six complementarity-determining (CDR) loops, three of which are contributed by each domain. On the basis of single Ig domains, the binding stoichiometry of antibodies is therefore 2:1, since two V-domains combine to form the binding site for one antigen molecule. On a per molecule basis, each Ig molecule contains two Fv (Fab) fragments, and hence two antigen-binding sites.

The interaction of hGH with GHR is currently the best characterized receptor/ligand interaction involving non-antibody IgSF members. The extracellular region of GHR consists of two Ig domains, both of which are similar to, but distinct from, classical C-domains (Harpaz *et al.*, 1994). The hGH/GHR interaction is sequential. In the first step, a 1:1 high affinity hGH/GHR complex is formed. In the second step, this complex engages another GHR molecule in a low affinity interaction to form a final 1:2 complex (Cunningham *et al.*, 1991) Thus, the number of Ig domains per binding site of GHR is the same as a Fab fragment. hGH/GHR interactions are asymmetrical in the sense that the two receptors recognize different sites on hGH (Cunningham *et al.*, 1991).

CD2 (like CD4) is stable as a monomer. Its extracellular region consists of an N-terminal V-domain followed by a C-domain. Mutagenesis has shown that the CD2 V-domain includes the binding site for CD58, a CD2 ligand. The binding site is formed by residues of the GFCC'C'' face of the domain (Arulanandam *et al.*, 1993). The stoichiometry of the CD2/CD58 interaction is thought to be 1:1.

CD8 comprises a single extracellular V-domain, connected to its transmembrane segment by a hinge region. However, this molecule is usually expressed as a (α,β-) heterodimer with disulphide bonds connecting the hinge regions. The X-ray structure of the α-CD8 V-domain showed that CD8 formed a non-covalent Fv-like homodimer, analogous to the one seen in antibodies (Leahy *et al.*, 1992). Mutagenesis

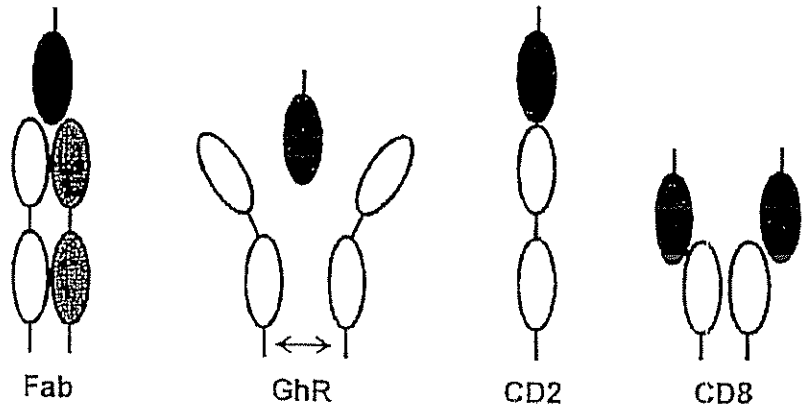


Fig. 1. Models of different binding stoichiometries by IgSF receptors.

Shown are schematic examples of different binding stoichiometries of IgSF members, as discussed in the text. Domains involved in binding are depicted as open ovals; light shaded ovals indicate that two different chains comprise the binding sites of Ig Fab fragments. Ligands are depicted by dark filled ovals.

studies have suggested that, in contrast to antibody Fvs, a CD8 dimer has two binding sites for class I molecules (Giblin *et al.*, 1994). Each binding site includes parts of the CDR-like loops and β-strands A and B of a monomeric CD8 subunit. Thus, the Fv-like CD8 dimer includes two equivalent binding sites and the stoichiometry of its interaction with class I is thought to be 2:2.

Receptor dimerization and/or oligomerization are generally critical for initiating signal transduction (Metzger, 1992; Schlessinger, 1988; Lemmon *et al.*, 1994). Their importance is illustrated by the variety of mechanisms which have evolved to achieve dimerization and/or oligomerization following ligand binding (reviewed in (Lemmon *et al.*, 1994)). The analysis of the hGH/GHR interaction has elucidated a common theme of signalling via cytokine receptors. Ligand binding triggers receptor dimerization, which is essential for signalling (Cunningham *et al.*, 1991). In the case of the platelet-derived growth factor (PDGF) binding to its receptor (PDGFR), dimeric PDGF can cross-link adjacent PDGFR monomers (Bishayee *et al.*, 1989; Heldin *et al.*, 1989; Hart *et al.*, 1988). Fibroblast growth factor (FGF) forms a 1:1 complex with its receptor (FGFR), but oligomerization of FGF molecules by binding to heparan sulphate leads to oligomerization of FGFR (Spivak-Kroizman *et al.*, 1994). Finally, insulin receptor exists as a disulphide-linked hetero-tetramer of α and β subunits. In this case, signal transduction may be initiated by an allosteric change within a preexisting tetrameric structure (Ullrich *et al.*, 1990).

Structures of CD28/CTLA-4/B7 receptors

The domain structures of the CD28/CTLA-4 and B7 families are schematically shown in figure 2. CD28/CTLA-4 have single extracellular V-like domains, and are expressed as disulphide-linked homodimers. CD80/CD86 each include two extracellular domains, a V(like)-domain and a C-type domain. Thus, the extracellular domain arrangements of CD28/CTLA-4 and CD80/CD86 are analogous to CD8 and CD2, respectively.

The common structural features of IgSF molecules sometimes permit comparative molecular modeling studies where known 3D structures are used as templates to build models of related proteins with unknown structure (Bajorath *et al.*, 1993). However, IgSF molecules often display only weak sequence relations, and only a portion of the Ig core structure is conserved (Bork *et al.*, 1994). Thus, the identification of suitable template structures for modeling can be difficult, and it may often not be possible to build reasonably accurate models of IgSF molecules. In the absence of 3D

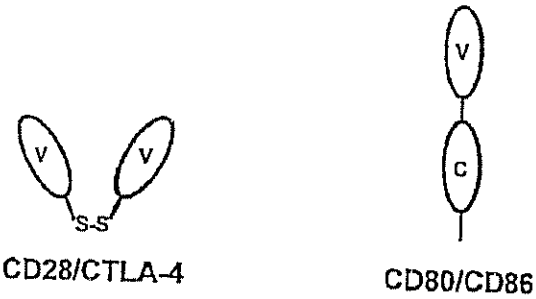


Fig. 2. Domain structures of CTLA-4, CD28 and B7 receptors.

A schematic representation of the domain structures of CD28/CTLA-4 and CD80/CD86. The position of the interchain disulphide bond determined for CTLA-4 and predicted for CD28 is illustrated. V, Ig (V)ariable-like domains; C, Ig (C)onstant-like domains.

structures for CD28/CTLA-4 and CD80/CD86, we have attempted to generate approximate molecular models for the Ig domains in CTLA-4 (Peach *et al.*, 1994) and CD80 (Bajaroth *et al.*, 1994). Figure 3 shows a model of the CTLA-4 V-domain and figure 4 a model of the C-like domain in CD80. Since we have not been able to identify a meaningful structural template for model building of the CD80 V(like-) domain (Bajaroth *et al.*, 1994), we can only approximately map residues of the CD80 N-terminal domain on a representative Ig V-fold. The predicted location of non-IgSF consensus residues which are conserved within the CD28/CTLA-4 family are shown in figure 3. The highest concentration of conserved residues is the hexapeptide MYPPPY motif, which maps to the CDR3-like loop region (Peach *et al.*, 1994). These residues are important for binding to B7, since mutations in the MYPPPY loop reduce binding activity of both CD28 and CTLA-4 (Peach *et al.*, 1994). The predicted location of non-IgSF consensus residues which are conserved within the CD80/CD86 family are shown in figure 4. Conserved residues are scattered throughout the V-like and C domains. Unlike the CD28/CTLA-4 family, conserved residues in the B7 family are not concentrated in the CDR-analogous loop regions. Conserved residues are instead concentrated on the A, B, E, D face of the C-type domain, and the G, F, C, C', C" face of the V-like domain. Thus, residue conservation within the CD80/CD86 extracellular domains is predominantly found on opposite faces of the V-like and C-type domains. Residues of both domains are critical for binding activity of CD80 to CD28 and CTLA-4 (unpublished observations).

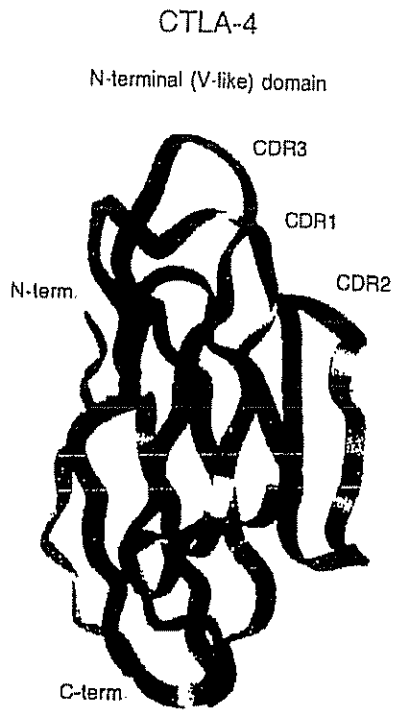


Fig. 3. Conservation of residues in the CD28/CTLA-4 receptor family.

Shown is a ribbon representation of an approximate molecular model (Peach *et al.*, 1994) of the extracellular domain of CTLA-4. The termini and predicted β-strands are labelled. The location of residues which are rigorously conserved in the CD28/CTLA-4 receptor families, but which are not IgSF consensus residues, is shown in black. Mutagenesis studies suggest that rigorously conserved, but also non-conserved, residues are determinants of the CD28/CTLA-4:B7 interactions (unpublished observations).

Stoichiometry of CTLA-4/B7 interactions

We have investigated the stoichiometry of CTLA-4 binding to B7 molecules (Linsley *et al.*, 1995) using soluble, monodisperse fragments of the extracellular domains of CTLA-4 and CD86. CTLA-4 is a homodimer interconnected by one disulphide bond in the extracellular domain at cysteine residue 120 (Linsley *et al.*, 1995). CTLA-4 was stably expressed as a monomer when cysteine 120 was mutated to serine, and monomeric CTLA-4 contained a high affinity binding site for B7 molecules. Thus, the chains of CTLA-4 have no particular affinity for each other, in contrast to Fv-like dimers such as CD8, which exist as dimers even in the absence of disulphide bonding. Dimeric CTLA-4 formed complexes with CD86 which contained equimolar amounts of CTLA-4 and CD86 (i.e., each homodimer bound two CD86 molecules, yielding a 2:2 molecular complex). Thus, CTLA-4 and probably CD28, have a receptor structure consisting of preexisting covalent homodimers with two equivalent binding sites.

Binding characteristics of CD28/CTLA-4 receptors

CD80 and CD86 showed very similar (within ~3-fold) equilibrium binding properties for CD28 and CTLA-4 receptors (Linsley *et al.*, 1994). CD28 is a low avidity receptor for both CD80 and CD86, whereas CTLA-4 is a high avidity receptor (Linsley *et al.*, 1994). The similarities in binding between CD80 and CD86 were surprising, given the limited sequence similarity between these molecules. CD80 and CD86 differed more in their rates of dissociation from ^{125}I-CTLA4Ig than they did in overall binding avidity. Bound ^{125}I-CTLA4Ig dissociated from CD86 CHO cells 5-8-fold faster than from CD80 CHO cells. In recent experiments (Cosand *et al.*, unpublished), we have measured kinetic and equilibrium properties of these CD28/CTLA-4:B7 interactions by surface plasmon resonance. These experiments have provided precise kinetic and equilibrium constants for CD28/CTLA-4:B7 interactions. Both CD28 and CTLA-4 have extremely fast off-rates of binding to CD80 and CD86 ($t_{1/2}$<~10 sec). The high avidity of CTLA-4 binding is primarily due to its very fast on-rate. This was previously suggested by the tendency of ^{125}I-CTLA4Ig to rebind during dissociation (Linsley *et al.*, 1994).

These data may be compared with recently published kinetic constants for other IgSF receptor ligand pairs. T-cell receptor (TCR)-MHC class I peptide interactions were studied by Corr *et al.* (Maripat *et al.*, 1994). These investigators measured a moderate association rate constant and affinity (~10^{-7} M or 100 nM), and a rapid dissociation rate constant (half time of dissociation or $t_{1/2}$ ~30 sec) for TCR-MHC class I peptide complexes. In another study (Van der Merwe *et al.*, 1994), the $t_{1/2}$ for CD2/CD58 interactions was determined to be ≤~0.2 sec. Thus, these IgSF receptors which mediate intercellular interactions between lymphocytes have very fast kinetic constants of ligand binding. In contrast, IgSF receptors which bind cytokines tend to have slower off-rates ($t_{1/2}$~2000 s for hGH/GHR interactions (Lowman *et al.*, 1993) and ~40 sec for human erythropoietin receptor (Harris *et al.*, 1992)). The binding stoichiometries and kinetic off-rates for CD28/CTLA-4 binding are schematically compared with those of cytokine receptors in figure 5.

Why do these lymphocyte surface receptors have fast kinetic off-rates? Probably because they

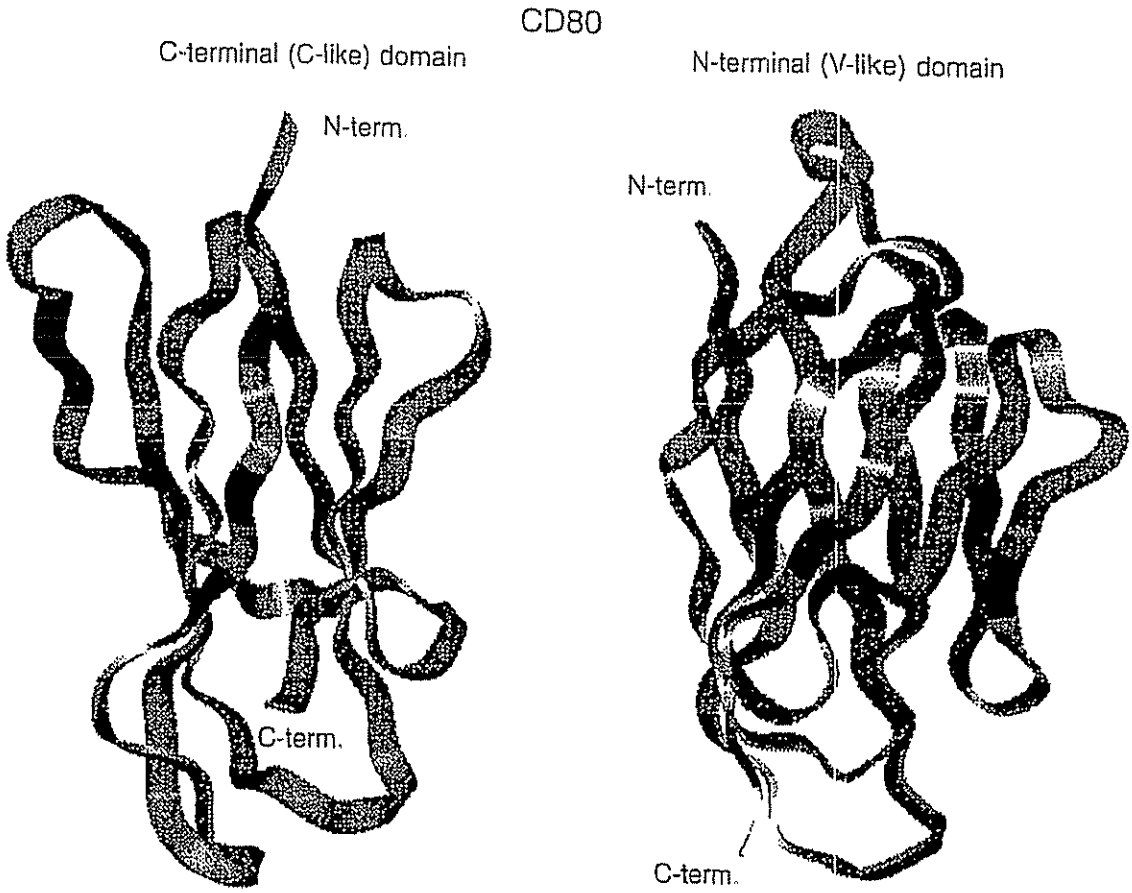


Fig. 4. Conservation of residues in the B7 family.

On the left is shown an approximate molecular model of the extracellular C-like domain of CD80 (Bajaroth *et al.*, 1993). On the right, a typical V-domain is used to represent the N-terminal domain in CD80 for which no meaningful molecular model could be generated (Bajaroth *et al.*, 1993). The termini and predicted β-strands of each domain are labeled. The location of residues which are rigorously conserved in the B7 family, but which are not IGSF consensus residues, is shown in black. The mapping of residues to the N-terminal domain of CD80 is only approximate.

favour disengagement of T cell/APC conjugates, and thereby allow the transient interactions characteristic of lymphocytes. However, fast kinetic off-rates present the problem of how to maintain receptor occupation by ligand for a sufficient period of time to permit productive signalling. This problem is circumvented by TCR and CD2 by their organization on the cell surface membrane as a complex containing several different coreceptors (Beyers *et al.*, 1992), which upon ligand binding, may increase the overall T cell:APC avidity and increase the probability of productive signalling. However, CD28/CTLA-4 receptors are not known to exist in complexes on the cell surface and may therefore utilize different mechanisms to ensure productive signaling.

How do CD28/CTLA-4 receptors signal despite having rapid kinetic off-rates?

Productive receptor signaling depends upon receptor occupancy by ligand and the transduction time required to engage the signal transduc-

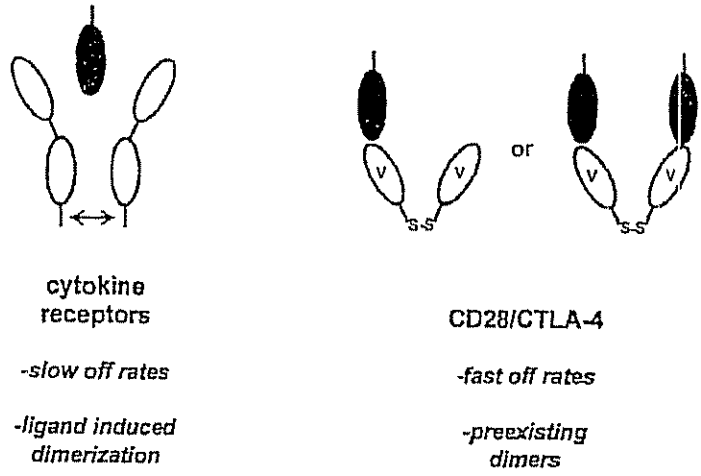


Fig. 5. Comparison of CTLA-4/CD28:B7 receptor binding with cytokine receptor binding. Characteristics of binding by different dimeric receptors are indicated. See text for details.

tion machinery (DeLisi, 1980). Receptor occupancy is determined by receptor and ligand expression levels, and their kinetic and equilibrium binding properties. If the time of receptor occupancy is short compared with the transduction time, the probability of signalling decreases (DeLisi, 1980).

Two characteristics of the CD28/CTLA-4:B7 system may facilitate receptor occupancy, and thereby the probability of signalling. The first of these is the availability of pre-existing covalent dimers of CD28 and/or CTLA-4 receptors. A homodimeric receptor with two binding sites has increased binding avidity for B7 molecules, since the probability of rebinding ligand after its dissociation is increased. The effective time of occupancy of CD28/CTLA-4 receptors by B7 molecules would thereby be prolonged.

The second characteristic is the apparent oligomerization of CD80 and/or CD86 molecules. Since B7 molecules are not disulphide-linked, full occupancy of CTLA-4/CD28 receptors would require that B7 molecules either preexist as non-covalent oligomers on the APC, or that they oligomerize as the result of binding to CTLA-4/CD28. Evidence for the former possibility was provided by immunofluorescence microscopy studies showing that CTLA4Ig-binding molecules on human Langerhans cells assume a non-uniform distribution (Symington *et al.*, 1993). These clusters would favour multiple engagements with CD28 and/or CTLA-4 receptors.

Evidence for CD28 aggregation during signal transduction

Since CD28/CTLA-4 are preexisting homodimers, signal transduction is most likely initiated by either allosteric changes in receptor structure (Ullrich *et al.*, 1990; Milligan and Koshland, 1991), or receptor oligomerization (Metzger, 1992; Schlessinger, 1988; Lemmon *et al.*, 1994). With CD28/CTLA-4, there is no evidence to support ligand-induced conformational changes, whereas several sets of experiments support a role for oligomerization in signal transduction.

Evidence that the CD28 receptor requires aggregation for signal transduction came from some of the earliest studies using mAbs. In bivalent form, soluble anti-CD28 mAb 9.3 was capable of potent synergy with other polyclonal mitogens such as PMA, PHA, or immobilized anti-CD3 mAb (Hara *et al.*, 1985; Moretta *et al.*, 1985; Martin *et al.*, 1986; Gmunder and Lesslauer, 1984; Ledbetter *et al.*, 1985). In contrast, Fab fragments of mAb 9.3 retained binding activity but did not augment or induce T-cell responses in these assays (Ledbetter *et al.*, 1985; Ledbetter *et al.*, 1990). Bivalent mAb binding to CD28 did not induce a detectable increase in cytoplasmic calcium concentration, suggesting the signals were calcium-independent (Ledbetter *et al.*, 1986).

Although soluble anti-CD28 mAbs were agonists in many assays, they were inhibitory for responses of antigen-specific T cells to peptides presented by

APC and in mixed lymphocyte reaction (MLR) (Lesslauer *et al.*, 1986; Damle *et al.*, 1988). In the MLR, cross-linking of anti-CD28 mAb using a secondary anti-κ mAb reversed the inhibition and resulted in direct restimulation of alloantigen-specific T cells without addition of stimulator cells (Damle *et al.*, 1988). Thus, bivalent anti-CD28 mAb, while capable of triggering some CD28 signals, is not sufficient to replace the function of CD80 and CD86 molecules during MLR. A more recent study showed that anti-CD28 mAbs and CD80 also differed in their abilities to activate ras (Nunes *et al.*, 1995). These results may indicate that signals through CD28 by engagement of natural ligands depends upon higher order CD28 aggregation.

The relationship of CD28 aggregation to activation of intracellular signals was further studied by comparing the effects of varying the degree of CD28 cross-linking on T-cell responses. Soluble mAb 9.3 was compared with 300-kDa homoconjugated mAb 9.3 and with more extensively cross-linked CD28 using mAb 9.3 followed by a secondary anti-κ mAb. Although soluble mAb 9.3 was synergistic with PMA or anti-CD3, these responses were enhanced by additional CD28 cross-linking (Ledbetter *et al.*, 1990). Only the most extensive cross-linking of CD28 induced responsiveness to IL2 and expression of IL2Rα chain by resting T cells. These results provide strong evidence that the CD28 receptor can deliver qualitatively different signals depending on its degree of aggregation.

There is also biochemical evidence suggesting that signals induced by anti-CD28 mAbs are altered by receptor cross-linking. Although soluble CD28 mAbs do not cause changes in cytoplasmic calcium concentration, extensive CD28 aggregation induces calcium mobilization through activation of PLCγ1 and production of IP3 (Ledbetter *et al.*, 1990; Ledbetter *et al.*, 1987; Ledbetter *et al.*, 1992). Activation of calcium-dependent CD28 signals through PLCγ1 is associated with its phosphorylation on tyrosine residues (Ledbetter *et al.*, 1992). The tyrosine kinase inhibitor, herbimycin A, inhibited the calcium signal triggered by CD28 cross-linking, consistent with the initiation of CD28 signals by tyrosine kinase activation (Lu *et al.*, 1992; Ohnishi *et al.*, 1995). Like the CD3/TCR pathway, multiple tyrosine kinases may be involved in transmitting CD28 signals. The src-family kinases lck and fyn are activated by CD28 crosslinking, and associate with CD28 in immunoprecipitates (August *et al.*, 1994; Hutchcroft and Bierer, 1994). Another T-cell-restricted kinase, EMT/ITK, has also been reported to be associated with CD28 and activated by CD28 cross-linking (August and Dupont. 1994). During receptor aggregation, CD28 itself becomes tyrosine-phosphorylated on cytoplasmic tyrosine residue(s) and associates with PI-3K through a specific SH2 interaction (Pages *et al.*, 1994; Prasad *et al.*, 1994; Stein *et al.*, 1994; Truitt *et al.*, 1994). The association with PI-3K is known to be essential for CD28 function (Stein *et al.*, 1994), but the relationship between CD28 aggregation and PI-3K association has not yet been well studied.

A model for CD28 receptor oligomerization

How may engagement of CD28/CTLA-4 receptors by B7 molecules trigger receptor oligomerization? Since the role of CTLA-4 in T-cell activation is not well understood, we will restrict the present discussion to CD28. On activated T cells, CD28 displayed a uniform distribution (Damle *et al.*, 1994). Thus, for oligomerization to occur, CD28 must redistribute on the T-cell membrane following B7 engagement. While there is no direct evidence that CD28/CTLA-4 receptors oligomerize, other initially dispersed membrane receptors cluster at sites of cell:cell contact following engagement with ligands on apposing cells ("mutual capping", (Singer, 1992)). With CD28, this tendency would be enhanced by the presence of clusters of B7 molecules on APC. Thus, engagement of dispersed CD28 receptors by B7 ligands should result in concentration and "capping" of CD28 on the cell surface. This would increase the local concentration of CD28, thereby shifting the equilibrium of binding towards greater CD28 receptor occupancy. Despite the concentration-driven increase in receptor occupancy, the rapid dissociation kinetics conditions of CD28:B7 complexes would still result in transiently unoccupied or "free" CD28 receptors. These free CD28 receptors could rebind B7 molecules because of the high local concentrations of CD28 and B7. However, a free CD28 receptor does not necessarily rebind the same B7 molecule from which it dissociated. The (predicted) divalent nature of CD28 and the clustering of B7 molecules on APC could result in the sequential engagement of more than one CD28 receptor by a single B7 molecule. Over time, the rapidity of dissociation would therefore lead to a quasi-simultaneous occupancy of many CD28 receptors by a few B7 molecules, an effect we term "virtual receptor occupancy". This would essentially cross-link different receptor molecules. A model for B7-induced clustering of CD28 molecules is shown in figure 6.

CD80 and CD86 have distinct binding characteristics for CD28 and CTLA-4 (Linsley *et al.*, 1994). These differences in binding properties of CD80 and CD86 may differentially regulate the dynamics of CD28 clustering and signalling. In most cellular interactions between T cells and APC, both CD80 and CD86 are expressed on the APC, and CD28 and CTLA-4 are coexpressed on T cells during the activation response. The presence of multiple ligands interacting simultaneously with common receptors

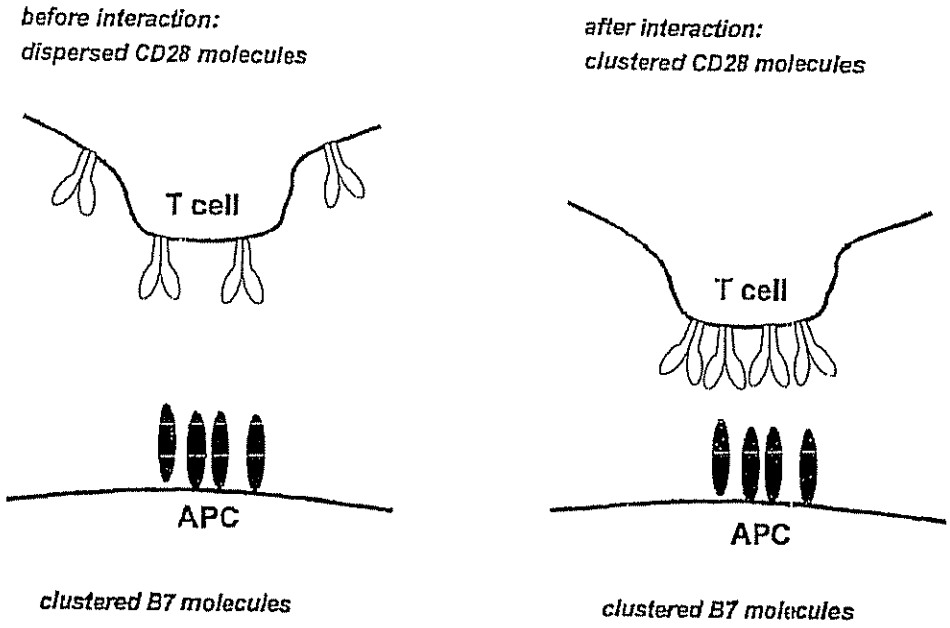


Fig. 6. Model for induced clustering of CD28/CTLA-4 receptors.

Model of predicted clustering of CD28 receptors by B7 molecules during cell:cell interactions. B7 molecules are shown as preexisting clusters (Symington *et al.*, 1993). See text for details.

suggests complex control of CD28 aggregation and signals during T cell activation by APC.

Concluding remarks

The model presented in figure 6 is hypothetical and based largely on analogy with other receptors. Further experiments are needed to determine how accurately this applies to CD28 signalling. We need to confirm that CD28 has the same binding stoichiometry as CTLA-4, and if so, to determine whether signal transduction requires occupancy of one or both subunits by B7 molecules. Another problem is to determine how many CD28/CTLA-4:B7 signalling complexes must form to productively costimulate T cells. It is also unknown whether CD28 receptors actually cluster during T cell:APC contact. How do CD28/CTLA-4:B7 complexes disengage upon completion of signalling? What tips the balance between occupancy and disengagement? Is this an active process, requiring the expenditure of energy to break contacts (perhaps by deforming the membrane at sites of contact, or by receptor or ligand internalization), or is it stochastic? Finally, what is the role of the high avidity binding to CTLA-4 in this process? We have presented data that CTLA-4 and CD28 cooperatively regulate T-cell adhesion and activation by B7 molecules (Damle *et al.*, 1994; Linsley *et al.*, 1992a), whereas others have suggested that CTLA-4 negatively regulates T-cell activation by CD28 (Walunas *et al.*, 1994). Since CD28 and CTLA-4 are coexpressed on the same cells (Linsley *et al.*, 1992a) and bind to the same ligands, they must affect each other's binding and/or signalling. Determining how CTLA-4 functions during T-cell activation remains a challenge. It is hoped that this discussion will help focus experiments on some of the unresolved issues in CD28/CTLA-4:B7 interactions and costimulatory signals.

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The critical role of CD28 signalling in the prevention of human T-cell anergy

V.A. Boussiotis, G.J. Freeman, J.G. Gribben, L.M. Nadler

Division of Hematologic Malignancies, Dana Farber Cancer Institute and Department of Medicine, Harvard Medical School, Boston, MA, 02115 (USA)

To clonally expand and differentiate into effectors, T cells require cellular interactions delivered by antigen-presenting cells (APC) including adhesion, MHC presentation of antigen, and costimulation. Although adhesion and antigen recognition are necessary, they are not sufficient to induce an immune response. Engagement of the T-cell receptor (TCR) by antigen in the absence of requisite costimulation fails to induce T-cell activation, but rather results in the induction of long term unresponsiveness, termed anergy. Costimulation is neither antigen-specific, nor MHC-restricted, and potentially can be mediated via a large number of cell surface molecules expressed on APCs. Among them, the B7 family appears to be unique, since ligation of its counter-receptor, CD28, is both necessary and sufficient to prevent the induction of anergy (Harding *et al.*, 1992; Lenschow *et al.*, 1992; Turka *et al.*, 1992; Boussiotis *et al.*; 1993a; Gimmi *et al.*, 1993; Lin *et al.*, 1993; Tan *et al.*, 1993; Boussiotis *et al.*, 1994b).

B7 family of costimulatory molecules

To date, two members of the B7 family of molecules, termed B7-1 and B7-2, have been molecularly

EXHIBIT 31

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SERIAL NUMBER	FILING DATE	NAMED INVENTOR	ATTY. DOCKET
07/347,291	05/02/89	SLEDZIEWSKI	A 990008446C2

ULM, J

SEED & BERRY
6300 COLUMBIA CENTER
SEATTLE, WA 98104-7092

ART UNIT 185 PAPER NUMBER 12

08/23/90

This is a communication from the examiner in charge of your application.
COMMISSIONER OF PATENTS AND TRADEMARKS

☒ This application has been examined ☒ Responsive to communication filed on 7-6-90 ☒ This action is made final

A shortened statutory period for response to this action is set to expire 3 month(s), 0 days from the date of this letter.
Failure to respond within the period for response will cause the application to become abandoned. 35 U.S.C. 133

Part I THE FOLLOWING ATTACHMENT(S) ARE PART OF THIS ACTION:

1. ☐ Notice of References Cited by Examiner, PTO-892
2. ☐ Notice re Patent Drawing, PTO-948.
3. ☒ Notice of Art Cited by Applicant, PTO-1449.
4. ☐ Notice of Informal Patent Application, Form PTO-152
5. ☐ Information on How to Effect Drawing Changes, PTO-1474.
6. ☐ _____

Part II SUMMARY OF ACTION

1. ☒ Claims 1-83 are pending in the application.
   Of the above, claims 1-21, 23-83 are withdrawn from consideration.
2. ☐ Claims _____ have been cancelled.
3. ☐ Claims _____ are allowed.
4. ☒ Claims 22 are rejected.
5. ☐ Claims _____ are objected to.
6. ☐ Claims _____ are subject to restriction or election requirement.
7. ☐ This application has been filed with informal drawings under 37 C.F.R. 1.85 which are acceptable for examination purposes.
8. ☐ Formal drawings are required in response to this Office action.
9. ☐ The corrected or substitute drawings have been received on _____. Under 37 C.F.R. 1.84 these drawings are ☐ acceptable; ☐ not acceptable (see explanation or Notice re Patent Drawing, PTO-948).
10. ☐ The proposed additional or substitute sheet(s) of drawings, filed on _____, has (have) been ☐ approved by the examiner; ☐ disapproved by the examiner (see explanation).
11. ☐ The proposed drawing correction, filed _____, has been ☐ approved; ☐ disapproved (see explanation)
12. ☒ Acknowledgement is made of the claim for priority under U.S.C. 119. The certified copy has ☒ been received ☐ not been received ☐ been filed in parent application, serial no. _____; filed on _____
13. ☐ Since this application appears to be in condition for allowance except for formal matters, prosecution as to the merits is closed in accordance with the practice under Ex parte Quayle, 1935 C.D. 11; 453 O.G. 213.
14. ☐ Other

EXAMINER'S ACTION

PTOL-326 (Rev. 9-89)

Your correspondence of June 28 of 1990 has been received and entered as amendment A, Paper Number 10, filed July 6 of 1990.

Receipt is acknowledged of papers submitted under 35 U.S.C. § 119, which papers have been placed of record in the file.

The text of those sections of Title 35, U.S. Code not included in this action can be found in a prior Office action.

Claim 22 is rejected under 35 U.S.C. § 102(e) as being anticipated by Boss (A) and Boss (L) for reasons of record.

Applicant's arguments filed July 6 of 1990 have been fully considered but they are not deemed to be persuasive. In response to applicants assertion that Boss et.al. do not teach or suggest the dimerized polypeptide fusions or methods of producing such fusions, the examiner would point out that Boss (L) does teach "that the present invention can be applied to the production of other multichain polypeptides or proteins". The term "multichain polypeptide or proteins" would be directly applicable to dimeric and multimeric forms of proteins. Applicants claim that Boss et.al. do not teach or suggest the use of the immunoglobulin constant region in the dimerization of non-immunoglobulin polypeptides. Boss (L) does teach that "it is also envisaged that the Ig molecule may be synthesized by a host cell with another peptide moiety attached to one of its constant domains." This passage teaches the production of multichain fusion proteins in which one component is an immunoglobulin chain. Whether the

peptide moiety becomes dimerized or not would be dictated by the moiety chosen.

THIS ACTION IS MADE FINAL. Applicant is reminded of the extension of time policy as set forth in 37 C.F.R. § 1.136(a). The practice of automatically extending the shortened statutory period an additional month upon the filing of a timely first response to a final rejection has been discontinued by the Office. See 1021 TMOG 35.

A SHORTENED STATUTORY PERIOD FOR RESPONSE TO THIS FINAL ACTION IS SET TO EXPIRE THREE MONTHS FROM THE DATE OF THIS ACTION. IN THE EVENT A FIRST RESPONSE IS FILED WITHIN TWO MONTHS OF THE MAILING DATE OF THIS FINAL ACTION AND THE ADVISORY ACTION IS NOT MAILED UNTIL AFTER THE END OF THE THREE-MONTH SHORTENED STATUTORY PERIOD, THEN THE SHORTENED STATUTORY PERIOD WILL EXPIRE ON THE DATE THE ADVISORY ACTION IS MAILED, AND ANY EXTENSION FEE PURSUANT TO 37 C.F.R. § 1.136(a) WILL BE CALCULATED FROM THE MAILING DATE OF THE ADVISORY ACTION. IN NO EVENT WILL THE STATUTORY PERIOD FOR RESPONSE EXPIRE LATER THAN SIX MONTHS FROM THE DATE OF THIS FINAL ACTION.

Any inquiry concerning this communication should be directed to John D. Ulm at telephone number (703) 557-5634.

Ulm/sg
August 22, 1990

RICHARD A. SCHWARTZ
PRIMARY EXAMINER
ART UNIT 121

EXHIBIT 32

ONLINE PROGRAMS
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Main Entry: **moi·e·ty**
Pronunciation: \'mȯi-ə-tē\
Function: *noun*
Inflected Form(s): *plural* **moi·e·ties**
Etymology: Middle English *moite,* from Anglo-French *meité, moité,* from Late Latin *medietat-, medietas,* from Latin *medius* middle — more at MID
Date: 15th century

1 a : one of two equal parts **:** HALF **b :** one of two approximately equal parts
2 : one of the portions into which something is divided **:** COMPONENT, PART <an ether molecule with a benzene *moiety*>
3 : one of two basic complementary tribal subdivisions

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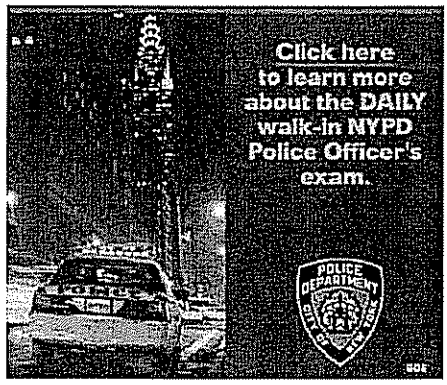
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PATENT

I hereby certify that on the date specified below, this correspondence is being deposited with the United States Postal Service as first-class mail in an envelope addressed to: Commissioner of Patents and Trademarks, Washington, DC 20231.

NOVEMBER 19, 1990
Date

David J. Maki

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Andrzej Z. Sledziewski et al.
Serial No. : 07/347,291
Filed : May 2, 1989
For : METHODS OF PRODUCING SECRETED RECEPTOR ANALOGS AND BIOLOGICALLY ACTIVE PEPTIDE DIMERS

GROUP 180 1990 NOV 23 AM 11:33

Examiner : ~~John~~ D. Ulm
Art Unit : 185
Docket No. : 990008.446C2
Date : November 19, 1990

Commissioner of Patents and Trademarks
Washington, DC 20231

AMENDMENT

Sir:

In response to the Office Action dated August 23, 1990, please amend the application as follows:

In the Claims:

Please amend claim 22 as follows:

~~22~~. (Twice Amended) A method for producing a secreted, biologically active, dimerized polypeptide fusion, comprising:

introducing into a eukaryotic host cell a first DNA construct comprising a transcriptional promoter operatively linked to a first secretory signal sequence followed downstream by and in proper reading frame with a first DNA sequence encoding a first polypeptide chain of a non-immunoglobulin polypeptide dimer requiring dimerization for biological activity joined to a DNA sequence encoding an immunoglobulin light chain constant region;

B1 Cont'd

(c) introducing into said eukaryotic host cell a second DNA construct comprising a transcriptional promoter operably linked to a second secretory signal sequence followed downstream by and in proper reading frame with a second DNA sequence encoding a second polypeptide chain of [a] said non-immunoglobulin polypeptide dimer joined to a DNA sequence encoding at least one immunoglobulin heavy chain constant region domain selected from the group consisting of C_H1, C_H2, C_H3 and C_H4;

(d) growing said host cell in an appropriate growth medium under physiological conditions to allow the secretion of a dimerized polypeptide fusion comprising [a] said first polypeptide chain of a non-immunoglobulin polypeptide dimer joined to [an] said immunoglobulin light chain constant region and [a] said second polypeptide chain of a non-immunoglobulin polypeptide dimer joined to at least one immunoglobulin heavy chain constant region domain, wherein said dimerized polypeptide fusion exhibits biological activity characteristic of said non-immunoglobulin polypeptide dimer; and

(e) isolating said biologically active, dimerized polypeptide fusion from said host cell.

REMARKS

Reconsideration of the application in view of the above amendment and following remarks is requested. Claim 22 is the only claim in this case, and has been amended.

Claim 22 was rejected under 35 U.S.C. §102(e) as being anticipated by the Boss references. As discussed with the Examiner during a recent interview, applicants have amended claim 22 to recite that the first DNA sequence encodes a first polypeptide chain of a non-immunoglobulin polypeptide dimer requiring dimerization for biological activity. In addition, applicants have amended claim 22 to recite that the dimerized polypeptide fusion exhibits biological activity characteristic of the non-immunoglobulin polypeptide dimer. Neither of the Boss references discloses or suggests the production of such a biologically active, dimerized

polypeptide fusion. Therefore, applicants believe that the rejection under §102(e) has been overcome.

Consistent with applicants' duty of disclosure under 37 C.F.R. §1.56, applicants have enclosed herewith an Information Disclosure Statement setting forth PCT publication number WO 90/06953 by Genentech, Inc. As noted in the Information Disclosure Statement, applicants believe that the claimed invention is patentably distinct from this reference, which only recently came to the attention of applicants.

On the basis of the above amendments and remarks, reconsideration of this application and its allowance are requested. If the Examiner has any questions, he is encouraged to telephone the undersigned attorney at (206) 622-4900. Applicants wish to express their appreciation for the recent interview with the Examiner.

Respectfully submitted,
Andrzej Z. Sledziewski et al.
SEED and BERRY

David J. Mahl
Registration No. 31,392

DJM:js

Enclosures:
Form PTO 1083 (+ copy)
Information Disclosure Statement

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EXHIBIT 34

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