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.	C.A. No. 06-500-SLR **PUBLIC VERSION**

DECLARATION OF PAUL J. ANDRE IN SUPPORT OF PLAINTIFF ZYMOGENETICS, INC.'S OPENING CLAIM CONSTRUCTION BRIEF

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Dated: May 16, 2008
Public Version: May 16, 2008

I, PAUL J. ANDRE, declare:

1. I am a Partner with the law firm King & Spalding LLP, counsel of record for Plaintiff ZymoGenetics, Inc. ("ZymoGenetics"). I have personal knowledge of the facts set forth in this declaration and can testify competently to those facts.

2. Attached hereto as Exhibit 1 is a true and correct copy of United States Patent No. 5,843,725, bearing bates numbers ZG 000842-919.

3. Attached hereto as Exhibit 2 is a true and correct copy of United States Patent No. 6,018,026, bearing bates numbers ZG 00001-77.

4. Attached hereto as Exhibit 3 is a true and correct copy of a ZymoGenetics web page entitled "Corporate Fact Sheet," *available at* http://www.zymogenetics.com/about/fact-sheet.html, a ZymoGenetics web page entitled "ZymoGenetics History," *available* at http://www.zymogenetics.com/about/history.html, and a web page entitled "The Nobel Prize in Chemistry 1993," *available* at http://nobelprize.org/nobel_prizes/chemistry/laureates/1993/.

5. Attached hereto as Exhibit 4 is a true and correct copy of a Bristol-Myer Squibb Co. ("BMS") web page entitled "Pharmaceutical Research & Development - Biologics," *available at* http://www.bms.com/research/content/data/biologics.html.

6. Attached hereto as Exhibit 5 is a true and correct copy of a BMS web page entitled "News Release" dated May 5, 2006, *available at* http://newsroom.bms.com/article_display.cfm?article_id=5063.

7. Attached hereto as Exhibit 6 is a true and correct copy of a BMS' Orencia® Package Insert, bearing bates numbers ZG 001813-16.

8. Attached hereto as Exhibit 7 is a true and correct copy of an article entitled "The immunoglobulin superfamily -- domains for cell surface recognition," by Alan F. Williams & A. Neil Barclay dated 1988, bearing bates numbers BMS001054677-701.

9. Attached hereto as Exhibit 8 is a true and correct copy of excerpts from the textbook entitled "Immunology," by Leroy E. Hood, et al.

10. Attached hereto as Exhibit 9 is a true and correct copy of the Expert Rebuttal Report of Jeffrey V. Ravetch, M.D., Ph.D. Regarding Non-Infringement of U.S. Patent Nos. 5,842,725 and 6,018,026.

I declare under penalty of perjury under the laws of the State of California and the United States of America that each of the above statements is true and correct.

Executed in Redwood City, California on May 16, 2008.

By: _____
Paul J. André

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I, Philip A. Rovner, hereby certify that on May 22, 2008, the within document was filed with the Clerk of the Court using CM/ECF which will send notification of such filing(s) to the following; that the document was served on the following counsel as indicated; and that the document is available for viewing and downloading from CM/ECF.

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EXHIBIT 1
Part 1

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

(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. 90/006,970, Mar. 16, 2004

Reexamination Certificate for:
Patent No.: 5,843,725
Issued: Dec. 1, 1998
Appl. No.: 08/475,458
Filed: Jun. 7, 1995

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

(51) Int. Cl.
C12N 15/10 (2006.01)
C12N 5/10 (2006.01)

(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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Primary Examiner—Bennett Celsa

(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

[22] Filed: **Jun. 7, 1995**

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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[51] **Int. Cl.**[6] **C12N 15/10**; C12N 5/10; C07K 16/46; C07K 14/705

[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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(List continued on next page.)

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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 GGCCCCTCAGCCCTGCTGCCCAGCACGAGCCTGTGCTCGCCCTGCCCAACGCAGACAGCCAGACCCAGG   69
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 346 GCAAGGACACCATGCGGCTTCCGGGTGCGATGCCAGCTCTGGCCCTCAAAGGCGAGCTGCTGTTGCTGT  414
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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

```
1312 TGGGAGAGGTGGGCACACTACAATTTGCTGAGCTGCATCGGAGCCGGACACTGCAGGTAGTGTTCGAGG 1380
       G  E  V  G  T  L  Q  F  A  E  L  H  R  S  R  T  L  Q  V  V  F  E  A  342

1381 CCTACCCAGCGCCCACTGTCCTGTGGTTCAAAGACAACCGCACCCTGGGCGACTCCAGCGCTGGCGAAA 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  M  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 TGACGTACTGGGAGGAGGAGCAGGAGTTTGAGGTGGTGAGCACACTGCGTCTGCAGCACGTGGATCGGC 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
       S  L  P  F  K  V  V  V  I  S  A  I  L  A  L  V  V  L  T  I  I  S  L  549

2002 TTATCATCCTCATCATGCTTTGGCAGAAGAAGCCACGTTACGAGATCCGATGGAAGGTGATTGAGTCTG 2070
       I  I  L  I  M  L  W  Q  K  K  P  R  Y  E  I  R  W  K  V  I  E  S  V  572

2071 TGAGCTCTGACGGCCATGAGTACATCTACGTGGACCCCATGCAGCTGCCCTATGACTCCACGTGGGAGC 2139
       S  S  D  G  H  E  Y  I  Y  V  D  P  M  Q  L  P  Y  D  S  T  W  E  L  595

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
       A  H  G  L  S  H  S  Q  A  T  M  K  V  A  V  K  M  L  K  S  T  A  R  641

2278 GCAGCAGTGAGAAGCAAGCCCTTATGTCGGAGCTGAAGATCATGAGTCACCTTGGGCCCCACCTGAACG 2346
       S  S  E  K  Q  A  L  M  S  E  L  K  I  M  S  H  L  G  P  H  L  N  V  664

2347 TGGTCAACCTGTTGGGGGCCTGCACCAAAGGAGGACCCATCTATATCATCACTGAGTACTGCCGCTACG 2415
       V  N  L  L  G  A  C  T  K  G  G  P  I  Y  I  I  T  E  Y  C  R  Y  G  687

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 CGCCCAGCGCGGAGCTCTACAGCAATGCTCTGCCCGTTGGGCTCCCCCTGCCCAGCCATGTGTCCTTGA 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
```

Fig. 1B

```
2554 CCGGGGAGAGCGACGGTGGCTACATGGACATGAGCAAGGACGAGTCGGTGGACTATGTGCCCATGCTGG 2622
       G  E  S  D  G  G  Y  M  D  M  S  K  D  E  S  V  D  Y  V  P  M  L  D  756

2623 ACATGAAAGGAGACGTCAAATATGCAGACATCGAGTCCTCCAACTACATGGCCCCTTACGATAACTACG 2691
       H  K  G  D  V  K  Y  A  D  I  E  S  S  N  Y  M  A  P  Y  D  N  Y  V  779

2692 TTCCCTCTGCCCCTGAGAGGACCTGCCGAGCAACTTTGATCAACGAGTCTCCAGTGCTAAGCTACATGG 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
       R  D  I  M  R  D  S  N  Y  I  S  K  G  S  T  F  L  P  L  K  W  M  A  871

2968 CTCCGGAGAGCATCTTCAACAGCCTCTACACCACCCTGAGCGACGTGTGGTCCTTCGGGATCCTGCTCT 3036
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3037 GGGAGATCTTCACCTTGGGTGGCACCCCTTACCCAGAGCTGCCCATGAACGAGCAGTTCTACAATGCCA 3105
       E  I  F  T  L  G  G  T  P  Y  P  E  L  P  M  N  E  Q  F  Y  N  A  I  917

3106 TCAAACGGGGTTACCGCATGGCCCAGCCTGCCCATGCCTCCGACGAGATCTATGAGATCATGCAGAAGT 3174
       K  R  G  Y  R  M  A  Q  P  A  H  A  S  D  E  I  Y  E  I  M  Q  K  C  940

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3244 GCGAAGGTTACAAAAAGAAGTACCAGCAGGTGGATGAGGAGTTTCTGAGGAGTGACCACCCAGCCATCC 3312
       E  G  Y  K  K  K  Y  Q  Q  V  D  E  E  F  L  R  S  D  H  P  A  I  L  986

3313 TTCGGTCCCAGGCCCGCTTGCCTGGGTTCCATGGCCTCCGATCTCCCCTGGACACCAGCTCCGTCCTCT 3381
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3382 ATACTGCCGTGCAGCCCAATGAGGGTGACAACGACTATATCATCCCCCTGCCTGACCCCAAACCCGAGG 3450
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3520 CCTCAACCATCTCCTGTGACAGCCCCCTGGAGCCCCAGGACGAACCAGAGCCAGAGCCCCAGCTTGAGC 3588
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3589 TCCAGGTGGAGCCGGAGCCAGAGCTGGAACAGTTGCCGGATTCGGGGTGCCCTGCGCCTCGGGCGGAAG 3657
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3658 CAGAGGATAGCTTCCTGTAGGGGGCTGGCCCCTACCCTGCCCTGCCTGAAGCTCCCCCCCTGCCAGCAC 3726
       E  D  S  F  L  .                                                    1106

3727 CCAGCATCTCCTGGCCTGGCCTGACCGGGCTTCCTGTCAGCCAGGCTGCCCTTATCAGCTGTCCCCTTC 3795
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Fig. 1C

```
3796 TGGAAGCTTTCTGCTCCTGACGTGTTGTGCCCCAAACCCTGGGGCTGGCTTAGGAGGCAAGAAAACTGC 3864
3865 AGGGGCCGTGACCAGCCCTCTGCCTCCAGGGAGGCCAACTGACTCTGAGCCAGGGTTCCCCCAGGGAAC 3933
3934 TCAGTTTTCCCATATGTAAGATGGGAAAGTTAGGCTTGATGACCCAGAATCTAGGATTCTCTCCCTGGC 4002
4003 TGACACGGTGGGGAGACCGAATCCCTCCCTGGGAAGATTCTTGGAGTTACTGAGGTGGTAAATTAACAT 4071
4072 TTTTTCTGTTCAGCCAGCTACCCCTCAAGGAATCATAGCTCTCTCCTCGCACTTTTTATCCACCCAGGA 4140
4141 GCTAGGGAAGAGACCCTAGCCTCCCTGGCTGCTGGCTGAGCTAGGGCCTAGCTTGAGCAGTGTTGCCTC 4209
4210 ATCCAGAAGAAAGCCAGTCTCCTCCCTATGATGCCAGTCCCTGCGTTCCCTGGCCCGAGCTGGTCTGGG 4278
4279 GCCATTAGGCAGCCTAATTAATGCTGGAGGCTGAGCCAAGTACAGGACACCCCCAGCCTGCAGCCCTTG 4347
4348 CCCAGGGCACTTGGAGCACACGCAGCCATAGCAAGTGCCTGTGTCCCTGTCCTTCAGGCCCATCAGTCC 4416
4417 TGGGGCTTTTTCTTTATCACCCTCAGTCTTAATCCATCCACCAGAGTCTAGAAGGCCAGACGGGCCCCG 4485
4486 CATCTGTGATGAGAATGTAAATGTGCCAGTGTGGAGTGGCCACGTGTGTGTGCCAGTATATGGCCCTGG 4554
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4624 AAAAAATGAATAAGTCGGACTTATTAACTCTGAGTGCCTTGCCAGCACTAACATTCTAGAGTATTCCAG 4692
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4762 CTGGGAGATTCCAGATCACACATCACACTCTGGGGACTCAGGAACCATGCCCCTTCCCCAGGCCCCCAG 4830
4831 CAAGTCTCAAGAACACAGCTGCACAGGCCTTGACTTAGAGTGACAGCCGGTGTCCTGGAAAGCCCCAAG 4899
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4969 CTGGGCAAAAGGGACAAAGAGGGCAAATGAGATCACCTCCTGCAGCCCACCACTCCAGCACCTGTGCCG 5037
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5176 CCCTGAGGCATGCGCTCCATGGGGGTATGGTTTTGTCACTGCCCAGACCTAGCAGTGACATCTCATTGT 5244
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5314 GGGTTGGGAAGGGGGTGCAGGAAGCTCAACCCCTCTGGGCACCAACCCTGCATTGCAGGTTGGCACCTT 5382
5383 ACTTCCCTGGGATCCCCAGAGTTGGTCCAAGGAGGGAGAGTGGGTTCTCAATACGGTACCAAAGATATA 5451
5452 ATCACCTAGGTTTACAAATATTTTTAGGACTCACGTTAACTCACATTTATACAGCAGAAATGCTATTTT 5520
5521 GTATGCTGTTAAGTTTTTCTATCTGTGTACTTTTTTTTAAGGGAAAGATTTT 5572
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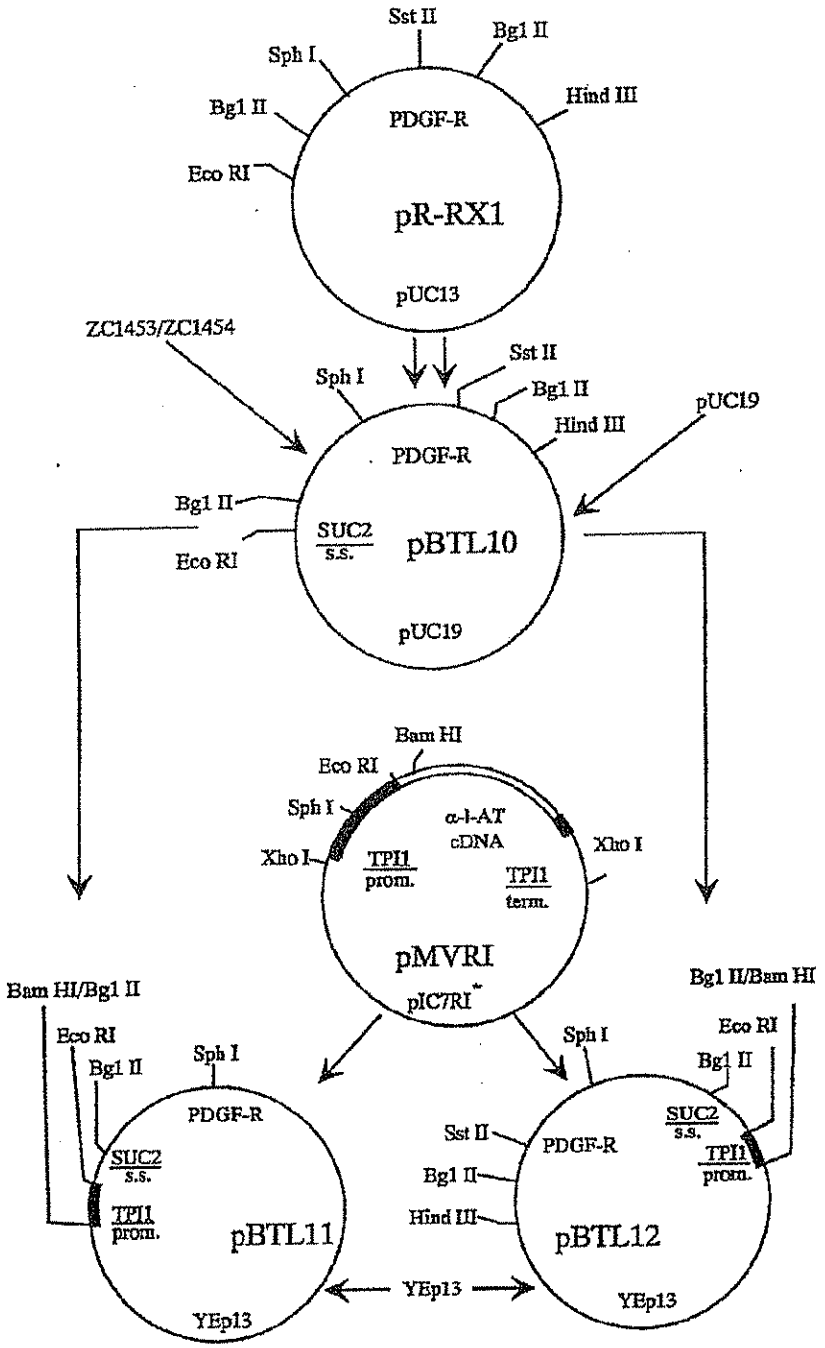
Fig. 1D

Figure 2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Eco RI
Sph I
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cDNA
Xho I
Xho I
TPI1
prom.
TPI1
term.
pMVRI
pIC7RI
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Bg1 II/Bam HI
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Sph I
Sph I
Eco RI
Bg1 II
Bg1 II
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SUC2
s.s.
Sst II
PDGF-R
SUC2
s.s.
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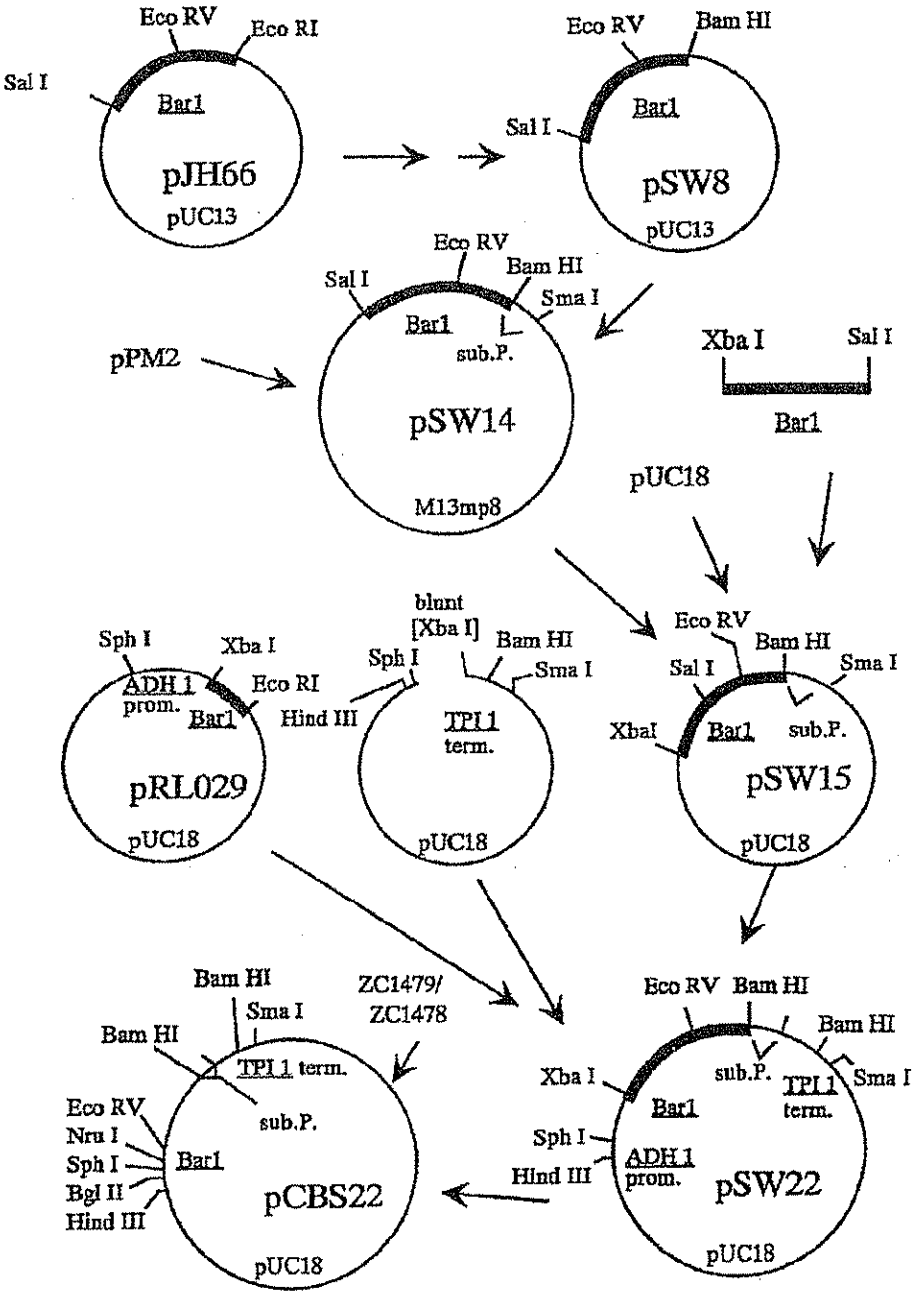
YEp13

Figure 3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M13mp8
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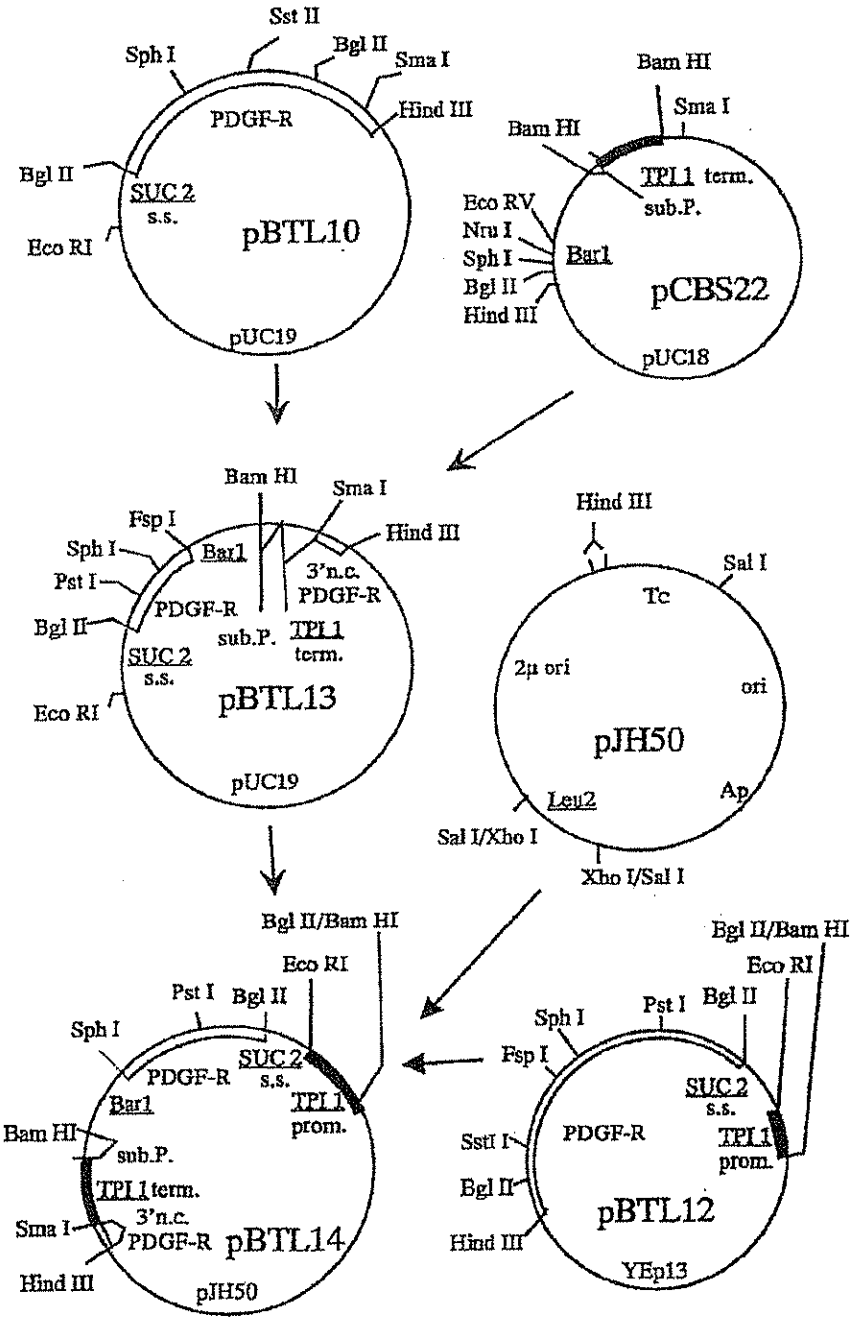
Figure 4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Bgl II
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Bar1
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Fsp I
Sph I
Bar1
Hind III
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PDGF-R
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term.
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2μ ori
ori
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Leu2
Ap
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Xho I/Sal I
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Bgl II
Sph I
SUC 2
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prom.
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Pst I
Bgl II
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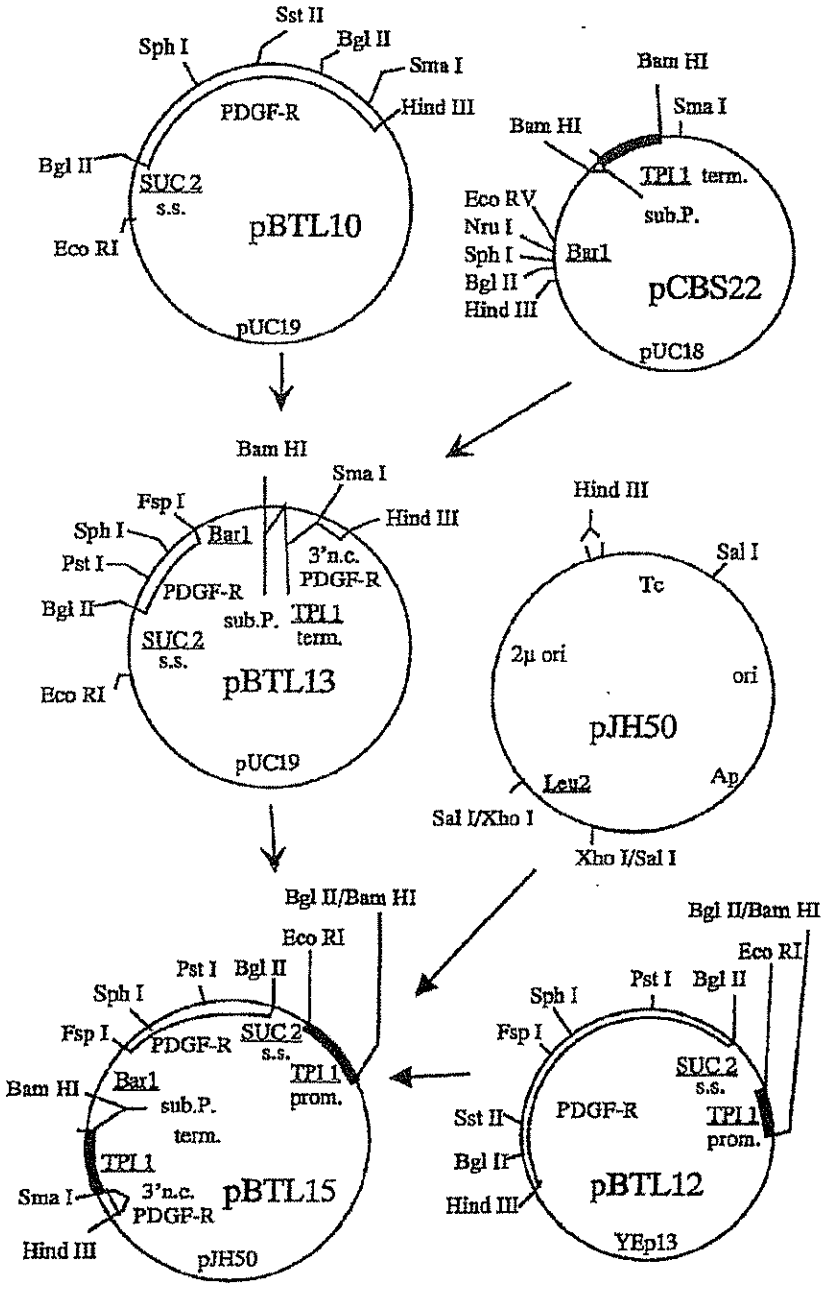
Hind III
YEp13

Figure 5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Bar1
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Fsp I
Sph I
Bar1
Hind III
Pst I
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PDGF-R
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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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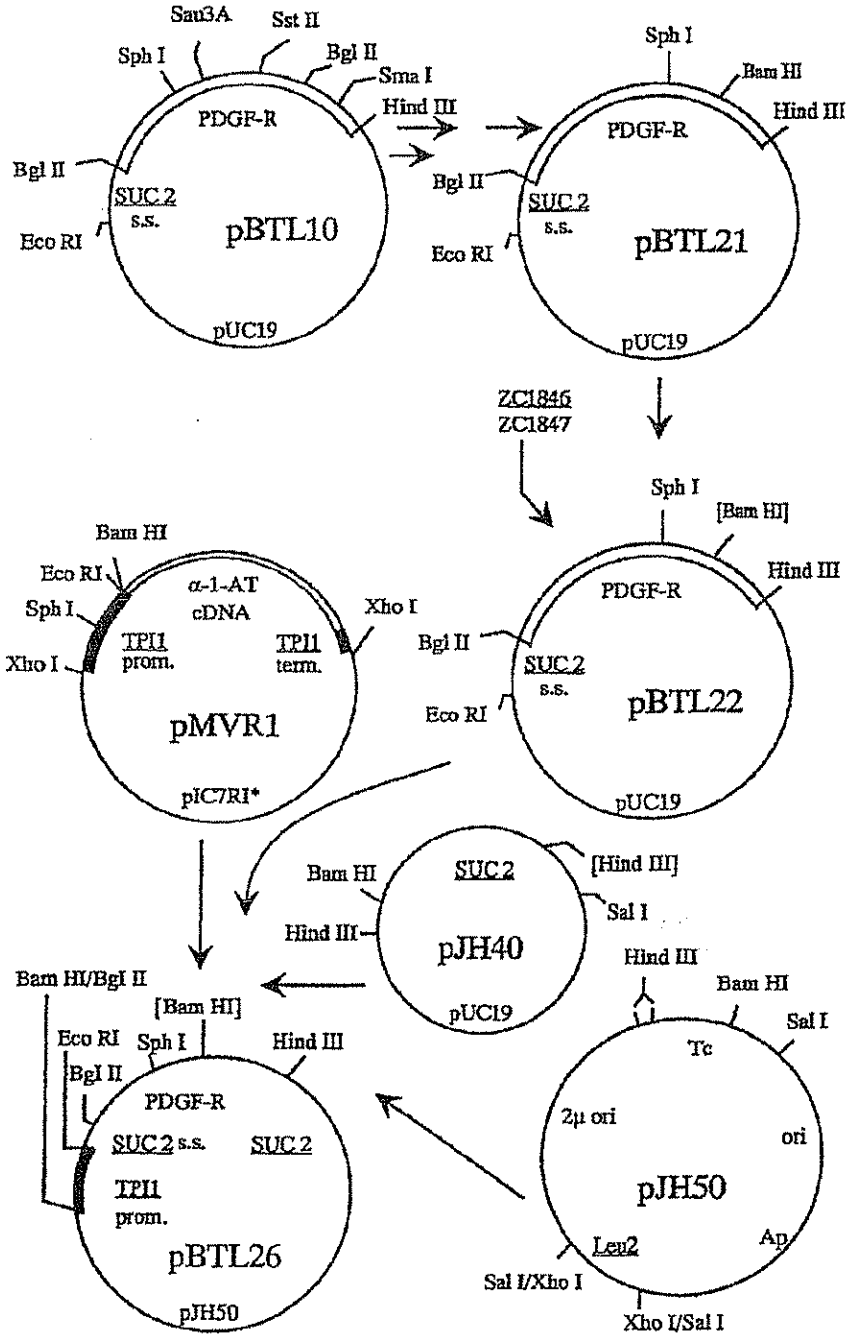
Figure 6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ori
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Leu2
Ap
Sal I/Xho I
Xho I/Sal I

Figure 7

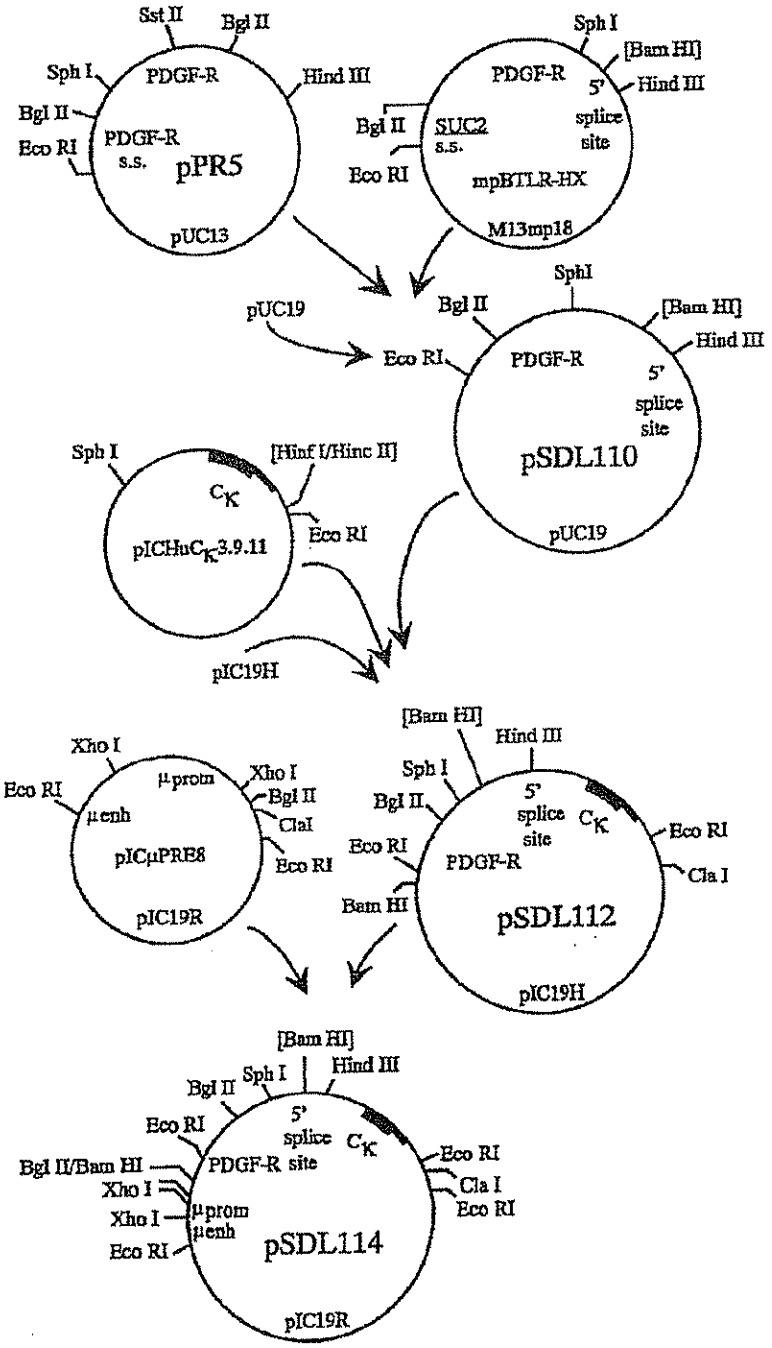
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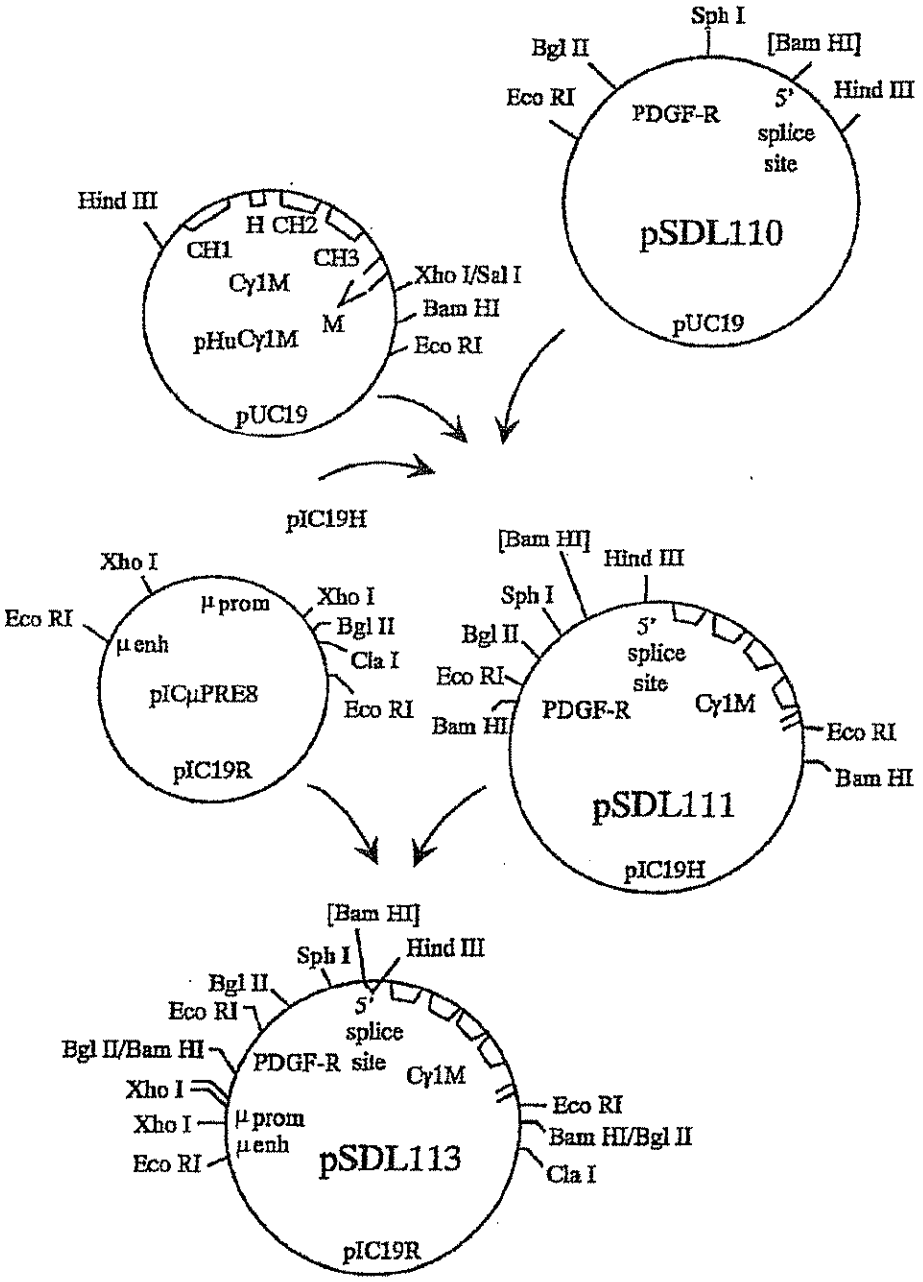
Figure 8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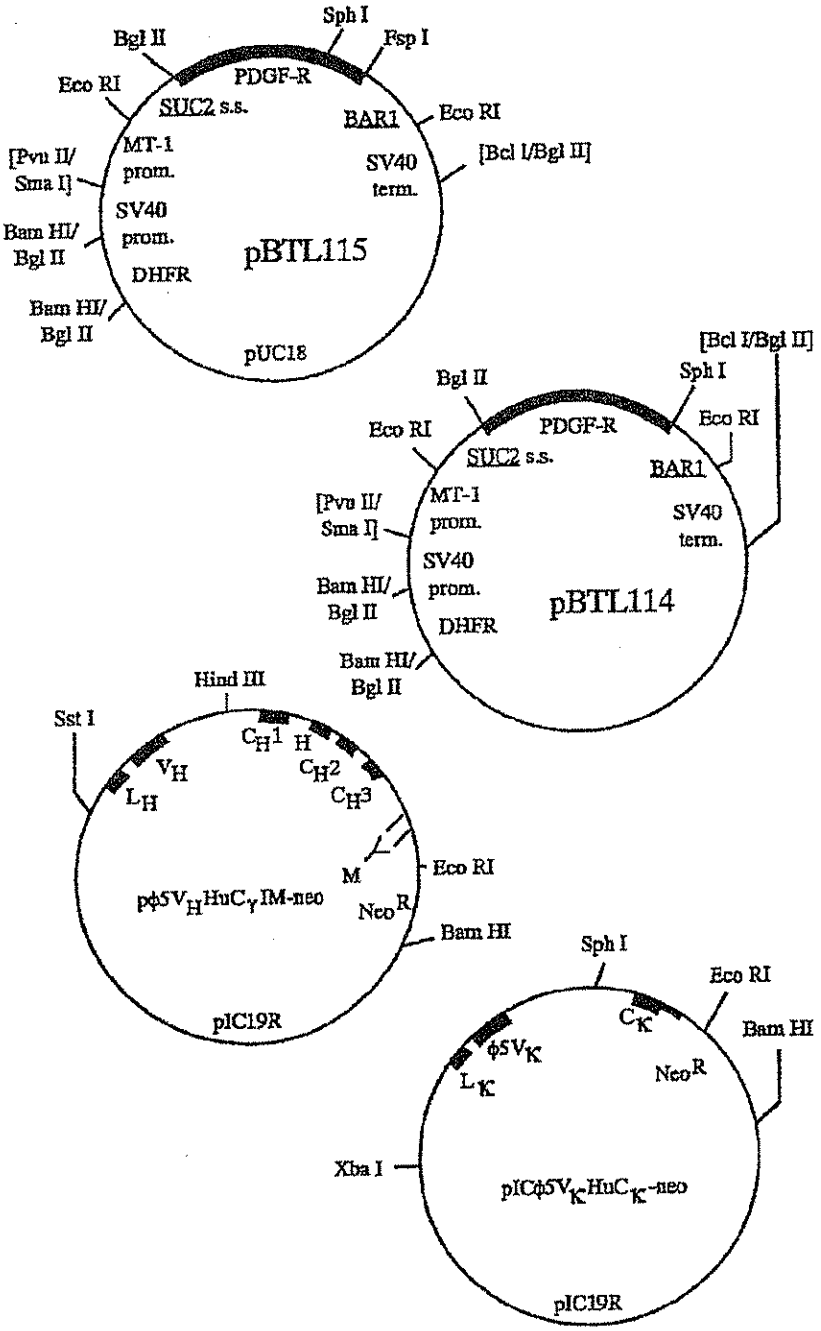
Bam HI
PDGF-R
Cγ1M
Eco RI
Bam HI
pSDL111
pIC19H
[Bam HI]
Sph I
Hind III
Bgl II
Eco RI
5'
splice
Bgl II/Bam HI
PDGF-R site
Cγ1M
Xho I
Eco RI
Xho I
μ prom
Bam HI/Bgl II
μ enh
pSDL113
Eco RI
Cla I
pIC19R

Figure 9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Bgl II
Fsp I
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Eco RI
SUC2 s.s.
BAR1
Eco RI
[Pvu II/
Sma I]
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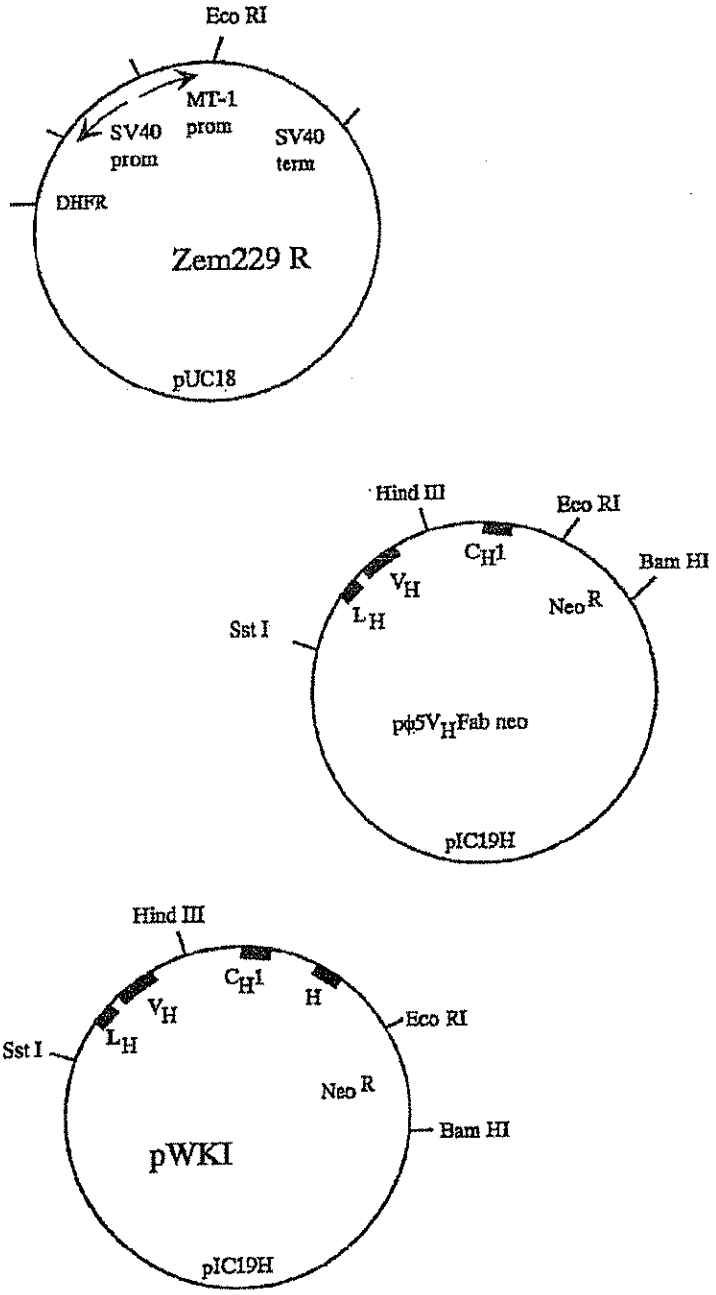
prom.
SV40
term.
[Bcl I/Bgl II]
SV40
prom.
Bam HI/
Bgl II
pBTL115
DHFR
Bam HI/
Bgl II
pUC18
[Bcl I/Bgl II]
Bgl II
Sph I
Eco RI
PDGF-R
Eco RI
SUC2 s.s.
BAR1
[Pvu II/
Sma I]
MT-1
prom.
SV40
term.
SV40
prom.
Bam HI/
Bgl II
pBTL114
DHFR
Bam HI/
Bgl II
Hind III
Sst I
C_H1
H
V_H
C_H2
L_H
C_H3
M
Eco RI
$p\phi5V_HHuC_\gamma IM$-neo
Neo^R
Bam HI
pIC19R
Sph I
Eco RI
C_K
Bam HI
$\phi5V_K$
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
Bam HI
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L_H
Neo^R
Sst I
pφ5V_HFab neo
pIC19H
Hind III
C_H1
H
V_H
Eco RI
L_H
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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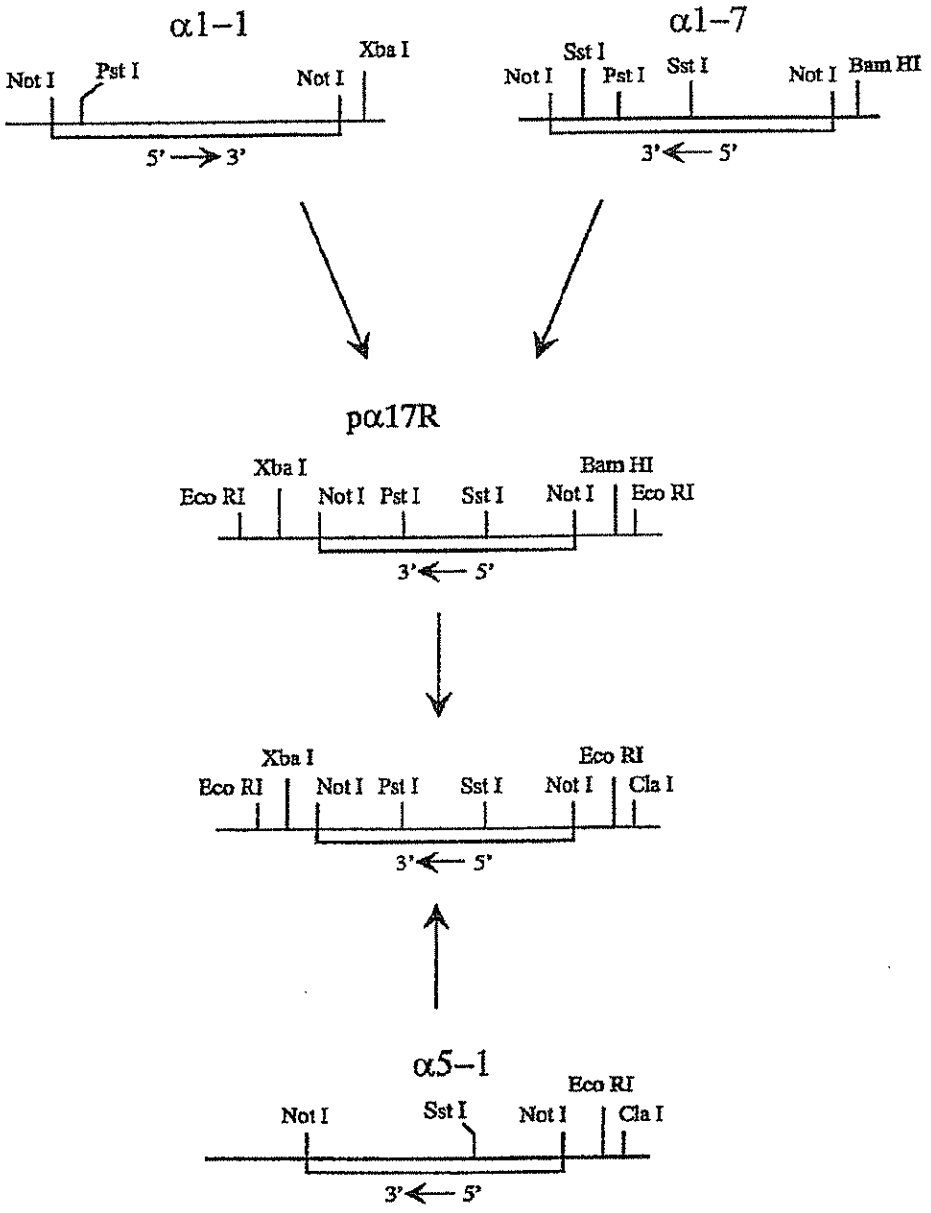
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Not I
Pst I
Not I
Xba I
5' → 3'
α1–7
Not I
Sst I
Pst I
Sst I
Not I
Bam HI
3' ← 5'
pα17R
Eco RI
Xba I
Not I
Pst I
Sst I
Not I
Bam HI
Eco RI
3' ← 5'
Eco RI
Xba I
Not I
Pst I
Sst I
Not I
Eco RI
Cla I
3' ← 5'
α5–1
Not I
Sst I
Not I
Eco RI
Cla I
3' ← 5'

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 4×10^5; 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_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 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_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_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 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_k, 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, $p\phi5V_HHuC_\gamma1M$-neo, $plC\phi5V_\kappa HuC_\kappa$-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_K, mouse immunoglobulin light chain signal sequence; $\phi5V_K$, 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 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 monomuclear 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 (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. calsberaensis* 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 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 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 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), 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" 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 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, 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

ZC871 (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 pUCtype 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

EXHIBIT 1
Part 2

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° OC. 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 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 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 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_{Tyr} 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_{Tyr} 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 100xPSN (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} tsl3 BHK Cells

Plasmids pBTL114 and pBTL115 were each transfected into tk^{13}-tsl3 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 pICHu$C_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 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 $V_H/D/J_H$ 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-i 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$_{65}$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$huC$_{65}$ 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$_{Tyr}$ (Example 18.F.) diluted in DMEM+10% fetal calf serum to the identical aliquots of receptor-bound beads to final PDGF-BB$_{Tyr}$ 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 pICu$_γ$-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_H1 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 immmunoglobulin 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 pWK1, which contained the Fab' portion of an immunoglobulin heavy chain gene. Plasmid pWK1 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 $p\phi 5V_H$ 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 pWK1 (FIG. 10).

An SP2/0-AG14 clone stably transfected with pSDL114 and p416 was transfected with Asp 718-linearized pWK1. 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 0.1M 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$_{Tyr}$ (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$_{Tyr}$ bound by the receptor analog. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-BB$_{Tyr}$ 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$_{Tyr}$, 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$_{Tyr}$ (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 PICμPRE8 (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 $_{\kappa}$ 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$_H$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 plCneo 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^{-11}
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 ^{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 0.1M 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 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 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, Melvern, 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.

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 0.1M 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:

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

(i i) MOLECULE TYPE: cDNA

(i i i) HYPOTHETICAL: N

(i v) ANTI-SENSE: N

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

(v i i) IMMEDIATE SOURCE:
(B) CLONE: pR-eX1

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

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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CCCTCAGCCC TGCTGCCCAG CACGAGCCTG TGCTCGCCCT GCCCAACGCA GACAGCCAGA      60
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
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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 GGT 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

ACC TGC AAT GTG ACG GAG AGT GTG AAT GAC CAT CAG GAT GAA AAG GCC         1268
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
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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 GAG TTT GAG GTG GTG AGC ACA CTG CGT CTG      1844
Tyr Trp Glu Glu Glu Gln Glu 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 ATC CTT TGG CAG AAG AAG CCA CGT          2036
Ile Ile Ser Leu Ile Ile Leu Ile Ile 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 Glu 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

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

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

Thr Tyr Trp Gln 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 Gln 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

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 Gln 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 Gln 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: 56 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: ZC904

(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: ZC906

(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: ZC906

(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 CTACCAGGAA 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: ZC1776

(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: ZC1846

(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) 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) 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



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(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: ZC21B4

(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: ZC23L1

(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: ZC23S1

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:28:

CGAATTCCAC 10

(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

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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: ZC2352

(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: ZC2392

(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: 205..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 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 CAG GAG ACC ATC GCC GTG CGA TGC CTG GCT AAG AAT      1719
Phe Ala Lys Val Gln 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 ACGTCTGGAT 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:

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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 Gln 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 Gln 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

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

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_H4 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_H4, 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_H4 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_H4 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 2
Part 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)

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Patent No.:	6,018,026
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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_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 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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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  GCGGCCCCTCTGGCGGCTCTGCTCCCTCCCGAAGGATGCTTGGGGAGTGAGGCGAAGCTGGGCGCTCCTC  138
 139  TCCCCTACAGCAGCCCCCTTCCTCCATCCCTCTGTTCTCCTGAGCCTTCAGGAGCCTGCACCAGTCCTG  207
 208  CCTGTCCTTCTACTCAGCTGTTACCCACTCTGGGACCAGCAGTCTTTCTGATAACTGGGAGAGGGCAGT  276
 277  AAGGAGGACTTCCTGGAGGGGGTGACTGTCCAGAGCCCTGGAACTGTGCCCACACCAGAAGCCATCAGCA  345

                M  R  L  P  G  A  M  P  A  L  L  A  L  K  G  E  L  L  L  L  L  S
 346  GCAAGGACACCATGCGGCTTCCGGGTGCGATGCCAGCTCTGGCCCTCAAAGGCGAGCTGCTGTTGCTGT  414
                L  L  L  L  E  P  Q  I  S  Q  G  L  V  V  T  P  P  G  P  E  L  V
 415  CTCTCCTGTTACTTCTGGAACCACAGATCTCTCAGGGCCTGGTCGTCACACCCCCGGGGCCAGAGCTTG  483
                L  N  V  S  S  T  F  V  L  T  C  S  G  S  A  P  V  V  W  E  R  M  S
 484  TCCTCAATGTCTCCAGCACCTTCGTTCTGACCTGCTCGGGTTCAGCTCCGGTGGTGTGGGAACGGATGT  552
                Q  E  P  P  Q  E  M  A  K  A  Q  D  G  T  F  S  S  V  L  T  L  T  N
 553  CCCAGGAGCCCCCACAGGAAATGGCCAAGGCCCAGGATGGCACCTTCTCCAGCGTGCTCACACTGACCA  621
                L  T  G  L  D  T  G  E  Y  F  C  T  H  N  D  S  R  G  L  E  T  D  E
 622  ACCTCACTGGGCTAGACACGGGAGAATACTTTTGCACCCACAATGACTCCCGTGGACTGGAGACCGATG  690
                R  K  R  L  Y  I  F  V  P  D  P  T  V  G  F  L  P  N  D  A  E  E  L
 691  AGCGGAAACGGCTCTACATCTTTGTGCCAGATCCCACCGTGGGCTTCCTCCCTAATGATGCCGAGGAAC  759
                F  I  F  L  T  E  I  T  E  I  T  I  P  C  R  V  T  D  P  Q  L  V  V
 760  TATTCATCTTTCTCACGGAAATAACTGAGATCACCATTCCATGCCGAGTAACAGACCCACAGCTGGTGG  828
```

Fig. 1A

```
829  TGACACTGCACGAGAAGAAAGGGGACGTTGCACTGCCTGTCCCCTATGATCACCAACGTGGCTTTCTCTG  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 CCCGCAAGAAAGTGGGCGGCTGGTGGAGCCGGTGACTGACTTCCTCTTGGATATGCCTTACCACATCC 1173
       R  K  E  S  G  R  L  V  E  P  V  T  D  F  L  L  D  H  P  Y  M  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

1312 TGGGAGAGGTGGGCACACTACAATTTGCTGAGCTGCATCGGAGCCGGACACTGCAGGTAGTGTTCGAGG 1380
       G  E  V  G  T  L  Q  F  A  E  L  H  R  S  R  T  L  Q  V  V  F  E  A  342

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
```

Fig. 1B

```
1519  TGGCAGAGGCTGGCCACTACACCATGCGGGCCTTCCATGAGGATGCTGAGGTCCAGCTCTCCTTCCTTCCAGC 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  M  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  TGACGTACTGGGAGGAGGAGCAGGAGTTTGAGGTGGTGAGCACACTGCGTCTGCAGCACGTGGATCGGC 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  ACTCCTTGCCCTTTAAGGTGGTGATCTCAGCCATCCTGGCCCTGGTGCTCACCATCATCTCCC 2001
        S  L  P  F  K  V  V  V  I  S  A  I  L  A  L  V  V  L  T  I  I  S  L  549

2002  TTATCATCCTCATCATGCTTTGGCAGAAGCCACGTTACGAGATCCGATGGAAGGTGATTGAGTCTG 2070
        I  I  L  I  M  L  W  Q  K  K  P  R  Y  E  I  R  W  K  V  I  E  S  V  572

2071  TGAGCTCTGACGGCCATGAGTACATCTACGTGGACCCCATGCAGCCTGCCCTATGACTCCACGTGGGAGC 2139
        S  S  D  G  H  E  Y  I  Y  V  D  P  M  Q  L  P  Y  D  S  T  W  E  L  595

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
```

Fig. 1C

```
2209  CGGCTCATGGCCTGAGCCATTCTCAGGCCACGATGAAAGTGGCCGTCAAGATGCTTAAATCCACAGCCC 2277
        A  H  G  L  S  H  S  Q  A  T  M  K  V  A  V  K  M  L  K  S  T  A  R  641

2278  GCAGCAGTGAGAAGCAAGCCCTTATGTCGGAGCTGAAGATCATGAGTCACCTTGGGCCCCACCTGAACG 2346
        S  S  E  K  Q  A  L  M  S  E  L  K  I  M  S  H  L  G  P  H  L  N  V  664

2347  TGGTCAACCTGTTGGGGGCCTGCACCAAAGGAGGACCCATCTATATCATCACTGAGTACTGCCGCTACG 2415
        V  N  L  L  G  A  C  T  K  G  G  P  I  Y  I  I  T  E  Y  C  R  Y  G  687

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  CGCCCAGCGCGGAGCTCTACAGCAATGCTCTGCCCGTTGGGCTCCCCCTGCCCAGCCATGTGTCCTTGA 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

2554  CCGGGGAGAGCGACGGTGGCTACATGGACATGAGCAAGGACGAGTCGGTGGACTATGTGCCCATGCTGG 2622
        G  E  S  D  G  G  Y  M  D  M  S  K  D  E  S  V  D  Y  V  P  M  L  D  756

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  TTCCCTCTGCCCCTGAGAGGACCTGCCGAGCAACTTTGATCAACGAGTCTCCAGTGCTAAGCTACATGG 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
```

Fig. 1D

```
2899  CTCGAGACATCATGCGGGACTCGAATTACATCTCCAAAGGCAGCACCTTTTTGCCTTTAAAGTGGATGG 2967
        R  D  I  M  R  D  S  N  Y  I  S  K  G  S  T  F  L  P  L  K  W  M  A  871

2968  CTCCGGAGAGCATCTTCAACAGCCTCTACACCACCCTGAGCGACGTGTGGTCCTTCGGGATCCTGCTCT 3036
        P  E  S  I  F  N  S  L  Y  T  T  L  S  D  V  W  S  F  G  I  L  L  W  894

3037  GGGAGATCTTCACCTTGGGTGGCACCCCTTACCCAGAGCTGCCCATGAACGAGCAGTTCTACAATGCCA 3105
        E  I  F  T  L  G  G  T  P  Y  P  E  L  P  M  N  E  Q  F  Y  N  A  I  917

3106  TCAAACGGGGTTACCGCATGGCCCAGCCTGCCCATGCCTCCCGACGAGATCTATGAGATCATGCAGAAGT 3174
        K  R  G  Y  R  M  A  Q  P  A  H  A  S  D  E  I  Y  E  I  H  Q  K  C  940

3175  GCTGGGAAGAGAAGTTTGAGATTCGGCCCCCCTTCTCCCAGCTGGTGCTGCTTCTCGAGAGACTGTTGG 3243
        W  E  E  K  F  E  I  R  P  P  F  S  Q  L  V  L  L  L  E  R  L  L  G  963

3244  GCGAAGGTTACAAAAAGAAGTACCAGCAGGTGGATGAGGAGTTTCTGAGGAGTGACCACCCAGCCATCC 3312
        E  G  Y  K  K  K  Y  Q  Q  V  D  E  E  F  L  R  S  D  H  P  A  I  L  986

3313  TTCGGTCCCAGGCCCGCTTGCCTGGGTTCCATGGCCTCCGATCTCCCCTGGACACCAGCTCCGTCCTCT 3381
        R  S  Q  A  R  L  P  G  F  H  G  L  R  S  P  L  D  T  S  S  V  L  Y  1009

3382  ATACTGCCGTGCAGCCCAATGAGGGTGACAACGACTATATCATCCCCCTGCCTGACCCCAAACCCGAGG 3450
        T  A  V  Q  P  N  E  G  D  N  D  Y  I  I  P  L  P  D  P  K  P  E  V  1032

3451  TTGCTGACGAGGGCCCACTGGAGGGTTCCCCCAGCCTAGCCAGCTCCACCCTGAATGAAGTCAACACCT 3519
        A  D  E  G  P  L  E  G  S  P  S  L  A  S  S  T  L  N  E  V  N  T  S  1055

3520  CCTCAACCATCTCCTGTGACAGCCCCCCTGGAGCCCCAGGACGAACCAGAGCCAGAGCCCCAGCTTGAGC 3588
        S  T  I  S  C  D  S  P  L  E  P  Q  D  E  P  E  P  E  P  Q  L  E  L  1078
```

Fig. 1E

```
3589  TCCAGGTGGAGCCGGAGCCAGAGCTGCCGGATTCGGGGTGCCCTGCGCCTCGGGCGGAAG 3657
        Q  V  E  P  E  P  E  L  E  Q  L  P  D  S  G  C  P  A  P  R  A  E  A 1101
3658  CAGAGGATAGCTTCCTGTAGGGGGCTGGCCCCTACCCTGCCCTGAAGCTCCCCCCCCTGCCAGCAC 3726
        E  D  S  F  L                                                    1106
3727  CCAGCATCTCCTGGCCCTGGCCCTGACCGGGCTTCCTGTCAGCCAGGCTGCCCTTATCAGCTGTCCCCTTC 3795
3796  TGGAAGCTTTCTGCTCCCTGACGTGTTGTGCCCCCAAACCCTGGGGCTGGCTTAGGAGGCAAGAAAACTGC 3864
3865  AGGGGCCGTGACCAGCCCCTCTGCCTCCAGGGAGGCCAACTGACTCTGAGCCAGGGTTCCCCCAGGGAAC 3933
3934  TCAGTTTTCCCATATGTAAGATGGGAAAGTTAGGCTTGATGACCCAGAATCTAGGATTCTCTCCCTGGC 4002
4003  TGACACGGTGGGGAGACCGAATCCCTCCCTGGGAAGATTCTTGGAGTTACTGAGGTGGTAAATTAACAT 4071
4072  TTTTTCTGTTCAGCCAGCTACCCCTCAAGGAATCATAGCTCTCTCCTCGCACTTTTTATCCACCCAGGA 4140
4141  GCTAGGGAAGAGACCCTAGCCTCCCTGGCTGCTGGCTGAGCTAGGGCCTAGCTTGAGCAGTGTTGCCTC 4209
4210  ATCCAGAAGAAAGCCAGTCTCCCTCCCTATGATGCCAGTCCCTGCGTTCCCTGGCCCGAGCTGGTCTGGG 4278
4279  GCCATTTAGGCAGCCTAATTAATGCTGGAGGCTGAGCCAAGTACAGGACACCCCCAGCCTGCAGCCCTTG 4347
4348  CCCAGGGCACTTGGAGCACACGCAGCCATAGCAAGTGCCTGTGTCCCTGTCCTTCAGGCCCATCAGTCC 4416
4417  TGGGGCTTTTTCTTTATCACCCTCAGTCTTAATCCATCCACCAGAGTCTAGAAGGCCAGACGGGCCCCG 4485
4486  CATCTGTGATGAGAATGTAAATGTGCCAGTGTGGAGTGGCCACGTGTGTGTGCCAGTATATGGCCCTGG 4554
```

Fig. 1F

```
4555  CTCTGCATTGGACCTGCTATGAGGCTTTGGAGGAATCCCTCACCCTCTCTGGGCCTCAGTTTCCCCTTC  4623

4624  AAAAAATGAATAAGTCGGACTTATTAACTCTGAGTGCCTTGCCAGCACTAACATTCTAGAGTATTCCAG  4692

4693  GTGGTGCACATTTGTCCAGATGAAGCAAGGCCTATACCCTAAACTTCATCCTGGGGGTCAGCTGGGCTC  4761

4762  CTGGGAGATTCCAGATCACACATCACACTCTGGGGACTCAGGAACCATGCCCCTTCCCCAGGCCCCCAG  4830

4831  CAAGTCTCAAGAACACAGCTGCACAGGCCTTGACTTAGAGTGACAGCCGGTGTCCTGGAAAGCCCCAAG  4899

4900  CAGCTGCCCCAGGGACATGGGAAGACCACGGGACCTCTTTCACTACCCACGATGACCTCCGGGGGGTATC  4968

4969  CTGGGCAAAAAGGGACAAAGAGGGCAAATGAGATCACCTCCTGCAGCCCACCACTCCAGCACCTGTGCCG  5037

5038  AGGTCTGCGTCGAAGACAGAATGGACAGTGAGGACAGTTATGTCTTGTAAAGACAAGAAGCTTCAGAT  5106

5107  GGTACCCCAAGAAGGATGTGAGAGGTGGCTGCTTTGGAGTTTGCCCCCTCACCCCACCAGCTGCCCCAT  5175

5176  CCCTGAGGCATGCGCTCCATGGGGGTATGGTTTTTGTCACTGCCCAGACCTAGCAGTGACATCTCATTGT  5244

5245  CCCCAGCCCAGTGGGCATTGGAGGTGCCAGGGGAGTCAGGGTTGTAGCCAAGACGCCCCCGCACGGGGA  5313

5314  GGGTTGGGAAGGGGGGTGCAGGAAGCTCAACCCCCTCTGGGCACCAACCCTGCATTGCAGGTTGGCACCTT  5382

5383  ACTTCCCTGGGATCCCCAGAGTTGGTCCAAGGAGGGAGAGTGGGTTCTCAATACGGTACCAAAGATATA  5451

5452  ATCACCTAGGTTTACAAATATTTTTAGGACTCACGTTAACTCACATTTATACAGCAGAAATGCTATTTT  5520

5521  GTATGCTGTTAAGTTTTTCTATCTGTGTACTTTTTTTTTAAGGGAAAGATTTT                  5572
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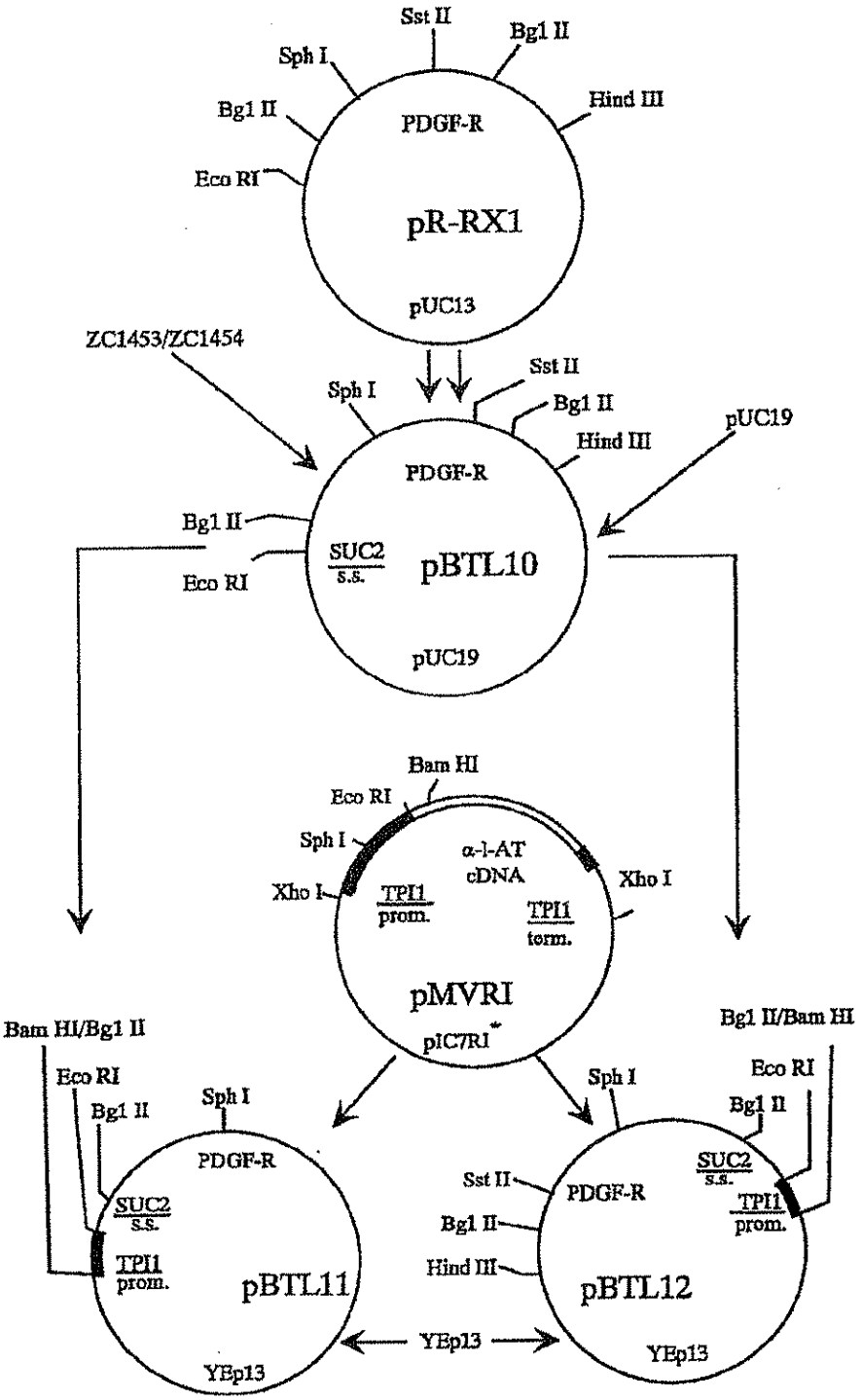
Fig. 1G

Figure 2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Xho I
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prom.
TPI1
term.
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Sph I
Eco RI
Bg1 II
Bg1 II
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S.S.
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SUC2
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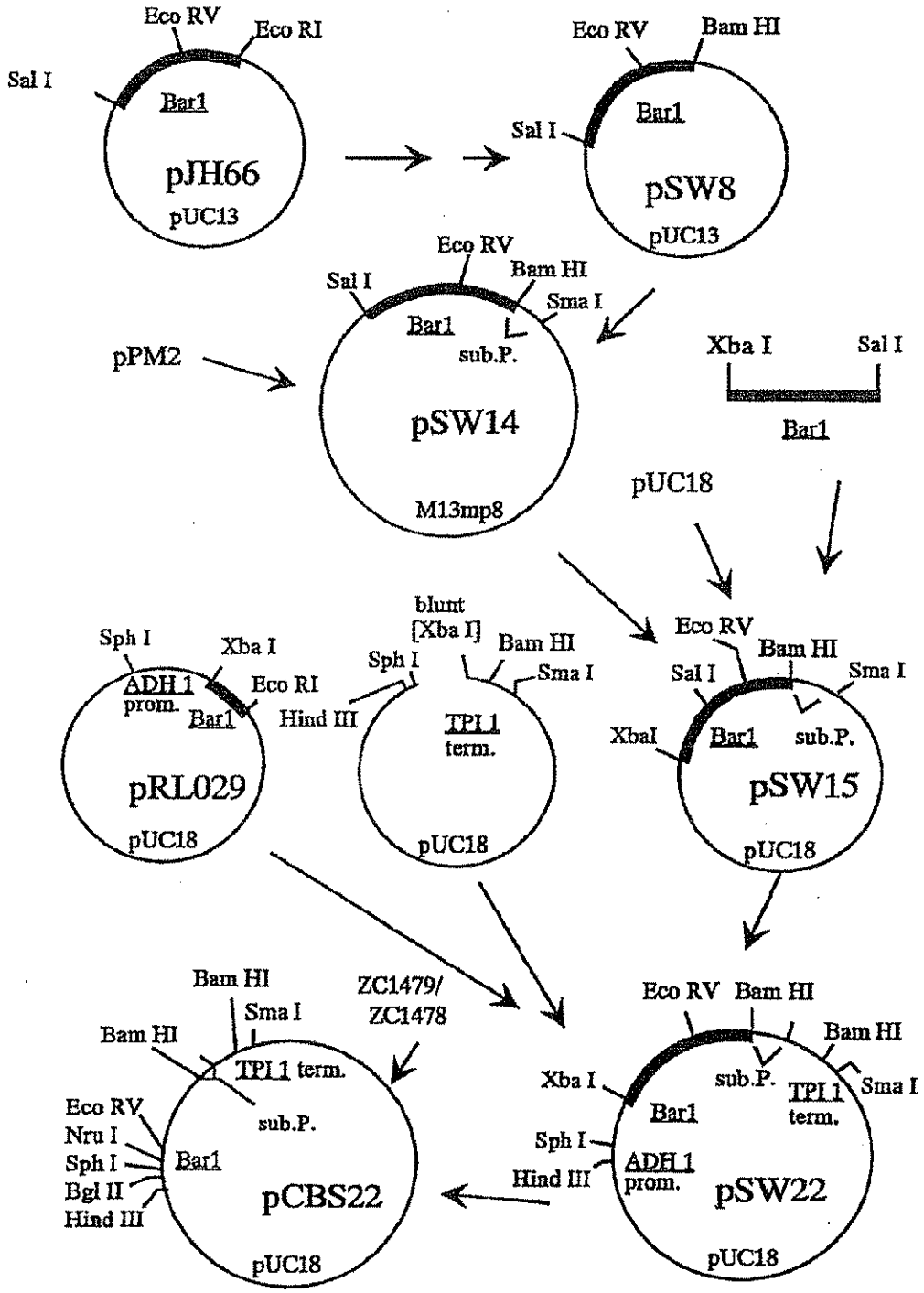
Figure 3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Bar1
sub.P.
pPM2
pSW14
M13mp8
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blunt
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pUC18
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Xba I
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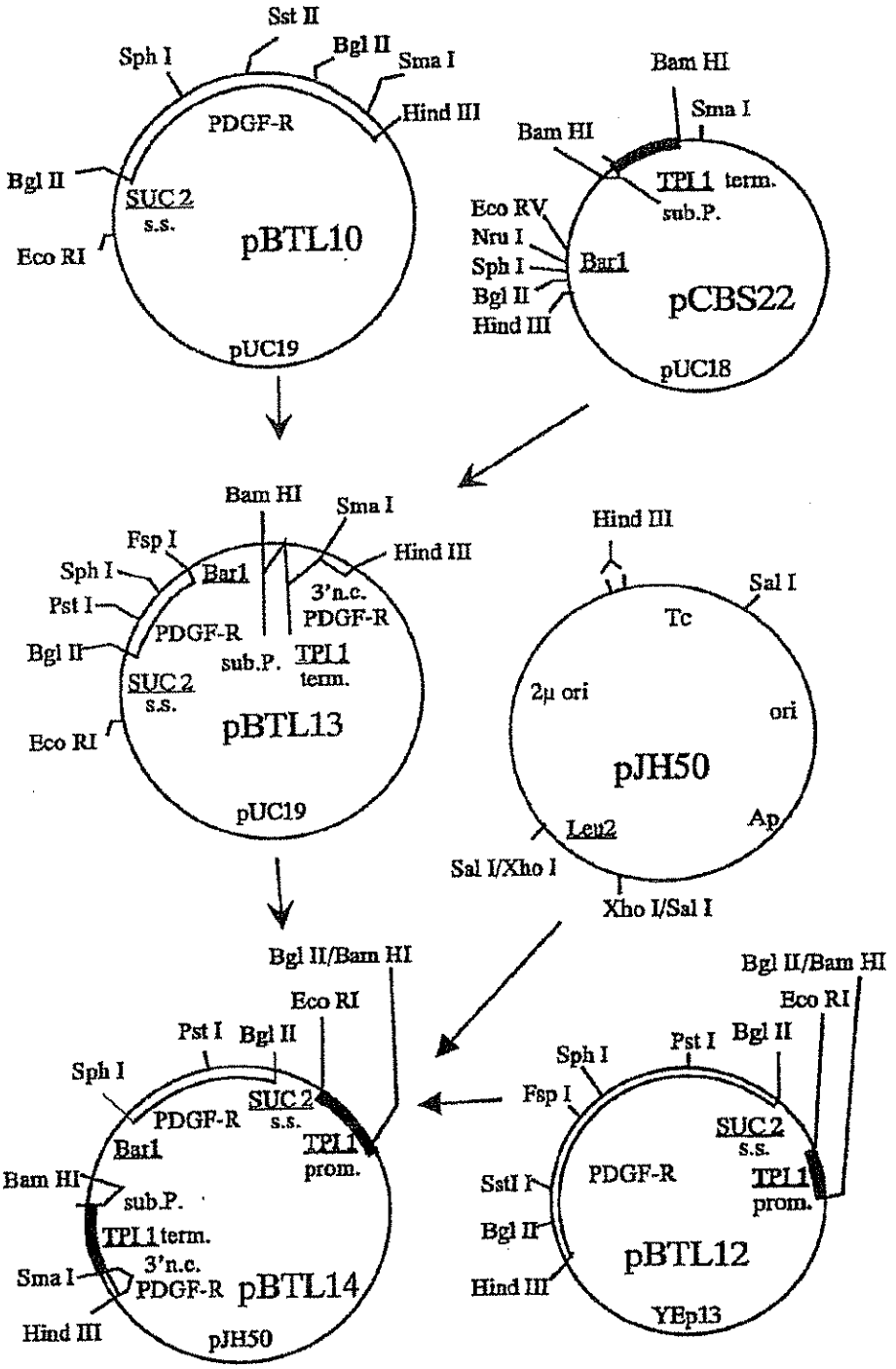
Figure 4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Sma I
Fsp I
Sph I
Pst I
Bgl II
Eco RI
Bar1
Hind III
3'n.c.
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PDGF-R
sub.P.
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term.
SUC 2
s.s.
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Tc
2μ ori
ori
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Leu2
Ap
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Xho I/Sal I
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Bgl II
Sph I
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prom.
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Bgl II
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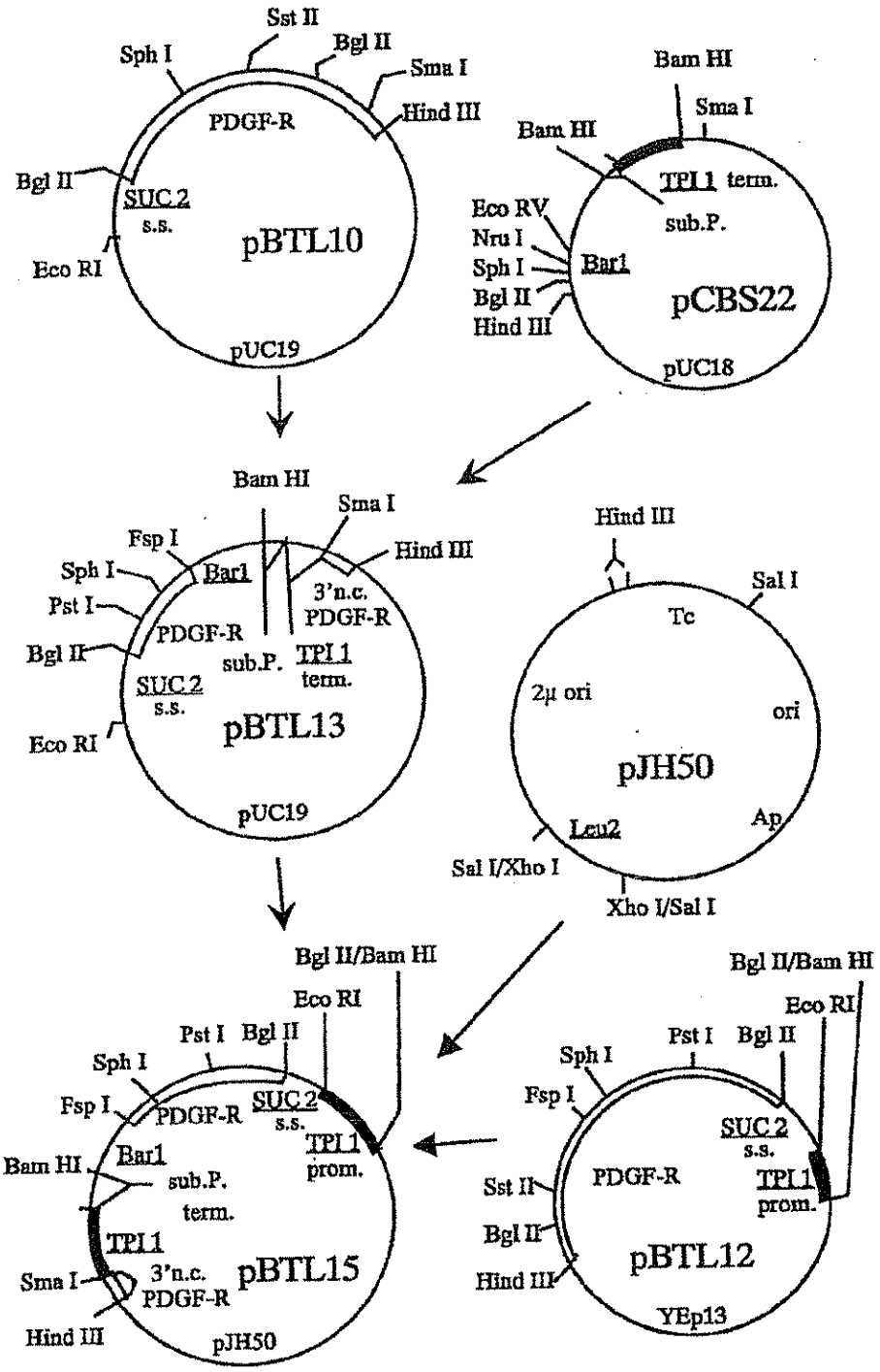
Figure 5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Sph I
Bar1
Hind III
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PDGF-R
Bgl II
sub.P.
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term.
SUC 2
s.s.
Eco RI
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Hind III
Sal I
Tc
2μ ori
ori
pJH50
Leu2
Ap
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Xho I/Sal I
Bgl II/Bam HI
Eco RI
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Bgl II
Sph I
Fsp I
PDGF-R
SUC 2
s.s.
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prom.
Bam HI
Bar1
sub.P.
term.
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Sma I
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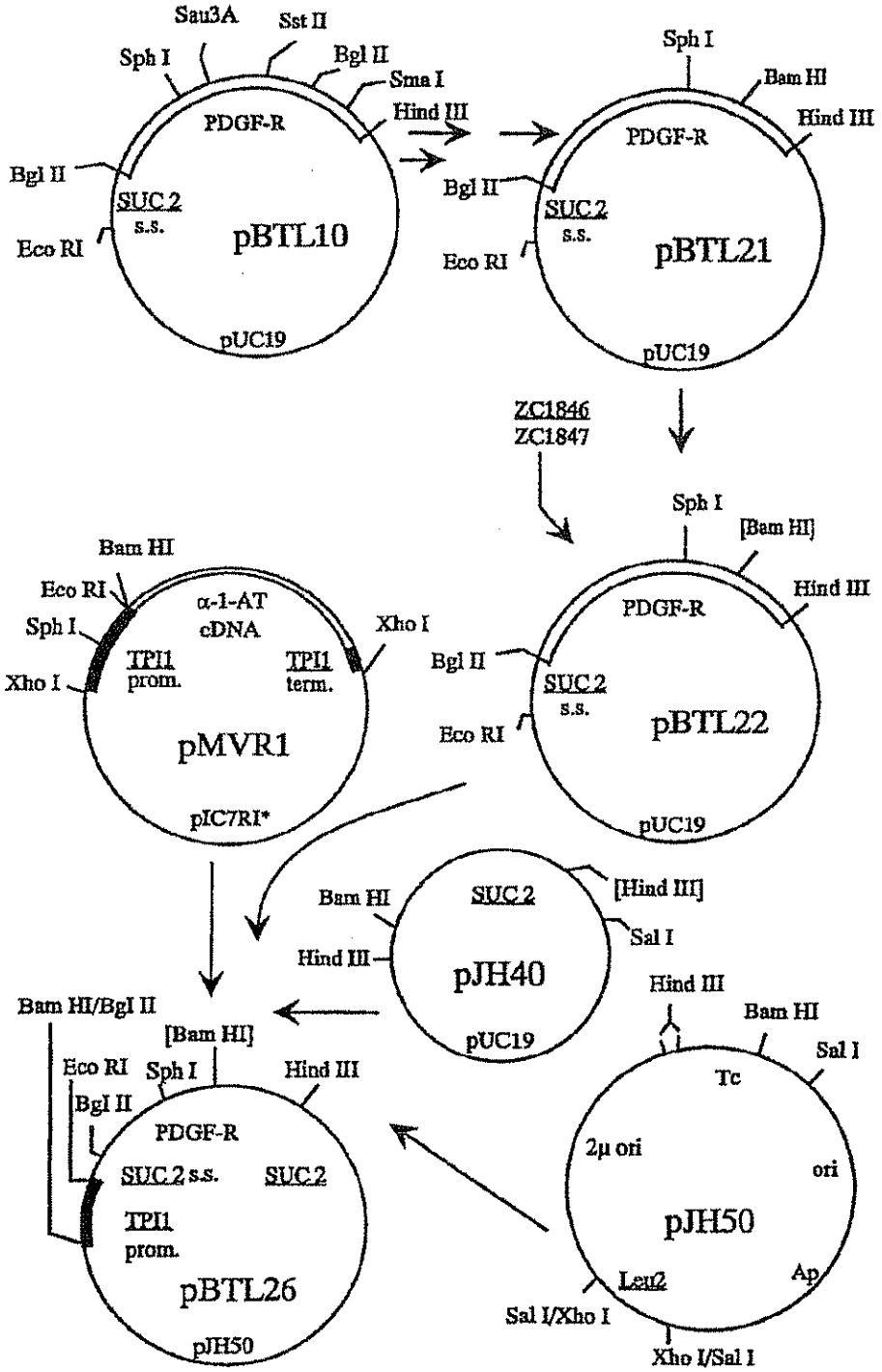
Figure 6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prom.
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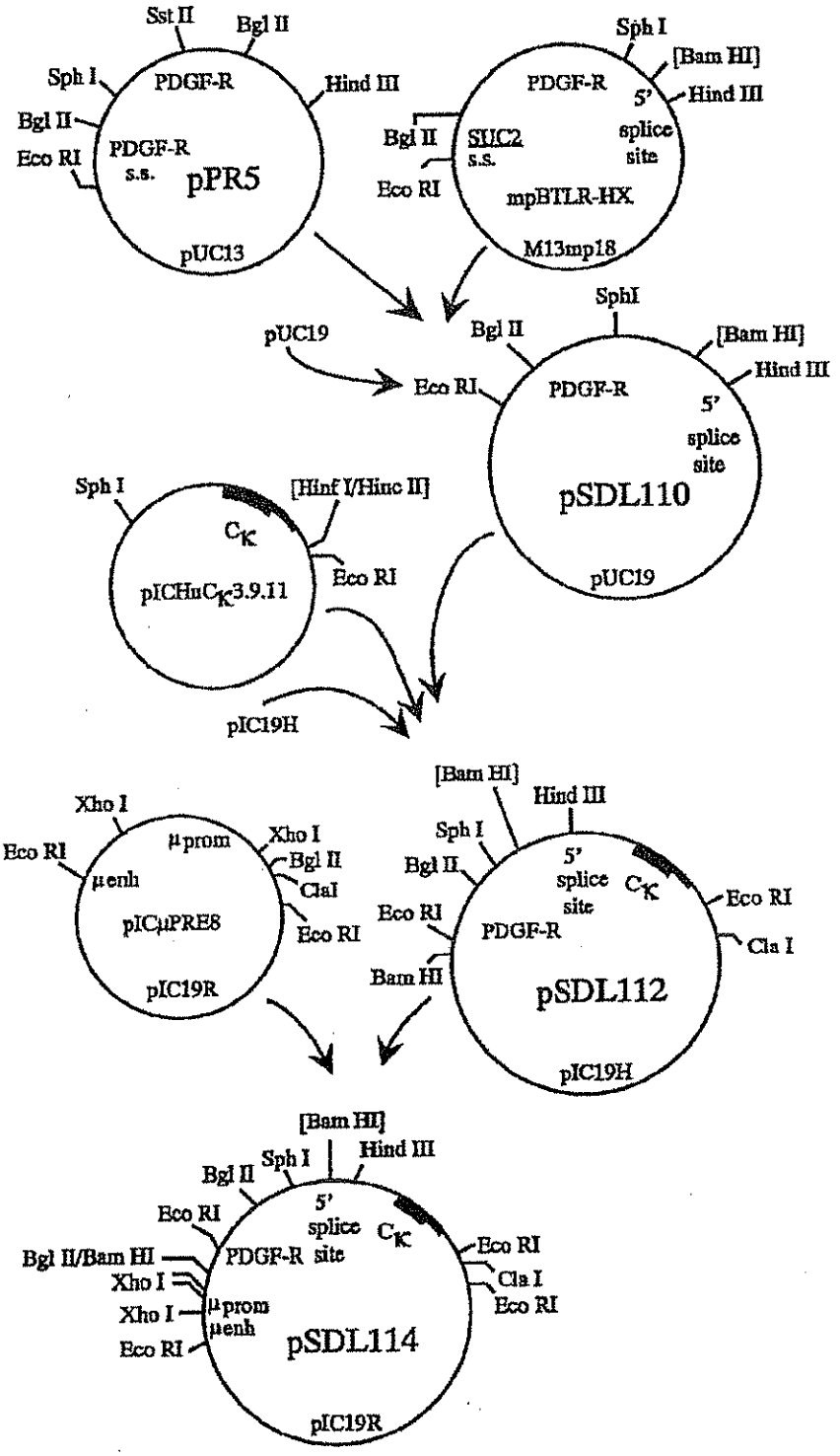
Bam HI
Sal I
Tc
2μ ori
ori
pJH50
Leu2
Ap
Sal I/Xho I
Xho I/Sal I

Figure 7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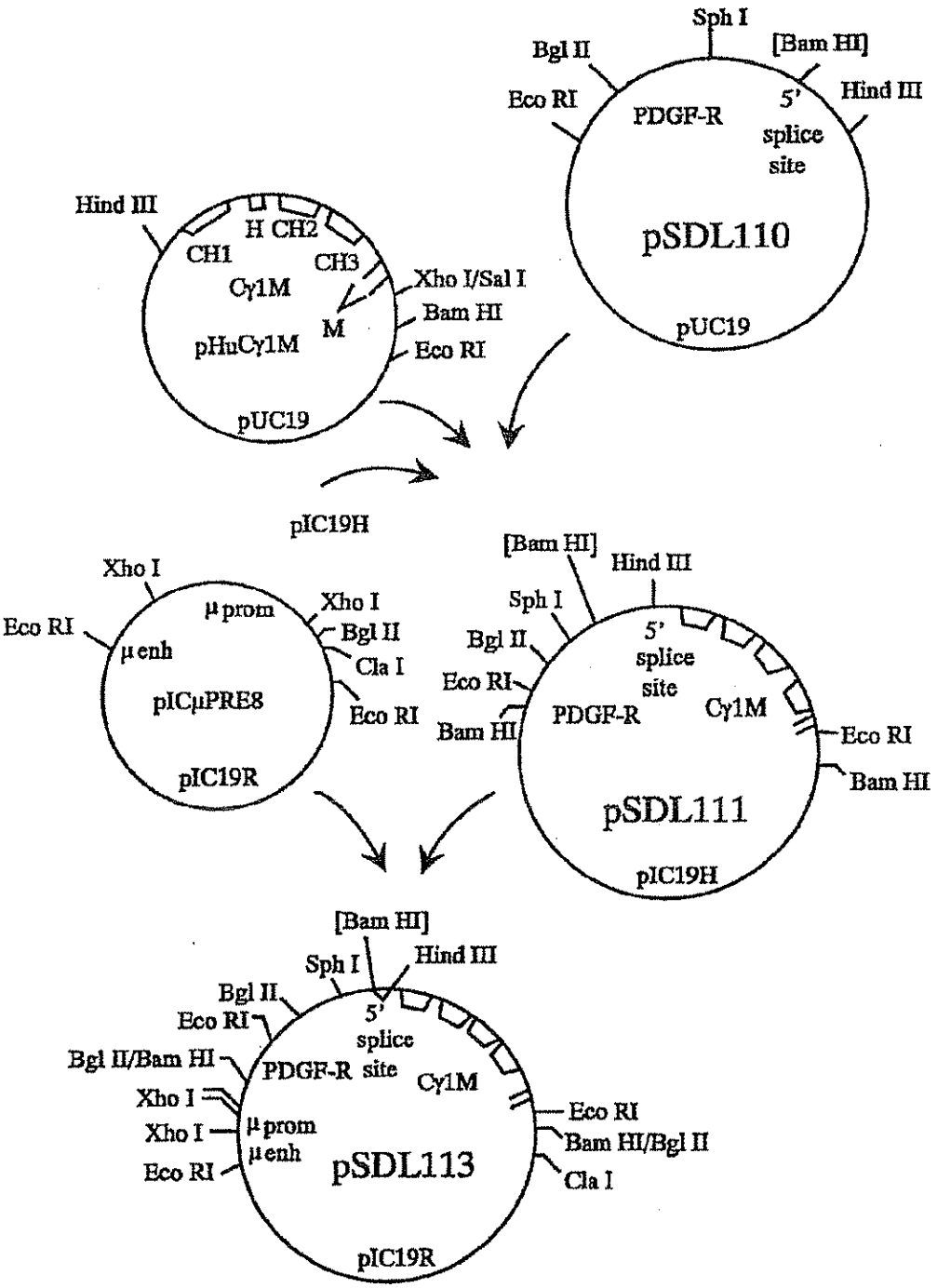
Sph I
PDGF-R
Hind III
Bgl II
PDGF-R
Eco RI
s.s.
pPR5
pUC13
Sph I
[Bam HI]
PDGF-R
5'
Hind III
Bgl II
SUC2
splice
site
s.s.
Eco RI
mpBTLR-HX
M13mp18
pUC19
SphI
Bgl II
[Bam HI]
Eco RI
PDGF-R
Hind III
5'
splice
site
pSDL110
pUC19
Sph I
[Hinf I/Hinc II]
C_K
Eco RI
pICHuC_K3.9.11
pIC19H
Xho I
Eco RI
μprom
Xho I
μenh
Bgl II
ClaI
Eco RI
pICμPRE8
pIC19R
[Bam HI]
Hind III
Sph I
Bgl II
5'
splice
site
C_K
Eco RI
Eco RI
PDGF-R
Cla I
Bam HI
pSDL112
pIC19H
[Bam HI]
Bgl II
Sph I
Hind III
5'
Eco RI
splice
C_K
Eco RI
Bgl II/Bam HI
PDGF-R
site
Cla I
Xho I
μprom
Eco RI
Xho I
μenh
pSDL114
Eco RI
pIC19R

Figure 8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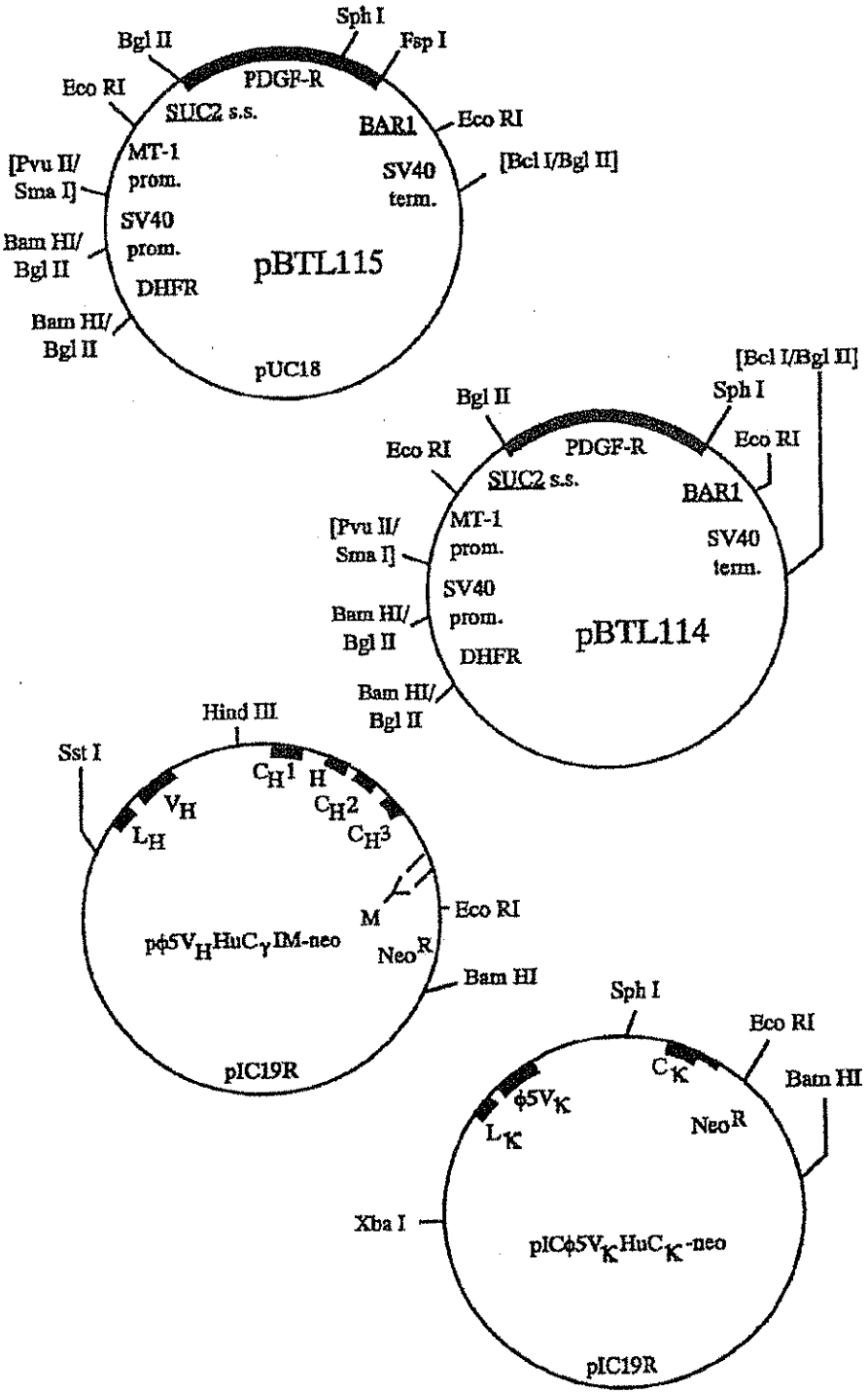
Bgl II
[Bam HI]
Eco RI
Hind III
PDGF-R
5'
splice
site
pSDL110
pUC19
Hind III
H CH2
CH1
CH3
Cγ1M
M
Xho I/Sal I
Bam HI
Eco RI
pHuCγ1M
pUC19
pIC19H
Xho I
Eco RI
μ prom
μ enh
Xho I
Bgl II
Cla I
Eco RI
pICμPRE8
pIC19R
[Bam HI]
Hind III
Sph I
Bgl II
Eco RI
Bam HI
5'
splice
site
PDGF-R
Cγ1M
Eco RI
Bam HI
pSDL111
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
μ prom
μ enh
Eco RI
Eco RI
Bam HI/Bgl II
Cla I
pSDL113
pIC19R

Figure 9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Eco RI
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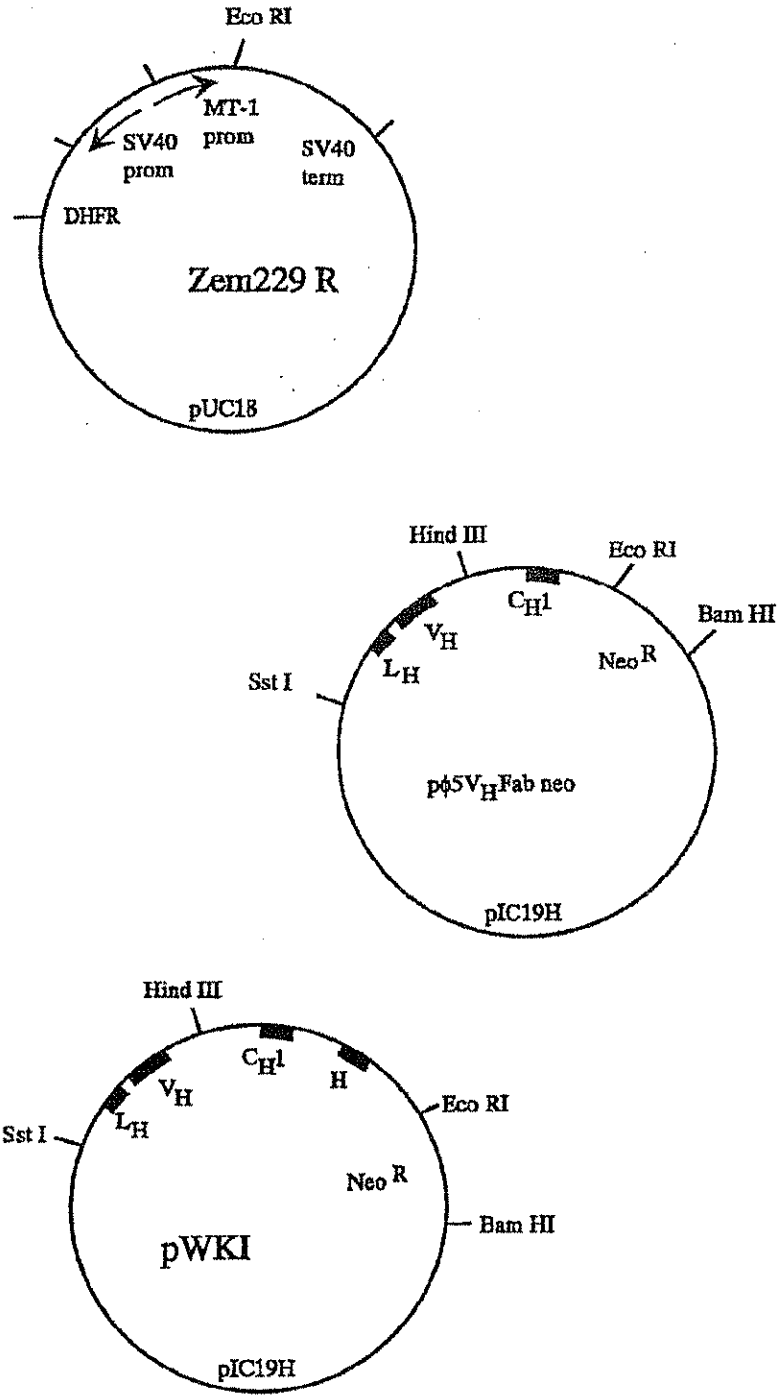
BAR1
Eco RI
MT-1
prom.
SV40
term.
[Bcl I/Bgl II]
[Pvu II/
Sma I]
SV40
prom.
Bam HI/
Bgl II
pBTL115
DHFR
Bam HI/
Bgl II
pUC18
[Bcl I/Bgl II]
Bgl II
Sph I
Eco RI
PDGF-R
Eco RI
SUC2 s.s.
BAR1
[Pvu II/
Sma I]
MT-1
prom.
SV40
term.
SV40
prom.
Bam HI/
Bgl II
pBTL114
DHFR
Bam HI/
Bgl II
Hind III
Sst I
C_H1
H
V_H
C_H2
L_H
C_H3
M
Eco RI
$p\phi5V_HHuC_\gamma IM$-neo
Neo^R
Bam HI
pIC19R
Sph I
Eco RI
C_K
Bam HI
$\phi5V_K$
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
Bam HI
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L_H
Neo^R
Sst I
pϕ5V_HFab neo
pIC19H
Hind III
C_H1
H
V_H
Eco RI
L_H
Sst I
Neo^R
Bam HI
pWKI
pIC19H

Figure 11A

```
  1 GCCCTGGGGACGGACCGTGGGCGGCGCGCAGCGGCGGGACGCGTTTTGGGGACGTGGTGGCCAGCGCCT
 70 TCCTGCAGACCCACAGGGAAGTACTCCCTTTGACCTCCGGGGAGCTGCGACCAGGTTATACGTTGCTGG

139 TGGAAAAGTGACAATTCTAGGAAAAGAGCTAAAAGCCGGATCGGTGACCGAAAGTTTCCCAGAGCTATG
                                                                      M
                                                                      I

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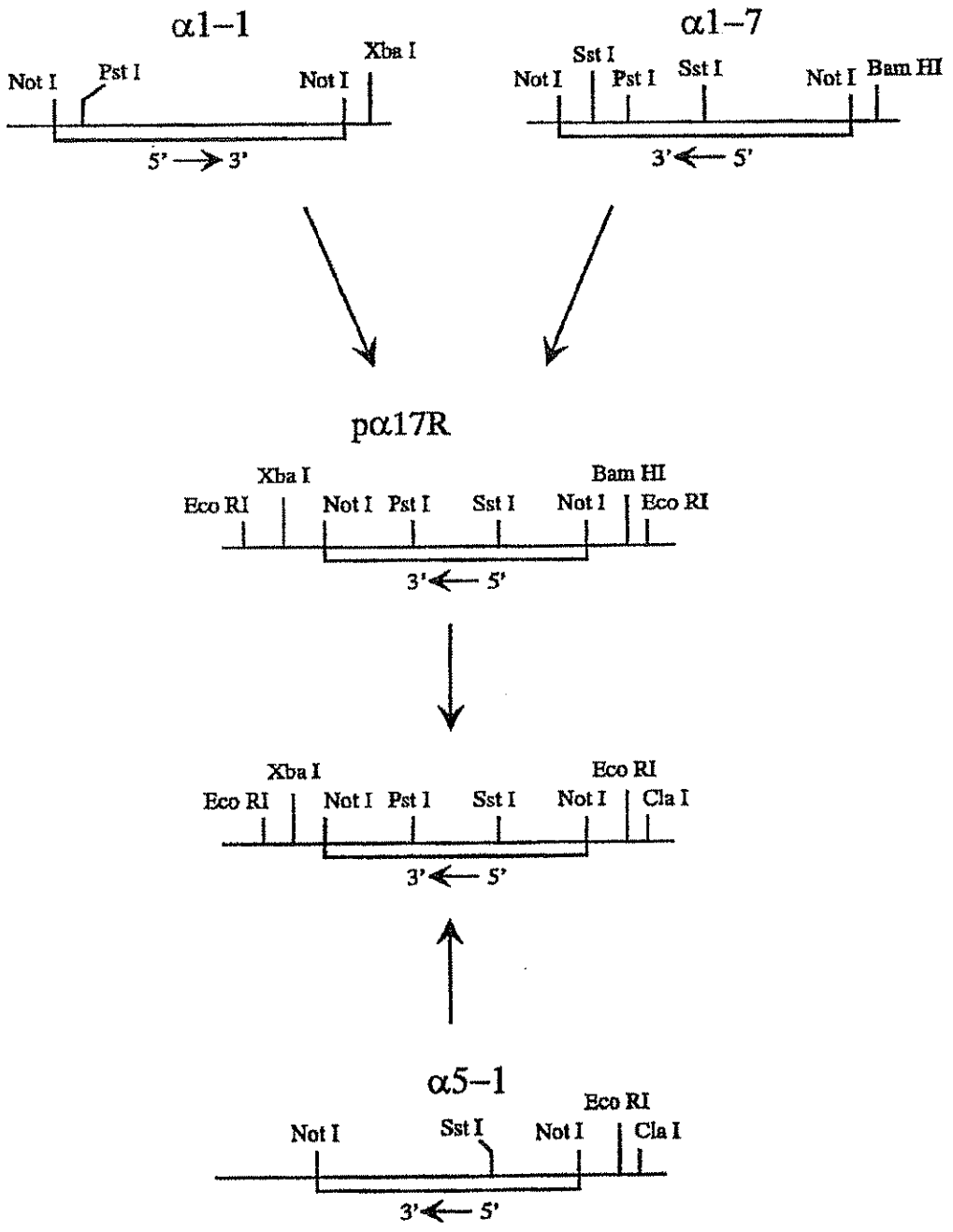
α1–1
Not I
Pst I
Not I
Xba I
5' → 3'
α1–7
Not I
Sst I
Pst I
Sst I
Not I
Bam HI
3' ← 5'
pα17R
Eco RI
Xba I
Not I
Pst I
Sst I
Not I
Bam HI
Eco RI
3' ← 5'
Eco RI
Xba I
Not I
Pst I
Sst I
Not I
Eco RI
Cla I
3' ← 5'
α5–1
Not I
Sst I
Not I
Eco RI
Cla I
3' ← 5'

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 4×10^3; 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 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 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_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 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_K, 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_γ1M, immunoglobulin heavy chain constant region and membrane anchor sequences.

FIG. 9 illustrates the constructions pBTL115, pBTL114, pϕ5V_HHuC_γ1M-neo, plCϕ5V_κHuC_κ-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–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 (Boni-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 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 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, e, 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' 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 CAG 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)
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'

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 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)

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 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 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 1.4×10^6 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 pUCtype 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-

EXHIBIT 2
Part 2

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 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-A9 ca12 pep4::TPI1prom-CAT) and ZY400 (MATa leu2-3.112 ade2-101 suc2-Δ9 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_{Tyr} 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_{Tyr} 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.

2× 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 ligaion 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, 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 pICpPRE8

The immunoglobulin μ heavy chain promoter and enhancer were subloned 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 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_{78} 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' 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 $p\phi5V_HhuC_\gamma1M$-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-$p\phi5V_HhuC_\gamma1M$-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-$p\phi5V_HhuC_\gamma1M$- 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-$p\phi5V_HhuC_\gamma1M$-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-$p\phi5V_HhuC_\gamma1M$-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 $PDGF\text{-}BB_{7,7r}$ (Example 18.F.) diluted in DMEM+10% fetal calf serum to the identical aliquots of receptor-bound beads to final $PDGF\text{-}BB_{7,7r}$ 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 $p\phi5V_HFab$-neo.

Plasmid $p\phi5V_HFab$-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_H1 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_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 $p\phi 5V_H$ 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 competetively 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_{Tyr} (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_{Tyr} bound by the receptor analog. Nonspecific binding for each concentration of ^{125}I-labeled PDGF-BB_{Tyr} 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_{Tyr}, 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_{Tyr} (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 ZC2181/2C2182/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 desginated 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/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 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 desginated 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 $p\phi 5V_H huC_\gamma 1M$-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 lot. 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^{-11}
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 0.1 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, Melvern, 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 replaing 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 derease 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 TCTGATAACT 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 GGT 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      1268
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 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
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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 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 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

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

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(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

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:

(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: ZC906

(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: ZC1380

(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: ZC1447

(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: ZC1453

(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

(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 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

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1777

(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: ZC1846

(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: ZC1847

(xi) 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

(ii) MOLECULE TYPE: Other nucleic acid

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(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC1886

(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: ZC1892

(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: ZC1893

(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: ZC1894

(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: ZC1895

(xi) 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

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2181

(xi) 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

(ii) MOLECULE TYPE: Other nucleic acid

(iii) HYPOTHETICAL: N

(iv) ANTI-SENSE: N

(vii) IMMEDIATE SOURCE:
(B) CLONE: ZC2182

(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: ZC2183

(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: ZC2184

(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: ZC2311

(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: ZC2351

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

CGAATTCCAC 10

(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: ZC2352

(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: ZC2392

(xi) 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

(ii) MOLECULE TYPE: peptide

(v) FRAGMENT TYPE: C-terminal

(vii) IMMEDIATE SOURCE:
(B) CLONE: pWK1

(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 TATAR 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: 205..3471
(D) OTHER INFORMATION:

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

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

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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 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
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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 Gln 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_H4 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 3

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CORPORATE FACT SHEET

ZymoGenetics creates novel protein drugs with the potential to significantly help patients fight their diseases.

History
ZymoGenetics, Inc. was founded in June 1981 by three noted university professors: Earl W. Davie, Professor of Biochemistry at the University of Washington; Benjamin D. Hall, Professor of Genetics at the University of Washington; and the late Michael Smith, Professor of Biochemistry at the University of British Columbia and 1993 Nobel Laureate. In 1988, Novo Nordisk acquired the company and for the next twelve years ZymoGenetics operated as the U.S. research arm of Novo Nordisk. In November of 2000, ZymoGenetics became an independent company, and in February of 2002, ZymoGenetics completed its initial public offering, raising $120M.

Chief Executive Officer: Bruce L.A. Carter, Ph.D.

President: Douglas E. Williams, Ph.D.

Cash Position: The Company's cash, cash equivalents and short-term investments totaled approximately $155 million (as of 3/31/08).

2007 Revenues: $38.5 million

Number of Shares Outstanding: approx. 68.7 million (as of 4/11/08)

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Stock Exchange Listing: NASDAQ: ZGEN

Number of Employees: 535 (as of 2/13/08)

Discovery efforts: ZymoGenetics has a growing pipeline of potential product candidates that includes proteins, antibodies and antibody-like molecules.

Product Candidates:
- Recombinant Thrombin (rThrombin) - for use as a general hemostat
- Atacicept (formerly referred to as TACI-Ig) - as a potential treatment for autoimmune diseases and B-cell malignancies
- IL-21 - as a potential treatment for cancer
- PEG-interferon lambda (IL-29) - as a potential treatment for hepatitis C

Key Partnerships:
- Partnered with Novo Nordisk to develop IL-21
- Co-development collaboration with Merck Serono for atacicept

Important Dates:
- 1981 Company founded
- 1988 ZymoGenetics acquired by Novo Nordisk
- 2000 Novo Nordisk spins off ZymoGenetics
- 2002 ZymoGenetics completes IPO
- 2003 Initiates clinical trials of atacicept and rThrombin
- 2004 Starts clinical development of IL-21
- 2004 Opens Earl Davie Building II with clinical manufacturing facility
- 2004 Enters into broad strategic alliance with Serono
- 2005 Designates PEG-interferon lambda as product candidate
- 2006 Completes rThrombin Phase 3 clinical trial and files BLA
- 2007 Enters into partnership with Bayer HealthCare to co-commercialize rThrombin
- 2008 FDA approves RECOTHROM™ Thrombin, topical (Recombinant) for sale

Corporate Headquarters
1201 Eastlake Ave. E, Seattle WA 98102 Tel: 206-442-6600

Web Site Address: www.zymogenetics.com

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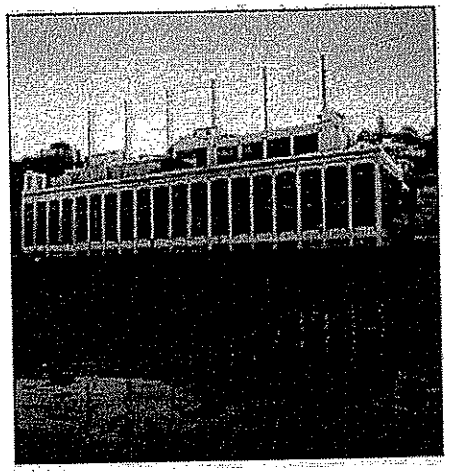
ZYMOGENETICS HISTORY



ZymoGenetics, Inc. was founded in 1981 by three noted university professors: Earl W. Davie, Professor of Biochemistry at the University of Washington; Benjamin D. Hall, Professor of Genetics at the University of Washington; and the late Michael Smith, Professor of Biochemistry at the University of British Columbia and 1993 Nobel Laureate in Chemistry.

ZymoGenetics' early focus on the development of production systems for recombinant proteins led to a 1982 collaboration with Novo Nordisk A/S, a Danish company, for the production of recombinant human insulin in yeast. In 1988, Novo Nordisk acquired ZymoGenetics and the Company became a wholly owned subsidiary. For 12 years, ZymoGenetics served as the primary U.S. discovery arm of Novo Nordisk, contributing to the development of several of Novo Nordisk's current products.

In November 2000, under the leadership of Dr. Bruce Carter, President and CEO, ZymoGenetics was reestablished as an independent biotechnology company, raising $150 million in a private placement financing. Subsequently, ZymoGenetics completed an initial public offering, and on February 1, 2002, began trading on the Nasdaq stock exchange under the ticker symbol ZGEN.

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Today, ZymoGenetics is one of the largest biotechnology companies in Washington State with over 500 employees. The Company's corporate headquarters, including its research and development facilities, are located on the south shore of Lake Union, in Seattle. In October 2002, ZymoGenetics completed a sale and leaseback of its corporate headquarters buildings, raising approximately $52 million. In addition to occupying the historic Lake Union Steam Plant building, and the adjacent Earl Davie Building, the Company, in partnership with its landlord, built additional research and development space as part of a 45,000 square foot expansion of the Earl Davie Building. The Earl Davie Building II expansion was completed in June 2004. ZymoGenetics now occupies approximately 200,000 square feet of laboratory and office space.

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Nobelprize.org

The Nobel Prize in Chemistry 1993

"for contributions to the developments of methods within DNA-based chemistry"

"for his invention of the polymerase chain reaction (PCR) method"	"for his fundamental contributions to the establishment of oligonucleotide-based, site-directed mutagenesis and its development for protein studies"
	
Kary B. Mullis	**Michael Smith**
½ of the prize	½ of the prize
USA	Canada
La Jolla, CA, USA	University of British Columbia Vancouver, Canada
b. 1944	b. 1932 (in Blackpool, United Kingdom) d. 2000

Titles, data and places given above refer to the time of the award.
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Exhibit 4

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Message From CSO & R&D President

Disease Areas of Focus

Recent Accomplishments

Pipeline

Biologics

R&D Facts

Clinical Trials Disclosure

Pharmaceutical Research & Development

Biologics

Most medicines today, such as aspirin, penicillin and chemotherapeutics, are compounds known as small molecules. Small molecules are a diverse group of natural and synthetic substances which generally have a low molecular weight. Small molecules are obtained either from natural sources – such as plants, fungi or microbes – or they are synthesized by organic chemistry.

But now a new kind of compound is delivering an increasing number of novel and targeted treatments. Whether they are called large molecules, protein therapeutics or biologics, they are proteins derived from living sources such as mammalian cells and grown in an artificial environment. Large molecules are aptly named. They can be 200 or more times the size of small molecules. Often designed to bind to specific cell surface receptors – which themselves are large molecules – biologics are generally more targeted than traditional small molecule drugs.

Biologics account for an increasing proportion of commercially significant products and as much as 25% of major new therapies could be biologics in the future. Biologics have the potential to treat a wide variety of diseases – we have certainly seen the impact in oncology and immunology, where biologics will play a major role with a growing number already on the horizon. Large molecules have also shown a higher probability of success than small molecules. In addition, it is often

difficult for small molecules to interact with large receptors and stimulate a response as well. At the same time, biologics represent specific challenges primarily in the area of complexity and cost of manufacturing.

Bristol-Myers Squibb's experience with marketed biologics began with the approval of its first biologic in 2004 and continued with the approval of its first internally discovered and developed biologic in 2005. Bristol-Myers Squibb is committed to increasing its biologics capability through technology acquisitions, licensing opportunities and in-house development.

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News Releases

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May 5, 2006
U.S. Food and Drug Administration Approves ORENCIA® (abatacept) Supplemental Biologics License Application (sBLA) for Third-Party Manufacturing Facility Lonza Biologics Inc.

PRINCETON, N.J., May 5 /PRNewswire-FirstCall/ -- Bristol-Myers Squibb Company (NYSE: BMY) today announced that the U.S. Food and Drug Administration (FDA) has approved a supplemental Biologics License Application (sBLA) for the licensure of Lonza Biologics Inc., a third-party manufacturing facility, to support increased production capacity for ORENCIA® (abatacept), enabling the company to meet expected long-term demand. This follows the December 23, 2005, FDA approval of ORENCIA, the first selective modulator of a co- stimulatory signal required for full T-cell activation, for the treatment of rheumatoid arthritis (RA).

ORENCIA is indicated for reducing signs and symptoms, inducing major clinical response, slowing the progression of structural damage, and improving physical function in adult patients with moderately to severely active rheumatoid arthritis who have had an inadequate response to one or more disease-modifying anti-rheumatic drugs (DMARDs), such as methotrexate or TNF antagonists. ORENCIA may be used as monotherapy or concomitantly with DMARDs other than TNF antagonists. ORENCIA should not be administered concomitantly with TNF antagonists and is not recommended for use concomitantly with anakinra.

ORENCIA became available for initial commercial use in February 2006. For more information about ORENCIA, healthcare providers and patients can call 1-800-ORENCIA® (673-6242) or visit http://www.orencia.com/.

Important Safety Information About ORENCIA® (abatacept)

Concurrent therapy with ORENCIA and a biologic DMARD is not recommended. In controlled clinical trials, patients receiving concomitant ORENCIA and TNF antagonist therapy experienced more infections (63 percent) and serious infections (4.4 percent) compared to patients treated with only TNF antagonists (43 percent and 0.8 percent, respectively), without an important enhancement of efficacy.

Caution should be exercised in patients with a history of infection or underlying conditions which predispose them to infections. Treatment with ORENCIA should be discontinued if a patient develops a serious infection. Patients should be screened for tuberculosis and if positive, should be treated with standard medical practice prior to therapy with ORENCIA.

Less than 1 percent of patients treated with ORENCIA experienced hypersensitivity reactions, including two cases of anaphylaxis or anaphylactoid reactions. Other events potentially associated with drug hypersensitivity, such as hypotension, urticaria, and dyspnea, each occurred in less than 0.9 percent of patients treated with ORENCIA and generally occurred within 24 hours of an infusion with ORENCIA. Appropriate medical support measures for the treatment of hypersensitivity reactions should be available for immediate use in the event of a reaction.

Live vaccines should not be given concurrently with ORENCIA or within 3 months of its discontinuation.

Chronic obstructive pulmonary disease (COPD) patients treated with ORENCIA developed adverse events more frequently than those treated with placebo (97 percent vs. 88 percent, respectively). Respiratory disorders occurred more frequently in patients treated with ORENCIA compared to placebo-treated patients (43 percent vs. 24 percent, respectively), including COPD exacerbations, cough, rhonchi, and dyspnea. A greater percentage of patients treated with ORENCIA developed a serious adverse event compared to placebo- treated patients (27 percent vs. 6 percent), including COPD exacerbation (3 of 37 patients (8 percent) and pneumonia (1 of 37 patients (3 percent)). Use of ORENCIA in patients with rheumatoid arthritis and COPD should be undertaken with caution and such patients should be monitored for worsening of their respiratory status.

ORENCIA® (abatacept) should be used during pregnancy only if clearly needed. Rats treated every three days with abatacept during early gestation throughout the lactation period showed no adverse effects in the offspring at doses up to 45 mg/kg. At a dose of 200 mg/kg, alterations of immune function consisted of a 9-fold increase in the T-cell dependent antibody response in female pups and inflammation of the thyroid in one female out of 10 male and 10 female pups evaluated. Whether these findings indicate a risk for development of autoimmune diseases in humans exposed in utero to abatacept has not been determined.

Nursing mothers should discuss with their healthcare practitioner the risk/benefit of continued breast-feeding or discontinuation of the drug.

The most serious adverse reactions were serious infections (3 percent ORENCIA vs. 1.9 percent placebo) and malignancies (1.3 percent ORENCIA vs. 1.1 percent placebo).

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The overall frequency of malignancies was similar in the patients treated with ORENCIA and placebo-treated patients. However, more cases of lung cancer were observed in patients treated with ORENCIA (0.2 percent) than placebo- treated patients (0 percent). A higher rate of lymphoma was seen compared to the general population; however, patients with RA, particularly those with highly active disease, are at a higher risk for the development of lymphoma. The potential role of ORENCIA in the development of malignancies in humans is unknown.

The most frequent adverse events occurring in greater than or equal 10 percent of patients treated with ORENCIA were headache, upper respiratory tract infection, nasopharyngitis, and nausea.

For full prescribing information, please visit http://www.orencia.com/ or http://www.bms.com/

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Print Article

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Exhibit 6

ORENCIA®

(abatacept)

Rx only

DESCRIPTION

ORENCIA® (abatacept) is a soluble fusion protein that consists of the extracellular domain of human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) linked to the modified Fc (hinge, CH2, and CH3 domains) portion of human immunoglobulin G1 (IgG1). Abatacept is produced by recombinant DNA technology in a mammalian cell expression system. The apparent molecular weight of abatacept is 92 kilodaltons.

ORENCIA is supplied as a sterile, white, preservative-free, lyophilized powder for parenteral administration. Following reconstitution with 10 mL of Sterile Water for Injection, USP, the solution of ORENCIA is clear, colorless to pale yellow, with a pH range of 7.0 to 8.0. Each single-use vial of ORENCIA provides 250 mg abatacept, 500 mg maltose, 17.2 mg monobasic sodium phosphate, and 14.6 mg sodium chloride for administration.

CLINICAL PHARMACOLOGY

Mechanism of Action

Abatacept, a selective costimulation modulator, inhibits T cell (T lymphocyte) activation by binding to CD80 and CD86, thereby blocking interaction with CD28. This interaction provides a costimulatory signal necessary for full activation of T lymphocytes, implicated in the pathogenesis of rheumatoid arthritis (RA). Activated T lymphocytes are found in the synovium of patients with RA.

In vitro, abatacept decreases T cell proliferation and inhibits the production of the cytokines tumor necrosis factor alpha (TNFα), interferon-γ, and interleukin-2. In a rat collagen-induced arthritis model, abatacept suppresses inflammation, decreases anti-collagen antibody production, and reduces antigen specific production of interferon-γ. The relationship of these biological response markers to the mechanisms by which ORENCIA exerts its effects in RA is unknown.

Pharmacodynamics

In clinical trials with ORENCIA at doses approximating 10 mg/kg, decreases were observed in serum levels of soluble interleukin-2 receptor (sIL-2r), interleukin-6 (IL-6), rheumatoid factor (RF), C-reactive protein (CRP), matrix metalloproteinase-3 (MMP3), and tumor necrosis factor alpha (TNFα). The relationship of these biological response markers to the mechanisms by which ORENCIA exerts its effects in RA is unknown.

Pharmacokinetics

The pharmacokinetics of abatacept were studied in healthy adult subjects after a single 10 mg/kg intravenous infusion and in RA patients after multiple 10 mg/kg intravenous infusions (see Table 1).

Table 1: Pharmacokinetic Parameters (Mean, Range) in Healthy Subjects and RA Patients After 10 mg/kg Intravenous Infusion(s)

PK Parameter	Healthy Subjects (After 10 mg/kg Single Dose) n=13	RA Patients (After 10 mg/kg Multiple Doses*) n=14
Peak Concentration (C_{max}) (mcg/mL)	292 (175-427)	295 (171-396)
Terminal half-life ($t_{1/2}$) (days)	16.7 (12-23)	13.1 (8-25)
Systemic clearance (CL) (mL/h/kg)	0.23 (0.16-0.30)	0.22 (0.13-0.47)
Volume of distribution (V_{ss}) (L/kg)	0.09 (0.06-0.13)	0.07 (0.02-0.13)

* Multiple intravenous infusions were administered at days 1, 15, 30, and monthly thereafter.

The pharmacokinetics of abatacept in RA patients and healthy subjects appeared to be comparable. In RA patients, after multiple intravenous infusions, the pharmacokinetics of abatacept showed proportional increases of C_{max} and AUC over the dose range of 2 mg/kg to 10 mg/kg. At 10 mg/kg, serum concentration appeared to reach a steady-state by day 60 with a mean (range) trough concentration of 24 (1-65) mcg/mL. No systemic accumulation of abatacept occurred upon continued repeated treatment with 10 mg/kg at monthly intervals in RA patients.

Population pharmacokinetic analyses in RA patients revealed that there was a trend toward higher clearance of abatacept with increasing body weight. Age and gender (when corrected for body weight) did not affect clearance. Concomitant methotrexate (MTX), nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, and TNF blocking agents did not influence abatacept clearance.

The pharmacokinetics of abatacept have not been studied in children and adolescents. No formal studies were conducted to examine the effects of either renal or hepatic impairment on the pharmacokinetics of abatacept.

CLINICAL STUDIES

The efficacy and safety of ORENCIA were assessed in five randomized, double-blind, placebo-controlled studies in patients ≥ age 18 with active RA diagnosed according to American College of Rheumatology (ACR) criteria. Studies I, II, III, and IV required patients to have at least 12 tender and 10 swollen joints at randomization. Study V did not require any specific number of tender or swollen joints. ORENCIA or placebo treatment was given intravenously at weeks 0, 2, and 4 and then every 4 weeks thereafter.

Study I evaluated ORENCIA as monotherapy in 122 patients with active RA who had failed at least one non-biologic, disease-modifying, anti-rheumatic drug (DMARD) or etanercept. In Study II and Study III, the efficacy and safety of ORENCIA were assessed in patients with an inadequate response to MTX and who were continued on their stable dose of MTX. In Study IV, the efficacy and safety of ORENCIA were assessed in patients with an inadequate response to a TNF blocking agent, with the TNF blocking agent discontinued prior to randomization; other DMARDs were permitted. Study V primarily assessed safety in patients with active RA requiring additional intervention in spite of current therapy with DMARDs; all DMARDs used at enrollment were continued. Patients in Study V were not excluded for comorbid medical conditions.

Study I patients were randomized to receive one of three doses of ORENCIA (0.5, 2, or 10 mg/kg) or placebo ending at week 8. Study II patients were randomized to receive ORENCIA 2 or 10 mg/kg or placebo for 12 months. Study III, IV, and V patients were randomized to receive a dose of ORENCIA based on weight range or placebo for 12 months (Studies III and V) or 6 months (Study IV). The dose of ORENCIA was 500 mg for patients weighing less than 60 kg, 750 mg for patients weighing 60 to 100 kg, and 1 gram for patients weighing greater than 100 kg.

Clinical Response

The percent of ORENCIA-treated patients achieving ACR 20, 50, and 70 responses and major clinical response in Studies I, III, and IV are shown in Table 2. ORENCIA-treated patients had higher ACR 20, 50, and 70 response rates at 6 months compared to placebo-treated patients. Month 6 ACR response rates in Study II for the 10 mg/kg group were similar to the ORENCIA group in Study III.

In Studies III and IV, improvement in the ACR 20 response rate versus placebo was observed within 15 days in some patients. In Studies II and III, ACR response rates were maintained to 12 months in ORENCIA-treated patients. ACR responses were maintained up to three years in the open-label extension of Study II.

Table 2: ACR Responses in Placebo-Controlled Trials

	Percent of Patients					
	Inadequate Response to DMARDs		Inadequate Response to MTX		Inadequate Response to TNF Blocking Agent	
	Study I		Study III		Study IV	
Response Rate	ORENCIA[a] (abatacept) n=32	Placebo n=32	ORENCIA[b] +MTX n=424	Placebo +MTX n=214	ORENCIA[b] +DMARDs n=256	Placebo +DMARDs n=133
ACR 20						
Month 3	53%	31%	62%***	37%	46%***	18%
Month 6	NA	NA	65%***	40%	50%***	20%
Month 12	NA	NA	73%***	40%	NA	NA
ACR 50						
Month 3	16%	6%	32%***	8%	16%**	6%
Month 6	NA	NA	40%***	17%	20%***	4%
Month 12	NA	NA	48%***	16%	NA	NA
ACR 70						
Month 3	6%	0	13%***	3%	6%*	1%
Month 6	NA	NA	20%***	7%	10%**	2%
Month 12	NA	NA	29%***	6%	NA	NA
Major Clinical Response[c]	NA	NA	14%***	2%	NA	NA

* p<0.05, ORENCIA vs placebo.
** p<0.01, ORENCIA vs placebo.
*** p<0.001, ORENCIA vs placebo.
[a] 10 mg/kg.
[b] Dosing based on weight range (see DOSAGE AND ADMINISTRATION).
[c] Major clinical response is defined as achieving an ACR 70 response for a continuous 6-month period.

The results of the components of the ACR response criteria for Studies III and IV are shown in Table 3. In ORENCIA-treated patients, greater improvement was seen in all ACR response criteria components through 6 and 12 months than in placebo-treated patients.

Table 3: Components of ACR Response at 6 Months

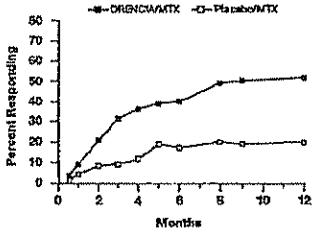
	Inadequate Response to MTX Study III				Inadequate Response to TNF Blocking Agent Study IV			
	ORENCIA +MTX n=424		Placebo +MTX n=214		ORENCIA +DMARDs n=256		Placebo +DMARDs n=133	
Component (median)	Baseline	Month 6	Baseline	Month 6	Baseline	Month 6	Baseline	Month 6
Number of tender joints (0-68)	28	7***	31	14	30	13***	31	24
Number of swollen joints (0-66)	19	5***	20	11	21	10***	20	14
Pain[a]	67	27***	70	50	73	43**	74	64
Patient global assessment[a]	65	29***	64	48	71	44***	73	63
Disability index[b]	1.75	1.13***	1.75	1.38	1.88	1.38***	2.00	1.75
Physician global assessment[a]	59	21***	68	40	71	32***	69	54
CRP (mg/dL)	2.2	0.9***	2.1	1.6	3.4	1.3***	2.8	2.3

** p<0.01, ORENCIA vs placebo, based on mean percent change from baseline.
*** p<0.001, ORENCIA vs placebo, based on mean percent change from baseline.
[a] Visual analog scale: 0 = best, 100 = worst.
[b] Health Assessment Questionnaire: 0 = best, 3 = worst; 20 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities.

The time course of ACR 50 response for Study III is shown in Figure 1. The time course for Study IV was similar.

Figure 1

Time Course of ACR 50 Response
Inadequate Response to MTX (Study III)



ORENCIA-treated patients experienced greater improvement than placebo-treated patients in morning stiffness.

Radiographic Response

Structural joint damage was assessed radiographically and expressed as change in Genant-modified Total Sharp Score[2] (TSS) and its components, the erosion score and Joint Space Narrowing (JSN) score, at month 12 compared to baseline. In Study III the baseline median TSS was 31.7 in ORENCIA-treated patients and 33.4 in placebo-treated patients. The results are shown in Table 4. ORENCIA/MTX slowed the progression of structural damage compared to placebo/MTX alone.

Table 4: Mean Radiographic Changes Over 12 Months in Study III

Parameter	ORENCIA/MTX n=391	Placebo/MTX n=195	Placebo/MTX – ORENCIA/MTX (95% Confidence Interval)	P-value[a]
Total Sharp score	1.21	2.32	1.11 (0.35, 1.88)	0.012
Erosion score	0.63	1.14	0.51 (0.08, 0.94)	0.029
JSN score	0.58	1.18	0.60 (0.21, 0.99)	0.009

[a] Based on non-parametric analysis.

Physical Function Response and Health-Related Outcomes

Improvement in physical function was measured by the Health Assessment Questionnaire Disability Index (HAQ-DI).[1,2] In Studies II-V, ORENCIA (abatacept) demonstrated greater improvement from baseline than placebo in the HAQ-DI. The results from Studies II and III are shown in Table 5. Similar results were observed in Study V. During the open-label period of Study II, the improvement in physical function has been maintained for up to 3 years.

Table 5: Mean Improvement from Baseline in Health Assessment Questionnaire Disability Index (HAQ-DI)

	Inadequate Response to Methotrexate			
	Study II		Study III	
HAQ Disability Index	ORENCIA[a] +MTX (n=115)	Placebo +MTX (n=119)	ORENCIA[b] +MTX (n=422)	Placebo +MTX (n=212)
Baseline (Mean)	0.98[c]	0.97[c]	1.69[d]	1.69[d]
Mean Improvement Year 1	0.40[c,***]	0.15[c]	0.66[d,***]	0.37[d]

*** p<0.001, ORENCIA vs placebo.

[a] 10 mg/kg.

[b] Dosing based on weight range (see DOSAGE AND ADMINISTRATION).

[c] Modified Health Assessment Questionnaire[2]: 0 = best, 3 = worst; 8 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities.

[d] Health Assessment Questionnaire[1]: 0 = best, 3 = worst; 20 questions; 8 categories: dressing and grooming, arising, eating, walking, hygiene, reach, grip, and activities.

Health-related quality of life was assessed by the SF-36 questionnaire[4] at 6 months in Studies II, III, and IV and at 12 months in Studies II and III. In these studies, improvement was observed in the ORENCIA group as compared with the placebo group in all 8 domains of the SF-36 as well as the Physical Component Summary (PCS) and the Mental Component Summary (MCS).

INDICATIONS AND USAGE

ORENCIA is indicated for reducing signs and symptoms, inducing major clinical response, slowing the progression of structural damage, and improving physical function in adult patients with moderately to severely active rheumatoid arthritis who have had an inadequate response to one or more DMARDs, such as methotrexate or TNF antagonists. ORENCIA may be used as monotherapy or concomitantly with DMARDs other than TNF antagonists.

ORENCIA should not be administered concomitantly with TNF antagonists. ORENCIA is not recommended for use concomitantly with anakinra.

CONTRAINDICATIONS

ORENCIA should not be administered to patients with known hypersensitivity to ORENCIA or any of its components.

WARNINGS

Concomitant Use with TNF Antagonists

In controlled clinical trials, patients receiving concomitant ORENCIA and TNF antagonist therapy experienced more infections (63%) and serious infections (4.4%) compared to patients treated with only TNF antagonists (43% and 0.8%, respectively) (see ADVERSE REACTIONS: Infections). These trials failed to demonstrate an important enhancement of efficacy with concomitant administration of ORENCIA with TNF antagonist; therefore, concurrent therapy with ORENCIA and a TNF antagonist is not recommended. While transitioning from TNF antagonist therapy to ORENCIA therapy, patients should be monitored for signs of infection.

PRECAUTIONS

Hypersensitivity

Of 2688 patients treated with ORENCIA in clinical trials, there were two cases of anaphylaxis or anaphylactoid reactions. Other events potentially associated with drug hypersensitivity, such as hypotension, urticaria, and dyspnea, each occurred in less than 0.5% of ORENCIA-treated patients. Appropriate medical support measures for the treatment of hypersensitivity reactions should be available for immediate use in the event of a reaction (see ADVERSE REACTIONS: Infusion-Related Reactions and Hypersensitivity Reactions).

Infections

Physicians should exercise caution when considering the use of ORENCIA in patients with a history of recurrent infections, underlying conditions which may predispose them to infections, or chronic, latent, or localized infections. Patients who develop a new infection while undergoing treatment with ORENCIA should be monitored closely. Administration of ORENCIA should be discontinued if a patient develops a serious infection (see ADVERSE REACTIONS: Infections). A higher rate of serious infections has been observed in patients treated with concurrent TNF antagonists and ORENCIA (see WARNINGS: Concomitant Use with TNF Antagonists).

Prior to initiating immunomodulatory therapies, including ORENCIA, patients should be screened for latent tuberculosis infection with a tuberculin skin test. ORENCIA has not been studied in patients with a positive tuberculosis screen, and the safety of ORENCIA in individuals with latent tuberculosis infection is unknown. Patients testing positive in tuberculosis screening should be treated by standard medical practice prior to therapy with ORENCIA.

Immunizations

Live vaccines should not be given concurrently with ORENCIA or within 3 months of its discontinuation. No data are available on the secondary transmission of infection from persons receiving live vaccines to patients receiving ORENCIA. The efficacy of vaccination in patients receiving ORENCIA is not known. Based on its mechanism of action, ORENCIA may blunt the effectiveness of some immunizations.

Use in Patients with Chronic Obstructive Pulmonary Disease (COPD)

COPD patients treated with ORENCIA developed adverse events more frequently than those treated with placebo, including COPD exacerbations, cough, rhonchi, and dyspnea. Use of ORENCIA in patients with rheumatoid arthritis and COPD should be undertaken with caution and such patients should be monitored for worsening of their respiratory status (see ADVERSE REACTIONS: Adverse Reactions in Patients with COPD).

Information for Patients

Patients should be provided the ORENCIA Patient Information leaflet and provided an opportunity to read it prior to each treatment session. Because caution should be exercised in administering ORENCIA to patients with active infections, it is important that the patient's overall health be assessed at each visit and any questions resulting from the patient's reading of the Patient Information be discussed.

Drug Interactions

Formal drug interaction studies have not been conducted with ORENCIA.

Population pharmacokinetic analyses revealed that MTX, NSAIDs, corticosteroids, and TNF blocking agents did not influence abatacept clearance (see CLINICAL PHARMACOLOGY: Pharmacokinetics). The majority of patients in RA clinical studies received one or more of the following concomitant medications with ORENCIA: MTX, NSAIDs, corticosteroids, TNF blocking agents, azathioprine, chloroquine, gold, hydroxychloroquine, leflunomide, sulfasalazine, and anakinra.

Concurrent administration of a TNF antagonist with ORENCIA has been associated with an increased risk of serious infections and no significant additional efficacy over use of the TNF antagonists alone. Concurrent therapy with ORENCIA and TNF antagonists is not recommended (see WARNINGS: Concomitant Use with TNF Antagonists).

There is insufficient experience to assess the safety and efficacy of ORENCIA (abatacept) administered concurrently with anakinra, and therefore such use is not recommended.

Immunosuppression

The possibility exists for drugs inhibiting T cell activation, including ORENCIA, to affect host defenses against infections and malignancies since T cells mediate cellular immune responses. The impact of treatment with ORENCIA on the development and course of malignancies is not fully understood (see ADVERSE REACTIONS: Malignancies). In clinical trials, a higher rate of infections was seen in ORENCIA-treated patients compared to placebo (see ADVERSE REACTIONS: Infections).

Carcinogenesis, Mutagenesis, Impairment of Fertility

In a mouse carcinogenicity study, weekly subcutaneous injections of 20, 65, or 200 mg/kg of abatacept administered each week for up to 84 weeks in males and 88 weeks in females were associated with increases in the incidence of malignant lymphomas (all doses) and mammary gland tumors (intermediate- and high-dose in females). The mice from this study were infected with murine leukemia virus and mouse mammary tumor virus. These viruses are associated with an increased incidence of lymphomas and mammary gland tumors, respectively, in immunosuppressed mice. The doses used in these studies were 0.6-, 2.0- and 3.0-fold, the human exposure at 10 mg/kg, respectively, based on AUC. The relevance of these findings to the clinical use of ORENCIA is unknown.

In a one-year toxicity study in cynomolgus monkeys, abatacept was administered intravenously once weekly at doses up to 50 mg/kg (9-fold the human exposure at 10 mg/kg dose based on AUC). Abatacept was not associated with any significant drug-related toxicity. Reversible pharmacological effects consisted of minimal transient decreases in serum IgG and minimal to severe lymphoid depletion of germinal centers in the spleen and/or lymph nodes. No evidence of lymphomas or preneoplastic morphologic changes was observed, despite the presence of a virus (lymphocryptovirus) known to cause these lesions in immunosuppressed monkeys within the time frame of this study. The relevance of these findings to the clinical use of ORENCIA is unknown.

No mutagenic potential of abatacept was observed in the *in vitro* reverse Ames or Chinese hamster ovary/hypoxanthine guanine phosphoribosyl-transferase (CHO/HGPRT) forward point mutation (with or without metabolic activation) assays, and no chromosomal aberrations were observed in human lymphocytes (with or without metabolic activation) treated with abatacept. In rats, abatacept had no adverse effects on male or female fertility at doses up to 200 mg/kg every three days (11-fold a human dose based on AUC).

Pregnancy Category C

Abatacept was found not to be teratogenic in mice at doses up to 300 mg/kg and in rats and rabbits at doses up to 200 mg/kg daily (29-fold a human 10 mg/kg dose based on AUC in rats and rabbits). Rats treated with abatacept every three days during early gestation throughout the lactation period showed no adverse effects in the offspring at doses up to 45 mg/kg (3-fold a human 10 mg/kg dose based on AUC). At a dose of 200 mg/kg (11-fold a human 10 mg/kg dose based on AUC), alterations of immune function consisted of a 9-fold increase in the T-cell dependent antibody response in female pups and inflammation of the thyroid in one female pup out of 10 males and 10 females evaluated. Whether these findings indicate a risk for development of autoimmune diseases in humans exposed *in utero* to abatacept has not been determined. Abatacept was shown to cross the placenta. Because animal reproduction studies are not always predictive of human response, ORENCIA should be used during pregnancy only if clearly needed. There are no adequate and well-controlled studies in pregnant women.

Nursing Mothers

Abatacept has been shown to be present in rat milk. It is not known whether abatacept is excreted in human milk or absorbed systemically after ingestion. Because many drugs are excreted in human milk, and because of the potential for serious adverse reactions in nursing infants from ORENCIA, possibly including effects on the developing immune system, a decision should be made whether to discontinue nursing or to discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use

Safety and effectiveness of ORENCIA in pediatric patients have not been established.

Geriatric Use

A total of 323 patients 65 years of age and older, including 53 patients 75 years and older, received ORENCIA in clinical studies. No overall differences in safety or effectiveness were observed between these patients and younger patients, but these numbers are too few to rule out differences. The frequency of serious infection and malignancy among ORENCIA-treated patients over age 65 was higher than for those under age 65. Because there is a higher incidence of infections and malignancies in the elderly population in general, caution should be used when treating the elderly.

ADVERSE REACTIONS

General

The most serious adverse reactions were serious infections and malignancies (see ADVERSE REACTIONS: Infections and ADVERSE REACTIONS: Malignancies).

The most commonly reported adverse events (occurring in ≥10% of patients treated with ORENCIA) were headache, upper respiratory tract infection, nasopharyngitis, and nausea.

The adverse events most frequently resulting in clinical intervention (interruption or discontinuation of ORENCIA) were due to infection. The most frequently reported infections resulting in dose interruption were upper respiratory tract infection (1.0%), bronchitis (0.7%), and herpes zoster (0.7%). The most frequent infections resulting in discontinuation were pneumonia (0.2%), localized infection (0.2%), and bronchitis (0.1%).

Because clinical trials are conducted under widely varying and controlled conditions, adverse reaction rates observed in clinical trials of a drug cannot be directly compared to rates in the clinical trials of another drug and may not predict the rates observed in a broader patient population in clinical practice.

The data described herein reflect exposure to ORENCIA in patients with active RA in placebo-controlled studies (1655 patients with ORENCIA, 989 with placebo). The studies had either a double-blind, placebo-controlled period of 6 months (258 patients with ORENCIA, 133 with placebo) or 1 year (1697 patients with ORENCIA, 856 with placebo). A subset of these patients received concomitant biologic DMARD therapy, such as a TNF blocking agent (284 patients with ORENCIA, 134 with placebo).

Infections

In the placebo-controlled trials, infections were reported in 54% of ORENCIA-treated patients and 48% of placebo-treated patients. The most commonly reported infections (reported in 5-13% of patients) were upper respiratory tract infection, nasopharyngitis, sinusitis, urinary tract infection, influenza, and bronchitis. Other infections reported in fewer than 5% of patients at a higher frequency (>0.5%) with ORENCIA compared to placebo, were rhinitis, herpes simplex, and pneumonia (see PRECAUTIONS: Infections).

Serious infections were reported in 3.0% of patients treated with ORENCIA and 1.9% of patients treated with placebo. The most common (0.2-0.5%) serious infections reported with ORENCIA were pneumonia, cellulitis, urinary tract infection, bronchitis, diverticulitis, and acute pyelonephritis (see PRECAUTIONS: Infections).

Malignancies

In the placebo-controlled portions of the clinical trials (1965 patients for a median of 12 months), the overall frequencies of malignancies were similar in the ORENCIA- and placebo-treated patients (1.3% and 1.1%, respectively). However, more cases of lung cancer were observed in ORENCIA-treated patients (4, 0.2%) than placebo-treated patients (0). In the cumulative ORENCIA clinical trials (placebo-controlled and uncontrolled, open-label) a total of 8 cases of lung cancer (0.21 cases per 100 patient-years) and 4 lymphomas (0.10 cases per 100 patient-years) were observed in 2688 patients (3827 patient-years). The rate observed for lymphoma is approximately 3.5-fold higher than expected in an age- and gender-matched general population based on the Surveillance, Epidemiology, and End Results Database.[3] Patients with RA, particularly those with highly active disease, are at a higher risk for the development of lymphoma. Other malignancies included skin, breast, bile duct, bladder, cervical, endometrial, lymphoma, melanoma, myelodysplastic syndrome, ovarian, prostate, renal, thyroid, and uterine cancers (see PRECAUTIONS: Immunosuppression). The potential role of ORENCIA in the development of malignancies in humans is unknown.

Infusion-Related Reactions and Hypersensitivity Reactions

Acute infusion-related events (adverse reactions occurring within 1 hour of the start of the infusion) in Studies III, IV, and V were more common in the ORENCIA (abatacept)-treated patients than the placebo patients (6% for ORENCIA, 6% for placebo). The most frequently reported events (1-2%) were dizziness, headache, and hypertension.

Acute infusion-related events that were reported in >0.1% and ≤1% of patients treated with ORENCIA included cardiopulmonary symptoms, such as hypotension, increased blood pressure, and dyspnea; other symptoms included nausea, flushing, urticaria, cough, hypersensitivity, pruritus, rash, and wheezing. Most of these reactions were mild to moderate. Fewer than 1% of ORENCIA-treated patients discontinued due to an acute infusion-related event. In controlled trials, 6 ORENCIA-treated patients compared to 2 placebo-treated patients discontinued study treatment due to acute infusion-related events.

Of 2688 patients treated with ORENCIA in clinical trials, there were two cases of anaphylaxis or anaphylactoid reactions. Other events potentially associated with drug hypersensitivity, such as hypotension, urticaria, and dyspnea, each occurred in less than 0.5% of ORENCIA-treated patients and generally occurred within 24 hours of ORENCIA infusion. Appropriate medical support measures for the treatment of hypersensitivity reactions should be available for immediate use in the event of a reaction (see **PRECAUTIONS: Hypersensitivity**).

Adverse Reactions in Patients with COPD

In Study V, there were 37 patients with chronic obstructive pulmonary disease (COPD) who were treated with ORENCIA and 17 COPD patients who were treated with placebo. The COPD patients treated with ORENCIA developed adverse events more frequently than those treated with placebo (97% vs 88%, respectively). Respiratory disorders occurred more frequently in ORENCIA-treated patients compared to placebo-treated patients (43% vs 24%, respectively) including COPD exacerbation, cough, rhonchi, and dyspnea. A greater percentage of ORENCIA-treated patients developed a serious adverse event compared to placebo-treated patients (27% vs 6%), including COPD exacerbation (3 of 37 patients [8%]) and pneumonia (1 of 37 patients [3%]).

Other Adverse Reactions

Adverse events occurring in 3% or more of patients and at least 1% more frequently in ORENCIA-treated patients during placebo-controlled RA studies are summarized in Table 6.

Table 6: Adverse Events Occurring in 3% or More of Patients and at Least 1% More Frequently in ORENCIA-Treated Patients During Placebo-Controlled RA Studies

Adverse Event (Preferred Term)	ORENCIA (n=1955)[a] Percentage	Placebo (n=589)[b] Percentage
Headache	18	13
Nasopharyngitis	12	9
Dizziness	9	7
Cough	8	7
Back pain	7	6
Hypertension	7	4
Dyspepsia	6	4
Urinary tract infection	6	5
Rash	4	3
Pain in extremity	3	2

[a] Includes 204 patients on concomitant biologic DMARDs (adalimumab, anakinra, etanercept, or infliximab).
[b] Includes 134 patients on concomitant biologic DMARDs (adalimumab, anakinra, etanercept, or infliximab).

Immunogenicity

Antibodies directed against the entire abatacept molecule or to the CTLA-4 portion of abatacept were assessed by ELISA assays in RA patients for up to 2 years following repeated treatment with ORENCIA. Thirty-four of 1993 (1.7%) patients developed binding antibodies to the entire abatacept molecule or to the CTLA-4 portion of abatacept. Because trough levels of abatacept can interfere with assay results, a subset analysis was performed. In this analysis it was observed that 9 of 154 (5.8%) patients that had discontinued treatment with ORENCIA for over 65 days developed antibodies.

Samples with confirmed binding activity to CTLA-4 were assessed for the presence of neutralizing antibodies in a cell-based luciferase reporter assay. Six of 9 (67%) evaluable patients were shown to possess neutralizing antibodies.

No correlation of antibody development to clinical response or adverse events was observed.

The data reflect the percentage of patients whose test results were positive for antibodies to abatacept in specific assays, and are highly dependent on the sensitivity and specificity of the assays. Additionally, the observed incidence of antibody positivity in an assay may be influenced by several factors, including sample handling, timing of sample collection, concomitant medication, and underlying disease. For these reasons, comparison of the incidence of antibodies to abatacept with the incidence of antibodies to other products may be misleading.

OVERDOSAGE

ORENCIA is administered as an intravenous infusion under medically controlled conditions. Doses up to 50 mg/kg have been administered without apparent toxic effect. In case of overdosage, it is recommended that the patient be monitored for any signs or symptoms of adverse reactions and appropriate symptomatic treatment instituted.

DOSAGE AND ADMINISTRATION

ORENCIA should be administered as a 30-minute intravenous infusion at the dose specified in Table 7. Following the initial administration, ORENCIA should be given at 2 and 4 weeks after the first infusion, then every 4 weeks thereafter. ORENCIA may be used as monotherapy or concomitantly with disease-modifying, anti-rheumatic drugs (DMARDs) other than TNF antagonists.

Table 7: Dose of ORENCIA

Body Weight of Patient	Dose	Number of Vials[a]
<60 kg	500 mg	2
60 to 100 kg	750 mg	3
>100 kg	1 gram	4

[a] Each vial provides 250 mg of abatacept for administration.

Preparation and Administration Instructions

Use aseptic technique.

ORENCIA is provided as a lyophilized powder in preservative-free, single-use vials. Refer to Table 7 for the dose and number of ORENCIA vials required. Each ORENCIA vial provides 250 mg of abatacept for administration. The ORENCIA powder in each vial must be reconstituted with 10 mL of Sterile Water for Injection, USP, using **ONLY** the **SILICONE-FREE DISPOSABLE SYRINGE PROVIDED WITH EACH VIAL** and an 18-21 gauge needle. If the ORENCIA powder is accidentally reconstituted using a siliconized syringe, the solution may develop a few translucent particles. Discard any solutions prepared using siliconized syringes.

If the **SILICONE-FREE DISPOSABLE SYRINGE** is dropped or becomes contaminated, use a new **SILICONE-FREE DISPOSABLE SYRINGE** from inventory. For information on obtaining additional **SILICONE-FREE DISPOSABLE SYRINGES**, contact Bristol-Myers Squibb 1-800-ORENCIA™.

During reconstitution, to minimize foam formation in solutions of ORENCIA, the vial should be rotated with gentle swirling until the contents are completely dissolved. Avoid prolonged or vigorous agitation. **DO NOT SHAKE.** Upon complete dissolution of the lyophilized powder, the vial should be vented with a needle to dissipate any foam that may be present. The solution should be clear and colorless to pale yellow. Do not use if opaque particles, discoloration, or other foreign particles are present.

1) To reconstitute the ORENCIA (abatacept) powder, remove the flip-top from the vial and wipe the top with an alcohol swab. Insert the syringe needle into the vial through the center of the rubber stopper and direct the stream of Sterile Water for Injection, USP, to the glass wall of the vial. Do not use the vial if the vacuum is not present. Rotate the vial with gentle swirling until the contents are completely dissolved.
2) Upon complete dissolution of the lyophilized powder, the vial should be vented with a needle to dissipate any foam that may be present. After reconstitution, each milliliter will contain 25 mg (250 mg/10 mL).
3) The reconstituted ORENCIA solution must be further diluted to 100 mL as follows. From a 100 mL infusion bag or bottle, withdraw a volume of 0.9% Sodium Chloride Injection, USP, equal to the volume of the reconstituted ORENCIA vials (for 2 vials remove 20 mL, for 3 vials remove 30 mL, for 4 vials remove 40 mL). Slowly add the reconstituted ORENCIA solution from each vial into the infusion bag or bottle using the same **SILICONE-FREE DISPOSABLE SYRINGE PROVIDED WITH EACH VIAL.** Gently mix. The concentration of the fully diluted ORENCIA solution in the infusion bag or bottle will be approximately 5, 7.5, or 10 mg of abatacept per mL of solution depending on whether 2, 3, or 4 vials of ORENCIA are used. Any unused portion in the vials must be immediately discarded.
4) Prior to administration, the ORENCIA solution should be inspected visually for particulate matter and discoloration. Discard the solution if any particulate matter or discoloration is observed.
5) The entire, fully diluted ORENCIA solution should be administered over a period of 30 minutes and must be administered with an infusion set and a **STERILE, NON-PYROGENIC, LOW-PROTEIN-BINDING FILTER** (pore size of 0.2 μm to 1.2 μm).
6) The infusion of the fully diluted ORENCIA solution must be completed within 24 hours of reconstitution of the ORENCIA vials. The fully diluted ORENCIA solution may be stored at room temperature or refrigerated at 2°C to 8°C (36°F to 46°F) before use.
7) ORENCIA should not be infused concomitantly in the same intravenous line with other agents. No physical or biochemical compatibility studies have been conducted to evaluate the coadministration of ORENCIA with other agents.

Storage and Stability

ORENCIA lyophilized powder must be refrigerated at 2°C to 8°C (36°F to 46°F). Do not use beyond the expiration date. Protect the vials from light by storing in the original package until time of use.

HOW SUPPLIED

ORENCIA lyophilized powder for intravenous infusion is supplied as an individually packaged, single-use vial with a silicone-free disposable syringe. The product is available in the following strength:

NDC 0003-2187-10 providing 250 mg of abatacept in a 15-mL vial.

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ORENCIA®

Rx only

(abatacept)

Patient Information

This leaflet tells you about ORENCIA (pronounced ob-REN-see-ah). Please read this information before you start using ORENCIA and each time before you are scheduled to receive ORENCIA, in case something has changed. The information in this leaflet does not take the place of talking with your doctor before you start receiving this medicine and at check ups. Talk to your doctor if you have any questions about your treatment with ORENCIA.

What is ORENCIA?
ORENCIA (abatacept) is a medicine that is used to treat adults with moderate to severe rheumatoid arthritis (RA) who have not been helped by other medicines for RA. RA is a disease that causes pain and joint inflammation (tenderness and swelling). RA can also cause joint damage. Your doctor has decided to treat you with ORENCIA because your disease is still active even though you may have tried other treatments.

How does ORENCIA work?
ORENCIA is a medicine that keeps the immune system from attacking healthy tissues in the body. The immune system defends the body against infections caused by bacteria and viruses. A normal immune system leaves healthy body tissues alone. In people with RA, the immune system attacks normal body tissues causing damage and inflammation especially in the tissues of your joints. ORENCIA interferes with an important step in this attack. By decreasing the immune system's attack on normal tissues, ORENCIA can reduce pain and joint inflammation, and slow the damage to your bones and cartilage. However, ORENCIA can also lower your body's ability to fight infection. ORENCIA treatment can make you more prone to getting infections or make any infection you have worse. It is important to tell your doctor if you think you have any infections.

Who should not receive ORENCIA?
You should not receive ORENCIA if you have ever had an allergic reaction to ORENCIA.

What should I tell my doctor before treatment with ORENCIA?
Before you receive treatment with ORENCIA you should tell your doctor if you:
- are taking a TNF blocker to treat RA. You may have a higher chance of getting a serious infection if you take ORENCIA with other biologic medications for RA.
- have any kind of infection including an infection that is in only one place in your body (such as an open cut or sore), or an infection that is in your whole body (such as the flu). Having an infection could put you at risk for serious side effects from ORENCIA. If you are unsure, please ask your doctor.
- have an infection that won't go away or a history of infections that keep coming back.
- have had tuberculosis (TB), a positive skin test for TB, or if you recently have been in close contact with someone who has had TB. If you develop any of the symptoms of TB (a dry cough that doesn't go away, weight loss, fever, night sweats) call your doctor right away. Before you start ORENCIA, your doctor may examine you for TB or perform a skin test.
- are scheduled to have surgery.
- recently received a vaccination or are scheduled for any vaccination.
- are pregnant or planning to become pregnant. It is not known if ORENCIA can harm your unborn baby.
- are breast-feeding. ORENCIA can pass into breast milk. You will need to decide to either breast-feed or receive treatment with ORENCIA, but not both.

If you are not sure or have any questions about any of this information, ask your doctor.

What important information do I need to know about side effects with ORENCIA?
Like all medicines that affect your immune system, ORENCIA can cause serious side effects. The possible serious side effects include:
- **Serious infections.** Patients taking ORENCIA are at increased risk for developing infections including pneumonia, and other infections caused by viruses, bacteria, or fungi. Call your doctor immediately if you feel sick or get any infection during treatment with ORENCIA.
- **Allergic reactions.** These reactions are usually mild or moderate and include hives, swollen face, eyelids, lips, tongue, throat, or trouble breathing.
- **Malignancies.** There have been rare cases of certain kinds of cancer in patients receiving ORENCIA. The role of ORENCIA in the development of cancer is not known.

What are the more common side effects with ORENCIA?
- The more common side effects with ORENCIA are headache, upper respiratory tract infection, sore throat, and nausea.

Can I receive ORENCIA if I am pregnant or breast-feeding?
ORENCIA has not been studied in pregnant women or nursing mothers, so we don't know what the effects are on pregnant women or nursing babies. You should tell your doctor if you are pregnant, become pregnant, or are thinking about becoming pregnant.

Can I receive ORENCIA if I am taking other medicines for my RA or other conditions?
Yes, you can take other medicines if your doctor has prescribed them or has told you it is okay to take them while you are receiving ORENCIA. It is important to tell your doctor if you are taking any other medicines including hormones, over the counter medicines, vitamins, supplements, or herbal products before you are treated with ORENCIA. If you start taking or plan to start taking any new medicine while you are receiving ORENCIA, tell your doctor. Taking ORENCIA with TNF blocker medications (Enbrel®, Humira®, Remicade®) is not recommended.

How will ORENCIA be given to me?
ORENCIA will be given to you by a healthcare professional using an IV. This means the medicine will be given to you through a needle placed in a vein in your arm. It will take about 30 minutes to give you the full dose of medicine.

How often will I receive ORENCIA?
You will receive your first dose of ORENCIA followed by additional doses at 2 and 4 weeks after the first dose. You will then receive a dose every 4 weeks.

What should I do if I miss a dose of ORENCIA?
If you miss receiving ORENCIA when you are supposed to, ask your doctor when to schedule your next dose.

What if I still have questions?
If you have any questions or problems, always talk with your doctor. You can also call 1-800-ORENCIA™ toll-free or visit the ORENCIA Internet site at www.ORENCIA.com or the company Internet site at www.BMS.com.

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Exhibit 7

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THE IMMUNOGLOBULIN SUPERFAMILY—DOMAINS FOR CELL SURFACE RECOGNITION[1,2]

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ENCOUNTERING THE Ig SUPERFAMILY

When Ig chains were first sequenced, segments within the constant regions of H and L chains showed sequence similarities, and this led to the idea that the Ig chains had all evolved from a primordial gene coding for about 100 amino acids (1). The domains within the Ig chains all contained a characteristic intrachain disulfide bond, and the idea of the domain as an independent structural unit was proposed (2). The domain hypothesis was firmly established when the structures of V and C domains were determined to reveal a common fold forming a sandwich of two β-sheets that was stabilized by the conserved disulfide bond (3, 4).

Beta-2 microglobulin (β_2-m), identified in the urine of patients with kidney disease, was the first nonimmunoglobulin structure found to share sequence similarities with Ig-domains. The β_2-m sequence looked like an Ig C-domain (5, 6). β_2-m was subsequently discovered to be part of the major histocompatibility complex (MHC) class I structure, and sequencing of the class I heavy chain showed that a segment of sequence adjacent to the transmembrane region was also similar to Ig C-domains (7, 7a). MHC antigens were known to play some role in the specificity of T lymphocyte

[1] Abbreviations: NBRF data base is a protein sequence data base from Protein Identification Resource (1987) National Biomedical Research Foundation, Washington, DC. Terms are defined in Table 1.

[2] Because of limited space, full referencing for this article has not been possible. Recent key references that lead to the full literature on each topic are cited.

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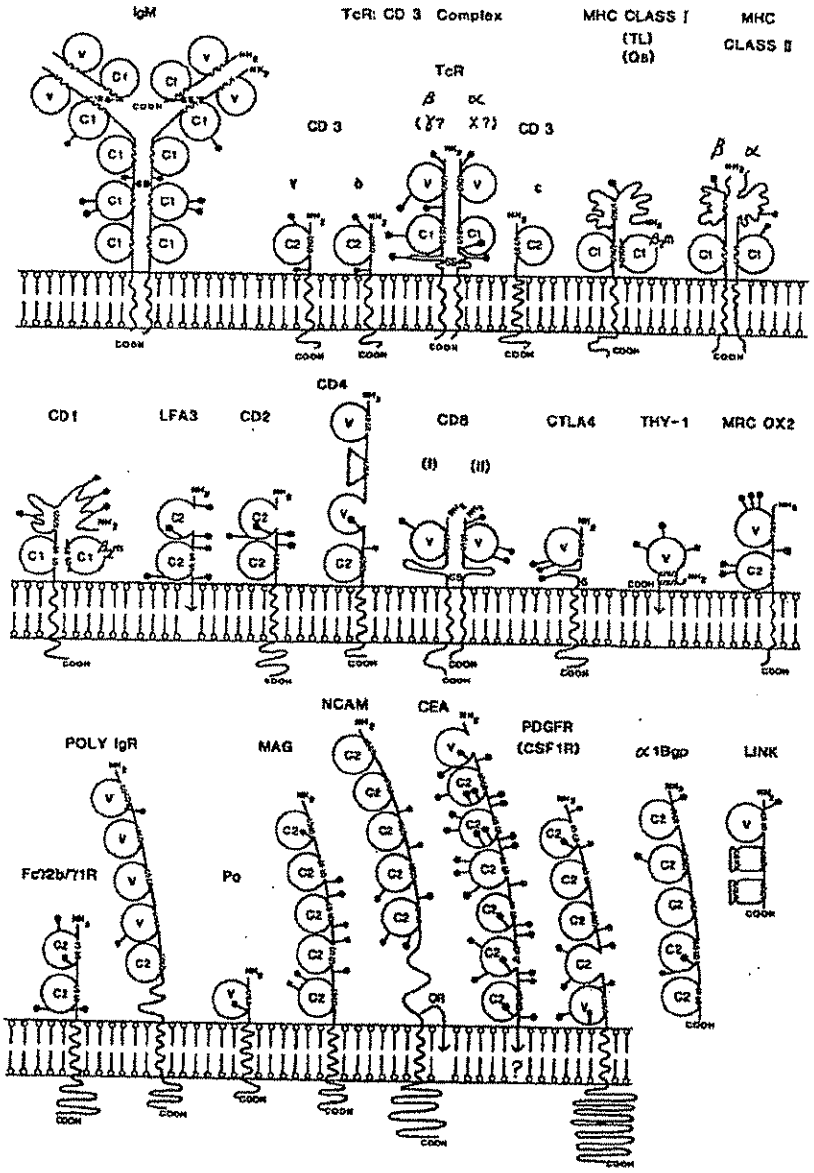
recognition, and thus the finding that the MHC class I was Ig-related was in accord with the concept that Ig domains were uniquely concerned with immune recognition. This view was clearly dominant in the 1970s.

The Thy-1 differentiation antigen was sequenced at the same time as MHC class I H chain, and the finding that this molecule was like a single Ig V domain was not consistent with the immune recognition concept (8). Thy-1 was expressed in large amounts in neural tissue and thymocytes in rodents, but expression in lymphoid cells was not conserved in all species. It thus seemed likely that Ig-related structures would have a general role in cell surface recognition (9).

In the 1980s, many new cell surface structures have been identified and sequenced, and the Ig-related family of molecules can now be argued to include the structures shown in Figure 1 and Table 1. The molecules have a diversity of functions (Table 2), but in most cases the common denominator is a recognition role at the cell surface. The genetic loci are widely spread throughout the chromosomes, but a number of interesting linkage groups are seen. This family of molecules is undoubtedly one of the key groups not only in immunity but also in the mediation of cell surface recognition to control the behavior of cells in various tissues.

Figure 1 Models for molecules in the Ig-superfamily. One model is shown for each main molecular type from one species (Table 1), and in some cases the same model suffices for the additional structures named in brackets. The circles show sequence segments that fold as for an Ig domain or are predicted to do so at least to the extent of two sheets with β-strands ABE:GFC (Figure 2). Segments labelled V, C1, and C2 are in the categories indicated in Figure 2 and in the text. Domain numbers as used in the text and figures are from the NH_2-terminus. In CD4 four domains are counted even though the second domain is not typically Ig-related. In the MHC class I H chain and related structures, the three obvious segments starting from the NH_2-terminus are called α_1, α_2, and α_3 while in MHC class II β and α chains, the segments are β_1, β_2 and α_1, α_2. CD1 is shown independently from class I to indicate that it has much lower sequence identity to class I than do Qa and T1 (full CD1a data by personal communication from L. H. Martin, F. Calabi, and C. Milstein). Intrachain disulfide bonds that are like the conserved Ig disulfide bond are shown by $\overset{S}{S}$ symbols within the circles, and cases where these are confirmed are given in Table 1. Other intrachain bonds also exist, but these are mostly not shown. Interchain bonds are indicated by SS between chains known to be disulfide linked. CTLA4 could well exist in a dimer since a free sulphydryl is predicted in a membrane proximal position. N-linked carbohydrate sites as indicated by the presence of an Asn × Thr or Ser sequence are shown by the symbol (ψ) unless absence of glycosylation is known. The presence of glycophospholipid anchors is indicated by an arrow for Thy-1, LFA-3, and one NCAM form, and the possibility of this is indicated for CEA by an arrow plus ?. The Qa2 antigen also has a lipid tail (see text). A second form of LFA3 with a protein anchor has also been identified (84). References are in Table 1.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(TL)
(Qa)
MHC
CLASS II
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β α
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β α
CD4
CD1
LFA3
CD2
CD8
CTLA4
THY-1
MRC OX2
(I) (II)
NCAM
CEA
POLY IgR
MAG
PDGFR
(CSF1R)
α1Bgp
LINK
Fc?2b/?1R
Po
OR
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Table 1 Molecules of the Ig superfamily

Category	Sequence number	Human chromosome	Ig-like disulfide bonds	References
Immunoglobulins				
H chains (IgM)	572 (M)	14q32.33	Y	(2, 10)
L chain kappa	214 (M)	2p12	Y	
L chain lambda	213 (M)	22q11.12	Y	
T cell receptor (Tcr) complex				
Tcr α-chain	250 (M)	14q11.2	ND	(11)
β-chain	282 (M)	7q35	ND	
γ-chain	286 (M)	7p15	ND	
X-chain	272 (M)	ND	ND	(12)
CD3 γ-chain	160 (H)	11q23	ND	(13, 14, 15)
δ-chain	150 (H)	11q23	ND	
ε-chain	185 (H)	11q23	ND	
Major histocompatibility complex (MHC antigens)				
Class I H-chain	339 (H)	6p21.3	Y	(7, 16)
β_2-m	99 (H)	15q21-q22	Y	(6)
Class II α	229 (H)	6p21.3	Y	(17)
β	237 (H)	6p21.3	Y	
β_2-m associated antigens				
TL H chain	335 (M)	mouse only	ND	(18)
Qa H chain	313 (M)	mouse only	ND	(19, 20)
CD1a H chain	311 (H)	1	ND	(21)
T cell adhesion molecules				
CD2	322 (R)	1p13	ND	(22, 23, 24)
LFA-3	207 (H)	1	ND	(22, 25, 26)
T subset antigens				
CD4	435 (H)	12pter.p12	Y	(27, 28)
CD8 chain I	210 (R)	2p12	ND	(27)
chain II	187 (R)	2p12	ND	(27, 29)
CTLA4	188 (M)	ND	ND	(30)
Brain/lymphoid antigens				
Thy-1	111 (R)	11q23	Y	(8, 31, 32)
MRC OX-2	248 (R)	3	ND	(33)
Immunoglobulin receptors				
Poly Ig R	755 (RB)	ND	Y	(34, 35, 36)
Fcγ2b/γ1R	351 (M)	ND	Y	(37, 38, 39)
Neural molecules				
Neural adhesion molecule (NCAM)	1072 (CH)	11q23	ND	(40, 41)
Myelin associated gp (MAG)	610 (R)	ND	ND	(42, 43)
P_0 myelin protein	219 (R)	ND	ND	(42, 44)

Table 1 (*continued*)

Category	Sequence number	Human chromosome	Ig-like disulfide bonds	References
Tumor antigen				
Carcinoembryonic antigen (CEA)	668 (H)	ND	ND	(45)
Growth factor receptors				
Platelet-derived growth factor (PDGF) receptor	1067 (M)	5q31-q32	ND	(42, 46)
Colony stimulating factor-1 (CSF1) receptor	953 (H)	5q33.2-q33.3	ND	(42, 47)
Non-cell surface molecules				
α_1B-glycoprotein	474 (H)	ND	Y	(48)
Basement membrane link protein	339 (R)	ND	Y	(49, 50)

Most sequences are predicted from cDNA, but all have been also characterized as proteins except Tcr X-chain and CTLA4. The sequence length is for the fully processed form of one sequence in each category, and most sequence data is from the NBRF data base. For LFA-3 and CEA, the residue number includes the hydrophobic COOH-terminal sequence that will be processed off if a lipid tail is present. Differential exon splicing of genes, giving alternative products, is seen for Igs (membrane and secreted forms), poly IgR (2 forms), class I MHC and related molecules, NCAM (3 forms), MAG (2 forms), and CD8 chain 1 (2 forms). The letters in brackets after the sequence number indicate species as follows: M, mouse; H, human; R, rat; RB, rabbit; CH, chicken. The human chromosome assignments are from Ref. (51) or other references given in the table. Under the heading *Ig-like disulfide bonds* a Y indicates the presence of conserved disulfide bonds as expected from sequence similarities, and ND denotes "not determined."

CHARACTERISTICS OF THE Ig-FOLD AND SEQUENCE PATTERNS

The two β-sheets of the Ig-fold consist of anti-parallel β-strands containing 5–10 amino acids. Between the sheets a hydrophobic interior is formed from in-pointing hydrophobic amino acids that alternate in the β-strands with out-pointing hydrophilic residues (3, 4). The interaction between the sheets is further stabilized by the conserved disulfide bond. The Ig fold for V- and C-domains is shown in Figure 2, and the core of the fold consists of β-strands A, B, E in one sheet and G, F, C in the other (52). These strands come from the first and last parts of the domain sequence, while in the middle considerable variation in sequence length occurs. V and V-related domains have about 65–75 amino acid residues between the conserved disulfide bond, and there are four β-strands in each β-sheet plus a short β-strand segment across the top of the domain. In C-domains the sequence between the disulfide bond is shorter at 55–60 residues, yielding sheets with 4 and 3 β-strands. In some non-Ig domains (see below) as few as 40 residues exist between the disulfide bond, and in some cases the fold

Table 2 Functions of the Ig-related molecules (References in Table 1)

Molecules and tissue expression	Functions	Recognition within the superfamily
Immunoglobulins: B lymphocytes only	B lymphocyte antigen receptors and in secreted form, antibodies	No, antibodies recognize antigen without involvement of other molecules
T cell receptors: T lymphocytes and thymocytes	T lymphocyte antigen receptors: no known soluble forms	Yes, heterophilic: Tcr binds MHC antigens plus peptide but recognition does not involve Ig-related MHC segments
CD3 chains: T lymphocytes and thymocytes	Part of the Tcr complex; role in signal transduction?	CD3 associates with Tcr but no known recognition of other molecules
MHC antigens: Many cell types, induced by interferon	Present peptides from foreign antigen to the Tcr; some soluble forms	Yes, heterophilic; Tcr interacts with class I and class II MHC antigens
β_2-m associated antigens: Subsets of lymphoid cells	Functions not known	No natural ligands known
T lymphocyte adhesion molecules: CD2, thymocytes and T cells (some macrophages in rat); LFA-3, widespread expression	CD2 of T cells interacts with LFA-3 on other cells in adhesion reactions, Anti-CD2 antibodies can trigger T cell division	Yes, heterophilic; CD2 binds LFA-3
T subset markers: CD4 and CD8 on thymocytes and T subsets, CD4 on macrophages, CD8 on NK cells; CTLA4, activated T cells	CD4 and CD8 appear to control the bias of T cells towards interaction with class I or class II MHC. CTLA4 function unknown	Perhaps heterophilic: CD4 and CD8 may bind class II and class I MHC antigens respectively. CTLA4 unknown
Brain/lymphoid antigens: Thy-1, neurons, fibroblasts, various lymphoid; MRC OX-2, neurons, endothelium, various lymphoid	Anti-Thy-1 antibody triggers mouse T lymphocyte division; MRC OX-2 function unknown	No natural ligands known
Immunoglobulin receptors: PolyIgR, gut and liver epithelium; Fcγ2b/γ1R, macrophages	PolyIgR transports multimeric IgA or IgM across epithelium; macrophage Fcγ2b/γ1R binds aggregated IgG	Yes, heterophilic for both PolyIgr and Fcγ2b/γ1R. First domain of PolyIgR binds IgA
Neural-associated molecules: NCAM, neurons and glia, early embryo; MAG, peripheral and central myelin, some neurons; P_0 peripheral myelin	NCAM mediates adhesion of neural cells. MAG may function in myelination. P_0 constitutes 50% of peripheral myelin protein	Yes, homophilic for NCAM via Ig-related parts and perhaps for P_0. MAG not known
CEA: Epithelial cells and their tumors, early embryos	Tumor marker but function unknown	Natural ligand unknown
Growth factor receptors: PDGFR, widespread on mesenchymal cells; CSF1R, monocyte lineage	Interact with growth factors to trigger cell division and other activities	No, PDGFR and CSF1R not known to react with molecules other than growth factors
Link protein: Basement membrane	Acts as a binding molecule between proteoglycan and hyaluronate chain	No
α,β-glycoprotein: Found in serum	Function unknown	Natural ligands unknown

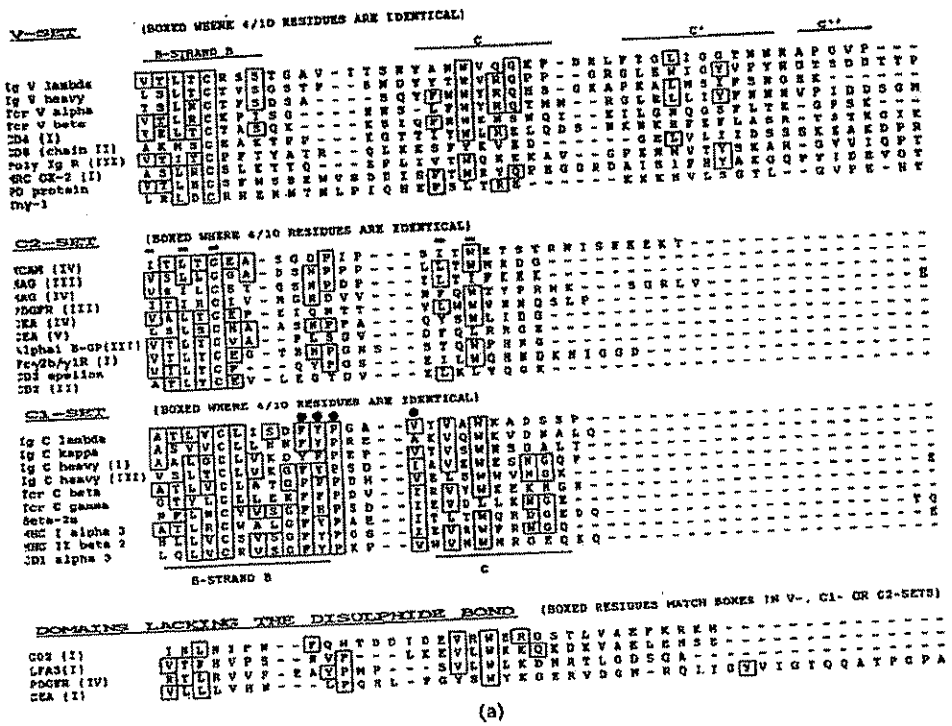
may consist of 3 β-strands in each sheet, plus a short connecting sequence across the top of the β-sheet sandwich.

Interactions between domains in Igs occur between the faces of the β-sheets, while in the interaction between antibody and antigen the contact residues consist of sequences in bends at the end of the V-domains (53). Thus, it seems that Ig-domains can interact with other molecules via any accessible part of their surface.

The Ig-related domains of non-Ig molecules are described as being V- or C-like according to whether they are likely to have a pattern of β-strands approximating to a V- or C-domain. A designation of a sequence as being V-like does not indicate sequence variation in the molecule concerned.

Conserved patterns of sequence are seen among the Ig-superfamily domains, and some alignments are shown in Figure 2. The first and last β-strands have been omitted to simplify the data, but this does not imply that conserved patterns are absent in these regions. Also, the whole of the domain is included in statistical analyses. The sequences are grouped into three categories called the V-SET, C1-SET, and C2-SET (54). The V-SET includes antigen receptor V-domains and other sequences likely to have a V-type fold. The extra sequences that form or are thought to form the C′ and C″ β-strands are obvious in Figure 2. The C1-SET includes mostly receptor C-domains and MHC antigen domains, and these are distinguished from the C2-SET in some conserved sequence patterns. However, both C1-SET and C2-SET sequences are likely to be folded as for Ig C-domains.

Across all these sequences, identities or conservative amino acid substitutions are seen in β-strands B, C, E, and F; in particular, the alternating hydrophobic residues are evident. In regions outside the β-strands, conserved patterns characterize V-SET and C1-SET sequences (marked in Figure 2). The C2-SET sequences seem somehow in between the V-SET and C1-SET since they are likely to have a C-type fold, but their sequence patterns in the region of β-strands E and F are like those of the V-SET. In Figure 1 all the domains are labelled as V, C1, or C2, and these assignments are mostly clear-cut. However, in some cases the designation is somewhat arbitrary, and among the C-like domains, sequences are placed in the C2-SET unless they clearly show the conserved C1-SET residues. The one exception to this is the C domain of TCR alpha chain which is not typical of sequences in the C1 or C2-SET. In this case, assignment to the C1-SET was made on the basis that the alpha chain is part of the heterodimer that dictates antigen specificity in one category of Tcrs. In general, statistical analysis of sequence similarities (see below) supports the domain assignments shown in Figure 1.



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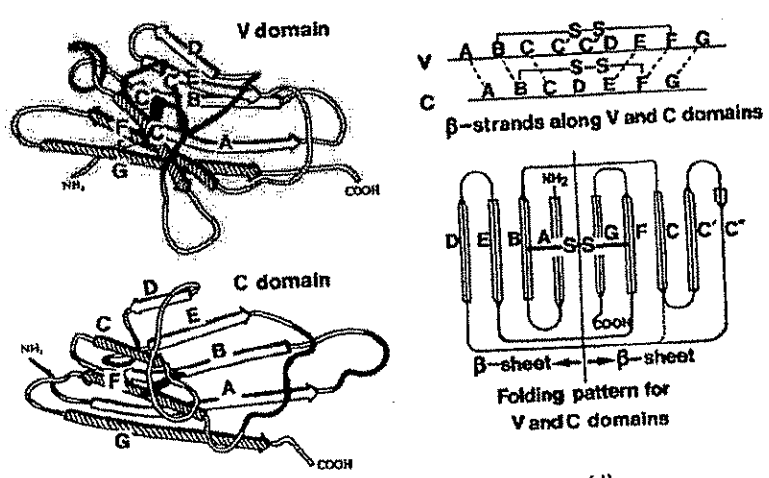
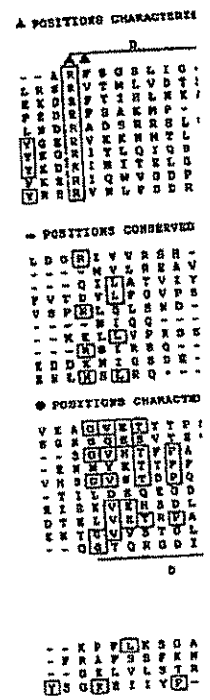


(c) (d)



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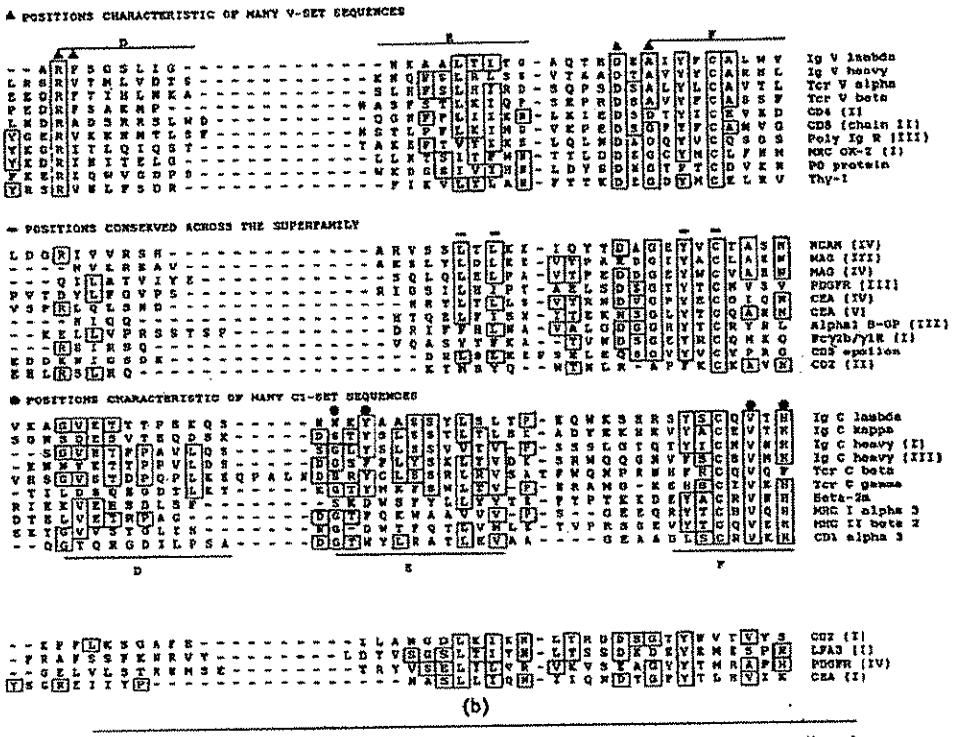

Figure 2 Alignments of Ig-related sequences and Ig-folding patterns. Sequences are aligned by eye and with the use of the ALIGN program (55), and sequence categories are defined in the text. The positions of the β-strands known for Ig V and C domains are indicated above and below the V-SET and C1-SET sequences, respectively. Sequences in regions corresponding to β-strands A and G have been omitted to simplify the data. Some conserved sequence positions are indicated by symbols, and these often involve conservation of amino acids of similar type rather than identities. The sequences are referenced by NBRF protein data base code in square brackets or literature references in round brackets. Ig V lambda, Mouse [L1MS4E]; Ig VH, human [G1HUNM]; Tcr V alpha, mouse [RWMSAV]; Tcr V beta, human [RWHUVY]; CD4, human [RWHUTA]; CD8 chain II, rat (56); PolyIgR, rabbit [QRRBG]; MRC OX-2, rat [TDRTOX]; P_0, rat (44); Thy-1, rat [TDRT]; NCAM, chicken (40); MAG, rat (42); PDGFR, mouse (46); CEA, human (45); Alpha1V-gp, human [OMHU1B]; Fcγ2b/γ1R, mouse (37); CD3 epsilon, human (14); CD2, rat (24); Ig C lambda, human [L2HU]; Ig C kappa, human [K3HU]; Ig C heavy, human [GHHU]; TcR C beta, human [RWHUCY]; TcR C gamma, mouse [RWMSC1]; β_2-M, human [MGHUB2]; MHC I alpha 3, human [HLHUB2]; MHC II beta 2, human [HLHU3D]; CD1a alpha 3, human (21); LFA-3, human (25).

Below the sequence alignments diagrams of the folding patterns of V and C domains are shown. The folds as determined for an Ig V_L and C_L domain are from Ref. (3), and the labelling of the β-strands along the domain is illustrated in the schematic diagram. The schematic view of the fold at bottom right is adapted from Ref. (4).

Although conserved sequence patterns are seen in Figure 2, no residue is invariant in all Ig-related domains. The conserved disulfide bond was once considered the hallmark of Ig domains, but recently a functional antibody has been described that has a Tyr residue instead of Cys in β-strand F of the V_H domain (57). Also, it can be argued that domains in CD2, LFA3, CD4, CEA, PDGFR, and CSF1R may be Ig-related even though they have no Cys residues in putative β-strands B and F (54, 58). Four of these sequences are shown below the C1-SET in Figure 2. In these and the other cases the Cys residues are replaced by hydrophobic amino acids that would presumably be suitable as inpointing residues that stabilize an Ig-like fold, just as do the other hydrophobic residues in the β-strands.

There is great diversity of sequence in the Ig-related molecules, and the question arises—why is a conserved pattern seen at all? The biological functions (see below) require unique recognition specificities, and these cannot directly be responsible for conserved sequence patterns. The conserved sequences are mostly seen in the β-strands that make up the core of the fold. It can be argued both that the fold itself is selected for, because it is stable to proteolysis, and that this is an essential feature for molecules operating in the extracellular environment (54).

CRITERIA FOR INCLUSION OF MOLECULES IN THE Ig SUPERFAMILY

The initial argument for related domains within the Ig chains was based on sequence similarities. However, an evolutionary relationship between V and C domains was only accepted when similarities in tertiary structures were established. It remains a consensus that the first criterion for an Ig relationship should be the presence of a domain-sized sequence with significant similarity to Ig or Ig-related domains, but in addition there should also be the probability that the sequence shares key structural features of the Ig-fold. If evolutionary selection acts on the basis of domain stability, then a rationale for requiring sequence and structural similarity is evident.

To evaluate sequence similarities, a statistical test must be used, and the ALIGN program of Dayhoff and colleagues (55) is now widely available as part of the NBRF data base package [Protein Identification Resource (1987) Protein Sequence Database, National Biomedical Research Foundation, Washington, DC]. The ALIGN program scores the best match between two sequences on the basis of a scoring matrix derived by determining the frequency of amino acid replacements in equivalent molecules

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between widely divergent species. Then the sequences are scrambled and rescored a number of times (e.g. 150) to yield a mean best random score and standard deviation (SD). The score for the real sequences is expressed as the number of SD units away from the random mean score. Assuming a normal distribution and no effect of sequence selection, scores of 3.1, 4.3, and 5.2 SD units indicate chance probabilities of 10^{-3}, 10^{-5}, and 10^{-7}, respectively.

In choosing sequences for ALIGN analyses, a putative domain is defined by taking sequence within positions that are 20 residues before and after Cys residues that might approximate to the conserved Ig disulfide bond. If Cys residues are absent, then possible replacements are identified and used to define the domain segment. The selection of Cys residues to define the domain carries the possible problem that this will bias ALIGN scores since matches between Cys residues carry a high value in the Dayhoff scoring matrix. To check this effect, 11 segments from membrane molecules that are not Ig-related were chosen on the basis of the presence of a suitable pair of Cys residues. These were scored against Ig-related domains (54). From 682 scores, a mean and standard deviation of 0.6 ± 1 SD units was obtained. In the controls there were three scores of >4 (4.1, 4.2, 4.2) and 13 of $>3<4$ SD units.

In testing a new sequence, ALIGN scores should be determined with as many distinct sequences as possible from the V-SET, C1-SET, and C2-SET. This overcomes the problem that the ALIGN program scores throughout the sequence and takes no account of the conserved sequence patterns that have great significance when assessing sequence similarities by eye (Figure 2). By chance a reasonable score might result in one comparison, but repeated good scores should indicate that a test sequence contains a conserved Ig-related pattern of sequence, since this is the only common denominator between the sequences in Figure 2 (54, 58).

In Table 3 some ALIGN scores are shown for β_2-m, Thy-1, NCAM, and LCA (control) against sequences from the V-SET and C1-SET. β_2-m scores well with the sequences from the C1-SET, but a significant relationship is not seen with V-SET sequences, and THY-1 shows good scores in the opposite direction. NCAM gives good scores with both C1-SET and V-SET sequences because, although its length matches with C-domains, it has some of the conserved sequence patterns of the V-SET. The control sequence from LCA gives no good scores even though the sequence has two Cys residues that can match with those in the Ig-domains and it also has a Trp that can match the conserved Trp of β-strand C in Figure 2.

Some molecules that have been claimed to be Ig-related fail the ALIGN test against the sets of domains (54, 58). These include the adenoviral E3 glycoprotein (59) and the CD5 antigen (60). Also, the enzyme superoxide

Table 3 ALIGN scores for comparisons of Ig related sequences

V or V-related	β_2-m	NCAM Thy-1	NCAM (III)	LCA	C or C-related	β_2-m	NCAM Thy-1	NCAM (III)	LCA
Ig lambda	−0.9	7.4	3.3	−0.6	Ig lambda	5.6	1.4	4.7	−1.0
Ig kappa	1.7	3.7	5.4	−0.1	Ig kappa	6.0	1.3	4.0	−1.5
Ig heavy	1.1	3.9	3.9	1.5	Ig CH1	4.0	3.0	4.1	0.7
Tcr beta	1.8	3.3	4.6	−0.2	Ig CH2	2.4	2.9	3.8	0
Tcr alpha	2.1	2.3	4.4	−1.5	Ig CH3	6.3	3.1	3.7	0.7
Tcr gamma	−0.2	1.6	3.9	1.4	Tcr beta	4.4	2.3	3.0	2.2
CD8 (chain I)	2.6	4.5	4.7	−0.3	Tcr alpha	2.1	−0.3	1.7	−0.7
CD4 (I)	2.4	2.5	5.5	−0.1	Tcr gamma	1.9	0.8	3.6	−0.6
PolyIgR (I)	1.5	5.7	2.7	0	MHC I α3	8.2	2.2	2.9	1.3
PolyIgR (III)	1.7	5.8	4.3	0.9	MHC II α2	11.2	3.7	4.9	1.2
MRC OX-2 (I)	−1.1	5.0	5.3	−0.6	MHC II β2	11.3	2.4	4.3	1.6
P_0 Protein	0.7	3.5	6.0	−0.4	CD1 α3	9.1	1.3	5.4	1.2

Domains were defined from a position 20 residues before the first Cys to 20 residues after the second Cys of a putative Ig-like disulfide bond. The leucocyte common antigen (LCA) sequence is a control and includes residues 88-189 from the partial rat LCA sequence (54). All other sequences are referenced in Figure 2 except the following with NBRF data base code given in square brackets or reference in parentheses: Ig kappa [K1HURY], Tcr gamma V [RWMSV1], CD8 chain I (56), Tcr alpha C [RWHUAC], MHC class I [HLHU12], MHC class II beta [HLHU3D]. The ALIGN program (55) was run with a bias of 6 and a break penalty of 6, and 150 random runs were performed.

dismutase which has a fold like an Ig-domain (61) shows no sequence similarity and thus is not regarded as being in the Ig-superfamily (58). Other domains with no statistically significant relationship are the α_1 and α_2 domains of MHC class I antigen and the α_1 and β_1 domains of MHC class II antigens. A possible Ig-related segment in the sequence of HIV glycoprotein gp110 does not include sequence that can be matched with a full domain (62). However, there are matches with Ig C-domains over 44 residues that give ALIGN scores of 7–9 SD. This is sufficiently high to raise the possibility that the viral sequence has been captured from Igs without the maintenance of the full domain. A sequence similarity might remain if it were selected for unknown reasons or if the capture of a piece of Ig-related sequence by virus was a recent event.

Structural proof for an Ig-related domain can only convincingly be established by tertiary structure determined by X-ray crystallography. Thus far, this is only reported for Ig V and C domains and for the β_2-m and α3 domains of MHC class I antigens which have structures exactly like Ig C_H3 domains (6, 7a). Unambiguous but limited evidence comes from the determination of disulfide bonds. Thus far in all cases where this has been determined the bonding pattern is in accord with the Ig-fold (Table 1). Circular dichroism can convincingly establish the presence of pure β-structure without α-helix, and this has been shown for Thy-1 (63).

Secondary structure prediction can be used, but this remains imprecise (64) and in our view should be used as a test to see whether a domain assignment indicated by sequence similarities might be improbable on structural grounds. Finally, the exon pattern in the genes can support domain assignments, and this is discussed below.

OVERALL MOLECULAR CHARACTERISTICS

The Ig superfamily is notable because no extracellular sequence includes an enzymatic activity or segments from more than one protein superfamily. This generalization does not apply to intracellular parts since the PDGFR and CSF1R molecules have cytoplasmic domains that have tyrosine kinase enzymatic activities and are related in sequence to other tyrosine kinase domains (46). Mixing of segments from different superfamilies is commonly seen in other cell or immune-related surface molecules. For example, in the complement proteins, repeats of a disulfide-linked domain of about 60 amino acids can be found together with serine protease domains, plus in some cases epidermal growth factor-like segments (65).

The Ig-related molecules commonly form dimers (Figure 1), and binding between domains can be homophilic, as seen between the C_H3 domains of Igs, or heterophilic, as in interactions between V domains and Ig C_H1 and C_L domains. Stable dimers are often disulfide-linked, but this is not the case for the MHC antigens and β_2-m associated antigens. Metastable interactions can also occur, as is seen in the Tcr complex where the α and β chains in the disulfide-linked dimer associate with the CD3 ε, γ, δ, and ξ chains (13). (The ξ chain is not shown in Figure 1 since the sequence is unknown.) This precedent raises the possibility that even weaker interactions that cannot be detected by conventional techniques might occur between Ig-related molecules at the cell surface during functional responses.

Carbohydrate structures can be dominant features in some Ig-superfamily molecules, and in CEA up to six possible N-linked sites have been observed on one Ig-related domain (Figure 1). Between tissues the same protein can be differentially glycosylated, and all the complex N-linked structures of Thy-1 differ between brain and thymus (66). Also on NCAM differences occur in glycosylation between fetal and adult forms (40). The fetal forms show extensive polysialation, and this is thought to modulate the adhesive potential of NCAM molecules.

The transmembrane sequences and cytoplasmic domains of Ig-related molecules show great diversity. Most molecules have a hydrophobic transmembrane protein sequence, but for Thy-1 (31), one form of NCAM (40, 41), and of LFA-3 (25, 84) and Qa-2 antigen (20, 20a) membrane

attachment via a glycophospholipid anchor has been established. Such an attachment may also be the case for CEA, since this molecule has hydrophobic residues at its COOH-terminus, but there are no basic residues (as is to be expected) on the cytoplasmic side of an authentic transmembrane sequence (45). The COOH-terminal sequence of CEA is like that of Thy-1, which is absent from the mature molecule and is presumed to be cleaved off when the glycophospholipid tail is attached (31, 67). A similar sequence is also predicted from NCAM cDNA clones that are thought to encode the form of NCAM that has a glycophospholipid anchor (40, 41; reviewed in 85).

Ig-superfamily molecules with predicted transmembrane sequences show only one such sequence per chain, and in most cases these sequences show no amino acids with amide or charged residues. However, in all the CD3 chains there is one acidic residue in the midst of the hydrophobic domain (13, 14), and in Tcr chains basic residues are found in similar positions (11). In Tcr γ and X chains two basic residues and an Asn are found in the 22 residues most likely to cross the bilayer (12). The charged residues in the hydrophobic domains of the Tcr complex may stabilize interactions between CD3 and Tcr chains, or alternatively, they may be involved in signal transduction. Cytoplasmic domains of Ig-superfamily sequences are mostly completely unrelated, and they vary in length from 3 amino acids for IgM (10) to 543 residues for PDGFR (46). Their roles in general are a mystery, although a function in intracellular traffic is known for the cytoplasmic domain of PolyIgR (68). The unknown aspects of signal transduction are illustrated by considering Thy-1, IgM, and PDGFR, all of which can act as targets in the triggering of mitogenesis in various circumstances (Table 2). Thy-1 has no transmembrane protein sequence; IgM has such a sequence but has almost no cytoplasmic domain; and PDGFR has both a transmembrane sequence and a very large cytoplasmic domain with a tyrosine kinase activity.

GENETIC LINKAGE AND EXON STRUCTURE

It can be seen from Table 1 that loci for Ig-superfamily molecules are spread across the chromosomes. Genetic separation is common for loci that are coregulated and whose products are found in molecular complexes, but four notable cases of linkage are seen.

Firstly, in all cases the loci for V, J, and D segments of antigen receptors are linked to the C domain genes to which they can be rearranged (11, 69). Presumably a relatively close *cis* orientation is essential for the gene rearrangement mechanism.

Secondly, all the polymorphic MHC antigens are found in one large

chromosomal region, and this has been found in all species that have been investigated (16). Also in the mouse, the Qa and T1 products that are very closely related to MHC class I chains are coded by loci linked to the MHC. In contrast the CD1 antigens of humans are not MHC linked (21). The H chains of CD1 antigens are associated with β_2-m, but their sequences are much less closely related to class I MHC than are the Qa or T1 antigen sequences. The linkage of the polymorphic MHC antigens may be due to selective advantages that result if a set of polymorphic variants favorable for antigen presentation are inherited on one chromosome.

Thirdly, the two chains of the CD8 antigens are closely linked to each other and to the V_κ loci in human and mouse (27). No functional reason for this linkage is obvious. The CD8 antigens seem particularly closely related to the V-domains of antigen receptors (56). It may be that the V_κ and CD8 genes have remained together in a region of chromosome where extensive duplication occurred of genes for heterodimer structures related to immunity.

Finally, the loci for the CD3 chains, NCAM, and Thy-1 are all linked on the q23 band of chromosome 11, and these loci are also linked in the mouse (15). The CD3 δ and γ chain genes are arranged in reverse orientation and are separated by only 1.4 kb of sequence (70, 71). These genes are within 400 kb of the CD3 ε gene. The distance between the CD3 genes and NCAM and Thy-1 genes is not known.

There is no obvious functional reason for the chromosome 11 linkage group. Thy-1 and NCAM have in common their expression in neural tissues and also the fact that both molecules can be attached to the membrane by a glycophospholipid tail. The CD3 chains are found only on cells in the T lymphocyte lineage, but among Ig-related sequences the CD3 domains seem to match best with NCAM domains (14). Extensive gene duplication and divergence of Ig-related sequences may have occurred in the region now constituted by band q23 of chromosome 11, and Ig-related genes now found in this position may be those that have not moved to other chromosomes in evolution.

The most characteristic aspect of gene structure for Ig-superfamily molecules is that the majority of the domain sequence is often encoded within one exon. This is true for all domains in Igs and Tcrs and for 20 out of 30 domains in nonreceptor genes whose structures are shown in Figure 3A. However, a number of examples now exist of introns found between sequences coding for the Cys residues of the conserved disulfide bonds. The positions of these introns in relation to the postulated folding pattern of the domains are shown in Figure 3B. One other exception to the one domain: one exon rule is seen in the PolyIgR where the domains (II) and (III) are both encoded in one exon (76). In one PolyIgR mRNA form, this

A. ORGANIZATION OF EXONS

CD3 δ(70,71)	L		D		T	Y Y
CD3 γ(70,71)	L L		D		T	Y Y
MHC Class I (16)	L	O O	D		T	Y Y Y
MHC Class II α (72)	L	O	D		TY	
MHC Class II β (72)	L	O	D		T	Y
TL (18)	L	O O	D		T	Y
Qa (Q7) (19,20A)	L	O O	D		G	
B_{gp} (73)	L		D	O		
CD4 (74)	L		dd D D D		T	Y Y
CD8 (27)	L		D	O	T	Y
Thy-1 (75)	L		D		G	
MRC OX-2 (33)	L		D D		TY	
Poly Ig R (76)	-		dd (DD) - -		-	-
P_0 (77)	L		dd		T	Y Y
MAG (43)	L		D D D D D		T	Y Y
NCAM (40)	-		dd dd dd dd dd	O O O O	GT	Y Y Y

B. IG-LIKE DOMAINS ENCODED BY TWO EXONS

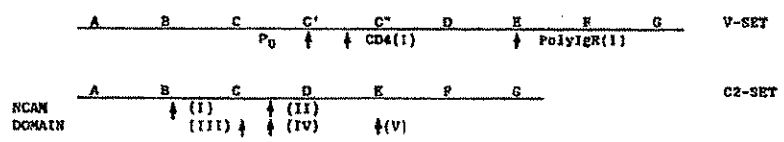

Figure 3 Organization of coding exons from genes for Ig-superfamily molecules excepting antigen receptor genes. *A: Organization of exons.* References to each gene structure are given after the molecule name. The letters showing exon organization are coded: L, exon for leader sequence; O, an exon encoding extracellular sequence that is not Ig-related; D, an exon encoding an Ig-related domain with no introns between codons for the conserved cys residues; dd, two exons encoding an Ig-related domain with an intron between codons for the conserved cys residues; (DD), an exon encoding two Ig-related domains; G, an exon for a hydrophobic sequence that is or may be cleaved from the protein when lipid is attached at the COOH-terminus; T, an exon for a transmembrane sequence; TY, an exon for a transmembrane plus cytoplasmic sequence; Y, an exon for cytoplasmic region sequence; — indicates exon structure was not established. *Comments:* the second D in CD4 applies to the second domain in Figure 1 which will not form a standard Ig-fold but has some sequence similarities to Ig-domains (28). The exon marked G in Thy-1 is established to encode a sequence that is cleaved in processing, and this is likely to be so for NCAM in which the shortest mRNA form has the exon marked G but not the TYYY exons. The longer NCAM mRNA forms splice out the G exon and include two possible combinations of the other exons. No attempt is made in this figure to show the alternative splicing events that can be seen, and noncoding exons are not shown. *B. Ig-like domains encoded by two exons.* The arrows indicate the positions of introns within the Ig-like domains. The letters A, B, C, C', C", D, E, F, G indicate the positions of putative β-strands determined by sequence similarity as indicated in Figure 2. (Data for PolyIgR from J. Harris and K. Mostov, personal communication.)

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exon is spliced out and the mRNA is translated to give the small form of rabbit PolyIgR (76).

FUNCTIONAL ASPECTS

Known functions of Ig-superfamily molecules are given in Table 2; in all cases they include adhesion functions or binding functions that trigger a subsequent event at the cell surface. A key functional feature is that homophilic or heterophilic binding occurs between Ig-related molecules; this is often between molecules on opposed membrane surfaces. Most of the Ig-superfamily molecules function at cell surfaces; the exceptions are antibody, the link protein, and α_1B-glycoprotein. The functions of antibody involve interactions with antigen and then with effector molecules via the Fc regions to trigger subsequent events. The link protein could be considered an adhesion molecule for binding together hyaluronic acid and proteoglycan; the function of α_1B-gp is unknown. It remains possible that α_1B-gp is a cleaved product from a cell surface receptor in much the same way that secretory component is a product derived by proteolysis from PolyIgR.

In all functions in which Ig-related domains are known to be involved, the domain can be considered as providing a stable platform for the display of specific determinants for recognition reactions on the faces of β-sheets or at the bends between β-strands (9, 52). The determinants involved are likely to be mostly protein in nature, but there is always the possibility that the chemical entities recognized are carbohydrate structures.

EVOLUTION

It is commonly accepted that all the Ig-related sequences shown in Figure 1 will have been derived by gene duplication and divergence from one primordial domain. Preceding this there may have been a half domain structure (78) that formed a homodimer with a structure like the V-domain (79). The half domain fold is postulated to be like that of β-strands ABCC' or GFED in Figure 2, and to associate to form a homodimer in the same way that the ABCC' and GFED β-strand loops associate in the V-domains (79). The half-domain idea seems to be supported by the existence of genes that have introns in the midst of sequence coding for residues between the conserved disulfide bonds, and it is notable that in a number of cases these come in a position that would roughly demarcate a half domain as

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proposed by McLachlan (79) (Figure 3B). Genes with the intra-domain introns may be thought more directly derived from ancient genes than those lacking this feature, but this is not a reliable conclusion since the probability of intron loss is unknown. Equally unknown is the probability of intron acquisition.

The possibility that the V-like fold is the most ancient is supported by the fact that Thy-1 and P_0 are the only single domain molecules thought to exist without association with other chains, and both of these are in the V-SET. P_0 also has an intron in the middle of the domain. If the V-fold were the most ancient type, then the C-like domain would be derived by loss of sequence from the middle of a V-type fold. A possible lineage would be V-type to C2-type to C1-type. However, one could argue alternatively that the C2-type is the most primitive and that both V-type and C1-type sequences were derived from this. A start from a C-type fold would not fit with the idea that the primordial domain is derived from a homodimer of half-domain structures. The C1-type seems an unlikely candidate for the most primitive domain since thus far C1-set sequences have been seen only in structures associated with the immune system and mostly with immune recognition. Also no intradomain introns have been seen in the C1-set sequences.

It is difficult to suggest detailed evolutionary trees for the Ig-related molecules. Some molecules can be grouped because they show greater than average similarity within the superfamily. Some such groupings might be: (IgV, TcrV, CD8); (IgC, TcrC, MHC, CD1); (CD3 ε, δ, γ); (CD2, LFA-3); (MAG, NCAM); (PDGFR, CSF1R). The difficulty in trying to connect up these groups and the other molecules is that the Ig-related molecules appear to be diverging very rapidly, as assessed by the percentage of identity for equivalent chains between species. This is as low as 42% for the V-like domain of CD8 chain I between rodents and humans (27) which suggests that when new genes are created they might rapidly diverge to a level where only the basic conserved patterns remain. Intermediate stages in evolution may be hard to detect in the contemporary sequences. In addition, multichain sequences have probably arisen repeatedly, and perhaps sometimes single domain structures have been rederived from multidomain forms. Examples of differences in evolution of multidomain structures can be seen with PDGFR and CSF1R in one case and CEA in another. PDGFR and CSF1R have similar 5 domain patterns (46); these were probably derived from an immediate common precursor with the same domain pattern. In contrast, CEA has seven domains among which the last 6 appear to have been derived by a recent double duplication of a two domain segment (45). This is likely because within domains II, IV, VI and III, V, VII there is about 70% identity, while the level of identity

between these two sets is about 25%. The nature of the immediate precursor of CEA is thus unpredictable. In the case of rabbit PolyIgR, reduction in size can be seen in the alternative splicing that produces a variant lacking domains II and III of the structure as shown in Figure 1 (76).

In terms of functional evolution the phenomenon of interactions within the superfamily (Table 2) is strongly suggestive that the primordial function involved a single domain interacting with itself probably between opposed membranes. Such a function has been suggested for the P_0 myelin protein (44). If correct, this may be an interesting model for the function of the primordial domain. Heterophilic receptor pairs presumably evolved from a homophilic interaction system, and highly specific interactions become possible with heterophilic recognition between different cell types. It seems likely that the first functions were related to adhesion or triggering at cell surfaces to control the behavior of cells within a multicellular organism. The Ig-related molecules that function in neural tissues may be mediating functions of the primitive type. A scheme for the evolution of heterophilic interactions from a homophilic adhesion system between cells is illustrated in Figure 4A.

Another possibility is that heterophilic pairs giving recognition between cells may have originated from chains that interacted on one cell to form a heterodimer (80). This is illustrated in Figure 4B starting from a homodimer diverging to a heterodimer and then to modified forms of the heterodimer chains interacting between cells. The LFA-3: CD2 adhesion molecules may have evolved in this way from a chain that formed a homodimer and contained one Ig-related domain with a disulfide bond and one without (NH_2 terminal). From this type of origin the result could be a heterophilic pair, each member of which is more closely related to the other than to other members of the superfamily (as is the case for LFA-3 and CD2) (25).

If it is accepted that involvement in cell recognition was the primary role of the Ig-superfamily, the question then arises—how might the vertebrate immune system be derived from this? One possible functional antecedent is the phenomenon of programmed cell death. In the invertebrate *Caenorhabditis elegans*, 25% of developing neural cells die in a predictable way, and this commonly involves an apparent differentiation to cell death followed by phagocytosis (81, 82). In some cases, however, one cell appears to kill another, and this function is of the type that might be turned outwards to produce an immune system. Figure 4C suggests that Ig-related molecules control the specificity of a primitive natural killer cell and that modification of this specificity to include determinants of a common pathogen resulted in a killer system to eliminate pathogen-infected cells.

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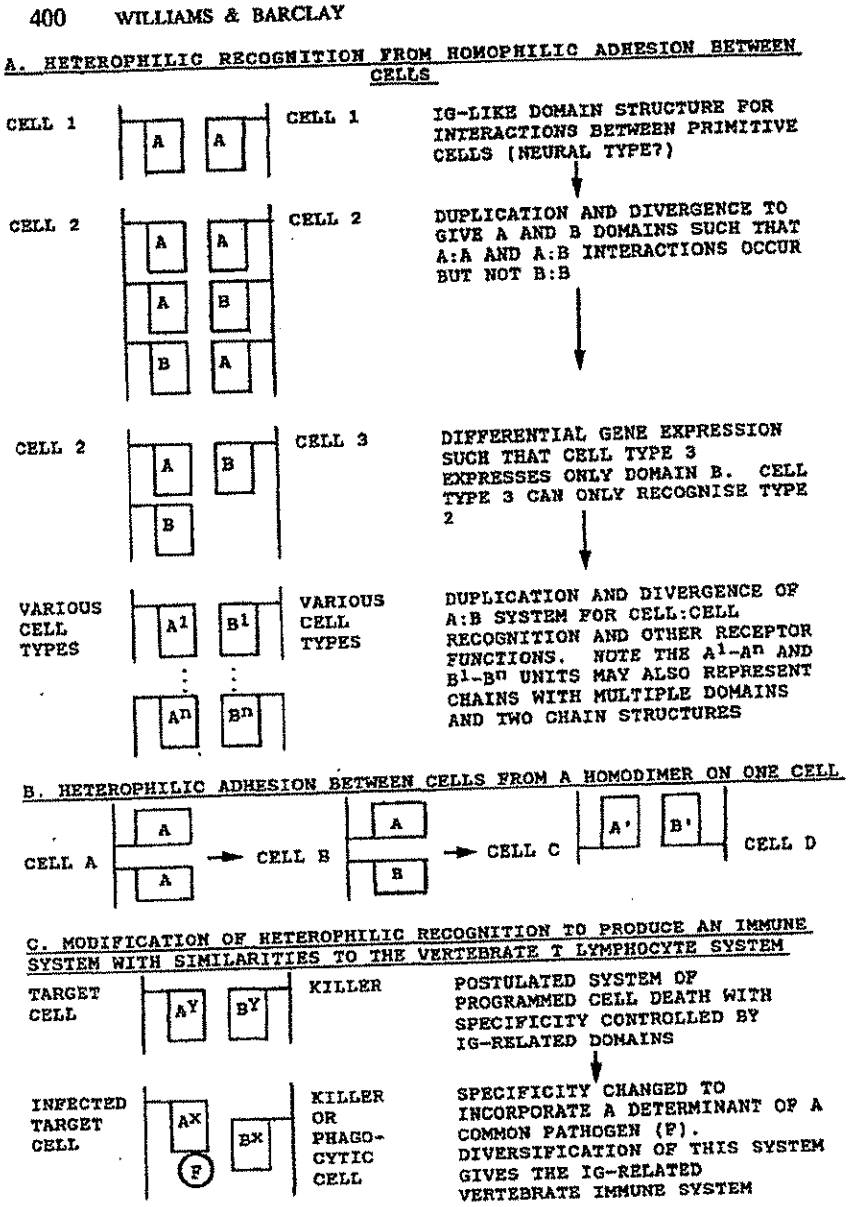

Figure 4 A possible scheme for structural and functional evolution in the Ig superfamily.

Duplication and divergence of this system could lead to an immune system with the properties of the vertebrate T lymphocyte system. The B lymphocyte arm of the immune response may have evolved from this as a recognition system freed from the constraint of interaction with MHC antigens. The possibility that T cell immunity is the more primitive is supported by the finding that the T cell CD8 antigen chain II has a sequence that is very like receptor J pieces without an intron or other intervening genomic sequence between the main V-like exon and the J-related piece (56). The CD8 heterodimer may be similar in its V-like domains to the structure that gave rise to the antigen receptor heterodimers.

Structural and functional evolution would be greatly illuminated if invertebrate Ig-related molecules were identified. Thus far only one invertebrate sequence that might be Ig-related is known, and this is a glycoprotein of 84 amino acids with a glycophospholipid tail that was identified in a search for Thy-1-like molecules from squid neural tissue (83). The squid glycoprotein has some interesting sequence similarities to Thy-1 and Ig V-domains but does not have a standard domain pattern. Thus, it cannot at this stage be added to the Ig-superfamily with the level of confidence that was applied for molecules in Figure 1. Are Ig-related structures common in invertebrate neural cells and do invertebrate immune systems use Ig-related molecules at all? Answers to questions of this type are needed to further elucidate the structural and functional evolution of the Ig-superfamily.

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Note Added in Proof

Additional Ig-related structures include the CD7 and CD28 T lymphocyte antigens, one of the chains of the mast cell Fc receptor for IgE, BLAST-1, a leucocyte antigen and the proto-oncogene c-*kit*. CD7 and CD28 antigens have a single V-like domain with transmembrane sequence and cytoplasmic domain. The CD28 chain exists as a disulfide linked homodimer. In structural architecture the FcRε chain is the same as the FcRγ chain, BLAST-1 is like LFA-3 and CD2. The proto-oncogene c-*kit* is the same as CSF1R and PDGFR.

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Exhibit 8

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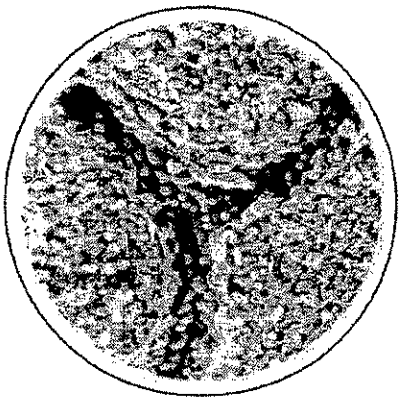

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Front Cover:

Natural transplantation in Tunicates

Among some nonvertebrates, such as tunicates, individuals can fuse to form a multi-individual colony. *Colonial tunicates* form colonies by a complex recognition system involving fusion or rejection of extracorporeal blood vessels between two individuals or two colonies. When fused, the two colonies share blood cells. On the cover a colony bearing orange pigment cells (lower right) has fused with a colony bearing purple pigment cells (upper left), resulting in a mixing of the blood cells. Fusion or rejection is genetically controlled in this species by a single gene locus which has many alleles; two individuals must share the same allele to fuse. The same locus (or a closely linked locus) also controls *fertilization* (sperm-egg fusion) between two individuals; in this case sperm and egg must *not* share alleles to fuse.

This type of genetic control of "transplantation" extends into all vertebrates, where a genetic region called the *major histocompatibility complex* (MHC) encodes transplantation antigens (cell-surface glycoproteins) which prevent the acceptance of the type of unnatural transplants tried by transplantation biologists and physicians. The products of the MHC are also used as signals for intercellular communications between immunity cells within an individual, and through these signals control our susceptibility to infections. Because the biology and molecular genetics of the MHC are recurring themes throughout this book, we have chosen this cover to illustrate an example of the unity of important biological principles, including the possibility that tunicate fusion/rejection genes may be forerunners of the vertebrate MHC. (Photo courtesy of Virginia Scofield and Irving Weissman).

Back Cover:

Structure of an Antibody Molecule

This computer graphic representation by Arthur J. Olson illustrates the protein backbone of the human myeloma IgG Dob. This representation is based on the X-ray crystal studies of M. Navia, E. Silverton, V. R. Sarma, G. H. Cohen, and D. R. Davies. The image was produced using software developed by Olson, T. J. O'Donnell, and Michael L. Connolly.

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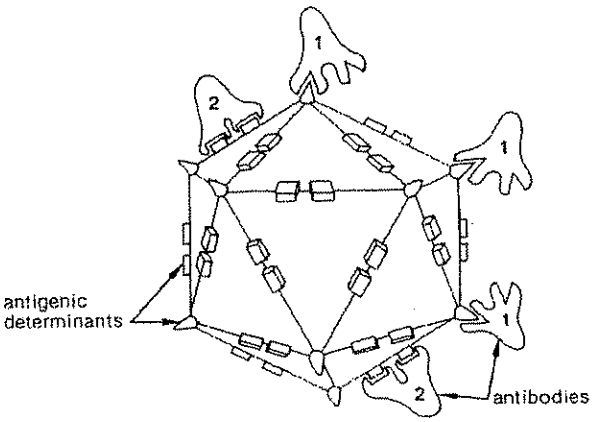

Figure 1-4
A virus interacting with antibodies specific for two types of antigen determinants.

entities that they recognize are termed *antigens*. That portion of the antigen to which an antibody binds is called an *antigenic determinant* (Figure 1-4). Antibodies recognize and bind antigens by molecular complementarity, which permits multiple noncovalent interactions of the same types that confer specificity on enzyme-substrate binding. An antigen complementary to a specific antibody is called a *cognate antigen*.

Immunogens and immunity

An antigen that elicits a response from the immune system is referred to as an *immunogen*. Macromolecules such as foreign proteins, nucleic acids, and carbohydrates are usually effective immunogens; molecules with molecular weights of less than 5000 are usually not. However, many small nonimmunogenic molecules, termed *haptens*, can stimulate an immune response if covalently attached to a large *carrier* molecule. For example, the 2,4-dinitrophenyl group is not immunogenic unless attached to a carrier protein such as serum albumin (Figure 1-5).

Animals that have an appropriate number of activated specific T cells or an appropriate concentration of specific antibody in their blood are *immune* to the cognate antigens.

Immunoglobulin molecules

Antibodies form the class of proteins called *immunoglobulins*. The basic unit of immunoglobulin structure is a complex of four polypeptides, two identical "light" (low molecular weight) chains and two identical "heavy" (high molecular weight) chains, linked together by disulfide bonds (Figure 1-6).

The amino-terminal (N-terminal) portions of the light and heavy chains differ substantially in amino acid sequence between individual species of antibody. These *variable* regions of the light and heavy chains combine to form the *antigen-binding sites* of an antibody mole-

The carboxyl-terminal (C-terminal) portions of the light and heavy chains, termed the *constant* regions, are identical or nearly identical for antibodies of the same class. The constant regions of circulating antibodies are responsible for the variety of characteristic *effector functions* that are involved in the elimination of foreign antigens. Immunoglobulins in mammalian serum can be divided into five classes on the basis of amino acid sequence differences in the constant regions of their heavy chains. These classes, designated IgM, IgG, IgA, IgD, and IgE, correspond to antibodies with different effector functions. One example of the diverse effector functions specified by heavy-chain constant regions is the differential transport of antibodies across tissues (Figure 1-7). IgA molecules (but not IgG molecules) may be transported across the intestinal lining into the lumen, whereas IgG molecules (but not IgA molecules) may be transported across the placenta to a fetus. The structures and molecular functions of antibody molecules are discussed in more detail in Chapter 2.

Antigen-binding sites

The antigen-binding sites of individual antibodies are unique combinations of light-chain and heavy-chain variable regions, and consequently these binding sites exhibit unique protein structures. If a specific antibody from one animal is injected as an immunogen into a suitable second animal, the injected antibody will elicit production of host antibodies. Some of these antibodies will be specific for the unique determinants of the variable regions of the injected antibody. Such antigenic determinants are known collectively as the *idiotype* of the injected antibody. Every species of antibody will exhibit unique idiotypic determinants, some of which are determined by its antigen-binding site and some of which are determined by structural features outside the binding site.

1-4 Each lymphocyte is predetermined to express a homogeneous set of membrane-bound receptors with a single specificity for antigen

The molecular nature of receptors

The cell surfaces of lymphocytes carry membrane-bound antibodies or antibodylike molecules that function as antigen receptors. The receptors of B cells are antibodies that are present on the membrane in a quantity of about 10^5 molecules per cell. The receptors of T cells appear to have similar properties, but their molecular nature is less well known. Each lymphocyte carries only one kind of specific receptor, and therefore it will respond to only a few closely related antigenic determinants. Binding of an antigen to a receptor initiates a humoral or a cellular immune response, depending upon whether a B-cell or a T-cell receptor is stimulated.

and complement fixation), whereas others are not (placental transfer, epithelial transfer, and mast-cell fixation). It is likely, although unproven, that each of the C domains contains the active site for at least one effector function. There is some evidence that the C_H2 domain of the IgG molecule plays a role in complement activation. The C_H3 domain will bind to lymphocyte and certain macrophage membranes. Accordingly, the V domain must communicate with the C_H2 and C_H3 domains to trigger appropriate effector functions. Presumably this communication involves conformational changes that occur throughout the antibody molecule when an antigen is bound by the V domain. However, these conformational changes probably require binding of antigen to more than one V domain. Binding of univalent haptens does not activate complement or trigger B-cell activation. Apparently these effector functions are initiated by cross-linking the arms of a single antibody or by cross-linking two or more antibody molecules to each other.

2-6 The five classes of immunoglobulin molecules differ in the structures and functions of their heavy-chain constant regions

Heavy- and light-chain classes and structural differences

The five immunoglobulin classes are distinguished structurally by differences in their heavy-chain constant regions. Comparisons of C_H-region amino acid sequences show that there are five major heavy-chain classes, designated α, γ, δ, ϵ, and μ. These heavy-chain classes define the corresponding immunoglobulin classes IgA, IgG, IgD, IgE, and IgM, respectively (Table 2-4). The C_H amino acid sequences of these classes are homologous, but they differ by more than 60% of their residues. Some classes can be divided into subclasses, defined by C_H regions that are distinct but more similar in amino acid sequence; for example, in humans the γ class can be divided into $\gamma1$, $\gamma2$, $\gamma3$, and $\gamma4$ subclasses. The genes that code for heavy chains are known collectively as the *heavy-chain (H) gene family*.

In addition there are two major types of light chains based on comparisons of C-region amino acid sequence. These two types, designated κ and λ, differ by about 60% in their C_L amino acid sequences (Table 2-5). Like some heavy-chain classes, the λ-type light chains may be further divided into subtypes defined by distinct but very similar C_L sequences. Immunoglobulin light chains from various mammals can be assigned readily to the κ and λ types, based on sequence homology. The genes that code for these light-chain types comprise the κ gene family and the λ gene family, respectively.

Exhibit 9

THIS EXHIBIT HAS BEEN REDACTED IN ITS ENTIRETY