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-MPT **PUBLIC VERSION**

PLAINTIFF ZYMOGENETICS, INC.'S RESPONSE STATEMENT OF MATERIAL FACTS AS TO WHICH THERE IS A GENUINE ISSUE TO BE TRIED AND LEGAL ISSUES IN OPPOSITION TO DEFENDANT BRISTOL-MYERS SQUIBB COMPANY'S MOTION FOR SUMMARY JUDGMENT OF NO WILLFULNESS

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ZymoGenetics, Inc. ("ZymoGenetics") has asserted that Bristol-Myers Squibb Co. ("Bristol") infringes Claims 1-3, 5, 6, 21, 22, 24, 25, 50, and 51 of U.S. Patent No. 5,843,725 ("the '725 Patent") and Claims 1-4, 7, and 30 of U.S. Patent No. 6,018,026 ("the '026 Patent") (collectively, "Patents-in-Suit"). In accordance with Paragraph 3 of the Court's Revised Scheduling Order of March 13, 2008 (D.I. 148), ZymoGenetics submits this Response Statement of Material Facts As To Which There is a Genuine Issue to Be Tried and Legal Issues in Opposition to Defendant Bristol-Myers Squibb Company's Motion for Summary Judgment of No Willfulness. With reasons set forth below, ZymoGenetics states whether the facts set forth by Bristol in its Statement are "undisputed" or "disputed." Bristol seeks to include opinion testimony and legal conclusion as alleged undisputed fact, including arguments and responses to hypothetical questions. In addition, Bristol refers to nonexistent claim limitations of the Patents-in-Suit as undisputed fact. Bristol also improperly attempts to characterize opinion testimony as fact by using the word "admitted" throughout its statement.

Pursuant to the Court's Revised Scheduling Order dated March 13, 2008, Paragraph 3, the record evidence cited by ZymoGenetics below constitutes only that evidence which directly rebuts the facts which Bristol alleges are undisputed in its underlying summary judgment motion. Bristol's statement of undisputed facts in support of its summary judgment motion is not comprehensive, and therefore the evidence cited below is not exhaustive of the evidence which ZymoGenetics would include in any opposition to Bristol's summary judgment motion. ZymoGenetics reserves the right to rely on record evidence not cited below in any such opposition. These disputed facts pertain to the legal issue of whether Orencia satisfies the claim elements in the asserted claims of the Patents-in-Suit.

STATEMENT OF DISPUTED FACTS

1. Not disputed. The Patents-in-Suit require that the nonimmunoglobulin polypeptides requiring dimerization for biological activity be joined to a dimerizing protein. Declaration of John W. Kirkland in Support of Bristol-Myers Squibb Company's Motion for

Summary Judgment of No Willfulness ("Kirkland Decl."), Ex. 1 at ZG 000003; Ex. 2 at ZG 000844.

2. Not disputed. This is a legal contention, not a factual statement, and is not in dispute. The claim term "dimerizing protein" should be construed as the Patents-in-Suit have explicitly defined them. *See* Joint Claim Construction Chart (D.I. 153) at 3-4; *see also* ZymoGenetics' Opening Claim Construction Brief (D.I. 156) at 11-12.

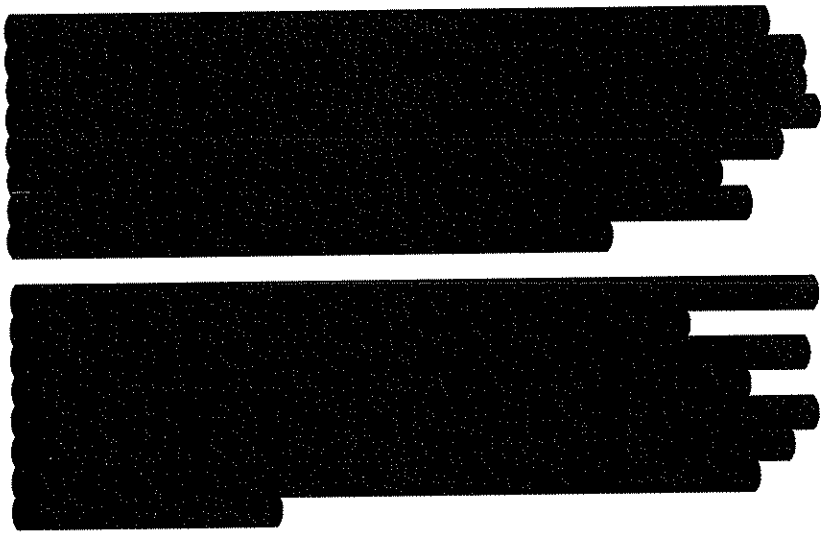
3. Not disputed.

4. Not disputed. *See* Kirkland Decl., Ex. 1 at ZG 000003; Ex. 2 at ZG 000844.

5. Disputed. ZymoGenetics stated that its claim construction position is that "requiring dimerization for biological activity" should be afforded its ordinary meaning within the context of the claims of the Patents-in-suit. Joint Claim Construction Chart (D.I. 153), Ex. A at 2. In addition, ZymoGenetics provided specific definitions for the terms contained in this statement: biological activity and dimerizing protein.

6. Not disputed.

7. Disputed. ZymoGenetics' response to Bristol's Interrogatory No. 7 states:



Kirkland Decl., Ex. 6 at 13.

8. Not disputed.

9. Disputed. This is a nonsensical statement of fact: either an immunoglobulin in its native form is a tetramer, [redacted] *id.*, Ex. 4 at 1. Moreover, a paper written by Bristol scientists states that the Ig portion of Bristol's CTLA4-Ig was mutated for the purpose of "reduc[ing] binding to Fc receptors." *See* article entitled "The Immunoglobulin Superfamily -- Domains for Cell Surface Recognition" by Alan Williams and A. Neil Barclay ("Williams Article") at BMS001054678, attached hereto as Exhibit 1. Dr. Kirk Leister, [redacted] *See* Andre Decl., Ex. 6 at 53:8-12; *see also* testimony from Dr. David Smolin, [redacted] ("Smolin Depo.") at 37:12-17, attached hereto as Exhibit 2; *see also* Andre Decl., Ex. 11 at 27:19-25, testimony from Dr. Gary Nadler, [redacted] for Bristol and 30(b)(6) witness.

10. Disputed. Dr. Gascoigne stated that [redacted] Deposition of Nicholas Gascoigne at 204:21-205:2, attached hereto as Exhibit 3. In fact, experiments by [redacted] Andre Decl., Ex. 14 at 262:24-264:1, 271:14-17, 274:8-14; Disclosure of Expert Testimony of David A. Ostrov, Ph.D. ("Ostrov Expert Report") at ¶20, attached hereto as Exhibit 4; *see also* Deposition of Peter S. Linsley, Ph.D. ("Linsley Depo.") at BMS004256417-418 (69:24-70:10), attached hereto as Exhibit 5; paper entitled [redacted] by James K. Tamura [redacted] at BMS003504382-84, attached hereto as Exhibit 6; [redacted] at BMS001596163-67, attached hereto as Exhibit 7; [redacted] at BMS001463910, attached hereto as Exhibit 8; Kirkland Decl., Ex. 10 at BMS 003700202; Andre Decl., Ex. 6 at 30:25-31:9; *id.*, Ex. 18 at 63:12-21; article entitled "Cleavable CD40Ig

fusion proteins and the binding of sgp39" by Diane Hollenbaugh, et al. ("Hollenbaugh Article") at BMS 003700192-198, attached hereto as Exhibit 9; Andre Decl., Ex. 5 at BMS001290162; Disclosure of Expert Testimony of Nicholas R.J. Gascoigne ("Gascoigne Expert Report") at ¶17-19, attached hereto as Exhibit 10.

11. Disputed. This statement is nonsensical because there [redacted] Dr. Gascoigne offered his opinion that [redacted] *See* Gascoigne Depo. at 16:20-17:1, 202:20. Dr. Gascoigne directly relied upon [redacted] *See* Gascoigne Expert Report at ¶3. In fact, experiments prove that [redacted] *See e.g.* Andre Decl., Ex. 14 at 262:24-264:1, 271:14-17, 274:8-14; *see also* Ostrov Expert Report at ¶20; Linsley Depo. at BMS004256417-418 (69:24-70:10); Tamura Paper at BMS003504382-84; [redacted] at BMS001596163-67; [redacted] at BMS001463910; Kirkland Decl., Ex. 12 at BMS 003700202; Andre Decl., Ex. 6 at 30:25-31:9; *id.*, Ex. 18 at 63:12-21; Hollenbaugh Article at BMS 003700192-198; Andre Decl., Ex. 5 at BMS001290162; Ostrov Expert Report at ¶17-19.

12. Disputed. The Patents-in-Suit define "biological activity" as

> 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 activity 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.

Kirkland Decl., Ex. 2 at col. 9, ll. 36-45; *id.*, Ex. 1 at col. 9, ll. 52-63.

13. Disputed. The Interrogatory response in question was to an Interrogatory relating to U.S. Patent No. 6,291,646 B1, and is not a Patent-in-Suit.

14. Disputed. The Interrogatory response in question was to an Interrogatory relating to U.S. Patent No. 6,291,646 B1, and is not a Patent-in-Suit.

15. Disputed. The quote from the scientific article is taken out-of-context and is misleading, and should include the following text in order to understand that binding to a protein expressed from an artificial monomer construct is not representative of how the native dimeric CTLA-4 exists, nor how the native form binds ligand on the surface of the T cell:

> CD28 and CTLA-4 are homologous T cell receptors of the immunoglobulin (Ig) superfamily, which bind B7 molecules (CD80 and CD86) on antigen-presenting cells and transmit important costimulatory signals during T cell activation…We demonstrate CTLA-4 is a homodimer interconnected by one disulfide bond in the extracellular domain at cysteine residue 120…Thus, CTLA-4 and probably CD28 have a receptor structure consisting of preexisting covalent homodimers with two binding sites.
>
> * * *
>
> Some studies have suggested that CTLA-4 is also a disulfide-liked homodimer (10, 13, 15), but another suggested that it exists as a monomer (16)…We show that CTLA-4 is a disulfide-linked homodimer containing two binding sites for its ligands.
>
> * * *
>
> Our data show that although full-length CTLA-4 is predominantly homodimeric, removal of the transmembrane and cytoplasmic domains (CTLA4X) leads to predominantly monomeric protein. Thus, the presence of C120 does not ensure dimerization. This suggests that dimerization of CTLA-4 and formation of the disulfide bond at Cys120 requires the transmembrane and/or cytoplasmic domains of the two chains…Monomeric CTLA-4 had reduced binding activity when compared to dimeric CTLA-4…30 - 90-fold less active…

Kirkland Decl., Ex. 12 at BMS 003700199, 003700205.

16. Disputed. The quote from the scientific article is taken out-of-context and is misleading, and should include the following text in order to understand that binding to a protein expressed from an artificial monomer construct is not representative of how the native dimeric CTLA-4 form exists, nor how the native form binds ligand on the surface of the T cell:

> Antibodies (or immunoglobulin, Ig)…Receptor dimerization and/or oligomerization are generally critical for initiating signal transduction…CTLA-4 is a homodimer interconnected by one disulphide bond…Thus CTLA-4…have a receptor structure consisting of preexisting homodimers with two equivalent binding sites…The first of these is the

> availability of preexisting covalent dimers of…CTLA4 receptors…Since CD28/CTLA-4 are preexisting homodimers, signal transduction is most likely initiated by either allosteric changes in receptor structure…CD28 and CTLA-4 are coexpressed on T cells during the activation response…

Kirkland Decl., Ex. 13 at ZG 054279-86.

17. Disputed. The quote from the scientific article is taken out-of-context and is misleading, and should include the following text in order to understand that binding to a protein expressed from an artificial monomer construct is not representative of how the native dimeric CTLA-4 form exists, nor how the native form binds ligand on the surface of the T cell:

> CD28 and CTLA-4 are homologous T cell receptors of the immunoglobulin (Ig) superfamily, which bind B7 molecules (CD80 and CD86) on antigen-presenting cells and transmit important costimulatory signals during T cell activation…We demonstrate CTLA-4 is a homodimer interconnected by one disulfide bond in the extracellular domain at cysteine residue 120…Thus, CTLA-4 and probably CD28 have a receptor structure consisting of preexisting covalent homodimers with two binding sites.
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> * * *
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> Some studies have suggested that CTLA-4 is also a disulfide-liked homodimer (10, 13, 15), but another suggested that it exists as a monomer (16)…We show that CTLA-4 is a disulfide-linked homodimer containing two binding sites for its ligands.
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> * * *
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> Our data show that although full-length CTLA-4 is predominantly homodimeric, removal of the transmembrane and cytoplasmic domains (CTLA4X) leads to predominantly monomeric protein. Thus, the presence of C120 does not ensure dimerization. This suggests that dimerization of CTLA-4 and formation of the disulfide bond at Cys120 requires the transmembrane and/or cytoplasmic domains of the two chains…Monomeric CTLA-4 had reduced binding activity when compared to dimeric CTLA-4…30 - 90-fold less active…

Kirkland Decl., Ex. 12 at BMS 003700199, 003700205.

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Exhibit 1

THIS EXHIBIT HAS BEEN
REDACTED IN ITS ENTIRETY

Exhibit 2

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Exhibit 3

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Exhibit 4

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Exhibit 7

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Exhibit 8

THIS EXHIBIT HAS BEEN
REDACTED IN ITS ENTIRETY

Exhibit 9

ELSEVIER

Journal of Immunological Methods 188 (1995) 1–7

JOURNAL OF IMMUNOLOGICAL METHODS

Cleavable CD40Ig fusion proteins and the binding to sgp39

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Abstract

Recombinant immunoglobulin (Ig) fusion proteins of cell surface and intracellular proteins have wide applications. For example, fusion proteins have been used in the isolation, identification and study of ligands and the effects of binding or blocking a receptor-ligand pair, either in vivo or in vitro. For some applications, removal of the immunoglobulin Fc region is advantageous. We have developed two vectors for the expression of Ig fusion proteins that contain recognition sequences for protease cleavage using thrombin. In one vector, the sequence encoding the thrombin cleavage site is located at the junction of the DNA fragment encoding the protein or protein fragment to be studied and the hinge and constant regions of the immunoglobulin, allowing the generation of a monomeric form of the protein of interest. In the second vector, the sequence encoding the thrombin cleavage site is located between the sequences encoding the hinge and constant regions of the immunoglobulin, allowing for the generation of covalent dimers of the recombinant protein without the constant Fc domains. We have used these vectors to produce the constructs encoding two forms of the extracellular domain of CD40, CD40ThrIg and CD40HinThrIg, allowing production of a monomeric and dimeric form of recombinant CD40. Cleavage is efficient and complete. Following cleavage, there was no detectable binding of the monomeric form of CD40 to a soluble form of gp39, the ligand of CD40, while the dimeric form was able to bind. These vectors have been constructed to allow facile substitution with other sequences to generate cleavable forms of other proteins of interest.

Keywords: CD40; gp39; Fusion protein; Protease cleavage

1. Introduction

An immunoglobulin (Ig) fusion protein, CD40Ig, consisting of the extracellular domain of CD40 fused to the hinge and constant domains of human IgG1, has been used extensively as a tool to study this receptor and its ligand. Though originally identified as a B cell antigen, CD40 has been found to be expressed by thymic epithelium, basal epithelium, some melanoma and carcinoma derived cell lines, monocytes, follicular dendritic cells, T cells, and endothelial cells (Clark and Ledbetter, 1986; Paulie et al., 1985; Ledbetter et al., 1987; Young et al., 1989; Galy and Spits, 1992; Alderson et al., 1993; Armitage et al., 1993; Hollenbaugh et al., 1995). The cDNA encoding CD40 was isolated using mammalian cell based expression cloning, and found to encode a type I membrane bound protein, which consists of a 22 amino acid NH_2-terminus secretory signal sequence, a 171 amino acid extracellular domain followed by a 22 amino acid hydrophobic transmembrane domain and a 62 amino acid cyto-

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plasmic sequence (Stamenkovic et al., 1988). Early work using antibodies directed against the CD40 protein showed that signaling through this receptor led to alterations in B cell function. For example, in the presence of an appropriate costimulatory signal, stimulation of B cells with anti-CD40 will result in B cell proliferation and isotype switching (Clark and Ledbetter, 1986; Gordon et al., 1987; Gascan et al., 1991; Aruffo et al., 1993).

Membranes isolated from activated T cells were also known to provide this help in B cell activation. CD40Ig was found to inhibit the stimulation of B cells by membranes isolated from activated T cells (Noelle et al., 1992). CD40Ig recognized a ~34 kDa cell surface protein expressed by activated peripheral blood T cells. The murine ligand of CD40 was cloned using a CD40Ig fusion protein to isolate a subpopulation of the EL4 cell line with increased levels of CD40Ig binding, followed by mammalian cell based expression cloning to isolate a cDNA encoding the ligand (Armitage et al., 1992). The ligand of CD40, known as gp39 (T-BAM, TRAP, CD40L) is a type II membrane protein consisting of 22 amino acids predicted to be intracellular, a 24 amino acid hydrophobic region consistent with a transmembrane domain and a 215 amino acid extracellular domain. Expression of gp39 has been shown to be tightly regulated on T cells, where expression is detected only after activation.

The CD40Ig has further been used to demonstrate that defects in the gene encoding gp39 are the cause of the X-linked hyper-IgM syndrome, a primary immunodeficiency resulting in absence or near absence of all immunoglobulin isotypes other than IgM despite normal numbers of both T and B cells. T cells isolated from patients with this disease do not bind CD40Ig under conditions of stimulation that lead to binding of CD40Ig in normal control cells. The defects in the gp39 gene, leading to amino acid substitutions or deletions in the protein, destroy the ability of encoded gp39 protein to bind to CD40. The disease apparently results from the inability of T cells expressing the defective gp39 to provide appropriate help to B cells (Allen et al., 1993; Aruffo et al., 1993; Di Santo et al., 1993; Fuleihan et al., 1993; Korthauer et al., 1993; Ramesh et al., 1993).

The effect of blocking the CD40-gp39 interaction in vivo has been studied both by the use of an anti-murine gp39 mAb, MR1, as well as the CD40Ig fusion protein. Using MR1, blocking of the gp39-CD40 interaction has been shown to block antibody production to a T-dependent antigen (Foy et al., 1993). More recently, administration of the antibody has been shown to block disease progression in several models of disease including collagen induced arthritis and graft vs. host disease (Durie et al., 1993; Durie et al., 1994). In the case of the CD40Ig, in vivo studies have made use of both injection of the purified fusion protein as well as the generation of transgenic mice expressing the CD40Ig (Gray et al., 1994; Lane et al., 1994).

In the present study, we sought to develop vectors whereby Ig fusion proteins could be readily produced in a format that allowed facile removal of the Ig tail. In this way the fusion proteins generated would contain the Fc region for initial purification and all the previously identified applications, and allow other applications that are precluded, or made more difficult, by the presence of the Ig hinge and constant domains. This is analogous to the production of F(ab) and $F(ab')_2$ fragments of antibodies. An example of one such application is the study of cells expressing Fc receptors, where the presence of the Fc might introduce a stimulatory or inhibitory signal unrelated to the receptor/ligand pair of interest. Another example is the production of polyclonal antisera or monoclonal antibodies, where the response to the Ig may mask the response to the desired protein, interfere in the use of a polyclonal sera or introduce complications in the screening for a desired monoclonal antibody. In particular, we sought to develop these vectors for the study of the CD40/gp39 receptor ligand pair.

2. Material and methods

The vectors were constructed by PCR, with the sequences encoding the cleavage site and linker synthesized as part of the PCR primer. Linker and cleavage sequences were adapted from Hakes and Dixon (Hakes and Dixon, 1992). PCR was performed using as the template a vector containing the extracellular domain of CD40 fused to the hinge and two constant domains of human IgG1, with a *Bam*HI site at the junction, in the parent plasmid CDM7B$^-$.

In these constructs, the IgG1 contains three point mutations designed to reduce binding to Fc receptors as previously described (Hollenbaugh et al., 1992). For both constructs, the forward PCR primer used primes in the promoter region of the vector. This primer is 5′-GTACGGGCCAGATATACGCGT-TGACATTGATTA-3′. The underlined sequence is the recognition site for the enzyme *Mlu*I, used to insert the PCR products into the vector.

The CD40ThrIg was constructed using the primer 5′-CGGAAGATCTCCGGTACCGAAGCCACGAG-GAACCAGGCGGCCGCCGCCGCCGCCGGGAT-CCT**GGGGACCACAGAC**-3′. Underlined sequences are recognition sequences of *Bgl*II, used to clone the PCR product, *Kpn*I and *Eag*I, used as diagnostic sites in the construction of the plasmids, and *Bam*HI, used to allow digestion of the final product to insert other sequences to generate cleavable fusion proteins other than CD40. The region in bold is complimentary to CD40. PCR reactions using CD40Ig were performed using the polymerase *Pfu* according to the manufacturers recommendations. The PCR product was extracted with phenol, precipitated with ethanol and digested with *Mlu*I and *Bgl*II. The resulting fragment was ligated into a vector containing the human IgG1 with the three point mutations described above that was digested with *Mlu*I and *Bam*HI to create complimentary ends with the *Mlu*I/*Bgl*II digested PCR fragment.

The CD40HinThrIg vector was constructed using the PCR primer 5′-GCCCAATTGAG-GTGCGCCGGTACCGAAGCCACGAGGAACCA-GGCGGCCGCCGCCGCCGCCT**GGGCACGGT-GGGCATGTGTG**-3′. The underlined sequences are recognition sites for *Mun*I, used to clone the PCR product and *Kpn*I and *Eag*I, used as diagnostic sites in the construction of the plasmids. The region in bold is complimentary to the hinge of the human IgG1. PCR reactions were performed using the polymerase *Pfu* according to the manufacturers recommendations. The PCR product was extracted with phenol, precipitated with ethanol and digested with *Mlu*I and *Mun*I. The resulting fragment was ligated into a vector containing the human IgG1 digested with *Mlu*I and *Eco*RI to create complimentary ends with the *Mlu*I/*Mun*I digested PCR fragment.

Protein was produced from COS cells by transfection of the mammalian expression plasmids as previously described (Hollenbaugh and Aruffo, 1994). Briefly, COS cells are transfected using DEAE-dextran, then grown in cysteine- and methionine-free DMEM to which Tran^{35}S label (ICN, Costa Mesa, CA) had been added. Cell supernatants were harvested and centrifuged to clarify. Immunoprecipitations were performed using Protein A-agarose (Repligen,) or anti-rat Ig-agarose (Sigma Chemical Co., St. Louis, MO) as indicated. 500 μl–1 ml COS cell supernatant was incubated with 100 μl Protein A-agarose slurry and rotated at room temperature for 1–2 h or at 4°C overnight. The agarose was then washed five times with PBS containing 0.1% NP-40. Samples to be analyzed by SDS-PAGE without digestion were resuspended in gel loading buffer with or without 5% β-mercaptoethanol. Samples to be digested were resuspended in 25 μl thrombin digestion buffer (50 mM Tris pH8, 100 mM NaCl, 2.5 mM $CaCl_2$, 0.1% β-mercaptoethanol) and 300 U thrombin was added (Calbiochem). Protein was digested for 1 h at room temperature. 5 μl of gel loading buffer was added to samples to be analyzed by SDS-PAGE without further treatment. For samples to be precipitated further, each sample was resuspended in 120 μl PBS/0.1% NP-40 and 40 μl of the wash transferred to each of three fresh tubes. This was repeated three times, so that each tube contained 120 μl. Reagents for precipitation were added and immunoprecipitation carried out as above. For immunoprecipitation with sgp39, a recombinant fusion protein of the ligand of CD40 (Hollenbaugh et al., 1992), 250 μl of supernatant from COS cells transfected with the sgp39 expression plasmid and 50 μl of anti-rat Ig-agarose were added to the CD40 fragments.

Digestion and removal of the IgG has been performed on a preparative scale. Fusion protein in PBS was digested with human thrombin (Sigma, cat. # T-7009) at a ratio of 100:1 (w/w; 10 U thrombin per mg fusion protein) for 1 h at room temperature. Free IgG was removed by passing over a protein A-Sepharose column three times.

3. Results and discussion

Fusion proteins have been used to study a variety of proteins in a wide array of applications. The use

of a fusion protein may allow ease of purification, development of assays in which the protein of interest can be followed either by allowing binding of another protein or by an enzymatic activity, or a fusion protein may allow expression of a protein that might otherwise be rapidly degraded. One common form of fusion protein is the immunoglobulin fusion protein, in which the protein of interest is fused to the hinge and constant region of an antibody. In this way, the extensive collection of reagents developed for the isolation and studies of, and with, antibodies, can be applied to the study of an unrelated protein. For example, the immunoglobulin fusion protein can be easily purified by affinity chromatography using immobilized protein A and used in staining, Western blot or ELISA experiments with readily available conjugated anti-Ig reagents. Ig fusion proteins have been used for several applications. Like antibodies, they have been used in the staining of cells and tissue sections, in the identification and isolation of ligands, in the study of the expression of ligands and in the study of the effects of binding or blocking a receptor ligand pair, both in vitro and in vivo.

In the present study, two chimeric CD40Ig genes containing a recognition site for thrombin were generated. In the first, CD40ThrIg, the thrombin site was placed between the extracellular domain of CD40 and the hinge and second (CH_2) and third (CH_3) constant regions of the human IgG1. In the second, CD40HinThrIg, the thrombin cleavage site was placed between the hinge and CH_2 domains of the Ig. In this way, the extracellular domain of CD40 can be produced either as a monomeric protein, or as a dimeric protein, linked by the hinge domain (Fig. 1).

The CD40Ig proteins containing the recognition site for thrombin were expressed by transfection of COS cells with the appropriate vectors. The proteins

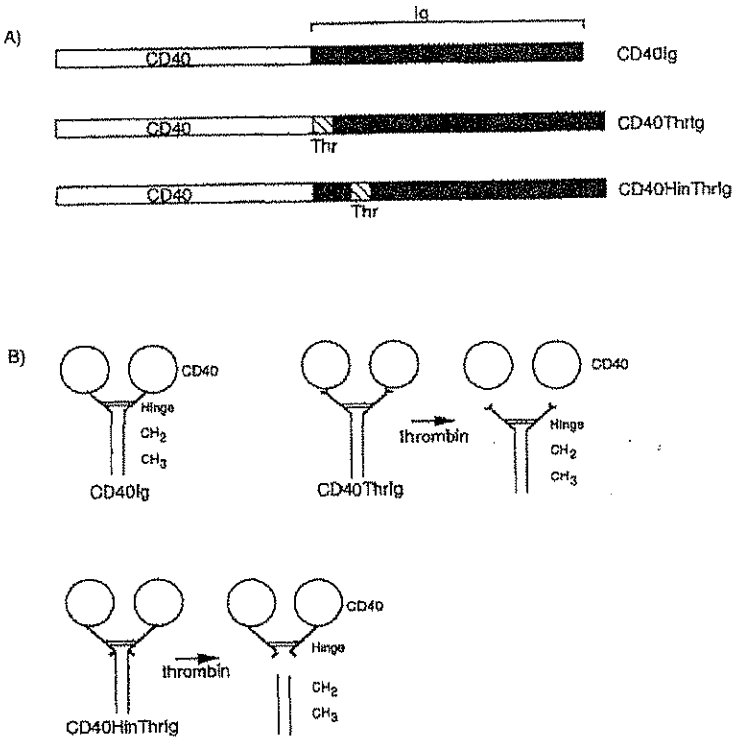

Fig. 1. Schematic representation of the CD40Ig, CD40ThrIg and CD40HinThrIg and products resulting from digestion. *A*: schematic of the gene construct. Sequence encoding the thrombin recognition site is shown hatched. *B*: schematic of the resulting proteins and the products obtained following cleavage with thrombin.

were then recovered from the supernatant by immobilized protein A (Fig. 2A). Following purification from the supernatants, the protein can be readily cleaved by the addition of thrombin under mild conditions. Under reducing conditions, the thrombin-cleaved CD40ThrIg yielded two bands at approximately 24 kDa and 36 kDa, corresponding to the CD40 extracellular domain and the Fc portion, respectively. The CD40HinThrIg yielded two fragments which appear as one broad band under reducing conditions at approximately 30 kDa, because the CD40-hinge and CH_2-CH_3 fragments are approximately the same size and are not resolved on the gel (Fig. 2B). As expected, the thrombin treatment did not cleave the CD40Ig. Under non-reducing conditions, the thrombin-cleaved CD40ThrIg yields bands at approximately 35 kDa and 60 kDa, corresponding to the monomeric CD40 fragment and the dimeric hinge-CH_2-CH_3 fragment, respectively. Thrombin cleavage of the CD40HinThrIg yields bands at approximately 30 kDa and 70 kDa, corresponding to the monomeric CH_2-CH_3 fragment and the dimeric CD40-hinge fragment, respectively (Fig. 2C).

The ability of these fragments to bind sgp39, a recombinant fusion protein of the ligand of CD40, was then tested by immunoprecipitation. The CD40 immunoglobulin fusion proteins were purified and digested with thrombin. The released fragments, consisting of the extracellular domain of CD40 alone or fused with the hinge of the human Ig, were then precipitated with either anti-CD40, to confirm the presence of the fragment, or the soluble recombinant gp39 fusion protein. This fusion protein consists of the extracellular domain of gp39 fused to the extracellular domain of murine CD8. Immunoprecipitations were performed using a rat antibody to the murine CD8 and anti-rat Ig agarose. In each experiment, and small amount of ^{35}S-labeled sgp39 was included in the precipitation to confirm the presence of the sgp39. The sgp39 is seen as a band at approximately 50 kDa. In addition, a control in which no sgp39 was added was included to verify the specificity of the precipitation. As seen in Fig. 3, the CD40 monomeric fragment was not precipitated with the sgp39. However, the dimeric CD40 did bind the sgp39 with sufficient avidity to be precipitated (Fig. 3).

These cleavable Ig vectors will provide the ability to produce soluble forms of pure proteins in a simple format that can be used with many different proteins. The ability to generate fragments analogous to the F(ab) and $F(ab')_2$ fragments of antibodies will allow the use of Ig fusion proteins in systems in which the Fc domain may interfere. The use of the two forms

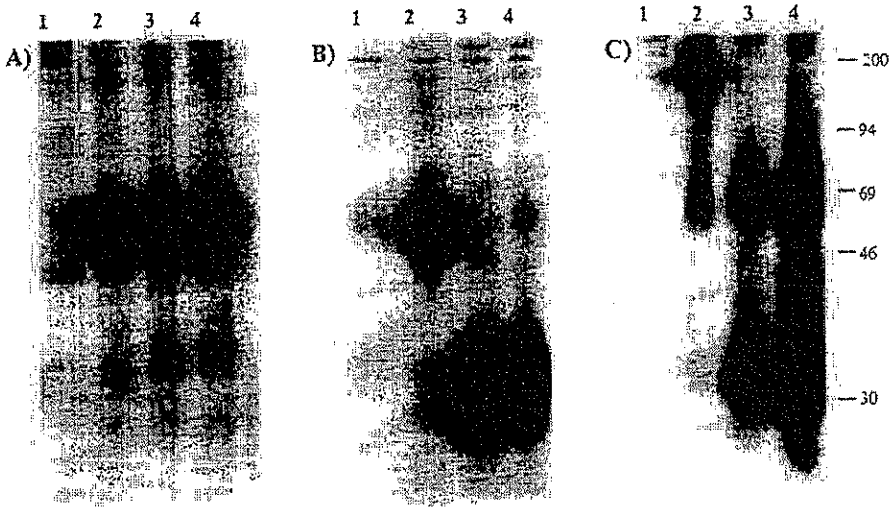

Fig. 2. Autoradiograph of immunoprecipitated CD40Ig, CD40ThrIg and CD40HinThrIg, and products obtained following digestion with thrombin. *A*: reduced, undigested fusion protein. *B*: reduced fragments after thrombin digestion. *C*: nonreduced fragments after thrombin digestion. In each panel: lane 1, mock transfected COS cell supernatant; lane 2, CD40Ig; lane 3, CD40ThrIg; lane 4, CD40HinThrIg.

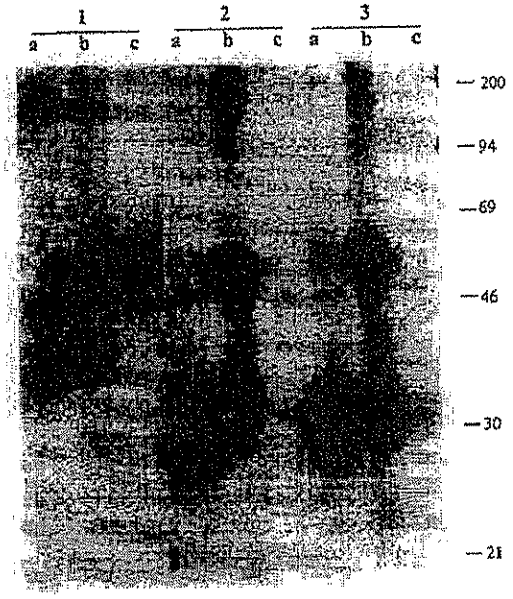

Fig. 3. Autoradiograph of immunoprecipitated CD40ThrIg and CD40HinThrIg fragments under reducing conditions. Fusion proteins were immunoprecipitated with protein A and treated with thrombin. Supernatants following digestion were then immunoprecipitated with *A*: anti-CD40 and Protein A agarose; *B*: sgp39 fusion protein with the rat mAb 53-6 (anti-Ly-2 to bind the Ly-2-gp39 fusion protein) and anti-rat Ig agarose; *C*: anti-rat Ig agarose alone as control. Group 1, mock transfected COS cell supernatants; group 2, CD40ThrIg; group 3, CD40ThrHinIg.

of the vector demonstrated that the monomeric CD40 did not bind sgp39 while the dimeric form did. This suggests that the interaction between the soluble forms of these proteins is not strong.

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Exhibit 10

THIS EXHIBIT HAS BEEN
REDACTED IN ITS ENTIRETY

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CERTIFICATE OF SERVICE

I, Philip A. Rovner, hereby certify that on June 9, 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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