

**(19) AUSTRALIAN PATENT OFFICE**

(54) Title  
Modified binding molecules comprising connecting peptides

(51)<sup>6</sup> International Patent Classification(s)  
**C07K** 16/00 (2006.01) 16/28  
**C07K** 16/18 (2006.01) 20060101ALI2005100  
**C07K** 16/28 (2006.01) <sup>8BMEP</sup> **C07K**  
**C07K** 16/30 (2006.01) 16/30  
**C12N** 15/62 (2006.01) 20060101ALI2005100  
**A61K** 39/00 (2006.01) <sup>8BMEP</sup> **C12N**  
C07K 16/00 15/62  
20060101AFI2005100 20060101ALI2005100  
<sup>8BMEP</sup> **C07K** <sup>8BMEP</sup> **A61K**  
16/18 39/00  
20060101ALI2005100 20060101ALN200510  
<sup>8BMEP</sup> **C07K** 08BMEP  
PCT/US2004/020945

(21) Application No: 2004252171 (22) Application Date: 2004 .06 .28

(87) WIPO No: W005/000899

(30) Priority Data

(31) Number	(32) Date	(33) Country
60/516,030	2003 .10 .30	US
60/515,351	2003 .10 .28	US
60/483,877	2003 .06 .27	US
60/508,810	2003 .10 .03	US

(43) Publication Date : 2005 .01 .06

(71) Applicant(s)  
Biogen Idec MA Inc.

(72) Inventor(s)  
Glaser, Scott, Chinn, Paul, Reff, Mitchell, Yang, Tzung-Horng, Wu, Xiufeng

(74) Agent/Attorney  
Cullens Patent and Trade Mark Attorneys, Level 32 239 George Street, Brisbane, QLD, 4000

(56) Related Art  
Hu et al (1996) Cancer Research, 56:3055-3061  
Bloom et al (1997) Protein Science, 6(2):407-415  
Alt et al (1999) FEBS Letters, 454:90-94  
Shuurman et al (2001) Molecular Immunology, 38:1-8  
WO 1999/015549 (Celltech Therapeutics Limited)  
Slavin-Chiorini et al (1995) Cancer Research Supple., 55(23):5957S-5967S  
Gillies et al (1990) Human Antibodies and Hybridomas, 1(1):47-54  
Reff et al (2001) Critical Reviews in Oncology/Hematology, 40:23-35  
Angal et al (1993) Molecular Immunology, 31(1):105-108

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
6 January 2005 (06.01.2005)

PCT

(10) International Publication Number  
WO 2005/000899 A3

- (51) International Patent Classification<sup>7</sup>: **C07K 16/00**,  
16/28, 16/30, C12N 15/62
- (21) International Application Number:  
PCT/US2004/020945
- (22) International Filing Date: 28 June 2004 (28.06.2004)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:  
60/483,877 27 June 2003 (27.06.2003) US  
60/508,810 3 October 2003 (03.10.2003) US  
60/515,351 28 October 2003 (28.10.2003) US  
60/516,030 30 October 2003 (30.10.2003) US
- (71) Applicant (for all designated States except US): **BIOGEN IDEC MA INC.** [US/US]; 14 Cambridge Center, Cambridge, MA 02142 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **GLASER, Scott** [US/US]; 4649 Granger Street, San Diego, CA 92107 (US). **REFF, Mitchell** [US/US]; 4166 Combe Way, San Diego, CA 92122 (US). **YANG, Tsung-Hong** [—/US]; 3962 St. Augustine Way, San Diego, CA 92130 (US). **WU, Xinfeng** [US/US]; 12312 Briardale Way, San Diego, CA 92128 (US). **CHINN, Paul**.
- (74) Agents: **SMITH, DeAnn, F.** et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).
- (81) Designated States (unless otherwise indicated, for every kind of national protection available): AF, AG, AI, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CI, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SI, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:  
— with international search report  
— before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments
- (88) Date of publication of the international search report:  
11 August 2005

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.



WO 2005/000899 A3

(54) Title: MODIFIED BINDING MOLECULES COMPRISING CONNECTING PEPTIDES

(57) Abstract: The instant invention describes methods of separating or preferentially synthesizing dimers which are linked via at least one interchain disulfide linkage from dimers which are not linked via at least one interchain disulfide linkage from a mixture comprising the two types of polypeptide dimers. These forms can be separated from each other using hydrophobic interaction chromatography. In addition, the invention pertains to connecting peptides that result in the preferential biosynthesis of dimers that are linked via at least one interchain disulfide linkage or that are not linked via at least one interchain disulfide linkage. The invention also pertains to compositions in which a majority of the dimers are linked via at least one interchain disulfide linkage or are not linked via at least one interchain disulfide linkage. The invention still further pertains to novel binding molecules, e.g., comprising: connecting peptides of the invention.

2004252171 30 Mar 2011

MODIFIED BINDING MOLECULES  
COMPRISING CONNECTING PEPTIDES

Related Applications

5 This application claims priority to USSN 60/483877, titled "Purification and Preferential Synthesis of Polypeptides," filed on June 27,2003 and to USSN 60/508, 810, titled "Purification and Preferential Synthesis of Antigen Binding Polypeptides," filed October 3,2003. This application also claims priority to USSN 60/515, 351, titled "Modified Antibody Molecules Comprising Connecting Peptides," October 28,2003 and to USSN 60/516,030, titled "Modified Antibody Molecules Comprising Connecting Peptides," filed October 30,2003. This application is also related to USSN 10/880,320, titled "Purification and preferential synthesis of Binding Polypeptides" filed on June 28,2004. The contents of these applications are incorporated in their entirety by this reference.

Background of the Invention

15 Antibodies are dimeric molecules; each monomer making up the dimer comprises one light and one heavy chain. Solutions of antibody molecules exist in two forms associated with hinge heterogeneity. Using SDS-PAGE analysis of purified Mab Mab, typically the two forms are observed as two protein bands, a major band (MW approximately 150-160 kDa) and a minor band (MW approximately 75-80 kDa). This latter form is typically  
20 observed after SDS-PAGE analysis of purified IgG4 preparations, but can be identified at much lower frequencies in all IgG isotypes, including purified, recombinant MAbs (Angal et al. 1993, *Mol. Immunol.* 30: 105; Norderhaug et al. 1990. *Eur. J. Immunol.* 21: 2370). The larger molecular weight isoform, referred to as Form A, contains covalent interchain disulfide bonds at positions corresponding to 239 and 242, Kabat numbering system (positions 226 and 229, EU  
25 numbering system) (Kabat, E, Wu, TT, Perry, HM, Gottesman, KS, Foeller, C: Sequences of Proteins of Immunological Interest. Bethesda, US Department of Health and Human Services, NIH, 1991). The second isoform, Form B, is thought to contain no covalent linkages between the two heavy chains and an intrachain disulfide bond between the two neighboring cysteine residues as evidenced by the 75-80 kDa seen in non-reducing SDS-PAGE electrophoresis. The  
30 two heavy chains of Form B are presumably held together by strong non-covalent (e. g. , ionic) interactions associated with the CH3 domain region of

the molecule. These mixtures of A and B forms are not present in solutions of MAb fragments that contain an intact hinge, but lack a CH3 domain, such as, for example, F(ab)<sub>2</sub> fragments. Typically, genetically engineered or enzymatically digested F(ab)<sub>2</sub> MAb preparations lack the B-form, since the molecule lacks the necessary domains for maintaining non-covalent interactions (e.g., hydrogen bonding). However, they are present in MAb preparations that do contain a CH3 domain, such as IgG4, CH2 domain deleted MAb fragments (e.g., as described in 02/060955 A2) and minibodies (see, e.g., Hu et al. 1996. Cancer Research 56:3055).

The application of protein engineering techniques to therapeutic antibody design has also produced a number of antibody formats that have been shown to have altered, and in some cases, improved pharmacodynamic, biodistribution, and activity profiles. Some altered antibody molecules have been made in which the number of cysteine residues in the hinge region is reduced to one to facilitate assembly of antibody molecules as it is only necessary to form a single disulfide bond. This also provides a specific target for attaching the hinge region either to another hinge region or to an effector or reporter molecule (U.S. Patent 5,677,425). The number of cysteine residues in the antibody hinge has also been increased (U.S. Patent 5,677,425). Other mutated antibodies have been constructed in which the IgG1 hinge region and the CH2 domain have been replaced with the human IgG3 hinge region. (WO 97/11370). These molecules contain 11 sulfhydryl groups for substitution of multiple haptens via thiol groups.

CH2 domain deleted antibodies have a molecular mass of approximately 120 kDa and have been shown to penetrate tumors significantly better than full length IgG. Minibodies, which also have deletion of the CH2 domain, have similar characteristics. These domain deleted molecules accumulate at tumor sites more efficiently than other MAb fragments, such as F(ab)<sub>2</sub>s, but without the unfavorable pharmacodynamic profiles seen with intact IgG antibody. CH2 domain deleted antibodies consist of a VLCL light chain and a VH1 heavy chain domain and a portion of the hinge region (e.g., the upper and middle hinge) genetically fused (either directly or through a modified peptide spacer) to a CH3 domain. As an example, the biosynthesis of recombinant CH2 domain deleted ddCC49, a domain deleted antibody that recognizes the tumor associated TAG72 antigen expressed on a variety of human carcinomas, produces the A and B isoforms in approximately 50:50 distribution in cell cultures. Cells engineered to express alternative forms of CH2 domain deleted antibodies, for example, tetravalent CH2 domain deleted antibodies, minibodies, or tetravalent minibodies also express a mixture consisting of A and B isoforms and/or monomeric half-mer molecules.

Form A and Form B are extremely difficult to separate even after MAb purification, since they are composed of identical amino acids and, therefore, have identical molecular weight and similar physical and chemical properties. They cannot be separated by standard gel filtration, affinity chromatography, or ion exchange chromatography typically used to purify antibody molecules, including recombinant MAb proteins. Current manufacturing processes discard at least 50% of the total antibody produced, having a negative impact on overall yield. Moreover, the presence of the two isoforms increases efforts required for downstream processing. Thus, a method of separating forms A and B or of increasing biosynthesis of one or the other form of antibody would be of great benefit.

#### Summary of the Invention

The invention is based, at least in part, on the finding that in a composition comprising a mixture of dimeric polypeptide molecules comprising different isoforms (molecules comprising two heavy chain portions in which a fraction of the molecules comprise two heavy chain portions that are linked via at least one interchain disulfide linkage (Form A) and a portion of the molecules comprise two heavy chain portions that are not linked via at least one interchain disulfide linkage (Form B)) one form or the other can be preferentially obtained, e.g., by separation using hydrophobic interaction chromatography or by inclusion of synthetic connecting peptides which result in the preferential biosynthesis of either Form A or Form B.

In one embodiment, the binding molecules of the invention are tetravalent. The connecting peptides of the invention can be included in any dimeric molecule that tends to form both Form A and Form B, e.g., antibody molecules, domain deleted antibody molecules (e.g., lacking all or part of a CH2 domain), minibodies, diabodies, fusion proteins, etc. In a preferred embodiment, the formation of Form A is enhanced.

In another embodiment, the invention pertains to a composition comprising polypeptide dimers comprising at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one heavy chain portion and a synthetic connecting peptide, and wherein greater than about 50% of the dimers are linked via at least one interchain disulfide linkage.

In another embodiment, greater than about 90% of the dimers are linked via at least one interchain disulfide linkage.

In another embodiment, at least one of the polypeptide chains comprises a CH3 domain linked to a VL, VH or CH1 domain via the connecting peptide.

In another embodiment, the polypeptide chains lack all or part of a CH2 domain.

In another embodiment, the dimers are linked via two or more interchain disulfide linkages.

5 In another embodiment, the heavy chain portion is derived from an antibody of an isotype selected from the group consisting of: IgG1, IgG2, IgG3, and IgG4.

In another embodiment, the heavy chain portion comprises an amino acid sequence derived from a hinge region selected from the group consisting of: a  $\gamma$ 1 hinge,  
10 a  $\gamma$ 2 hinge, a  $\gamma$ 3 hinge, and a  $\gamma$ 4 hinge.

In another embodiment, the molecules are bispecific.

In another embodiment, the molecules comprise at least one binding site specific for a soluble ligand.

In another embodiment, the molecules comprise at least one binding site  
15 specific for a cell surface molecule.

In another embodiment, the molecules comprise two binding sites specific for a tumor cell antigen and two binding sites specific for a prodrug.

In another embodiment, the binding sites specific for the prodrug are catalytic.

20 In another embodiment, the synthetic connecting peptide comprises a proline residue at position 243, Kabat numbering system.

In another embodiment, the synthetic connecting peptide further comprises an alanine residue at position 244 and a proline residue at position 245, Kabat numbering system.

25 In another embodiment, the heavy chain portion comprises a chimeric hinge.

In another embodiment, the synthetic connecting peptide comprises at least a portion of an IgG1 hinge domain, at least a portion of an IgG3 hinge domain.

In another embodiment, the connecting peptide comprises an amino acid  
30 sequence selected from the group consisting of: SEQ ID NOs: 8-15 and 48.

In a second aspect, the invention provides a method of treating a subject that would benefit from treatment with an antigen binding molecule comprising administering to the subject a composition comprising a mixture of dimeric polypeptide molecules comprising different isoforms, wherein one isoform or the other is  
35 preferentially obtained, such that treatment occurs.

In one embodiment, the subject is suffering from cancer.

In another embodiment, the subject is suffering from lymphoma.

In another embodiment, the subject is suffering from an autoimmune disease or disorder.

In another embodiment, the subject is suffering from an inflammatory disease or disorder.

5 In a third aspect, the invention provides a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide molecule comprising different isoforms (molecules comprising two heavy chain portions in which a fraction of the molecules comprise two heavy chain portions that are linked via at least one interchain disulfide linkage (Form A) and a portion of the molecules comprise two heavy chain  
10 portions that are not linked via at least one interchain disulfide linkage (Form B)) such that one form or the other can be preferentially obtained.

In one embodiment, the polypeptide dimers comprise four polypeptide chains and wherein two of the polypeptide chains comprise at least one heavy chain portion and a synthetic connecting peptide.

15 In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 8B (SEQ ID NO: 17).

In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 8C (SEQ ID NO: 18).

20 In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 10B (SEQ ID NO: 23).

In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 12A (SEQ ID NO:26).

In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 12B (SEQ ID NO:27).

25 In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 14 (SEQ ID NO:30).

In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 15 (SEQ ID NO:31).

30 In another embodiment, the nucleic acid molecule is in a vector. In still another embodiment, the vector is in a host cell.

In a fourth aspect, the invention provides binding molecules comprising an amino acid sequence encoding a polypeptide molecule comprising different isoforms (molecules comprising two heavy chain portions in which a fraction of the molecules comprise two heavy chain portions that are linked via at least one interchain disulfide  
35 linkage (Form A) and a portion of the molecules comprise two heavy chain portions that are not linked via at least one interchain disulfide linkage (Form B)) such that one form or the other can be preferentially obtained.

In one embodiment, the binding molecule comprises the amino acid sequence of Figure 9B (SEQ ID NO: 20).

In another embodiment, the binding molecule comprises the amino acid sequence of SEQ ID NO: 21.

In another embodiment, the binding molecule comprises the amino acid sequence of Figure 11B (SEQ ID NO: 25).

In another embodiment, the binding molecule comprises the amino acid sequence of Figure 13A (SEQ ID NO: 28).

In another embodiment, the binding molecule comprises the amino acid sequence of Figure 13B (SEQ ID NO: 29).

In another embodiment, the binding molecule comprises the amino acid sequence of Figure 16 (SEQ ID NO: 32).

In another embodiment, the binding molecule comprises the amino acid sequence of Figure 17 (SEQ ID NO: 33).

In a fifth aspect, the invention provides a composition comprising a mixture of dimeric polypeptide molecules comprising different isoforms (molecules comprising two heavy chain portions in which a fraction of the molecules comprise two heavy chain portions that are linked via at least one interchain disulfide linkage (Form A) and a portion of the molecules comprise two heavy chain portions that are not linked via at least one interchain disulfide linkage (Form B)) one form or the other can be preferentially obtained, and wherein said binding sites are individually selected from the group consisting of: an antigen binding site, a ligand binding portion of a receptor, and a receptor binding portion of a ligand.

In one embodiment, the polypeptide chains have at least one binding site derived from an antibody selected from the group consisting of: 2B8, Lym 1, Lym 2, LL2, Her2, B1, MB1, BH3, B4, B72.3, CC49, 5E8, B3F6, and 5E10.

In another embodiment, the polypeptide dimers are tetravalent minibody molecules.

In another embodiment, the polypeptide dimers are tetravalent domain deleted antibody molecules.

In another embodiment, the polypeptide dimers are diabodies.

In a sixth aspect, the invention provides a composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise a heavy chain portion and a synthetic connecting peptide, wherein the polypeptide chains lack all or part of a CH2 domain, and wherein greater than about 50% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.

SUBSTITUTE SHEET 6

AMENDED SHEET



In one embodiment, greater than about 90% of the dimers are linked via at least one interchain disulfide linkage.

In another embodiment, at least one of the polypeptide chains comprises a CH3 domain genetically fused to a VL, VH or CH1 domain via the connecting peptide.

5 In another embodiment, the polypeptide chains lack the entire CH2 domain.

In another embodiment, the dimers are linked via two or more interchain disulfide linkages.

10 In another embodiment, the heavy chain portion is derived from an antibody of an isotype selected from the group consisting of: IgG1, IgG2, IgG3, and IgG4.

In another embodiment, the heavy chain portion comprises an amino acid sequence is derived from a hinge region selected from the group consisting of: a  $\gamma$ 1 hinge, a  $\gamma$ 2 hinge, a  $\gamma$ 3 hinge, and a  $\gamma$ 4 hinge.

15 In another embodiment, the binding sites are individually selected from the group consisting of: an antigen binding site, a ligand binding portion of a receptor, and a receptor binding portion of a ligand.

In another embodiment, the molecules are bispecific.

20 In another embodiment, the connecting peptide comprises a proline residue at position 243, Kabat numbering system.

In another embodiment, the synthetic connecting peptide comprises a chimeric hinge.

In another embodiment, the synthetic connecting peptide comprises at least a portion of an IgG1 hinge domain, at least a portion of an IgG3 hinge domain.

25 In a seventh aspect, the invention provides a method of treating a subject that would benefit from treatment with an antigen binding molecule comprising administering to the subject a composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise a heavy chain portion and a synthetic connecting peptide, wherein the polypeptide chains lack all or part of a CH2 domain, and wherein greater than about 50% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage, such that treatment occurs.

30 In another aspect, the invention provides a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide chain comprising a heavy chain portion and a synthetic connecting peptide, wherein the polypeptide chains lack all or part of a CH2 domain, and wherein greater than about 50% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.

2004252171 30 Mar 2011

In still another aspect, the invention provides a composition comprising polypeptide dimers having at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one heavy chain portion and lacks all or part of a CH2 domain, wherein greater than 50% of the polypeptide dimers are

5 linked via at least one interchain disulfide linkage.

Definitions of the specific embodiments of the invention as claimed herein follow.

According to a first embodiment of the invention, there is provided a composition comprising polypeptide dimers comprising at least four binding sites and at least

10 two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion and a synthetic connecting peptide comprising a chimeric hinge, wherein said synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system) and at least a portion of an IgG3 hinge region, and wherein greater than 90% of the dimers comprise

15 polypeptide chains that are linked via at least one interchain disulfide linkage.

According to a second embodiment of the invention, there is provided a composition comprising polypeptide dimers comprising at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion from an IgG1 antibody isotype and a synthetic connecting

20 peptide comprising a chimeric hinge, and wherein greater than 90% of the dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

According to a third embodiment of the invention, there is provided a composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise an immunoglobulin heavy chain portion and a synthetic

25 connecting peptide comprising a chimeric hinge, wherein said synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system) and at least a portion of an IgG3 hinge region and at least four binding sites,, wherein the polypeptide chains lack all or part of a CH2 domain, and wherein greater than 90% of the molecules are present in a form in which one of the

30 polypeptide chains are linked via at least one interchain disulfide linkage.

According to a fourth embodiment of the invention, there is provided a composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise at least one immunoglobulin heavy chain portion from an IgG1 antibody isotype and a synthetic connecting peptide comprising a chimeric hinge, wherein said

2004252171 06 Apr 2011

synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system), at least four binding sites, and at least a portion of an IgG3 hinge region, and wherein greater than 90% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.

According to a fifth embodiment of the invention, there is provided a composition comprising polypeptide dimers having at least four antigen binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains each comprise a complete Ig heavy chain, and a chimeric hinge,

wherein said chimeric hinge connects the CH1 and the CH2 domain of the Ig heavy chain, and

wherein greater than about 90% of the dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage, and

wherein amino acids at positions 226-242 (Kabat numbering) comprise: (i) the human IgG1 upper hinge region sequence EPKSCDKTHT (SEQ ID NO:2) or the human IgG4 upper hinge region sequence ESKYGPP (SEQ ID NO:45) at Kabat hinge positions 226-238; (ii) a cysteine residue (C) at Kabat hinge position 239; (iii) a proline residue (P) at Kabat hinge position 240; (iv) a proline (P) or serine (S) residue at Kabat hinge position 241; (v) the human IgG3 middle hinge sequence CPEPKSCDTTPPCPR (SEQ ID NO:49) at Kabat hinge positions 241EE-241SS; and (vi) a cysteine residue (C) at Kabat hinge position 242.

According to a sixth embodiment of the invention, there is provided a composition comprising polypeptide dimers having at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion and lack all or part of a CH2 domain, wherein greater than 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

According to a seventh embodiment of the invention, there is provided a composition comprising polypeptide dimers having at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise (i) an Ig heavy chain lacking a CH2 domain, and (ii) a chimeric hinge,

wherein said chimeric hinge comprises an amino acid sequence selected from the group consisting of SEQ ID NOS: 8-15 and wherein said chimeric hinge connects said heavy chain to at least one of said binding sites, and

2004252171 06 Apr 2011

wherein greater than 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

According to an eighth embodiment of the invention, there is provided a method of treating a subject that would benefit from treatment with an antigen binding molecule comprising administering a composition of any one of the first to seventh embodiments to the subject such that treatment occurs.

According to a ninth embodiment of the invention, there is provided a nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide chain of the composition of any one of the first to seventh embodiments.

According to a tenth embodiment of the invention, there is provided a vector comprising the nucleic acid molecule of the ninth embodiment.

According to an eleventh embodiment of the invention, there is provided a host cell comprising the vector of the tenth embodiment.

#### Brief Description of the Drawings

- 15 Figure 1 shows Form A which appears as a 120kDa dimer and Form B which appears as a 60kDa monomer in domain deleted antibodies.

Figure 2 shows a schematic diagram of an exemplary two chain dimeric minibody and an exemplary two chain dimeric tetravalent minibody each comprising a hinge connecting peptide (HCP). Other configurations are also possible, for example, the two chain dimeric tetravalent minibody comprising a connecting peptide (HCP) can also be constructed to be bispecific. In another embodiment, the orientation of the VH and VL domains in the scFv may be changed.

Figure 3 shows a schematic diagram of a four chain dimeric diabody comprising a hinge connecting peptide (HCP). The four chain dimeric diabody comprising a hinge connecting peptide (HCP) can also be constructed to be bispecific. The orientation of the VH and VL domains may be changed.

Figure 4 shows a schematic diagram of a four chain dimeric tetravalent scFv antibody (C-scFv tetravalent antibody) and a four chain tetravalent scFv CH2 domain deleted antibody (C-scFv tetravalent CH2 domain deleted antibody) each comprising a scFv appended to the carboxyl terminus of CH3 and a hinge connecting peptide. The orientation of the VH and VL domains in the scFv may be changed.

- 8b -

2004252171 06 Apr 2011

Figure 5 shows a schematic diagram of a four chain dimeric CH2 domain deleted tetravalent (NL-scFv CH2 domain deleted tetravalent) antibody comprising a hinge connecting peptide (HCP). The four chain dimeric CH2 domain deleted tetravalent antibody comprising a connecting peptide (HCP) can also be constructed to be bispecific. The orientation of the VH and VL domains in the scFv appended to the light chain may be changed.

[Text continues on page 9.]

- 8c -

Figure 6 shows a schematic diagram of a four chain dimeric CH2 domain deleted tetraivalent (N<sub>H</sub>-scFv CH2 domain deleted tetraivalent) antibody comprising a hinge connecting peptide (HCP). The four chain dimeric CH2 domain deleted tetraivalent antibody comprising a connecting peptide (HCP) can also be constructed to be bispecific. The orientation of the VH and VL domains in the scFv appended to the heavy chain may be changed.

Figure 7 shows a schematic diagram of a two chain dimeric tetraivalent minibody (C-scFv tetraivalent minibody) comprising a hinge connecting peptide (HCP). The two chain dimeric tetraivalent minibody comprising a connecting peptide (HCP) can also be constructed to be bispecific. The orientation of the VH and VL domains in both scFvs may be changed.

Figure 8A (SEQ ID NO:16) shows the single-stranded DNA sequence of heavy chain CH2 domain-deleted sc(Fv)<sub>2</sub> tetraivalent (C-scFv tetraivalent CH2 domain deleted) huCC49 gene. Figure 8B (SEQ ID NO:17) shows the single-stranded DNA sequence of heavy chain tetraivalent CH2 domain-deleted sc(Fv)<sub>2</sub> tetraivalent (C-scFv tetraivalent CH2 domain deleted) huCC49 gene containing the synthetic G1/G3:/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 8C (SEQ ID NO:18) shows the single-stranded DNA sequence of light chain CH2 domain-deleted sc(Fv)<sub>2</sub> tetraivalent (C-scFv tetraivalent CH2 domain deleted) huCC49.

Figure 9A (SEQ ID NO:19) shows the amino acid sequence of heavy chainCH2 domain-deleted sc(Fv)<sub>2</sub> tetraivalent (C-scFv tetraivalent CH2 domain deleted) huCC49. Figure 9B (SEQ ID NO:20) shows the amino acid sequence of heavy chain CH2 domain-deleted sc(Fv)<sub>2</sub> tetraivalent (C-scFv tetraivalent CH2 domain deleted) huCC49 containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

Figure 10A (SEQ ID NO:22) shows the single-stranded DNA sequence of CH2 domain-deleted huCC49 tetraivalent (N- scFv tetraivalent) minibody gene. Figure 10B (SEQ ID NO:23) shows the single-stranded DNA sequence of tetraivalent CH2 domain-deleted (N-scFv tetraivalent) huCC49 tetraivalent minibody gene containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

Figure 11A (SEQ ID NO:24) shows the amino acid sequence of tetraivalent CH2 domain-deleted (N- scFv tetraivalent) huCC49 minibody. Figure 11B (SEQ ID NO:25)

SUBSTITUTE SHEET 9

AMENDED SHEET

shows the amino acid sequence of tetravalent CH2 domain-deleted (N- scFv tetravalent) huCC49 minibody containing the synthetic G1/G3/ Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

- 5 Figure 12A (SEQ ID NO:26) shows the single-stranded DNA sequence of heavy chain tetravalent CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 (C-scFv tetravalent CH2 domain deleted) antibody gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.
- 10 Figure 12B (SEQ ID NO:27) shows the single-stranded DNA sequence of light chain tetravalent CH2 domain-deleted (C-scFv tetravalent CH2 domain deleted) PRIMATIZED® p5E8 sc(Fv)2 gene.

- Figure 13A (SEQ ID NO:28) shows the amino acid sequence of heavy chain tetravalent
- 15 CH2 domain-deleted (C-scFv tetravalent CH2 domain deleted) PRIMATIZED® p5E8 sc(Fv)2 antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.
- Figure 13B (SEQ ID NO:29) shows the amino acid sequence of light chain tetravalent CH2 domain-deleted (C-scFv tetravalent CH2 domain deleted) PRIMATIZED® p5E8
- 20 sc(Fv)2 antibody .

- Figure 14 (SEQ ID NO:30) shows the single-stranded DNA sequence of CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody gene containing the
- 25 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

- Figure 15 (SEQ ID NO:31) shows the single-stranded DNA sequence of CH2 domain-deleted PRIMATIZED® p5E8 VH/VL minibody gene containing the
- G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

- 30 Figure 16 (SEQ ID NO:32) shows the amino acid sequence of CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

- Figure 17 (SEQ ID NO:33) shows the amino acid sequence of CH2 domain-deleted
- 35 PRIMATIZED® p5E8 VH/VL minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

- Figure 18 shows a Western Blot of supernatants from five independent clones producing huCC49 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody. Each supernatant was electrophoresed under both reducing and non-reducing conditions.
- 5 Figure 19 shows a Coomassie Blue stained gel of purified Forms A and B huCC49 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibodies. Each antibody was electrophoresed under reducing and non-reducing conditions.
- 10 Figure 20 shows that purified Form A huCC49 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody predominantly elutes as a single peak by HPLC size-exclusion chromatography.
- Figure 21 shows the results of a competitive binding assay of Form A huCC49 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved fluorometric immunoassay using a Delphia fluorimeter (Wallac Inc, Gaithersburg, MD).
- 15 Figure 22 shows a Western Blot of supernatant from a representative clone producing huCC49 CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.
- 20 Figure 23 shows a Coomassie Blue stained gel of purified Form A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP (C-scFv tetravalent CH2 domain deleted) antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.
- 25 Figure 24 shows that purified Form A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP (C-scFv tetravalent CH2 domain deleted) antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide predominantly elutes as a single peak by HPLC size-exclusion chromatography.
- 30 Figure 25 shows the results of a competitive binding assay of Form A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP (C-scFv tetravalent CH2 domain deleted) antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide to bovine submaxillary mucine, a source of the TAG-72 antigen, by
- 35



time-resolved fluorometric immunoassay using a Delphia fluorimeter (Wallac Inc, Gaithersburg, MD).

Figure 26 shows Western Blots of supernatants from representative clones producing  
 5 huCC49 minibody, huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent huCC49 minibody), and huCC49 CH2 domain deleted antibody. A Western Blot was performed on supernatants that were electrophoresed under reducing and non-reducing conditions.

Figure 27 shows a Western Blot of supernatants from representative clones producing  
 10 huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent huCC49 minibody) and huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent huCC49 minibody) containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

Figure 28 shows a Coomassie Blue stained gel of purified Form A huCC49 domain-deleted sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody) containing the  
 15 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

Figure 29 shows purified Form A huCC49 domain-deleted sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody) containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser]  
 20 hinge connecting peptide predominantly elutes as a single peak by HPLC size-exclusion chromatography.

Figure 30 shows the results of a competitive binding assay of huCC49 domain-deleted sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody) containing the  
 25 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide, huCC49 minibody, huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody (C-scFv tetravalent antibody) containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide, and control parent CH2 domain-deleted huCC49 (referred to as HuCC49 or IDEC 159) to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved  
 30 fluorometric immunoassay using a Delphia fluorimeter (Wallac Inc, Gaithersburg, MD).

Figure 31 shows a Western Blot of supernatant from a cell line producing tetravalent CH2 domain-deleted (C-scFv tetravalent CH2 domain deleted) PRIMATIZED® p5E8 sc(Fv)2 antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge  
 35 connecting peptide.

Figure 32 shows a Western Blot of supernatants from five independent cell lines producing CH2 domain-deleted huCC49 VL/VH minibody. Minibody samples were

analyzed under non-reducing, denaturing conditions revealing the presence of Form A and Form B isoforms.

Figure 33 show the results of an ELISA binding assay of PRIMATIZED® p5E8 VH/VL and VL/VH minibodies containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide binding to the CD23 antigen. p5E8G1 is intact full length PRIMATIZED® IgG1.

Figure 34 shows a Western Blot of supernatant from cell lines producing PRIMATIZED® p5E8 VH/VL and VL/VH minibodies containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide.

Figure 35 A shows tumor retention for control parent huCC49 and huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody as measured by %ID/gm. Figure 35 B shows the same tumor retention data normalized to peak antibody accumulation.

#### Detailed Description of the Invention

Human immunoglobulins (Igs), including monoclonal antibodies (MAbs), can exist in two forms that are associated with hinge heterogeneity. In native solutions, both of these forms are present as dimeric proteins (each monomer comprising one heavy chain and one light chain). One immunoglobulin molecule comprises a stable four chain construct of approximately 150-160 kDa in which the dimers are held together by an interchain heavy chain disulfide bond (Form A) and one comprises form in which the dimers are not linked via interchain disulfide bonds (Form B). Form B also forms a stable dimer under native conditions, but can be identified under denaturing, non-reducing conditions, in which the heavy chains dissociate yielding a 75-80 kDa molecule. These forms have been extremely difficult to separate, even after MAb affinity purification.

The frequency of appearance of the B form in various intact IgG isotypes is due to, but not limited to, structural differences associated with the hinge region isotype of the MAb molecule. In fact, a single amino acid substitution in the hinge region of the human IgG4 hinge can significantly reduce the appearance of the B form (Angal *et al.* 1993. *Molecular Immunology* 30:105) to levels typically observed using a human IgG1 hinge. However, applying this same amino acid substitution to MAb fragments in which the CH3 domain was retained did not eliminate Form B from preparations. Typically, all recombinant CH2 domain deleted antibodies produced in cell cultures often result in hinge heterogeneity which is not corrected via similar molecular mutations in the hinge.

The instant invention advances the state of the art by providing methods of, e.g., separating a first dimeric polypeptide from a second dimeric polypeptide wherein the first and second polypeptides comprise at least two polypeptide chains and at least two of the polypeptide chains comprise at least one heavy chain portion. In one  
5 embodiment, the polypeptides of the invention lack all or part of a CH2 domain. The monomers are linked via at least one interchain disulfide linkage (referred to herein as "Form A") and the monomers of the second polypeptide are not linked via at least one interchain disulfide linkage (referred to herein as "Form B"). These forms can be separated from each other using hydrophobic interaction chromatography. In addition,  
10 the invention pertains to polypeptides that comprise connecting peptides. The inclusion of certain connecting peptides results in the preferential biosynthesis of polypeptide dimers comprising polypeptide chains that are linked via at least one interchain disulfide linkage or that are not linked via at least one interchain disulfide linkage.

Before further description of the invention, for convenience, certain terms  
15 are described below:

#### I. Definitions

20 The polypeptides of the invention are binding molecules, i.e., polypeptide molecules or the nucleic acid molecules that encode them, that comprise at least one binding domain which comprises a binding site that specifically binds to a target molecule (such as an antigen or binding partner). For example, in one embodiment, a binding molecule of the invention comprises an immunoglobulin antigen binding site or  
25 the portion of a receptor molecule responsible for ligand binding or the portion of a ligand molecule that is responsible for receptor binding. The binding molecules of the invention are polypeptides or the nucleic acid molecules which encode them.

In one embodiment, the binding molecules comprise at least two binding sites. In one embodiment, the binding molecules comprise two binding sites. In one  
30 embodiment, the binding molecules comprise three binding sites. In another embodiment, the binding molecules comprise four binding sites.

The polypeptides of the invention are multimers. For example, in one embodiment, the polypeptides of the invention are dimers. In one embodiment, the dimers of the invention are homodimers, comprising two identical monomeric subunits.  
35 In another embodiment, the dimers of the invention are heterodimers, comprising two non-identical monomeric subunits. The subunits of the dimer may comprise one or more polypeptide chains. For example, in one embodiment, the dimers comprise at least two polypeptide chains. In one embodiment, the dimers comprise two polypeptide

chains. In another embodiment, the dimers comprise four polypeptide chains (e.g., as in the case of antibody molecules).

The polypeptides of the invention comprise at least one amino acid sequence derived from an immunoglobulin domain. A polypeptide or amino acid sequence "derived from" a designated protein refers to the origin of the polypeptide. Preferably, the polypeptide or amino acid sequence which is derived from a particular starting polypeptide or amino acid sequence has an amino acid sequence that is essentially identical to that of the starting sequence, or a portion thereof wherein the portion consists of at least 10-20 amino acids, preferably at least 20-30 amino acids, more preferably at least 30-50 amino acids, or which is otherwise identifiable to one of ordinary skill in the art as having its origin in the starting sequence.

Preferred binding polypeptides comprise an amino acid sequence derived from a human amino acid sequence. However, binding polypeptides may comprise one or more amino acids from another mammalian species. For example, a primate heavy chain portion, hinge portion, or binding site may be included in the subject binding polypeptides and/or connecting polypeptides. Alternatively, one or more murine amino acids may be present in a binding polypeptide, e.g., in an antigen binding site of a binding molecule. Preferred binding molecules of the invention are not immunogenic.

It will also be understood by one of ordinary skill in the art that the binding molecules of the invention (e.g., the heavy chain or light chain portions or binding portions of the subject polypeptides) may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule from which they were derived. For example, nucleotide or amino acid substitutions leading to conservative substitutions or changes at "non-essential" amino acid residues may be made.

An isolated nucleic acid molecule encoding a non-natural variant of a polypeptide derived from an immunoglobulin (e.g., an immunoglobulin heavy chain portion or light chain portion) can be created by introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence of the immunoglobulin such that one or more amino acid substitutions, additions or deletions are introduced into the encoded protein. Mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine,

tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in an immunoglobulin polypeptide is preferably replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members.

Alternatively, in another embodiment, mutations may be introduced randomly along all or part of the immunoglobulin coding sequence, such as by saturation mutagenesis, and the resultant mutants can be incorporated into polypeptides of the invention and screened for their ability to bind to the desired antigen.

As used herein, the term "heavy chain portion" includes amino acid sequences derived from an immunoglobulin heavy chain. A polypeptide comprising a heavy chain portion comprises at least one of: a CH1 domain, a hinge (*e.g.*, upper, middle, and/or lower hinge region) domain, a CH2 domain, a CH3 domain, or a variant or fragment thereof. In one embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH2 domain. In another embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a CH1 domain and a CH3 domain. In another embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a CH1 domain, at least a portion of a hinge domain, and a CH3 domain. In another embodiment, a polypeptide of the invention comprises a polypeptide chain comprising a CH3 domain. In one embodiment, a polypeptide of the invention lacks at least a portion of a CH2 domain (*e.g.*, all or part of a CH2 domain). In another embodiment, a polypeptide of the invention comprises a complete Ig heavy chain. As set forth above, it will be understood by one of ordinary skill in the art that these domains (*e.g.*, the heavy chain portions) may be modified such that they vary in amino acid sequence from the naturally occurring immunoglobulin molecule.

In one embodiment, at least two of the polypeptide chains of a binding molecule of the invention comprise at least one heavy chain portion derived from an antibody or immunoglobulin molecule. In one embodiment, at least two heavy chain portions of a polypeptide of the invention are present on different polypeptide chains and interact, *e.g.*, via at least one disulfide linkage (Form A) or via non-covalent interactions (Form B) to form a dimeric polypeptide, each monomer of the dimer comprising at least one heavy chain portion.

In one embodiment, the heavy chain portions of one polypeptide chain of a dimer are identical to those on a second polypeptide chain of the dimer. In one

embodiment, the monomers (or half-mers) of a dimer of the invention are identical to each other. In another embodiment, they are not identical. For example, each monomer may comprise a different target binding site.

In one embodiment, a dimer of the invention is held together by covalent interactions, e.g., disulfide bonds. In one embodiment, a dimer of the invention is held together by one or more disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably two disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably three disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably four disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably five disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably six disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably seven disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably eight disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably nine disulfide bonds. In another embodiment, a dimer of the invention is held together by one or more, preferably ten disulfide bonds. In a further embodiment, a dimer of the invention is not held together by disulfide bonds, but is held together, e.g., by non-covalent interactions.

The heavy chain portions of a polypeptide may be derived from different immunoglobulin molecules. For example, a heavy chain portion of a polypeptide may comprise a CH1 domain derived from an IgG1 molecule and a hinge region derived from an IgG3 molecule. In another example, a heavy chain portion can comprise a hinge region derived, in part, from an IgG1 molecule and, in part, from an IgG3 molecule. In another example, a heavy chain portion can comprise a chimeric hinge derived, in part, from an IgG1 molecule and, in part, from an IgG4 molecule. As used herein, the term "light chain portion" includes amino acid sequences derived from an immunoglobulin light chain. Preferably, the light chain portion comprises at least one of a VL or CL domain.

In one embodiment a polypeptide of the invention comprises an amino acid sequence or one or more moieties not derived from an Ig molecule. Exemplary modifications are described in more detail below. For example, in one embodiment, a polypeptide of the invention may comprise a flexible linker sequence. In another embodiment, a polypeptide may be modified to add a functional moiety (e.g., PEG, a drug, or a label).

In one embodiment, a binding polypeptide of the invention is a fusion protein. Fusion proteins are chimeric molecules which comprise a binding domain

comprising at least one target binding site and at least one heavy chain portion. In one embodiment, a fusion protein further comprises a synthetic connecting peptide.

A "chimeric" protein comprises a first amino acid sequence linked to a second amino acid sequence with which it is not naturally linked in nature. The amino acid sequences may normally exist in separate proteins that are brought together in the fusion polypeptide or they may normally exist in the same protein but are placed in a new arrangement in the fusion polypeptide. A chimeric protein may be created, for example, by chemical synthesis, or by creating and translating a polynucleotide in which the peptide regions are encoded in the desired relationship. Exemplary chimeric polypeptides include fusion proteins and the chimeric hinge connecting peptides of the invention.

The term "heterologous" as applied to a polynucleotide or a polypeptide, means that the polynucleotide or polypeptide is derived from a genotypically distinct entity from that of the rest of the entity to which it is being compared. For instance, a heterologous polynucleotide or antigen may be derived from a different species origin, different cell type, or the same type of cell of distinct individuals.

The term "ligand binding domain" or "ligand binding portion of a receptor" as used herein refers to any native receptor (e.g., cell surface receptor) or any region or derivative thereof retaining at least a qualitative ligand binding ability, and preferably the biological activity of a corresponding native receptor.

The term "receptor binding domain" or "receptor binding portion of a ligand" as used herein refers to any native ligand or any region or derivative thereof retaining at least a qualitative receptor binding ability, and preferably the biological activity of a corresponding native ligand.

In one embodiment, a binding molecule of the invention is a fusion protein. A fusion protein of the invention is a chimeric molecule that comprises a binding domain (which comprises at least one binding site) and a dimerization domain (which comprises at least one heavy chain portion). The heavy chain portion may be from any immunoglobulin, such as IgG1, IgG2, IgG3, or IgG4 subtypes, IgA, IgE, IgD or IgM.

In another embodiment of the invention, a binding molecule is an "antibody-fusion protein chimera." Such molecules comprise a molecule which combines at least one binding domain of an antibody with at least one fusion protein. Preferably, the interface between the two polypeptides is a CH3 domain of an immunoglobulin molecule.

In one embodiment, the binding molecules of the invention are "antibody" or "immunoglobulin" molecules, e.g., naturally occurring antibody or immunoglobulin molecules or genetically engineered antibody molecules that bind

antigen in a manner similar to antibody molecules. As used herein, the term "immunoglobulin" includes a polypeptide having a combination of two heavy and two light chains whether or not it possesses any relevant specific immunoreactivity. "Antibodies" refers to such assemblies which have significant known specific immunoreactive activity to an antigen of interest (e.g. a tumor associated antigen). Antibodies and immunoglobulins comprise light and heavy chains, with or without an interchain covalent linkage between them. Basic immunoglobulin structures in vertebrate systems are relatively well understood.

As will be discussed in more detail below, the generic term "immunoglobulin" comprises five distinct classes of antibody that can be distinguished biochemically. All five classes of antibodies are clearly within the scope of the present invention, the following discussion will generally be directed to the IgG class of immunoglobulin molecules. With regard to IgG, immunoglobulins comprise two identical light polypeptide chains of molecular weight approximately 23,000 Daltons, and two identical heavy chains of molecular weight 53,000-70,000. The four chains are joined by disulfide bonds in a "Y" configuration wherein the light chains bracket the heavy chains starting at the mouth of the "Y" and continuing through the variable region.

Both the light and heavy chains are divided into regions of structural and functional homology. The terms "constant" and "variable" are used functionally. In this regard, it will be appreciated that the variable domains of both the light (VL) and heavy (VH) chain portions determine antigen recognition and specificity. Conversely, the constant domains of the light chain (CL) and the heavy chain (CH1, CH2 or CH3) confer important biological properties such as secretion, transplacental mobility, Fc receptor binding, complement binding, and the like. By convention the numbering of the constant region domains increases as they become more distal from the antigen binding site or amino-terminus of the antibody. The N-terminus is a variable region and at the C-terminus is a constant region; the CH3 and CL domains actually comprise the carboxy-terminus of the heavy and light chain, respectively.

Light chains are classified as either kappa or lambda ( $\kappa$ ,  $\lambda$ ). Each heavy chain class may be bound with either a kappa or lambda light chain. In general, the light and heavy chains are covalently bonded to each other, and the "tail" portions of the two heavy chains are bonded to each other by covalent disulfide linkages or non-covalent linkages when the immunoglobulins are generated either by hybridomas, B cells or genetically engineered host cells. In the heavy chain, the amino acid sequences run from an N-terminus at the forked ends of the Y configuration to the C-terminus at the bottom of each chain. Those skilled in the art will appreciate that heavy chains are classified as gamma, mu, alpha, delta, or epsilon, ( $\gamma$ ,  $\mu$ ,  $\alpha$ ,  $\delta$ ,  $\epsilon$ ) with some subclasses among them (e.g.,  $\gamma 1$ - $\gamma 4$ ). It is the nature of this chain that determines the "class" of the antibody as



IgG, IgM, IgA, IgG, or IgE, respectively. The immunoglobulin subclasses (isotypes) e.g., IgG<sub>1</sub>, IgG<sub>2</sub>, IgG<sub>3</sub>, IgG<sub>4</sub>, IgA<sub>1</sub>, etc. are well characterized and are known to confer functional specialization. Modified versions of each of these classes and isotypes are readily discernable to the skilled artisan in view of the instant disclosure and,  
5 accordingly, are within the scope of the instant invention.

As indicated above, the variable region allows the antibody to selectively recognize and specifically bind epitopes on antigens. That is, the V<sub>L</sub> domain and V<sub>H</sub> domain of an antibody combine to form the variable region that defines a three dimensional antigen binding site. This quaternary antibody structure forms the antigen  
10 binding site present at the end of each arm of the Y. More specifically, the antigen binding site is defined by three complementary determining regions (CDRs) on each of the V<sub>H</sub> and V<sub>L</sub> chains.

As used herein, the term "binding site" or "binding domain" comprises a region of a polypeptide which is responsible for selectively binding to a target molecule of interest (e.g. an antigen, ligand, receptor, substrate or inhibitor). Exemplary binding  
15 domains include an antibody variable domain, a receptor binding domain of a ligand, a ligand binding domain of a receptor or an enzymatic domain.

In one embodiment, the binding molecules have at least one binding site specific for a molecule targeted for reduction or elimination, e.g., a cell surface antigen  
20 or a soluble antigen.

In preferred embodiments, the binding domain is an antigen binding site. An antigen binding site is formed by variable regions that vary from one polypeptide to another. The polypeptides of the invention comprise at least two antigen binding sites. As used herein, the term "antigen binding site" includes a site that specifically binds  
25 (immunoreacts with) an antigen (e.g., a cell surface or soluble antigen). The antigen binding site includes an immunoglobulin heavy chain and light chain variable region and the binding site formed by these variable regions determines the specificity of the antibody. In one embodiment, an antigen binding molecule of the invention comprises at least one heavy or light chain CDR of an antibody molecule (e.g., the sequence of  
30 which is known in the art or described herein). In another embodiment, an antigen binding molecule of the invention comprises at least two CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least three CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least  
35 four CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least five CDRs from one or more antibody molecules. In another embodiment, an antigen binding molecule of the invention comprises at least six CDRs from one or more antibody molecules.

Exemplary antibody molecules comprising at least one CDR that can be included in the subject antigen binding molecules are known in the art and exemplary molecules are described herein.

The polypeptides comprising two heavy chain portions disclosed herein  
5 may be linked to form two associated Ys so there will be four binding sites forming a "tetravalent" molecule (see e.g., WO02/096948A2)). In another embodiment, tetravalent minibodies or domain deleted antibodies can be made.

The term "specificity" includes the number of potential binding sites which specifically bind (e.g., immunoreact with) a given target. A polypeptide may be  
10 monospecific and contain one or more binding sites which specifically bind a target or a polypeptide may be multispecific and contain two or more binding sites which specifically bind the same or different targets.

In one embodiment, a binding molecule of the invention is a bispecific molecule (e.g., antibody, minibody, domain deleted antibody, or fusion protein having  
15 binding specificity for more than one molecule, e.g., more than one antigen or more than one epitope on the same antigen. In one embodiment, the bispecific molecules have at least one target binding site specific for a molecule targeted for reduction or elimination and a targeting molecule on a cell. In another embodiment, the bispecific molecules have at least one target binding site specific for a molecule targeted for reduction or  
20 elimination and at least one target binding site specific for a drug. In yet another embodiment, the bispecific molecules have at least one target binding site specific for a molecule targeted for reduction or elimination and at least one target binding site specific for a prodrug. In a preferred embodiment, the bispecific molecules are tetravalent antibodies that have two target binding sites specific for one target and two  
25 target binding sites specific for the second target. A tetravalent bispecific molecule may be bivalent for each specificity. Further description of bispecific molecules is provided below.

As used herein the term "valency" refers to the number of potential target binding sites in a polypeptide. Each target binding site specifically binds one target  
30 molecule or specific site on a target molecule. When a polypeptide comprises more than one target binding site, each target binding site may specifically bind the same or different molecules (e.g., may bind to different ligands or different antigens, or different epitopes on the same antigen).

In naturally occurring antibodies, the six CDRs present on each  
35 monomeric antibody are short, non-contiguous sequences of amino acids that are specifically positioned to form the antigen binding site as the antibody assumes its three dimensional configuration in an aqueous environment. The remainder of the heavy and light variable domains show less inter-molecular variability in amino acid sequence and

are termed the framework regions. The framework regions largely adopt a  $\beta$ -sheet conformation and the CDRs form loops which connect, and in some cases form part of, the  $\beta$ -sheet structure. Thus, these framework regions act to form a scaffold that provides for positioning the six CDRs in correct orientation by inter-chain, non-covalent interactions. The antigen binding site formed by the positioned CDRs defines a surface complementary to the epitope on the immunoreactive antigen. This complementary surface promotes the non-covalent binding of the antibody to the immunoreactive antigen epitope. The position of CDRs can be readily identified by one of ordinary skill in the art.

As previously indicated, the subunit structures and three dimensional configuration of the constant regions of the various immunoglobulin classes are well known. As used herein, the term "VH domain" includes the amino terminal variable domain of an immunoglobulin heavy chain and the term "CH1 domain" includes the first (most amino terminal) constant region domain of an immunoglobulin heavy chain. The CH1 domain is adjacent to the VH domain and is amino terminal to the hinge region of an immunoglobulin heavy chain molecule.

As used herein the term "CH2 domain" includes the portion of a heavy chain molecule that extends, e.g., from about residue 244 to residue 360 of an antibody using conventional numbering schemes (residues 244 to 360, Kabat numbering system; and residues 231-340, EU numbering system; and Kabat EA et al. Sequences of Proteins of Immunological Interest. Bethesda, US Department of Health and Human Services, NIH. 1991). The CH2 domain is unique in that it is not closely paired with another domain. Rather, two N-linked branched carbohydrate chains are interposed between the two CH2 domains of an intact native IgG molecule. It is also well documented that the CH3 domain extends from the CH2 domain to the C-terminal of the IgG molecule and comprises approximately 108 residues.

As used herein, the term "hinge region" includes the portion of a heavy chain molecule that joins the CH1 domain to the CH2 domain. This hinge region comprises approximately 25 residues and is flexible, thus allowing the two N-terminal antigen binding regions to move independently. Hinge regions can be subdivided into three distinct domains: upper, middle, and lower hinge domains (Roux et al. J. Immunol. 1998 161:4083).

In one embodiment, a binding molecule of the invention comprises a connecting peptide. The connecting peptides of the invention are synthetic. As used herein the term "synthetic" with respect to polypeptides includes polypeptides which comprise an amino acid sequence that is not naturally occurring. For example, non-naturally occurring polypeptides which are modified forms of naturally occurring polypeptides (e.g., comprising a mutation such as an addition, substitution or deletion)

or which comprise a first amino acid sequence (which may or may not be naturally occurring) that is linked in a linear sequence of amino acids to a second amino acid sequence (which may or may not be naturally occurring) to which it is not naturally linked in nature.

5 Connecting peptides of the invention connect two domains (e.g., a binding domain and a dimerization domain) of a binding molecule of the invention. For example, connecting peptides connect a heavy chain portion to a binding domain comprising a binding site. In one embodiment, a connecting peptide connects two heavy chain constant region domains, such as CH1 and CH2 domains; CH1 and CH3 domains; 10 hinge and CH1 domains; hinge and CH3 domains; VH and hinge domains, or a CH3 domain and a non-immunoglobulin polypeptide) in a linear amino acid sequence of a polypeptide chain. Preferably, such connecting peptides provide flexibility to the polypeptide molecule and facilitate dimerization via disulfide bonding. In one embodiment, the connecting peptides of the invention are used to replace one or more 15 heavy chain domains (e.g., at least a portion of a constant region domain (e.g., at least a portion of a CH2 domain) and/or at least a portion of the hinge region (e.g., at least a portion of the lower hinge region domain) in a domain deleted construct). For example, in one embodiment, a VH domain is fused to a CH3 domain via a connecting peptide (the C-terminus of the connecting peptide is attached to the N-terminus of the CH3 20 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the VH domain). In another embodiment, a VL domain is fused to a CH3 domain via a connecting peptide (the C-terminus of the connecting peptide is attached to the N-terminus of the CH3 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the VL domain. In another embodiment, a CH1 domain is fused to a 25 CH3 domain via a connecting peptide (the C-terminus of the connecting peptide is attached to the N-terminus of the CH3 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the CH1 domain).

In one embodiment, a synthetic connecting peptide comprises a portion of a constant region domain. For example, in one embodiment, a connecting peptide that 30 replaces a CH2 domain can comprise a portion of the CH2 domain.

In one embodiment, a connecting peptide comprises or consists of a gly-ser linker. As used herein, the term "gly-ser linker" refers to a peptide that consists of glycine and serine residues. An exemplary gly/ser linker comprises the amino acid sequence GGGSSGGSG (SEQ ID NO:1). In one embodiment, a connecting peptide of 35 the invention comprises at least a portion of an upper hinge region (e.g., derived from an IgG1, IgG3, or IgG4 molecule), at least a portion of a middle hinge region (e.g., derived from an IgG1, IgG3, or IgG4 molecule) and a series of gly/ser amino acid residues (e.g., a gly/ser linker such as GGGSSGGSG (SEQ ID NO:1)). In one embodiment, the

connecting peptide comprises a substitution of one or more amino acids as compared to naturally occurring IgG1 or IgG3 hinge regions. In another embodiment, a connecting peptide comprises an amino acid sequence such as described in WO 02/060955.

Connecting peptides are described in more detail below.

5 As used herein the term "disulfide bond" includes the covalent bond formed between two sulfur atoms. The amino acid cysteine comprises a thiol group that can form a disulfide bond or bridge with a second thiol group. In most naturally occurring IgG molecules, the CH1 and CL regions are linked by a disulfide bond and the two heavy chains are linked by two disulfide bonds at positions corresponding to 239  
10 and 242 using the Kabat numbering system (position 226 or 229, EU numbering system).

It is known in the art that the constant region mediates several effector functions. For example, binding of the C1 component of complement to antibodies activates the complement system. Activation of complement is important in the  
15 opsonisation and lysis of cell pathogens. The activation of complement also stimulates the inflammatory response and may also be involved in autoimmune hypersensitivity. Further, antibodies bind to cells via the Fc region, with a Fc receptor site on the antibody Fc region binding to a Fc receptor (FcR) on a cell. There are a number of Fc receptors which are specific for different classes of antibody, including IgG (gamma receptors),  
20 IgE (epsilon receptors), IgA (alpha receptors) and IgM (mu receptors). Binding of antibody to Fc receptors on cell surfaces triggers a number of important and diverse biological responses including engulfment and destruction of antibody-coated particles, clearance of immune complexes, lysis of antibody-coated target cells by killer cells (called antibody-dependent cell-mediated cytotoxicity, or ADCC), release of  
25 inflammatory mediators, placental transfer and control of immunoglobulin production.

In one embodiment, the Fc portion may be mutated to decrease effector function using techniques known in the art. For example, the deletion or inactivation (through point mutations or other means) of a constant region domain may reduce Fc receptor binding of the circulating modified antibody thereby increasing tumor  
30 localization. In other cases it may be that constant region modifications consistent with the instant invention moderate complement binding and thus reduce the serum half life and nonspecific association of a conjugated cytotoxin. Yet other modifications of the constant region may be used to modify disulfide linkages or oligosaccharide moieties that allow for enhanced localization due to increased antigen specificity or antibody  
35 flexibility. More generally, those skilled in the art will realize that antibodies modified as described herein may exert a number of subtle effects that may or may not be readily appreciated. However the resulting physiological profile, bioavailability and other biochemical effects of the modifications, such as tumor localization, biodistribution and

serum half-life, may easily be measured and quantified using well know immunological techniques without undue experimentation.

In one embodiment, modified forms of antibodies can be made from a whole precursor or parent antibody using techniques known in the art. Exemplary techniques are discussed in more detail below. In particularly preferred embodiments both the variable and constant regions of polypeptides of the invention are human. In one embodiment, fully human antibodies can be made using techniques that are known in the art. For example, fully human antibodies against a specific antigen can be prepared by administering the antigen to a transgenic animal which has been modified to produce such antibodies in response to antigenic challenge, but whose endogenous loci have been disabled. Exemplary techniques that can be used to make antibodies are described in US patents: 6,150,584; 6,458,592; 6,420,140. Other techniques are known in the art.

A polypeptide comprising a heavy chain portion may or may not comprise other amino acid sequences or moieties not derived from an immunoglobulin molecule. Such modifications are described in more detail below. For example, in one embodiment, a polypeptide of the invention may comprise a flexible linker sequence. In another embodiment, a polypeptide may be modified to add a functional moiety such as PEG.

The polypeptides of the instant invention comprise at least two binding sites that provide for the association of the polypeptide with the selected target molecule.

In one embodiment, a binding molecule of the invention comprises an antibody molecule, e.g., an intact antibody molecule, or a fragment of an antibody molecule. In another embodiment, binding molecule of the invention is a modified or synthetic antibody molecule. In one embodiment, a binding molecule of the invention comprises all or a portion of (e.g., at least one antigen binding site from, at least one CDR from, or at least one heavy chain portion from) a monoclonal antibody, a humanized antibody, a chimeric antibody, or a recombinantly produced antibody.

In embodiments where the binding molecule is an antibody or modified antibody, the antigen binding site and the heavy chain portions need not be derived from the same immunoglobulin molecule. In this regard, the variable region may or be derived from any type of animal that can be induced to mount a humoral response and generate immunoglobulins against the desired antigen. As such, the variable region of the polypeptides may be, for example, of mammalian origin e.g., may be human, murine, non-human primate (such as cynomolgus monkeys, macaques, etc.), lupine, camelid (e.g., from camels, llamas and related species). In another embodiment, the variable region may be chondrichthoid in origin (e.g., from sharks).

Polypeptides of the invention can be made using techniques that are known in the art. In one embodiment, the polypeptides of the invention are antibody molecules that have been "recombinantly produced," i.e., are produced using recombinant DNA technology. Exemplary techniques for making antibody molecules are discussed in more detail below.

In one embodiment, the polypeptides of the invention are modified antibodies. As used herein, the term "modified antibody" includes synthetic forms of antibodies which are altered such that they are not naturally occurring, e.g., antibodies that comprise at least two heavy chain portions but not two complete heavy chains (such as, domain deleted antibodies or minibodies); multispecific forms of antibodies (e.g., bispecific, trispecific, etc.) altered to bind to two or more different antigens or to different epitopes on a single antigen); heavy chain molecules joined to scFv molecules and the like. ScFv molecules are known in the art and are described, e.g., in US patent 5,892,019. In addition, the term "modified antibody" includes multivalent forms of antibodies (e.g., trivalent, tetravalent, etc., antibodies that bind to three or more copies of the same antigen). In another embodiment, a binding molecule of the invention is a fusion protein comprising at least one heavy chain portion lacking a CH2 domain and comprising a binding domain of a polypeptide comprising the binding portion of one member of a ligand and its receptor.

In one embodiment, the term, "modified antibody" according to the present invention includes immunoglobulins, antibodies, or immunoreactive fragments or recombinants thereof, in which at least a fraction of one or more of the constant region domains has been deleted or otherwise altered so as to provide desired biochemical characteristics such as the ability to non-covalently dimerize, increased ability to localize at the site of a tumor, or reduced serum half-life when compared with a whole, unaltered antibody of approximately the same immunogenicity. In a preferred embodiment, the polypeptides of the present invention are domain deleted antibodies which comprise a polypeptide chain similar to an immunoglobulin heavy chain, but which lack at least a portion of one or more heavy chain domains. More preferably, one entire domain of the constant region of the modified antibody will be deleted and even more preferably all or part of the CH2 domain will be deleted.

In preferred embodiments, a polypeptide of the invention will not elicit a deleterious immune response in a human. Modifications to the constant region compatible with the instant invention comprise additions, deletions or substitutions of one or more amino acids in one or more domains. That is, the polypeptides of the invention disclosed herein may comprise alterations or modifications to one or more of the three heavy chain constant domains (CH1, CH2 or CH3) and/or to the light chain constant region domain (CL).

In one embodiment, the invention pertains to a modified antibody molecule comprising at least one CC49 binding site (specific for Tag72). For example, Figure 8A (SEQ ID NO:16) shows the single-stranded DNA sequence of sc(Fv)2 heavy chain tetravalent CH<sub>2</sub> domain-deleted huCC49 gene. Figure 8B (SEQ ID NO:17) shows the single-stranded DNA sequence of sc(Fv)2 heavy chain tetravalent CH<sub>2</sub> domain-deleted huCC49 gene containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 8C (SEQ ID NO:18) shows the single-stranded DNA sequence of sc(Fv)2 light chain CH<sub>2</sub> domain-deleted huCC49. Figure 9A (SEQ ID NO:19) shows the amino acid sequence of heavy chain sc(Fv)2 tetravalent CH<sub>2</sub> domain-deleted huCC49. Figure 9B (SEQ ID NO:20) shows the amino acid sequence of heavy chain tetravalent CH<sub>2</sub> domain-deleted sc(Fv)2 huCC49 containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. SEQ ID NO:21 shows the amino acid sequence of light chain CH<sub>2</sub> domain-deleted sc(Fv)2 huCC49. Figure 10A (SEQ ID NO:22) shows the single-stranded DNA sequence of tetravalent CH<sub>2</sub> domain-deleted 2sc(Fv)2 huCC49 minibody gene. Figure 10B (SEQ ID NO:23) shows the single-stranded DNA sequence of tetravalent CH<sub>2</sub> domain-deleted sc(Fv)2 huCC49 minibody gene containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 11A (SEQ ID NO:24) shows the amino acid sequence of tetravalent CH<sub>2</sub> domain-deleted sc(Fv)2 huCC49 minibody. Figure 11B (SEQ ID NO:25) shows the amino acid sequence of tetravalent CH<sub>2</sub> domain-deleted sc(Fv)2 huCC49 minibody containing the synthetic G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

In another embodiment, the invention pertains to a modified antibody molecule comprising at least one p5E8 binding site (specific for CD23). Figure 12A (SEQ ID NO:26) shows the single-stranded DNA sequence of heavy chain tetravalent CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 sc(Fv)2 antibody gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 12B (SEQ ID NO:27) shows the single-stranded DNA sequence of light chain tetravalent CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 sc(Fv)2 gene. Figure 13A (SEQ ID NO:28) shows the amino acid sequence of heavy chain tetravalent CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 sc(Fv)2 antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 13B (SEQ ID NO:29) shows the amino acid sequence of light chain tetravalent CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 sc(Fv)2 antibody. Figure 14 (SEQ ID NO:30) shows the single-stranded DNA sequence of CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 VL/VH minibody gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 15 (SEQ ID NO:31) shows the single-stranded DNA sequence of CH<sub>2</sub> domain-deleted PRIMATIZED® p5E8 VH/VL minibody gene containing the

SUBSTITUTE SHEET 27

AMENDED SHEET



G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 16 (SEQ ID NO:32) shows the amino acid sequence of CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide. Figure 17 (SEQ ID NO:33) shows the amino acid sequence of CH2 domain-deleted PRIMATIZED® p5E8 VH/VL minibody containing the  
 5 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

In one embodiment, the polypeptides of the invention may be modified to reduce their immunogenicity using art-recognized techniques. For example, antibodies or polypeptides of the invention can be humanized, deimmunized, or chimeric antibodies  
 10 can be made. These types of antibodies are derived from a non-human antibody, typically a murine antibody, that retains or substantially retains the antigen-binding properties of the parent antibody, but which is less immunogenic in humans. This may be achieved by various methods, including (a) grafting the entire non-human variable domains onto human constant regions to generate chimeric antibodies; (b) grafting at  
 15 least a part of one or more of the non-human complementarity determining regions (CDRs) into a human framework and constant regions with or without retention of critical framework residues; or (c) transplanting the entire non-human variable domains, but "cloaking" them with a human-like section by replacement of surface residues. Such methods are disclosed in Morrison *et al.*, *Proc. Natl. Acad. Sci.* 81: 6851-5 (1984);  
 20 Morrison *et al.*, *Adv. Immunol.* 44: 65-92 (1988); Verhoeven *et al.*, *Science* 239: 1534-1536 (1988); Padlan, *Molec. Immun.* 28: 489-498 (1991); Padlan, *Molec. Immun.* 31: 169-217 (1994), and U.S. Pat. Nos. 5,585,089, 5,693,761 and 5,693,762 all of which are hereby incorporated by reference in their entirety.

De-immunization can also be used to decrease the immunogenicity of an  
 25 antibody. As used herein, the term "de-immunization" includes alteration of an antibody to modify T cell epitopes (see, e.g., WO9852976A1, WO0034317A2). For example, VH and VL sequences from the starting antibody are analyzed and a human T cell epitope "map" from each V region showing the location of epitopes in relation to complementarity-determining regions (CDRs) and other key residues within the  
 30 sequence. Individual T cell epitopes from the T cell epitope map are analyzed in order to identify alternative amino acid substitutions with a low risk of altering activity of the final antibody. A range of alternative VH and VL sequences are designed comprising combinations of amino acid substitutions and these sequences are subsequently incorporated into a range of polypeptides of the invention that are tested for function.  
 35 Typically, between 12 and 24 variant antibodies are generated and tested. Complete heavy and light chain genes comprising modified V and human C regions are then cloned into expression vectors and the subsequent plasmids introduced into cell lines for

the production of whole antibody. The antibodies are then compared in appropriate biochemical and biological assays, and the optimal variant is identified.

In one embodiment, the binding molecule comprises a chimeric antibody. In the context of the present application the term "chimeric antibodies" will be held to mean any  
5 antibody wherein the immunoreactive region or site is obtained or derived from a first species and the constant region (which may be intact, partial or modified in accordance with the instant invention) is obtained from a second species. In preferred embodiments the target binding region or site will be from a non-human source (e.g. mouse) and the constant region is human. Preferably, the variable domains in both the heavy and light  
10 chains are altered by at least partial replacement of one or more CDRs and, if necessary, by partial framework region replacement and sequence changing. Although the CDRs may be derived from an antibody of the same class or even subclass as the antibody from which the framework regions are derived, it is envisaged that the CDRs will be derived from an antibody of different class and preferably from an antibody from a  
15 different species. It may not be necessary to replace all of the CDRs with the complete CDRs from the donor variable region to transfer the antigen binding capacity of one variable domain to another. Rather, it may only be necessary to transfer those residues that are necessary to maintain the activity of the target binding site. Given the explanations set forth in U. S. Pat. Nos. 5,585,089, 5,693,761 and 5,693,762, it will be  
20 well within the competence of those skilled in the art, either by carrying out routine experimentation or by trial and error testing to obtain a functional antibody with reduced immunogenicity.

As used herein the term "properly folded polypeptide" includes polypeptides (e.g., antigen binding molecules such as antibodies) in which all of the  
25 functional domains comprising the polypeptide are distinctly active. As used herein, the term "improperly folded polypeptide" includes polypeptides in which at least one of the functional domains of the polypeptide is not active. In one embodiment, a properly folded polypeptide comprises polypeptide chains linked by at least one disulfide bond and, conversely, an improperly folded polypeptide comprises polypeptide chains not  
30 linked by at least one disulfide bond.

As used herein, the term "malignancy" refers to a non-benign tumor or a cancer. As used herein, the term "cancer" includes a malignancy characterized by deregulated or uncontrolled cell growth. Exemplary cancers include: carcinomas, sarcomas, leukemias, and lymphomas. The term "cancer" includes primary malignant  
35 tumors (e.g., those whose cells have not migrated to sites in the subject's body other than the site of the original tumor) and secondary malignant tumors (e.g., those arising from metastasis, the migration of tumor cells to secondary sites that are different from the site of the original tumor).

In one embodiment, a binding molecule of the invention binds to a tumor cell. Exemplary antibodies which comprise antigen binding sites that bind to antigens expressed on tumor cells are known in the art and one or more CDRs from such antibodies can be included in a binding molecule of the invention. Exemplary antibodies include: 2B8, Lym 1, Lym 2, LL2, Her2, B1, MB1, BH3, B4, B72.3, 5E8, B3F6 and 5E10. In a preferred embodiment, a polypeptide of the invention is a C2B8 antibody which binds to CD20. In another preferred embodiment, a polypeptide of the invention is a CC49 antibody which recognizes TAG72.

In one embodiment, a binding molecule of the invention binds to a molecule which is useful in treating an autoimmune or inflammatory disease or disorder.

As used herein, the term "autoimmune disease or disorder" refers to disorders or conditions in a subject wherein the immune system attacks the body's own cells, causing tissue destruction. Autoimmune diseases include general autoimmune diseases, *i.e.*, in which the autoimmune reaction takes place simultaneously in a number of tissues, or organ specific autoimmune diseases, *i.e.*, in which the autoimmune reaction targets a single organ. Examples of autoimmune diseases that can be diagnosed, prevented or treated by the methods and compositions of the present invention include, but are not limited to, Crohn's disease; Inflammatory bowel disease (IBD); systemic lupus erythematosus; ulcerative colitis; rheumatoid arthritis; goodpasture's syndrome; Grave's disease; Hashimoto's thyroiditis; pemphigus vulgaris; myasthenia gravis; scleroderma; autoimmune hemolytic anemia; autoimmune thrombocytopenic purpura; polymyositis and dermatomyositis; pernicious anemia; Sjögren's syndrome; ankylosing spondylitis; vasculitis; type I diabetes mellitus; neurological disorders, multiple sclerosis, and secondary diseases caused as a result of autoimmune diseases.

As used herein the term "inflammatory disease or disorder" includes diseases or disorders which are caused, at least in part, or exacerbated by inflammation, *e.g.*, increased blood flow, edema, activation of immune cells (*e.g.*, proliferation, cytokine production, or enhanced phagocytosis). Exemplary disorders include those in which inflammation or inflammatory factors (*e.g.*, matrix metalloproteinases (MMPs), nitric oxide (NO), TNF, interleukins, plasma proteins, cellular defense systems, cytokines, lipid metabolites, proteases, toxic radicals, mitochondria, apoptosis, adhesion molecules, *etc.*) are involved or are present in an area in aberrant amounts, *e.g.*, in amounts which may be advantageous to alter, *e.g.*, to benefit the subject. The inflammatory process is the response of living tissue to damage. The cause of inflammation may be due to physical damage, chemical substances, micro-organisms, tissue necrosis, cancer or other agents. Acute inflammation is short-lasting, lasting only a few days. If it is longer lasting however, then it may be referred to as chronic inflammation.

Inflammatory disorders include acute inflammatory disorders, chronic inflammatory disorders, and recurrent inflammatory disorders. Acute inflammatory disorders are generally of relatively short duration, and last for from about a few minutes to about one to two days, although they may last several weeks. The main  
 5 characteristics of acute inflammatory disorders include increased blood flow, exudation of fluid and plasma proteins (edema) and emigration of leukocytes, such as neutrophils. Chronic inflammatory disorders, generally, are of longer duration, *e.g.*, weeks to months to years or even longer, and are associated histologically with the presence of lymphocytes and macrophages and with proliferation of blood vessels and connective  
 10 tissue. Recurrent inflammatory disorders include disorders which recur after a period of time or which have periodic episodes. Examples of recurrent inflammatory disorders include asthma and multiple sclerosis. Some disorders may fall within one or more categories.

Inflammatory disorders are generally characterized by heat, redness,  
 15 swelling, pain and loss of function. Examples of causes of inflammatory disorders include, but are not limited to, microbial infections (*e.g.*, bacterial, viral and fungal infections), physical agents (*e.g.*, burns, radiation, and trauma), chemical agents (*e.g.*, toxins and caustic substances), tissue necrosis and various types of immunologic reactions. Examples of inflammatory disorders include, but are not limited to,  
 20 osteoarthritis, rheumatoid arthritis, acute and chronic infections (bacterial, viral and fungal); acute and chronic bronchitis, sinusitis, and other respiratory infections, including the common cold; acute and chronic gastroenteritis and colitis; acute and chronic cystitis and urethritis; acute respiratory distress syndrome; cystic fibrosis; acute and chronic dermatitis; acute and chronic conjunctivitis; acute and chronic serositis  
 25 (pericarditis, peritonitis, synovitis, pleuritis and tendinitis); uremic pericarditis; acute and chronic cholecystitis; acute and chronic vaginitis; acute and chronic uveitis; drug reactions; and burns (thermal, chemical, and electrical).

As used herein the term "medium that separates polypeptides based on hydrophobic interaction" includes a medium comprising hydrophobic ligands (*e.g.*, alkyl  
 30 or aryl groups) covalently attached to a matrix. Such a medium can be used to separate polypeptides based on interaction between a solvent and accessible non-polar groups on the surface of the polypeptides and the hydrophobic ligands of the medium. An exemplary medium is Phenyl 5PW-HR available from Tosoh Bioscience.

As used herein, the term "conductivity" includes electrical conductivity  
 35 of a solution as measured in microSiemens/cm (formerly micromhos/cm). The greater the ion content of a solution, the greater the conductivity of the solution. Conductivity can be readily measured using techniques that are well known in the art (*e.g.*, by measuring the current passing between two electrodes).

The separation methods of the invention can be used with solutions having a pH ranging from acid to neutral, e.g., from about pH 3.5 to approximately neutral. As used herein, the term "approximately neutral pH" includes pH values of approximately 7. For example, in one embodiment, a separation method of the invention can be performed using a solution (e.g., a buffer) having a pH of about 3, about 4, about 5, about 6, about 7, or about 8. Preferably, the pH of the solution is about 6 or about 7. In one embodiment, the pH of the solution is about 4.0, about 4.1, about 4.2, about 4.3, about 4.4, about 4.5, about 4.6, about 4.7, about 4.8, about 4.9, about 5.0, about 5.1, about 5.2, about 5.3, about 5.4, about 5.5, about 5.6, about 5.7, about 5.8, about 5.9, about 6.0, about 6.1, about 6.2, about 6.3, about 6.4, about 6.5, about 6.6, about 6.7, about 6.8, about 6.9, about 7.0, about 7.1, about 7.2, about 7.3, about 7.4, about 7.5, about 7.6, about 7.7, about 7.8, about 7.9, or about 8.0.

As used herein the term "affinity matrix" includes a matrix, such as agarose, controlled pore glass, or poly (styrenedivinyl) benzene to which an affinity ligand is attached. The affinity ligand binds to the desired polypeptide and the contaminating polypeptides are not bound to the affinity ligand. The desired polypeptide can be eluted from the affinity matrix using known protocols.

As used herein the term "engineered" includes manipulation of nucleic acid or polypeptide molecules by synthetic means (e.g. by recombinant techniques, in vitro peptide synthesis, by enzymatic or chemical coupling of peptides or some combination of these techniques). Preferably, the binding molecules of the invention are engineered, e.g., to express a connecting peptide of the invention.

As used herein, the terms "linked," "fused" or "fusion" are used interchangeably. These terms refer to the joining together of two more elements or components, by whatever means including chemical conjugation or recombinant means. An "in-frame fusion" refers to the joining of two or more open reading frames (ORFs) to form a continuous longer ORF, in a manner that maintains the correct reading frame of the original ORFs. Thus, the resulting recombinant fusion protein is a single protein containing two or more segments that correspond to polypeptides encoded by the original ORFs (which segments are not normally so joined in nature.) Although the reading frame is thus made continuous throughout the fused segments, the segments may be physically or spatially separated by, for example, in-frame linker sequence.

In the context of polypeptides, a "linear sequence" or a "sequence" is an order of amino acids in a polypeptide in an amino to carboxyl terminal direction in which residues that neighbor each other in the sequence are contiguous in the primary structure of the polypeptide.

As used herein, the phrase "subject that would benefit from administration of a binding molecule" includes subjects, such as mammalian subjects,

that would benefit from administration of a binding molecule used, e.g., for detection of an antigen recognized by a binding molecule (e.g., for a diagnostic procedure) and/or from treatment with a binding molecule to reduce or eliminate the target recognized by the binding molecule. For example, in one embodiment, the subject may benefit from  
 5 reduction or elimination of a soluble or particulate molecule from the circulation or serum (e.g., a toxin or pathogen) or from reduction or elimination of a population of cells expressing the target (e.g., tumor cells). As described in more detail herein, the binding molecule can be used in unconjugated form or can be conjugated, e.g., to a drug, prodrug, or an isotope.

10

## II. Synthetic Connecting Peptides

At least one polypeptide chain of a dimer of the invention can comprise a synthetic connecting peptide of the invention. In one embodiment, at least two chains of a dimer of the invention comprise a connecting peptide. In a preferred embodiment, two  
 15 chains of a dimer of the invention comprise a connecting peptide.

In one embodiment, connecting peptides can be used to join two heavy chain portions in frame in a single polypeptide chain. For example, in one embodiment, a connecting peptide of the invention can be used to fuse a CH3 domain (or synthetic CH3 domain) to a hinge region (or synthetic hinge region). In another embodiment, a  
 20 connecting peptide of the invention can be used to fuse a CH3 domain (or synthetic CH3 domain) to a CH1 domain (or synthetic CH1 domain). In still another embodiment, a connecting peptide can act as a peptide spacer between the hinge region (or synthetic hinge region) and a CH2 domain (or a synthetic CH2 domain).

In another embodiment, a CH3 domain can be fused to an extracellular  
 25 protein domain (e.g., a VL domain (or synthetic domain), a VH domain (or synthetic domain), a CH1 domain (or synthetic domain), a hinge domain (or synthetic hinge), or to the ligand binding portion of a receptor or the receptor binding portion of a ligand). For example, in one embodiment, a VH or VL domain is fused to a CH3 domain via a connecting peptide (the C-terminus of the connecting peptide is attached to the N-terminus of the CH3 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the VH or VL domain). In another embodiment, a CH1 domain is fused to a CH3 domain via a connecting peptide (the C-terminus of the connecting peptide is attached to the N-terminus of the CH3 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the CH1 domain). In another  
 30 embodiment, a connecting peptide of the invention can be used to fuse a CH3 domain (or synthetic CH3 domain) to a hinge region (or synthetic hinge region) or portion thereof. In still another embodiment, a connecting peptide can act as a peptide spacer

between the hinge region (or synthetic hinge region) and a CH2 domain (or a synthetic CH2 domain).

In one embodiment, a connecting peptide can comprise or consist of a gly/ser spacer. For example, a domain deleted CC49 construct having a short amino acid spacer GGSSGGGGSG (SEQ. ID No. 1) substituted for the CH2 domain and the lower hinge region (CC49.ΔCH2 [gly/ser]) can be used. In another embodiment, a connecting peptide comprises the amino acid sequence IGKTISKKAK (SEQ ID NO:36).

In another embodiment, connecting peptide can comprise at least a portion of an immunoglobulin hinge region. For example, chimeric hinge domains can be constructed which combine hinge elements derived from different antibody isotypes. In one embodiment, a connecting peptide comprises at least a portion of an IgG1 hinge region. In another embodiment, a connecting peptide can comprise at least a portion of an IgG3 hinge region. In another embodiment, a connecting peptide can comprise at least a portion of an IgG1 hinge region and at least a portion of an IgG3 hinge region. In one embodiment, a connecting peptide can comprise an IgG1 upper and middle hinge and a single IgG3 middle hinge repeat motif.

Because the numbering of individual amino acids in such connecting peptides comprising an amino acid sequence derived from an immunoglobulin hinge region may vary depending upon the length of the connecting peptide, the numbering of amino acid positions in these molecules is given using Kabat numbering scheme, e.g., Table 2). Table 1 shows naturally occurring hinge sequence for IgG1, IgG3, and IgG4 molecules. Table 2 shows Kabat numbering for portions of these hinge molecules and also shows Kabat numbering for connecting peptide amino acid residues presented in that table.

In one embodiment, a connecting peptide of the invention comprises a non-naturally occurring immunoglobulin hinge region domain, e.g., a hinge region domain that is not naturally found in the polypeptide comprising the hinge region domain and/or a hinge region domain that has been altered so that it differs in amino acid sequence from a naturally occurring immunoglobulin hinge region domain. In one embodiment, mutations can be made to hinge region domains to make a connecting peptide of the invention. In one embodiment, a connecting peptide of the invention comprises a hinge domain which does not comprise a naturally occurring number of cysteines, i.e., the connecting peptide comprises either fewer cysteines or a greater number of cysteines than a naturally occurring hinge molecule. In a preferred embodiment, incorporation of a connecting peptide (e.g., comprising a non-naturally occurring number of cysteines) into a polypeptide results in a composition in which greater than 50%, 60%, 70%, 80% or 90% of the dimeric molecules present in a form in

which the two heavy chain portions are linked via at least one interchain disulfide linkage.

- In one embodiment of the invention, a connecting peptide comprises hinge region domain comprising a proline residue at an amino acid position
- 5 corresponding to amino acid position 243 in the Kabat numbering system (position 230, EU numbering system). In one embodiment, a connecting peptide comprises an alanine residue at an amino acid position corresponding to position 244, Kabat numbering system (position 246, EU numbering system). In another embodiment, a connecting peptide of the invention comprises a proline residue at an amino acid position
- 10 corresponding to position 245 (Kabat numbering system; position 247, EU numbering system)). In one embodiment, a connecting peptide comprises a cysteine residue at an amino acid position corresponding to position 239, Kabat numbering system (position 226, EU numbering system). In one embodiment, a connecting peptide comprises a serine residue at an amino acid position corresponding to position 239, Kabat numbering
- 15 system (position 226, EU numbering system). In one embodiment, a connecting peptide comprises a cysteine residue at an amino acid position corresponding to position 242, Kabat numbering system (position 229, EU numbering system). In one embodiment, a connecting peptide comprises a serine residue at an amino acid position corresponding to position 242, Kabat numbering system (position 229, EU numbering system).
- 20 In one embodiment, the connecting peptide can be chosen to result in the preferential synthesis of a particular isoform of polypeptide, e.g., in which the two heavy chain portions are linked via disulfide bonds or are not linked via disulfide bonds. For example, as described in the instant examples, the G1/G3/Pro243 + [gly/ser] linker (SEQ ID NO: 8), G1/G3/Pro243Ala244Pro245 + [gly/ser] linker (SEQ ID NO: 9), Pro243 +
- 25 [gly/ser] linker (SEQ ID NO:15), and Pro243Ala244Pro245 + [gly/ser] linker (SEQ ID NO: 14), connecting peptides resulted in the production of only Form A CH2 domain-deleted antibody with no detectable Form B. In contrast, CH2 domain-deleted Cys242Ser:Pro243 (SEQ ID NO: 12), and CH2 domain-deleted Cys242Ser:Pro243Ala244Pro245 (SEQ ID NO: 13), both resulted in a preference for
- 30 the Form B isoform. These synthetic hinge region connecting peptides would thus be useful for favoring synthesis of Form A or B isoform. This is true for any isotype of antibody, (e.g., IgG1, IgG2, IgG3, or IgG4) based on the high degree of homology among the CH3 domains for all four human isotypes. (Including identical and conserved amino acid residues, IgG1 CH3 domain is 98.13% homologous to IgG2 CH3,
- 35 97.20% homologous to IgG3 CH3, and 96.26% homologous to IgG4 CH3). The parentheticals referring to connecting peptides and various binding molecules of the invention represent equivalent terminology unless otherwise indicated.



- In one embodiment, a connecting peptide of the invention comprises a hinge region domain followed by a flexible gly/ser linker. Exemplary connecting peptides are shown in Table 2 and in SEQ ID NOs: 8-15, 48, and 49. It will be understood that variant forms of these exemplary connecting peptides can be created by
- 5 introducing one or more nucleotide substitutions, additions or deletions into the nucleotide sequence encoding a connecting peptide such that one or more amino acid substitutions, additions or deletions are introduced into the connecting peptide. For example, mutations may be introduced by standard techniques, such as site-directed mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid
- 10 substitutions are made at one or more non-essential amino acid residues such that the ability of the connecting peptide to preferentially enhance synthesis of Form A or Form B is not altered. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art, including
- 15 basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine,
- 20 phenylalanine, tryptophan, histidine). Thus, a nonessential amino acid residue in an immunoglobulin polypeptide is preferably replaced with another amino acid residue from the same side chain family. In another embodiment, a string of amino acids can be replaced with a structurally similar string that differs in order and/or composition of side chain family members.
- 25 Connecting peptides of the invention can be of varying lengths. In one embodiment, a connecting peptide of the invention is from about 15 to about 50 amino acids in length. In another embodiment, a connecting peptide of the invention is from about 20 to about 45 amino acids in length. In another embodiment, a connecting peptide of the invention is from about 25 to about 40 amino acids in length. In another
- 30 embodiment, a connecting peptide of the invention is from about 30 to about 35 amino acids in length. In another embodiment, a connecting peptide of the invention is from about 24 to about 27 amino acids in length. In another embodiment, a connecting peptide of the invention is from about 40 to about 42 amino acids in length.
- Connecting peptides can be introduced into polypeptide sequences using
- 35 techniques known in the art. For example, in one embodiment, the Splicing by Overlap Extension (SOE) method (Horton, R.M. 1993 *Methods in Molecular Biology*, Vol 15:PCR Protocols: Current Methods and applications. Ed. B.A. White) can be used.

Modifications can be confirmed by DNA sequence analysis. Plasmid DNA can be used to transform host cells for stable production of the polypeptides produced.

In one embodiment, incorporation of one of the subject connecting peptides into a polypeptide yields a composition comprising polypeptide molecules  
 5 having at least two binding sites and at least two polypeptide chains, wherein at least two of the polypeptide chains comprise a synthetic connecting peptide and wherein greater than 50% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment,  
 10 greater than 60% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than 70% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment,  
 15 greater than 80% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than 90% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage.

In one embodiment, incorporation of one of the subject connecting peptides into an IgG4 molecule yields a composition in which greater than 95% of the molecules are present in a form in which the two heavy chain portions are linked via at  
 20 least one interchain disulfide linkage.

### III. Binding molecules

25 The polypeptides of the invention comprise at least two binding sites which bind to a target molecule of interest. Exemplary binding sites include, e.g., sites which bind to an antigen (antigen binding sites), sites which bind to a receptor (receptor binding sites), or sites which bind to a ligand (ligand binding sites). In one embodiment,  
 30 the binding molecules comprise at least two binding sites. In one embodiment, the binding molecules comprise two binding sites. In one embodiment, the binding molecules comprise three binding sites. In another embodiment, the binding molecules comprise four binding sites.

In one embodiment, the binding molecules have at least one target  
 35 binding site specific for a molecule which mediates a biological effect (e.g., which modulates cellular activation (e.g., by binding to a cell surface receptor and resulting in transmission or inhibition of an activating or inhibitory signal), which results in death of the cell (e.g., by complement fixation or exposure to a payload present on the binding

molecule), or which modulates a disease or disorder in a subject (e.g., by promoting lysis of a fibrin clot or promoting clot formation, or by modulating the amount of a substance which is bioavailable (e.g., by enhancing or reducing the amount of a ligand such as TNF $\alpha$  in the subject)).

5 In one embodiment, the binding molecules have at least one target binding site specific for an molecule targeted for reduction or elimination, e.g., a cell surface antigen or a soluble antigen. In one embodiment, binding of the binding molecule to the target results in reduction or elimination of the target, e.g., from a tissue or from the circulation. In another embodiment, the binding molecules have at least one  
10 binding site specific for a molecule that can be used to detect the presence of a target molecule (e.g., to detect a contaminant or diagnose a condition or disorder). In yet another embodiment, a binding molecule of the invention comprises at least one binding site that targets the binding molecule to a specific site in a subject (e.g., to a tumor cell or blood clot).

15 Exemplary binding sites that can be included in the binding domain of a binding molecule of the invention include: the receptor binding portion of a ligand, the ligand binding portion of a receptor, the substrate binding portion of an enzyme, the enzyme binding portion of a substrate, or one or more antigen binding portions of an antibody.

20 In one embodiment, at least one target binding site of a binding molecule (e.g., an antibody molecule, a bispecific antibody, or a modified antibody) is catalytic (Shokat and Schultz. 1990. Annu. Rev. Immunol. 8:335).

In one embodiment, a heavy chain variable portion and a light chain variable portion of a binding molecule are present in the same polypeptide, e.g., as in a  
25 single chain antibody or a minibody (see e.g., US patent 5,837,821 or WO 94/09817A1). In another embodiment, the heavy chain portion and the light chain portion of a polypeptide are present in different polypeptide chains, e.g., as in antibody molecules.

The target binding polypeptides of the invention are multimeric molecules. In one embodiment, the target binding polypeptides are dimers. In one  
30 embodiment, the dimers of the invention are homodimers, comprising two identical monomeric subunits. In another embodiment, the dimers of the invention are heterodimers, comprising two non-identical monomeric subunits. The dimers comprise at least two polypeptide chains. In one embodiment, the binding molecules comprise two polypeptide chains. In another embodiment, the binding molecules comprise three  
35 polypeptide chains. In another embodiment, the binding molecules comprise four polypeptide chains.

In a preferred embodiment, a binding molecule of the invention comprises at least one CDR of an antibody, e.g., an antibody known in the art to bind to

a target of interest. In another embodiment, a binding molecule of the invention comprises at least two CDRs. In another embodiment, a binding molecule of the invention comprises at least three CDRs. In another embodiment, a binding molecule of the invention comprises at least four CDRs. In another embodiment, a binding molecule  
 5 of the invention comprises at least five CDRs. In another embodiment, a binding molecule of the invention comprises at least six CDRs. In a preferred embodiment, a binding molecule of the invention comprises at least one VH domain of an antibody, e.g., an antibody known in the art to bind to a target of interest. In a preferred  
 10 embodiment, a binding molecule of the invention comprises at least one VL domain. In another preferred embodiment, a binding molecule of the invention comprises at least one VH domain and one VL domain of an antibody.

In one embodiment, an antigen binding site consists of a VH domain, e.g., derived from camelids, which is stable in the absence of a VL chain (Hamers-Casterman et al. 1993. *Nature* 363:446; Desmyter et al. 1996. *Nat. Struct. Biol.* 3:803;  
 15 Desmyter, A., 1996. *Nat. Struct. Biol.* 3:803; Decanniere, K., et al. 1999. *Structure* 7:361; Davies et al. 1996. *Protein Eng.* 9:531; Kortt et al. 1995. *J. Protein Chem.* 14:167).

#### A. Fusion Proteins

20

The invention also pertains to binding molecules which comprise one or more immunoglobulin domains. The fusion proteins of the invention comprise a binding domain (which comprises at least one binding site) and a dimerization domain (which comprises at least one heavy chain portion). The subject fusion proteins may be  
 25 bispecific (with one binding site for a first target and a second binding site for a second target) or may be multivalent (with two binding sites for the same target).

Exemplary fusion proteins reported in the literature include fusions of the T cell receptor (Gascoigne et al., *Proc. Natl. Acad. Sci. USA* 84:2936-2940 (1987)); CD4 (Capon et al., *Nature* 337:525-531 (1989); Traunecker et al., *Nature* 339:68-70  
 30 (1989); Zettmeissl et al., *DNA Cell Biol.* USA 9:347-353 (1990); and Byrn et al., *Nature* 344:667-670 (1990)); L-selectin (homing receptor) (Watson et al., *J. Cell. Biol.* 110:2221-2229 (1990); and Watson et al., *Nature* 349:164-167 (1991)); CD44 (Aruffo et al., *Cell* 61:1303-1313 (1990)); CD28 and B7 (Linsley et al., *J. Exp. Med.* 173:721-730 (1991)); CTLA-4 (Lisley et al., *J. Exp. Med.* 174:561-569 (1991)); CD22 (Stamenkovic  
 35 et al., *Cell* 66:1133-1144 (1991)); TNF receptor (Ashkenazi et al., *Proc. Natl. Acad. Sci. USA* 88:10535-10539 (1991); Lesslauer et al., *Eur. J. Immunol.* 27:2883-2886 (1991); and Poppel et al., *J. Exp. Med.* 174:1483-1489 (1991)); and IgE receptor a (Ridgway and Gorman, *J. Cell. Biol.* Vol. 115, Abstract No. 1448 (1991)).

- 39 -

In one embodiment a fusion protein combines the binding domain(s) of the ligand or receptor (e.g. the extracellular domain (ECD) of a receptor) with at least one heavy chain domain and a synthetic connecting peptide. In one embodiment, when preparing the fusion proteins of the present invention, nucleic acid encoding the binding domain of the ligand or receptor will be fused C-terminally to nucleic acid encoding the N-terminus of an immunoglobulin constant domain sequence. N-terminal fusions are also possible. In one embodiment, a fusion protein includes a CH2 and a CH3 domain. Fusions may also be made to the C-terminus of the Fc portion of a constant domain, or immediately N-terminal to the CH1 of the heavy chain or the corresponding region of the light chain.

In one embodiment, the sequence of the ligand or receptor domain is fused to the N-terminus of the Fc domain of an immunoglobulin molecule. It is also possible to fuse the entire heavy chain constant region to the sequence of the ligand or receptor domain. In one embodiment, a sequence beginning in the hinge region just upstream of the papain cleavage site which defines IgG Fc chemically (i.e. residue 216, taking the first residue of heavy chain constant region to be 114), or analogous sites of other immunoglobulins is used in the fusion. The precise site at which the fusion is made is not critical; particular sites are well known and may be selected in order to optimize the biological activity, secretion, or binding characteristics of the molecule. Methods for making fusion proteins are known in the art.

For bispecific fusion proteins, the fusion proteins are assembled as multimers, and particularly as heterodimers or heterotetramers. Generally, these assembled immunoglobulins will have known unit structures. A basic four chain structural unit is the form in which IgG, IgD, and IgE exist. A four chain unit is repeated in the higher molecular weight immunoglobulins; IgM generally exists as a pentamer of four basic units held together by disulfide bonds. IgA globulin, and occasionally IgG globulin, may also exist in multimeric form in serum. In the case of multimer, each of the four units may be the same or different. Additional exemplary ligands and their receptors that may be included in the subject fusion proteins include the following:

#### Cytokines and Cytokine Receptors

Cytokines have pleiotropic effects on the proliferation, differentiation, and functional activation of lymphocytes. Various cytokines, or receptor binding portions thereof, can be utilized in the fusion proteins of the invention. Exemplary cytokines include the interleukins (e.g. IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-11, IL-12, IL-13, and IL-18), the colony stimulating factors (CSFs) (e.g. granulocyte CSF (G-CSF), granulocyte-macrophage CSF (GM-CSF), and monocyte

macrophage CSF (M-CSF)), tumor necrosis factor (TNF) alpha and beta, and interferons such as interferon- $\alpha$ ,  $\beta$ , or  $\gamma$  (US Patent Nos. 4,925,793 and 4,929,554).

Cytokine receptors typically consist of a ligand-specific alpha chain and a common beta chain. Exemplary cytokine receptors include those for GM-CSF, IL-3  
 5 (US Patent No. 5,639,605), IL-4 (US Patent No. 5,599,905), IL-5 (US Patent No. 5,453,491), IFN $\gamma$  (EP0240975), and the TNF family of receptors (e.g., TNF $\alpha$  (e.g. TNFR-1 (EP 417, 563), TNFR-2 (EP 417,014) lymphotoxin beta receptor).

#### Adhesion Proteins

10 Adhesion molecules are membrane-bound proteins that allow cells to interact with one another. Various adhesion proteins, including leukocyte homing receptors and cellular adhesion molecules, of receptor binding portions thereof, can be incorporated in a fusion protein of the invention. Leucocyte homing receptors are expressed on leucocyte cell surfaces during inflammation and include the  $\beta$ -1 integrins  
 15 (e.g. VLA-1, 2, 3, 4, 5, and 6) which mediate binding to extracellular matrix components, and the  $\beta$ 2-integrins (e.g. LFA-1, LPAM-1, CR3, and CR4) which bind cellular adhesion molecules (CAMs) on vascular endothelium. Exemplary CAMs include ICAM-1, ICAM-2, VCAM-1, and MadCAM-1. Other CAMs include those of the selectin family including E-selectin, L-selectin, and P-selectin.

20

#### Chemokines

Chemokines, chemotactic proteins which stimulate the migration of leucocytes towards a site of infection, can also be incorporated into a fusion protein of the invention. Exemplary chemokines include Macrophage inflammatory proteins  
 25 (MIP-1- $\alpha$  and MIP-1- $\beta$ ), neutrophil chemotactic factor, and RANTES (regulated on activation normally T-cell expressed and secreted).

#### Growth Factors and Growth Factor Receptors

Growth factors or their receptors (or receptor binding or ligand binding  
 30 portions thereof) may be incorporated in the fusion proteins of the invention. Exemplary growth factors include Vascular Endothelial Growth Factor (VEGF) and its isoforms (U.S. Pat. No. 5,194,596); Fibroblastic Growth Factors (FGF), including aFGF and bFGF; atrial natriuretic factor (ANF); hepatic growth factors (HGFs; US Patent Nos. 5,227,158 and 6,099,841), neurotrophic factors such as bone-derived neurotrophic factor  
 35 (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6), or a nerve growth factor such as NGF- $\beta$  platelet-derived growth factor (PDGF) (U.S. Pat. Nos. 4,889,919, 4,845,075, 5,910,574, and 5,877,016); transforming growth factors (TGF) such as TGF-alpha and TGF-beta (WO 90/14359), osteoinductive factors including bone

morphogenetic protein (BMP); insulin-like growth factors-I and -II (IGF-I and IGF-II; US Patent Nos. 6,403,764 and 6,506,874); Erythropoietin (EPO); stem-cell factor (SCF), thrombopoietin (c-*Mpl* ligand), and the Wnt polypeptides (US Patent No. 6,159,462).

Exemplary growth factor receptors which may be used as targeting receptor domains of the invention include EGF receptors; VEGF receptors (e.g. Flt1 or Flk1/KDR), PDGF receptors (WO 90/14425); HGF receptors (US Patent Nos. 5,648,273, and 5,686,292), and neurotrophic receptors including the low affinity receptor (LNGFR), also termed as p75<sup>NTR</sup> or p75, which binds NGF, BDNF, and NT-3, and high affinity receptors that are members of the trk family of the receptor tyrosine kinases (e.g. trkA, trkB (EP 455,460), trkC (EP 522,530)).

#### Hormones

Exemplary growth hormones for use as targeting agents in the fusion proteins of the invention include renin, human growth hormone (HGH; US Patent No. 5,834,598), N-methionyl human growth hormone; bovine growth hormone; growth hormone releasing factor; parathyroid hormone (PTH); thyroid stimulating hormone (TSH); thyroxine; proinsulin and insulin (US Patent Nos. 5,157,021 and 6,576,608); follicle stimulating hormone (FSH), calcitonin, luteinizing hormone (LH), leptin, glucagons; bombesin; somatropin; mullerian-inhibiting substance; relaxin and pror relaxin; gonadotropin-associated peptide; prolactin; placental lactogen; OB protein; or mullerian-inhibiting substance.

#### Clotting Factors

Exemplary blood coagulation factors for use as targeting agents in the fusion proteins of the invention include the clotting factors (e.g., factors V, VII, VIII, X, IX, XI, XII and XIII, von Willebrand factor); tissue factor (U.S. Pat. Nos. 5,346,991, 5,349,991, 5,726,147, and 6,596,84); thrombin and prothrombin; fibrin and fibrinogen; plasmin and plasminogen; plasminogen activators, such as urokinase or human urine or tissue-type plasminogen activator (t-PA).

Other exemplary fusion proteins are taught, e.g., in WO0069913A1 and WO0040615A2. Another exemplary molecule that may be included in a fusion protein of the invention is IGSF9.

Fusion proteins can be prepared using methods that are well known in the art (see for example US Patent Nos. 5,116,964 and 5,225,538). Ordinarily, the ligand or receptor domain is fused C-terminally to the N-terminus of the constant region of the heavy chain (or heavy chain portion) and in place of the variable region. Any

transmembrane regions or lipid or phospholipids anchor recognition sequences of ligand binding receptor are preferably inactivated or deleted prior to fusion. DNA encoding the ligand or receptor domain is cleaved by a restriction enzyme at or proximal to the 5' and 3' ends of the DNA encoding the desired ORF segment. The resultant DNA fragment is then readily inserted into DNA encoding a heavy chain constant region. The precise site at which the fusion is made may be selected empirically to optimize the secretion or binding characteristics of the soluble fusion protein. DNA encoding the fusion protein is then transfected into a host cell for expression.

10

#### B. Antibodies or portions thereof

In one embodiment, a binding molecule, e.g., antigen binding molecule, of the invention is an antibody molecule. Using art recognized protocols, for example, antibodies are preferably raised in mammals by multiple subcutaneous or intraperitoneal injections of the relevant antigen (e.g., purified tumor associated antigens or cells or cellular extracts comprising such antigens) and an adjuvant. This immunization typically elicits an immune response that comprises production of antigen-reactive antibodies from activated splenocytes or lymphocytes. While the resulting antibodies may be harvested from the serum of the animal to provide polyclonal preparations, it is often desirable to isolate individual lymphocytes from the spleen, lymph nodes or peripheral blood to provide homogenous preparations of monoclonal antibodies (MAbs). Preferably, the lymphocytes are obtained from the spleen.

In this well known process (Kohler et al., *Nature*, 256:495 (1975)) the relatively short-lived, or mortal, lymphocytes from a mammal which has been injected with antigen are fused with an immortal tumor cell line (e.g. a myeloma cell line), thus, producing hybrid cells or "hybridomas" which are both immortal and capable of producing the genetically coded antibody of the B cell. The resulting hybrids are segregated into single genetic strains by selection, dilution, and regrowth with each individual strain comprising specific genes for the formation of a single antibody. They produce antibodies which are homogeneous against a desired antigen and, in reference to their pure genetic parentage, are termed "monoclonal."

Hybridoma cells thus prepared are seeded and grown in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, parental myeloma cells. Those skilled in the art will appreciate that reagents, cell lines and media for the formation, selection and growth of hybridomas are commercially available from a number of sources and standardized protocols are well established. Generally, culture medium in which the hybridoma cells are growing is assayed for production of monoclonal antibodies against the desired antigen. Preferably,

- 43 -



the binding specificity of the monoclonal antibodies produced by hybridoma cells is determined by immunoprecipitation or by an *in vitro* assay, such as a radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). After hybridoma cells are identified that produce antibodies of the desired specificity, affinity and/or activity, the clones may be subcloned by limiting dilution procedures and grown by standard methods (Goding, *Monoclonal Antibodies: Principles and Practice*, pp 59-103 (Academic Press, 1986)). It will further be appreciated that the monoclonal antibodies secreted by the subclones may be separated from culture medium, ascites fluid or serum by conventional purification procedures such as, for example, protein-A, hydroxylapatite chromatography, gel electrophoresis, dialysis or affinity chromatography.

In another embodiment, DNA encoding a desired monoclonal antibodies may be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The isolated and subcloned hybridoma cells serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into prokaryotic or eukaryotic host cells such as *E. coli* cells, simian COS cells, Chinese Hamster Ovary (CHO) cells or myeloma cells that do not otherwise produce immunoglobulins. More particularly, the isolated DNA (which may be modified as described herein) may be used to clone constant and variable region sequences for the manufacture antibodies as described in Newman *et al.*, U.S. Pat. No. 5,658,570, filed January 25, 1995, which is incorporated by reference herein. Essentially, this entails extraction of RNA from the selected cells, conversion to cDNA, and amplification by PCR using Ig specific primers. Suitable primers for this purpose are also described in U.S. Pat. No. 5,658,570. As will be discussed in more detail below, transformed cells expressing the desired antibody may be grown up in relatively large quantities to provide clinical and commercial supplies of the immunoglobulin.

Those skilled in the art will also appreciate that DNA encoding antibodies or antibody fragments may also be derived from antibody phage libraries, e.g., using phage or Fd phagemid technology. Exemplary methods are set forth, for example, in EP 368 684 B1; U.S. patent. 5,969,108, Hoogenboom, H.R. and Chames. 2000. *Immunol. Today* 21:371; Nagy et al. 2002. *Nat. Med.* 8:801; Huie et al. 2001. *Proc. Natl. Acad. Sci. USA* 98:2682; Lui et al. 2002. *J. Mol. Biol.* 315:1063, each of which is incorporated herein by reference. Several publications (e.g., Marks et al. *Bio/Technology* 10:779-783 (1992)) have described the production of high affinity human antibodies by chain shuffling, as well as combinatorial infection and *in vivo* recombination as a strategy for constructing large phage libraries. In another embodiment, Ribosomal display can be used to replace bacteriophage as the display platform (see, e.g., Hanes et al. 2000. *Nat. Biotechnol.* 18:1287; Wilson et al. 2001. *Proc. Natl. Acad. Sci. USA* 98:3750; or Irving et al. 2001. *J.*

*Immunol. Methods* 248:31. In yet another embodiment, cell surface libraries can be screened for antibodies (Boder et al. 2000. *Proc. Natl. Acad. Sci. USA* 97:10701; Daugherty et al. 2000 *J. Immunol. Methods* 243:211. Such procedures provide alternatives to traditional hybridoma techniques for the isolation and subsequent cloning of monoclonal antibodies.

Yet other embodiments of the present invention comprise the generation of human or substantially human antibodies in transgenic animals (e.g., mice) that are incapable of endogenous immunoglobulin production (see e.g., U.S. Pat. Nos. 6,075,181, 5,939,598, 5,591,669 and 5,589,369 each of which is incorporated herein by reference). For example, it has been described that the homozygous deletion of the antibody heavy-chain joining region in chimeric and germ-line mutant mice results in complete inhibition of endogenous antibody production. Transfer of a human immunoglobulin gene array to such germ line mutant mice will result in the production of human antibodies upon antigen challenge. Another preferred means of generating human antibodies using SCID mice is disclosed in U.S. Pat. No. 5,811,524 which is incorporated herein by reference. It will be appreciated that the genetic material associated with these human antibodies may also be isolated and manipulated as described herein.

Yet another highly efficient means for generating recombinant antibodies is disclosed by Newman, *Biotechnology*, 10: 1455-1460 (1992). Specifically, this technique results in the generation of primatized antibodies that contain monkey variable domains and human constant sequences. This reference is incorporated by reference in its entirety herein. Moreover, this technique is also described in commonly assigned U.S. Pat. Nos. 5,658,570, 5,693,780 and 5,756,096 each of which is incorporated herein by reference.

In another embodiment, lymphocytes can be selected by micromanipulation and the variable genes isolated. For example, peripheral blood mononuclear cells can be isolated from an immunized mammal and cultured for about 7 days in vitro. The cultures can be screened for specific IgGs that meet the screening criteria. Cells from positive wells can be isolated. Individual Ig-producing B cells can be isolated by FACS or by identifying them in a complement-mediated hemolytic plaque assay. Ig-producing B cells can be micromanipulated into a tube and the V<sub>H</sub> and V<sub>L</sub> genes can be amplified using, e.g., RT-PCR. The V<sub>H</sub> and V<sub>L</sub> genes can be cloned into an antibody expression vector and transfected into cells (e.g., eukaryotic or prokaryotic cells) for expression.

Moreover, genetic sequences useful for producing the polypeptides of the present invention may be obtained from a number of different sources. For example, as discussed extensively above, a variety of human antibody genes are available in the form

of publicly accessible deposits. Many sequences of antibodies and antibody-encoding genes have been published and suitable antibody genes can be chemically synthesized from these sequences using art recognized techniques. Oligonucleotide synthesis techniques compatible with this aspect of the invention are well known to the skilled artisan and may be carried out using any of several commercially available automated synthesizers. In addition, DNA sequences encoding several types of heavy and light chains set forth herein can be obtained through the services of commercial DNA synthesis vendors. The genetic material obtained using any of the foregoing methods may then be altered or modified to provide obtain polypeptides of the present invention.

Alternatively, antibody-producing cell lines may be selected and cultured using techniques well known to the skilled artisan. Such techniques are described in a variety of laboratory manuals and primary publications. In this respect, techniques suitable for use in the invention as described below are described in *Current Protocols in Immunology*, Coligan et al., Eds., Green Publishing Associates and Wiley-Interscience, John Wiley and Sons, New York (1991) which is herein incorporated by reference in its entirety, including supplements.

It will further be appreciated that the scope of this invention further encompasses all alleles, variants and mutations of antigen binding DNA sequences.

As is well known, RNA may be isolated from the original hybridoma cells or from other transformed cells by standard techniques, such as guanidinium isothiocyanate extraction and precipitation followed by centrifugation or chromatography. Where desirable, mRNA may be isolated from total RNA by standard techniques such as chromatography on oligo dT cellulose. Suitable techniques are familiar in the art.

Variable and constant region domains can be obtained from any source and be incorporated into a modified antibody of the invention. To clone antibodies, mRNA can be isolated from hybridoma, spleen, or lymph cells, reverse transcribed into DNA, and antibody genes amplified by PCR. PCR may be initiated by consensus constant region primers or by more specific primers based on the published heavy and light chain DNA and amino acid sequences. As discussed above, PCR also may be used to isolate DNA clones encoding the antibody light and heavy chains. In this case the libraries may be screened by consensus primers or larger homologous probes, such as mouse constant region probes. Numerous primer sets suitable for amplification of antibody genes are known in the art (e.g., 5' primers based on the N-terminal sequence of purified antibodies (Benhar and Pastan. 1994. *Protein Engineering* 7:1509); rapid amplification of cDNA ends (Ruberti, F. et al. 1994. *J. Immunol. Methods* 173:33); antibody leader sequences (Larrick et al. 1989 *Biochem. Biophys. Res. Commun.* 160:1250); or based on known variable region framework amino acid sequences from

- the Kabat (Kabat et al. 1991. Sequences of Proteins of Immunological Interest. Bethesda, MD:JS Dep. Health Hum. Serv. 5<sup>th</sup> ed.) or the V-base databases (e.g., Orlandi et al. 1989. Proc. Natl. Acad. Sci. USA 86:3833; Sblattero et al. 1998. Immunotechnology 3:271; or Krebber et al. 1997. J. Immunol. Methods 201:35).
- 5 Constant region domains can be selected having a particular effector function (or lacking a particular effector function) or with a particular modification to reduce immunogenicity. Variable and constant domains can be cloned, e.g., using the polymerase chain reaction and primers which are selected to amplify the domain of interest. PCR amplification methods are described in detail in U.S. Pat. Nos. 4,683,195;
- 10 4,683,202; 4,800,159; 4,965,188; and in, e.g., "PCR Protocols: A Guide to Methods and Applications" Innis et al. eds., Academic Press, San Diego, CA (1990); Ho et al. 1989. Gene 77:51; Horton et al. 1993. Methods Enzymol. 217:270).

- Alternatively, V domains can be obtained from libraries of V gene sequences from an animal of choice. Libraries expressing random combinations of
- 15 domains, e.g., VH and VL domains, can be screened with a desired antigen to identify elements which have desired binding characteristics. Methods of such screening are well known in the art. For example, antibody gene repertoires can be cloned into a  $\lambda$  bacteriophage expression vector (Huse, WD et al. 1989. Science 247:1275). In addition, cells (Boder and Wittrup. 1997. Nat. Biotechnol. 15:553; Daugherty, P. et al.
- 20 2000. J. Immunol. Methods. 243:211; Francisco et al. 1994. Proc. Natl. Acad. Sci. USA 90:10444; Georgiou et al. 1997. Nature Biotechnology 15:29) or viruses (e.g., Hoogenboom, HR. 1998 Immunotechnology 4:1 Winter et al. 1994. Annu. Rev. Immunol. 12:433; Griffiths, AD. 1998. Curr. Opin. Biotechnol. 9:102) expressing antibodies on their surface can be screened. Ribosomal display can also be used to
- 25 screen antibody libraries (Hanes J., et al. 1998. Proc. Natl. Acad. Sci. USA 95:14130; Hanes, J. and Pluckthun. 1999. Curr. Top. Microbiol. Immunol. 243:107; He, M. and Taussig. 1997. Nucleic Acids Research 25:5132).

- Preferred libraries for screening are human V gene libraries. VL and VH domains from a non-human source may also be used. In one embodiment, such non-
- 30 human V domains can be altered to reduce their immunogenicity using art recognized techniques.

- Libraries can be naïve, from immunized subjects, or semi-synthetic (Hoogenboom, H.R. and Winter. 1992. J. Mol. Biol. 227:381; Griffiths, AD, et al. EMBO J. 13:3245; de Kruif, J. et al. 1995. J. Mol. Biol. 248:97; Barbas, C.F., et al.
- 35 1992. Proc. Natl. Acad. Sci. USA 89:4457).

In addition, the sequences of many antibody V and C domains are known and such domains can be synthesized using methods well known in the art.

In one embodiment, mutations can be made to immunoglobulin domains to create a library of nucleic acid molecules having greater heterogeneity (Thompson, J., et al. 1996. J. Mol. Biol. 256:77; Lamminmaki, U. et al. 1999. J. Mol. Biol. 291:589; Caldwell, R.C. and Joyce GF. 1992. PCR Methods Appl. 2:28; Caldwell RC and Joyce GF. 1994. PCR Methods Appl. 3:S136. Standard screening procedures can be used to select high affinity variants. In another embodiment, changes to VH and VL sequences can be made to increase antibody avidity, e.g., using information obtained from crystal structures using techniques known in the art.

10

#### C. Modified Antibodies

Exemplary constructs include, e.g., minibodies, diabodies, diabodies fused to CH3 molecules, tetravalent antibodies, intradiabodies (e.g., Jendreyko et al. 2003. J. Biol. Chem. 278:47813), bispecific antibodies, fusion proteins (e.g., antibody  
15 cytokine fusion proteins, proteins fused to at least a portion of an Fc receptor), bispecific antibodies. Other immunoglobulins (Ig) and certain variants thereof are described, for example in U.S. Pat. No. 4,745,055; EP 256,654; Faulkner et al., Nature 298:286 (1982); EP 120,694; EP 125,023; Morrison, J. Immun. 123:793 (1979); Kohler et al., Proc. Natl. Acad. Sci. USA 77:2197 (1980); Raso et al., Cancer Res. 41:2073 (1981); Morrison et al., Ann. Rev. Immunol. 2:239 (1984); Morrison, Science 229:1202 (1985); Morrison et al., Proc. Natl. Acad. Sci. USA 81:6851 (1984); EP 255,694; EP 266,663; and WO  
20 88/03559. Reassorted immunoglobulin chains also are known. See, for example, U.S. Pat. No. 4,444,878; WO 88/03565; and EP 68,763 and references cited therein.

In one embodiment, a polypeptide of the invention comprises an  
25 immunoglobulin heavy chain having deletion or substitution of at least one amino acid. For example, the mutation of one or more single amino acid in selected areas of the CH2 domain may be enough to substantially reduce Fc binding and thereby increase tumor localization. Similarly, it may be desirable to simply delete that part of one or more constant region domains that control the effector function (e.g. complement binding) to be  
30 modulated. Such partial deletions of the constant regions may improve selected characteristics of the antibody (serum half-life) while leaving other desirable functions associated with the subject constant region domain intact. Accordingly, in one embodiment, a binding molecule of the invention lacks all or part of a CH2 domain. Moreover, as alluded to above, the constant regions of the disclosed antibodies may be  
35 modified through the mutation or substitution of one or more amino acids that enhances the profile of the resulting construct. In this respect it may be possible to disrupt the activity provided by a conserved binding site (e.g. Fc binding) while substantially maintaining the configuration and immunogenic profile of the modified antibody. Yet other preferred

embodiments may comprise the addition of one or more amino acids to the constant region to enhance desirable characteristics such as effector function or provide for more cytotoxin or carbohydrate attachment. In such embodiments it may be desirable to insert or replicate specific sequences derived from selected constant region domains.

5 In another embodiment, mutations to naturally occurring hinge regions can be made. Such modifications to the constant region in accordance with the instant invention may easily be made using well known biochemical or molecular engineering techniques well within the skill of the art.

10 In one embodiment, polypeptides of the invention comprise modified constant regions wherein one or more domains are partially or entirely deleted ("domain deleted antibodies"). In especially preferred embodiments compatible modified antibodies will comprise domain deleted constructs or variants wherein the entire CH2 domain has been removed. A variety of modified antibody constructs are described in more detail below.

15

#### i. Minibodies

In one embodiment, the modified antibodies of the invention are minibodies. Minibodies are dimeric molecules made up of two polypeptide chains each comprising an ScFv molecule (a single polypeptide comprising one or more antigen  
20 binding sites, e.g., a VL domain linked by a flexible linker to a VH domain fused to a CH3 domain via a connecting peptide. An exemplary minibody construct is shown in Figure 2. In Figure 2 a CH3 domain is fused at its N-terminus to a connecting peptide which is fused at its N-terminus to a VH domain which is fused via its N-terminus to a flexible linker which is fused at its N-terminus to a VL domain.

25 ScFv molecules can be constructed in a VH-linker-VL orientation or VL-linker-VH orientation.

The flexible hinge that links the VL and VH domains that make up the antigen binding site preferably comprises from about 10 to about 50 amino acid residues. An exemplary connecting peptide for this purpose is (Gly4Ser)3 (SEQ ID  
30 NO:35) (Huston et al. 1988. *Proc. Natl. Acad. Sci. USA* 85:5879). Other connecting peptides are known in the art.

Methods of making single chain antibodies are well known in the art, e.g., Ho et al. 1989. *Gene* 77:51; Bird et al. 1988 *Science* 242:423; Pantoliano et al. 1991. *Biochemistry* 30:10117; Milenic et al. 1991. *Cancer Research* 51:6363;  
35 Takkinen et al. 1991. *Protein Engineering* 4:837.

Minibodies can be made by constructing an ScFv component and connecting peptide-CH3 component using methods described in the art (see, e.g., US patent 5,837,821 or WO 94/09817A1). These components can be isolated from separate

plasmids as restriction fragments and then ligated and recloned into an appropriate vector. Appropriate assembly can be verified by restriction digestion and DNA sequence analysis.

In one embodiment, a minibody of the invention comprises a connecting peptide. In one embodiment, the connecting peptide comprises a gly/ser linker, e.g., GGGSSGGSGG (SEQ ID NO: 1).

In another embodiment, a tetravalent minibody can be constructed. Tetravalent minibodies can be constructed in the same manner as minibodies, except that two ScFv molecules are linked using a flexible linker, e.g., having an amino acid sequence (G4S)<sub>4</sub>G3AS (SEQ ID NO: 36). An exemplary tetravalent minibody is illustrated in Figure 2.

## ii. Domain Deleted Antibodies

In another embodiment, the modified antibodies of the invention are CH2 domain deleted antibodies. Domain deleted constructs can be derived from a vector (e.g., from IDEC Pharmaceuticals, San Diego) encoding an IgG<sub>1</sub> human constant domain (see, e.g., WO 02/060955A2 and WO02/096948A2). Essentially, the vector was engineered to delete the CH2 domain and provide a modified vector expressing a domain deleted IgG<sub>1</sub> constant region. Genes encoding the murine variable region of the C2B8 antibody, 5E8 antibody, B3F6 antibody, or the variable region of the humanized CC49 antibody were then inserted in the modified vector and cloned. When expressed in transformed cells, these vectors provided C2B8.ΔCH2, 5E8.ΔCH2, B3F6.ΔCH2 or huCC49.ΔCH2 or respectively. These constructs exhibit a number of properties that make them particularly attractive candidates for monomeric subunits.

Besides the deletion of whole constant region domains, it will be appreciated that the antibodies of the present invention can be engineered to partially delete or substitute of a few amino acids or even a single amino acid. For example, the mutation of a single amino acid in selected areas of the C<sub>H</sub>2 domain may be enough to substantially reduce Fc binding and thereby increase tumor localization. Similarly, it may be desirable to simply delete that part of one or more constant region domains that control the effector function (e.g. complement C1Q binding). Such partial deletions of the constant regions may improve selected characteristics of the antibody (serum half-life) while leaving other desirable functions associated with the subject constant region domain intact.

Creation of a C<sub>H</sub>2 domain deleted version can be accomplished by way of overlapping PCR mutagenesis. The gamma 1 constant domain begins with a plasmid encoded Nhe I site which is in translational reading frame with the immunoglobulin sequence. A 5' PCR primer was constructed encoding the Nhe I site as well as sequence immediately

downstream. A 3' PCR primer mate was constructed such that it anneals with the 3' end to the immunoglobulin hinge region and encodes in frame the first several amino acids of the gamma 1 CH3 domain. A second PCR primer pair consisted of the reverse complement of the 3' PCR primer from the first pair (above) as the 5' primer and a 3' primer that anneals at a loci spanning the BsrG I restriction site within the C<sub>H</sub>3 domain. Following each PCR amplification, the resultant products were utilized as template with the Nhe I and BsrG I 5' and 3', respectively primers. The amplified product was then cloned back into N5KG1 to create the plasmid N5KG1ΔC<sub>H</sub>2. This construction places the intact CH3 domain immediately downstream and in frame with the intact hinge region. A similar procedure can be used to create a domain deleted construct in which the CH3 domain is immediately downstream of a connecting peptide. For example, a domain deleted version of the C2B8 antibody was created in this manner as described in U.S. Pat. Nos. 5,648,267 and 5,736,137 each of which is incorporated herein by reference.

In one embodiment, tetravalent domain-deleted antibodies can be produced by combining a DNA sequence encoding a domain deleted antibody with a ScFv molecule. For example, in one embodiment, these sequences are combined such that the ScFv molecule is linked at its N-terminus to the CH3 domain of the domain deleted antibody via a flexible linker (e.g., a gly/ser linker such as (Gly4Ser)<sub>3</sub> (SEQ ID NO: 35).

In another embodiment a tetravalent antibody can be made by fusing an ScFv molecule to a connecting peptide, which is fused to a CH1 domain to construct an ScFv – Fab tetravalent molecule. (Coloma and Morrison. 1997. Nature Biotechnology. 15:159; WO 95/09917).

25

### iii. Diabodies

Diabodies are similar to scFv molecules, but usually have a short (less than 10 and preferably 1-5) amino acid residue linker connecting both V-domains, such that the VL and VH domains on the same polypeptide chain can not interact. Instead, the VL and VH domain of one polypeptide chain interact with the VH and VL domain (respectively) on a second polypeptide chain (WO 02/02781). In one embodiment, a binding molecule of the invention is a diabody fused to at least one heavy chain portion. In a preferred embodiment, a binding molecule of the invention is a diabody fused to a CH3 domain.

35

In one embodiment a modified antibody of the invention a binding molecule comprises a tetravalent or bispecific tetravalent CH2 domain-deleted antibody



with a scFv appended to the N-terminus of the light chain. In another embodiment of the invention, a binding molecule comprises a tetravalent or bispecific tetravalent CH2 domain-deleted antibody with a scFv appended to the N-terminus of the heavy chain. In one embodiment, the attachment of the scFv to the N-terminus results in reduced  
 5 aggregation of the molecules as compared to molecules in which the scFv is attached at the carboxy-terminus. In one embodiment, less than about 30% aggregates are present in a composition of binding molecules produced by cells expressing a N-terminal fusion construct. In one embodiment, less than about 20% aggregates are present in a composition of binding molecules produced by cells expressing a N-terminal fusion  
 10 construct. In one embodiment, less than about 10% aggregates are present in a composition of binding molecules produced by cells expressing a N-terminal fusion construct. In one embodiment, less than about 5% aggregates are present in a composition of binding molecules produced by cells expressing a N-terminal fusion construct.

15

Other forms of modified antibodies are also within the scope of the instant invention (e.g., WO 02/02781 A1; 5,959,083; 6,476,198 B1; US 2002/0103345 A1; WO 00/06605; Bym et al. 1990. Nature. 344:667-70; Chamow and Ashkenazi. 1996. Trends Biotechnol. 14:52).

20

#### D. Catalytic antibodies

In one embodiment, at least one binding specificity of a modified antibody molecule of the invention is catalytic. Catalytic binding specificities can be  
 25 made using art recognized techniques (see, e.g., U.S. Pat. No. 6,590,080, U.S. Pat. No. 5,658,753). Catalytic binding specificities can work by a number of basic mechanisms similar to those identified for enzymes to stabilize the transition state, thereby reducing the free energy of activation. For example, general acid and base residues can be optimally positioned for participation in catalysis within catalytic active sites; covalent  
 30 enzyme-substrate intermediates can be formed; catalytic antibodies can also be in proper orientation for reaction and increase the effective concentration of reactants by at least seven orders of magnitude (Fersht, A. R., et al., Am. Chem. Soc. 90 (1968):5833) and thereby greatly reduce the entropy of a chemical reaction. Finally, catalytic antibodies can convert the energy obtained upon substrate binding to distort the reaction towards a  
 35 structure resembling the transition state.

In one embodiment, acid or base residues can be brought into the binding site by using a complementary charged molecule as an immunogen. This technique

proved successful for elicitation of antibodies with a hapten containing a positively-charged ammonium ion (Shokat, et al., Chem. Int. Ed. Engl. 27 (1988):269-271).

In another approach, antibodies can be elicited to stable compounds that resemble the size, shape, and charge of the transition state of a desired reaction (i.e., transition state analogs). See U.S. Pat. No. 4,792,446 and U.S. Pat. No. 4,963,355 which describe the use of transition state analogues to immunize animals and the production of catalytic antibodies. Both of these patents are hereby incorporated by reference. In one embodiment, such molecules can be administered as part of an immunoconjugate, e.g., with an immunogenic carrier molecule, such as KLH.

Exemplary catalytic binding specificities can have, e.g., esterase activity (involving a charged transition state whose electrostatic and shape characteristics can be mimicked by a phosphonate structure; Jacobs, et al., J. Am. Chem. Soc. 109 (1987):2174-2176; Durfor, et al., J. Am. Chem. Soc. 110 (1988):8713-8714; Tramontano, et al., J. Am. Chem. Soc. 110 (1988):2282; Pollack, et al., J. Am. Chem. Soc. 111 (1989):5961-5962); peptidase or amidase activity (Janda, et al., Science 241 (1988):1188-1191; Iverson, et al., Science 243 (1989):1184-1188; Paul, et al., Science 244 (1989):1158-1162); Claisen rearrangement (Jackson, et al., J. Am. Chem. Soc. 110 (1988):4841-4842; Hilvert, et al., Proc. Natl. Acad. Sci. USA 85 (1988):4953-4955; Hilvert, et al., J. Am. Chem. Soc. 110 (1988):5593-5594); redox reactions (Shokat, et al., Angew. Chem. Int. Ed. Engl. 27 (1989):269-271); photochemical cleavage of a thymine dimer (Cochran, et al., J. Am. Chem. Soc. 110 (1988):7888-7890); stereospecific transesterification rearrangements (Napper, et al., Science 237 (1987):1041-1043); or a bimolecular amide synthesis (Benkovic, et al., Proc. Natl. Acad. Sci. USA 85 (1988):5355-5358; Janda, et al., Science 241 (1988):1188-1191).

In another approach, conventional binding specificities can be mutated to render them catalytic.

Methods of screening for catalytic antibody activity are well known in the art (e.g., Reymond, J.L. 2002. Journal of Immunological Methods 269:125; Mouratou et al. 2002. J. of Immunological Methods. 269:147. In yet another embodiment, catalytic B cells can be selected, e.g., as described in US patent 6,590,080 using a molecule can be constructed which facilitates selection of catalytic B cells.

In another embodiment, catalytic binding specificities can be developed as part of a two step process. Catalytic antibodies can be selected only if displaying the following binding features: binding both the substrate and a reactive group in such a way that the two groups are in a reactive position towards each other. Second, the selected antibodies can be chemically engineered by covalently binding a reactive group into the binding pocket of the antibody. J Immunol Methods. 2002. 269:81-98.

In one embodiment, a catalytic binding specificity is specific for a prodrug. Such a binding specificity can be used to catalyze the conversion of a prodrug into a drug which is effective in vivo. Preferably, the reaction catalyzed is one that cannot be accomplished by natural enzymes in vivo. Examples of prodrug activation by antibodies are known in the art (see, e.g., Miyashita et al. 1993. Proc. Natl. Acad. Sci. USA 90:5337).

In one embodiment, a modified antibody molecule of the invention comprises at least one binding specificity for a target cell and at least one binding specificity for a prodrug. For example, in a preferred embodiment, an modified antibody molecule of the invention comprises at least one binding specificity for a tumor cell and at least one binding specificity for a prodrug which can be converted to cytotoxic drug. In one example, a modified antibody of the invention comprises a binding specificity for a carbamate prodrug 4-[N,N,-bis(2-chloroethyl)]aminophenyl-N-[(1S-(1,3-dicarboxy)propyl]carbamate and generates the corresponding cytotoxic nitrogen mustard (Wentworth et al. 1996. Proc Natl. Acad. Sci. USA. 93:799).

In one embodiment, the modified antibody is administered prior to administration of the prodrug to allow accumulation at the site of the target cell. Exemplary prodrugs are known in the art. Prodrugs can also be synthesized by incorporating a portion designed to be released by catalytic action, e.g., by sequential retro-aldol/retro-Michael reactions catalyzed by an antibody with aldolase activity. (Shabat et al. 2001. Proc. Natl. Acad. Sci. USA 98:7428). Such drug masking portions can be made, e.g., by modification of hydroxyl or thiol groups of drugs.

#### 25 E. Multispecific Binding Molecules

In one embodiment, a binding molecule of the invention is multispecific, i.e., has at least one binding site that binds to a first molecule or epitope of a molecule and at least one second binding site that binds to a second molecule or epitope of a molecule.

In one embodiment, a binding molecule of the invention is bispecific. Bispecific molecules can bind to two different target sites, e.g., on the same target molecule or on different target molecules. For example, in the case of antibodies, bispecific molecules can bind to two different epitopes, e.g., on the same antigen or on two different antigens. Bispecific molecules can be used, e.g., in diagnostic and therapeutic applications. For example, they can be used to immobilize enzymes for use in immunoassays. They can also be used in diagnosis and treatment of cancer, e.g., by binding both to a tumor associated molecule and a detectable marker (e.g., a chelator which tightly binds a radionuclide. Bispecific molecules can also be used for human

therapy, e.g., by directing cytotoxicity to a specific target (for example by binding to a pathogen or tumor cell and to a cytotoxic trigger molecule, such as the T cell receptor or the Fc $\gamma$  receptor. Bispecific antibodies can also be used, e.g., as fibrinolytic agents or vaccine adjuvants.

- 5           Examples of bispecific binding molecules include those with at least two arms directed against tumor cell antigens; bispecific binding molecules with at least one arm directed against a tumor cell antigen and the at least one arm directed against a cytotoxic trigger molecule (such as anti-Fc $\gamma$ .RI/anti-CD15, anti-p185.sup.HER2/Fc $\gamma$ .RIII (CD16), anti-CD3/anti-malignant B-cell (1D10), anti-  
10 CD3/anti-p185.sup.HER2, anti-CD3/anti-p97, anti-CD3/anti-renal cell carcinoma, anti-CD3/anti-OVCAR-3, anti-CD3/L-D1 (anti-colon carcinoma), anti-CD3/anti-melanocyte stimulating hormone analog, anti-EGF receptor/anti-CD3, anti-CD3/anti-CAMA1, anti-CD3/anti-CD19, anti-CD3/MoV18, anti-neural cell adhesion molecule (NCAM)/anti-CD3, anti-folate binding protein (FBP)/anti-CD3, anti-pan carcinoma associated antigen  
15 (AMOC-31)/anti-CD3); bispecific binding molecules with at least one which binds specifically to a tumor antigen and at least one which binds to a toxin (such as anti-saporin/anti-Id-1, anti-CD22/anti-saporin, anti-CD7/anti-saporin, anti-CD38/anti-saporin, anti-CEA/anti-ricin A chain, anti-interferon-.alpha.(IFN-.alpha.)/anti-hybridoma idiotype, anti-CEA/anti-vinca alkaloid); bispecific binding molecules for converting  
20 enzyme activated prodrugs (such as anti-CD30/anti-alkaline phosphatase (which catalyzes conversion of mitomycin phosphate prodrug to mitomycin alcohol)); bispecific binding molecules which can be used as fibrinolytic agents (such as anti-fibrin/anti-tissue plasminogen activator (tPA), anti-fibrin/anti-urokinase-type plasminogen activator (uPA)); bispecific binding molecules for targeting immune complexes to cell surface  
25 receptors (such as anti-low density lipoprotein (LDL)/anti-Fc receptor (e.g. Fc $\gamma$ .RI, Fc $\gamma$ .RII or Fc $\gamma$ .RIII)); bispecific binding molecules for use in therapy of infectious diseases (such as anti-CD3/anti-herpes simplex virus (HSV), anti-T-cell receptor:CD3 complex/anti-influenza, anti-Fc $\gamma$ .R/anti-HIV; bispecific binding molecules for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185HER2/anti- -haptent); bispecific binding  
30 molecules as vaccine adjuvants (see Fanger et al., supra); and bispecific binding molecules as diagnostic tools (such as anti-rabbit IgG/anti-ferritin, anti-horse radish peroxidase (HRP)/anti-hormone, anti-somatostatin/anti-substance P, anti-HRP/anti-FITC, anti-CEA/anti-.beta.-galactosidase (see Nolan et al., supra)). Examples of  
35 trispecific antibodies include anti-CD3/anti-CD4/anti-CD37, anti-CD3/anti-CD5/anti-CD37 and anti-CD3/anti-CD8/anti-CD37.

In a preferred embodiment, a bispecific molecule of the invention binds to CRIPTO-I.

Bispecific molecules may be monovalent for each specificity or be multivalent for each specificity. For example, an antibody molecule or fusion protein may comprise one binding site that reacts with a first target molecule and one binding site that reacts with a second target molecule or it may comprise two binding sites that react with a first target molecule and two binding sites that react with a second target molecule. Methods of producing bispecific molecules are well known in the art. For example, recombinant technology can be used to produce bispecific molecules, e.g., diabodies, single-chain diabodies, tandem scFvs, etc. Exemplary techniques for producing bispecific molecules are known in the art (e.g., Kontermann et al. Methods in Molecular Biology Vol. 248: Antibody Engineering: Methods and Protocols. Pp 227-242 US 2003/0207346 A1 and the references cited therein; ). In one embodiment, a multimeric bispecific molecules are prepared using methods such as those described e.g., in US 2003/0207346 A1 or US patent 5,821,333, or US2004/0058400.

As used herein the phrase "multispecific fusion protein" designates fusion proteins (as hereinabove defined) having at least two binding specificities (i.e. combining two or more binding domains of a ligand or receptor). Multispecific fusion proteins can be assembled as heterodimers, heterotrimers or heterotetramers, essentially as disclosed in WO 89/02922 (published Apr. 6, 1989), in EP 314, 317 (published May 3, 1989), and in U.S. Pat. No. 5,116,964 issued May 2, 1992. Preferred multispecific fusion proteins are bispecific. Examples of bispecific fusion proteins include CD4-IgG/TNFreceptor-IgG and CD4-IgG/L-selectin-IgG. The last mentioned molecule combines the lymph node binding function of the lymphocyte homing receptor (LHR, L-selectin), and the HIV binding function of CD4, and finds potential application in the prevention or treatment of HIV infection, related conditions, or as a diagnostic.

Target binding sites for the multispecific binding molecules of the invention can readily be selected by one of ordinary skill in the art. While not limiting in any way, exemplary binding sites include one or more epitopes of a tumor antigen. Other exemplary target molecules include one or more epitopes of, e.g., heparin sulfate, growth factors or their receptors (e.g., epidermal growth factor receptor, insulin-like growth factor receptor, hepatocyte growth factor (HGF/SF) receptor, MORE (See, e.g., Cao et al. Proc. Natl. Acad. Sci 2001. 98:7443; Lu et al. 2004. J. Biol. Chem. 279:2856).

In another embodiment, the invention pertains to bispecific molecules, e.g., antibodies, which incorporate at least one binding site that binds to a known target and at least one binding site which recognizes an unknown target (for example, in one embodiment, the bispecific molecule incorporates binding sites selected from a semi-synthetic antibody phage display library).

In one embodiment of the invention, one of ordinary skill in the art could start with a single chain antibody of known specificity and build a Fab library using techniques known in the art or, alternatively, the skilled artisan could start with an Fab fragment of known specificity and build a single chain library using techniques known in the art. It is known in the art that libraries from nonimmunized sources and prepared by synthetic recombination of V-gene sequences (preferably recombination of VH with DH and JH, and VL with JL sequences) can be used to isolate antibodies to any antigen. For example, patent application WO92/01047 teaches that antibody fragments can be displayed on the surface of bacteriophage and that they will bind antigen. Antibody fragments (e.g., Fab, Fv, ScFv and VH) can be directly selected using this characteristic. Other methods known in the art include those taught, e.g., in U.S. patent 5,698,426; 6,291,159; 5,658,727; 5,667,988; and 5,969,108.

In another embodiment, scFv which recognize a known target can be dimerized with scFv isolated from a semi-synthetic human phage antibody display library. (see, e.g., Kruif and Logtenberg 1996. J. Biol. Chem. 271:7630).

In one embodiment, the subject bispecific molecule is expressed in any expression system used to express antibody molecules, for example mammalian cells, yeast such as *Pichia*, *E. coli*, Bacculovirus, etc. In one embodiment, the subject bispecific molecule is expressed in the NEOSPLA vector system (see, e.g., U.S. patent 6,159,730). This vector contains the cytomegalovirus promoter/enhancer, the mouse beta globin major promoter, the SV40 origin of replication, the bovine growth hormone polyadenylation sequence, neomycin phosphotransferase exon 1 and exon 2, the dihydrofolate reductase gene and leader sequence.

In one embodiment, the subject bispecific molecules comprise a synthetic connecting peptide.

These bispecific molecules have one or more binding sites for a known target and express a library at one or more binding sites. Such bispecific molecules can be used, e.g., to identify molecules in close proximity to or associated with the known target. For example, the skilled artisan could use the subject bispecific molecules in an assay to select for those that induce a particular response, e.g., apoptosis or cellular activation, using screening methods well known in the art. The bispecific molecule identified as producing the response screened for can then be identified and its specificity determined. Using such methods it is possible to identify molecules in close association with particular targets of interest, e.g., T cell markers or other signaling molecules (such as CRIPTO-I, death domain molecules, or molecules involved in apoptosis). The proximity of the known target and the molecule newly identified as a "nearest neighbor" can be confirmed using immunoprecipitation or other techniques

2004252171 30 Mar 2011

known to those of skill in the art. Using these methods it is possible to identify molecules as targets for modulating a particular cellular response.

In one embodiment, a polypeptide molecule of the invention comprises an amino acid sequence encoded by a nucleic acid molecule comprising a nucleic acid sequence shown in Figure 8A (SEQ ID NO:16), Figure 8B (SEQ ID NO:17), Figure 8C (SEQ ID NO:18), Figure 10A (SEQ ID NO:22), Figure 10B (SEQ ID NO:23). In another embodiment, a nucleic acid molecule of the invention comprises a nucleotide sequence shown in Figure 8A (SEQ ID NO:16), Figure 8B (SEQ ID NO:17), Figure 8C (SEQ ID NO:18), Figure 10A (SEQ ID NO:22), Figure 10B (SEQ ID NO:23).

In another embodiment, a polypeptide of the invention comprises an amino acid sequence shown in Figure 9A (SEQ ID NO:19), Figure 9B (SEQ ID NO:20), SEQ ID NO:21, Figure 11A (SEQ ID NO:24), or Figure 11B (SEQ ID NO:25).

In another embodiment, a polypeptide of the invention is encoded by a nucleic acid molecule comprising the nucleotide sequence shown in Figure 12A (SEQ ID NO:26), Figure 12B (SEQ ID NO:27), Figure 14 (SEQ ID NO:30), or Figure 15 (SEQ ID NO:31). In another embodiment, a nucleic acid molecule of the invention comprises a nucleotide sequence shown in Figure 12A (SEQ ID NO:26), Figure 12B (SEQ ID NO:27), Figure 14 (SEQ ID NO:30), or Figure 15 (SEQ ID NO:31).

In one embodiment, a polypeptide molecule of the invention comprises an amino acid sequence shown in Figure 13A (SEQ ID NO:28), Figure 13B (SEQ ID NO:29), Figure 16 (SEQ ID NO:32), or Figure 17 (SEQ ID NO:33).

The other nucleic acid and amino acid sequences disclosed herein in the sequence listing and Figures are also embraced by the invention.

#### B. Expression of Polypeptides

Following manipulation of the isolated genetic material to provide polypeptides of the invention as set forth above, the genes are typically inserted in an expression vector for introduction into host cells that may be used to produce the desired quantity of modified antibody that, in turn, provides the claimed polypeptides.

The term "vector" or "expression vector" is used herein for the purposes of the specification and claims, to mean vectors used in accordance with the present invention as a vehicle for introducing into and expressing a desired gene in a cell. As known to those skilled in the art, such vectors may easily be selected from the group consisting of

plasmids, phages, viruses and retroviruses. In general, vectors compatible with the instant invention will comprise a selection marker, appropriate restriction sites to facilitate cloning of the desired gene and the ability to enter and/or replicate in eukaryotic or prokaryotic cells.

- 5 For the purposes of this invention, numerous expression vector systems may be employed. For example, one class of vector utilizes DNA elements which are derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, vaccinia virus, baculovirus, retroviruses (RSV, MMTV or MOMLV) or SV40 virus. Others involve the use of polycistronic systems with internal ribosome binding sites.
- 10 Exemplary vectors include those taught in U.S. patent no. 6,159,730 or 6,413,777 or US 2003 0157641 A1). Additionally, cells which have integrated the DNA into their chromosomes may be selected by introducing one or more markers which allow selection of transfected host cells. The marker may provide for prototrophy to an auxotrophic host, biocide resistance (e.g., antibiotics) or resistance to heavy metals such
- 15 as copper. The selectable marker gene can either be directly linked to the DNA sequences to be expressed, or introduced into the same cell by cotransformation.

In one embodiment, an inducible expression system can be employed.

- Additional elements may also be needed for optimal synthesis of mRNA. These elements may include signal sequences, splice signals, as well as transcriptional
- 20 promoters, enhancers, and termination signals.

- In one embodiment, a secretion signal, e.g., any one of several well characterized bacterial leader peptides (e.g., pelB, phoA, or ompA), can be fused in-frame to the N terminus of a polypeptide of the invention to obtain optimal secretion of the polypeptide. (Lei et al. 1988 Nature 331:543; Better et al. Science 1988. 240:1041;
- 25 Mullinax et al., 1990. Proc. Natl. Acad. Sci. USA 87:8095).

- In one embodiment, a vector can be used which comprises a nucleic acid sequence encoding a peptide linker. In another embodiment, it might be desirable to first assemble the desired coding sequences (e.g., secretion signal, VL, linker peptide, VH, etc.) into a single sequence, for example, by PCR amplification using overlapping
- 30 primers, followed by ligation into a plasmid or other vector.

- In particularly preferred embodiments the cloned variable region genes are inserted into an expression vector along with the heavy and light chain constant region genes (preferably human) modified as discussed above. Preferably, this is effected using a proprietary expression vector of IDEC, Inc., referred to as NEOSPLA
- 35 see U.S. patent 6,159,730. This vector contains the cytomegalovirus promoter/enhancer, the mouse beta globin major promoter, the SV40 origin of replication, the bovine growth hormone polyadenylation sequence, neomycin phosphotransferase exon 1 and exon 2, the dihydrofolate reductase gene and leader sequence. As seen in the examples below,

- 59 -



this vector has been found to result in very high level expression of antibodies upon incorporation of variable and constant region genes, transfection in CHO cells, followed by selection in G418 containing medium and methotrexate amplification. Vector systems are also taught in U.S. Pat. Nos. 5,736,137 and 5,658,570, each of which is  
5 incorporated by reference in its entirety herein. This system provides for high expression levels, e.g., > 30 pg/cell/day. Other exemplary vector systems are disclosed e.g., in U.S. patent 6,413,777.

In other preferred embodiments the polypeptides of the invention of the instant invention may be expressed using polycistronic constructs such as those  
10 disclosed in United States provisional application No. 60/331,481 filed November 16, 2001 and incorporated herein in its entirety. In these novel expression systems, multiple gene products of interest such as heavy and light chains of antibodies may be produced from a single polycistronic construct. These systems advantageously use an internal ribosome entry site (IRES) to provide relatively high levels of polypeptides of the  
15 invention in eukaryotic host cells. Compatible IRES sequences are disclosed in U.S. Pat. No. 6,193,980 which is also incorporated herein. Those skilled in the art will appreciate that such expression systems may be used to effectively produce the full range of polypeptides disclosed in the instant application.

More generally, once the vector or DNA sequence encoding a monomeric  
20 subunit of the polypeptide (e.g. a modified antibody) has been prepared, the expression vector may be introduced into an appropriate host cell. That is, the host cells may be transformed. Introduction of the plasmid into the host cell can be accomplished by various techniques well known to those of skill in the art. These include, but are not limited to, transfection (including electrophoresis and electroporation), protoplast fusion,  
25 calcium phosphate precipitation, cell fusion with enveloped DNA, microinjection, and infection with intact virus. See, Ridgway, A. A. G. "Mammalian Expression Vectors" Chapter 24.2, pp. 470-472 Vectors, Rodriguez and Denhardt, Eds. (Butterworths, Boston, Mass. 1988). Most preferably, plasmid introduction into the host is via electroporation. The transformed cells are grown under conditions appropriate to the  
30 production of the light chains and heavy chains, and assayed for heavy and/or light chain protein synthesis. Exemplary assay techniques include enzyme-linked immunosorbent assay (ELISA), radioimmunoassay (RIA), or fluorescence-activated cell sorter analysis (FACS), immunohistochemistry and the like.

As used herein, the term "transformation" shall be used in a broad sense  
35 to refer to any introduction of DNA into a recipient host cell that changes the genotype and consequently results in a change in the recipient cell.

Along those same lines, "host cells" refers to cells that have been transformed with vectors constructed using recombinant DNA techniques and encoding

at least one heterologous gene. In descriptions of processes for isolation of antibodies from recombinant hosts, the terms "cell" and "cell culture" are used interchangeably to denote the source of antibody unless it is clearly specified otherwise. In other words, recovery of polypeptide from the "cells" may mean either from spun down whole cells,  
 5 or from the cell culture containing both the medium and the suspended cells.

The host cell line used for protein expression is most preferably of mammalian origin; those skilled in the art are credited with ability to preferentially determine particular host cell lines which are best suited for the desired gene product to be expressed therein. Exemplary host cell lines include, but are not limited to, DG44  
 10 and DUXB11 (Chinese Hamster Ovary lines, DHFR minus), HELA (human cervical carcinoma), CVI (monkey kidney line), COS (a derivative of CVI with SV40 T antigen), R1610 (Chinese hamster fibroblast) BALBC/3T3 (mouse fibroblast), HAK (hamster kidney line), SP2/O (mouse myeloma), P3.times.63-Ag3.653 (mouse myeloma), BFA-1c1BPT (bovine endothelial cells), RAJI (human lymphocyte) and 293 (human kidney).  
 15 CHO cells are particularly preferred. Host cell lines are typically available from commercial services, the American Tissue Culture Collection or from published literature.

In another embodiment, a host cell is a prokaryotic cell, e.g., a strain which allows the formation of disulfide bonds (Derman, AI, et al. 1993. Science.  
 20 262:1744; Bessette, PH. Et al. 1999. Proc. Natl. Acad. Sci. USA 96:13703).

*In vitro* production allows scale-up to give large amounts of the desired polypeptides. Techniques for mammalian cell cultivation under tissue culture conditions are known in the art and include homogeneous suspension culture, e.g. in an airlift reactor or in a continuous stirrer reactor, or immobilized or entrapped cell culture, e.g. in  
 25 hollow fibers, microcapsules, on agarose microbeads or ceramic cartridges. If necessary and/or desired, the solutions of polypeptides can be purified by the customary chromatography methods, for example gel filtration, ion-exchange chromatography, chromatography over DEAE-cellulose or (immuno-)affinity chromatography, e.g., after preferential biosynthesis of a modified hinge region polypeptide or prior to or  
 30 subsequent to the HIC chromatography step described herein.

Genes encoding the polypeptide of the invention can also be expressed non-mammalian cells such as bacteria or yeast or plant cells. In this regard it will be appreciated that various unicellular non-mammalian microorganisms such as bacteria can also be transformed; i.e. those capable of being grown in cultures or fermentation.  
 35 Bacteria, which are susceptible to transformation, include members of the enterobacteriaceae, such as strains of *Escherichia coli* or *Salmonella*; *Bacillaceae*, such as *Bacillus subtilis*; *Pneumococcus*; *Streptococcus*, and *Haemophilus influenzae*. It will further be appreciated that, when expressed in bacteria, the polypeptides typically

become part of inclusion bodies. The polypeptides must be isolated, purified and then assembled into functional molecules. Where tetravalent forms of antibodies are desired, the subunits will then self-assemble into tetravalent antibodies (WO02/096948A2).

In addition to prokaryotes, eukaryotic microbes may also be used.

- 5 Saccharomyces cerevisiae, or common baker's yeast, is the most commonly used among eukaryotic microorganisms although a number of other strains are commonly available. For expression in Saccharomyces, the plasmid YRp7, for example, (Stinchcomb et al., Nature, 282:39 (1979); Kingsman et al., Gene, 7:141 (1979); Tschemper et al., Gene, 10:157 (1980)) is commonly used. This plasmid already contains the TRP1 gene which
- 10 provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC No. 44076 or PEP4-1 (Jones, Genetics, 85:12 (1977)). The presence of the trp1 lesion as a characteristic of the yeast host cell genome then provides an effective environment for detecting transformation by growth in the absence of tryptophan.

15

#### IV. Separation of Polypeptides Comprising at Least One Interchain Disulfide Linkage From Those Lacking Interchain Disulfide Linkages

- In one aspect, the invention pertains to separation of molecules having two heavy chain portions from a mixture, where a fraction of the molecules are present
- 20 in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage and a fraction of the molecules comprise heavy chain portions that are not linked via at least one disulfide linkage by hydrophobic interaction chromatography.

- Hydrophobic interaction chromatography was first developed following the observation that proteins could be retained on affinity gels which comprised
- 25 hydrocarbon spacer arms but lacked the affinity ligand. Elution from HIC supports can be effected by alterations in solvent, pH, ionic strength, or by the addition of chaotropic agents or organic modifiers, such as ethylene or propylene glycol. A description of the general principles of hydrophobic interaction chromatography can be found e.g., in U. S. Patent 3,917,527 and in U. S. Patent 4,000,098. HIC in the context of high performance
- 30 liquid chromatography (HPLC) has been used to separate antibody fragments lacking heavy chain portions (e.g., F(ab')<sub>2</sub>) from intact antibody molecules in a single step protocol. (Morimoto, K. et al., L Biochem. Biophys. Meth. 24: 107 (1992)).

- The separation method of the invention can be performed on an unpurified population of polypeptides (e.g., culture supernatants or preparations or
- 35 preparations of polypeptides isolated from prokaryotic inclusion bodies). Alternatively, the instant separation methods can be used on polypeptide mixtures obtained after one or more initial purification steps, e.g., after a preparation comprising forms A and B has been eluted from an affinity matrix.

In one embodiment, the binding molecules subjected to HIC chromatography comprise a connecting peptide of the invention.

In a preferred embodiment, HIC can be applied to mixtures that have been partially purified by other protein purification procedures. The term "partially purified" as used herein includes a protein preparation in which the protein of interest is present in at least 5 % by weight, more preferably at least 10% and most preferably at least 45%. Initial or subsequent purification steps can be used to remove, e.g., immunoglobulin aggregates, misfolded species, host cell protein, residue material from preceding chromatographic steps (such as Protein A when employed). In one embodiment, HIC can be performed on polypeptides comprising a connecting peptide of the invention. Accordingly, the application of HIC can also be appreciated in the context of an overall purification protocol. Exemplary purification steps that can be used prior to or subsequent to HIC include: affinity chromatography (for example, PROSEP-A® (BioProcessing Ltd., U.K.) which consists of Protein A covalently coupled to controlled pore glass or Protein A SEPHAROSE® Fast Flow (Pharmacia) or TOYOPEARL 650M Protein A (TosoHaas)). Protein A is preferred for human  $\gamma 1$ ,  $\gamma 2$ , or  $\gamma 4$  heavy chains and protein G for mouse isotypes. Bakerbond ABXtm resin can be used if the molecule comprises a CH3 domain. In addition or alternatively, ion exchange chromatography may be employed. In this regard various anionic or cationic substituents may be attached to matrices in order to form anionic or cationic supports for chromatography. Anionic exchange substituents include diethylaminoethyl(DEAE), quaternary aminoethyl(QAE) and quaternary amine(Q) groups. Cationic exchange substituents include carboxymethyl (CM), sulfoethyl(SE), sulfopropyl(SP), phosphate(P) and sulfonate(S). Cellulose ion exchange resins such as DE23, DE32, DE52, CM-23, CM-32 and CM-52 are available from Whatman Ltd. Maidstone, Kent, U.K. SEPHADEX®-based and -locross-linked ion exchangers are also known. For example, DEAE-, QAE-, CM-, and SP- SEPHADEX® and DEAE-, Q-, CM-and S- SEPHAROSE® and SEPHAROSE® Fast Flow are all available from Pharmacia AB. Further, both DEAE and CM derivitized ethylene glycol-methacrylate copolymer such as TOYOPEARL DEAE-650S or M and TOYOPEARL CM-650S or M are available from Toso Haas Co., Philadelphia, Pa. Because elution from ion exchange supports usually involves addition of salt and because HIC is enhanced under increased salt concentrations, the introduction of a HIC step following an ionic exchange chromatographic step or other salt mediated purification step is preferred. Additional purification protocols may be added including but not necessarily limited to: further ionic exchange chromatography, size exclusion chromatography, viral inactivation, concentration and freeze drying, hydroxylapatite chromatography, gel electrophoresis, dialysis, ethanol precipitation, reverse phase HPLC, chromatography on silica,

chromatography on heparin SEQHAROSE™, chromatofocusing, or ammonium sulfate precipitation.

Prior to purification using the subject methods, the composition comprising the mixture of polypeptides to be separated will preferably be placed in a buffer of acidic or approximately neutral pH. This can be done, for example, by adding concentrated buffer, resuspending the sample in the buffer, exchanging the buffer (e.g., using dialysis or ultrafiltration). Alternatively, the pH of the sample buffer can simply be adjusted to be within the desired range.

Hydrophobic interactions are strongest at high ionic strength, therefore, this form of separation is conveniently performed following salt precipitations or ion exchange procedures. Adsorption of the proteins to a HIC column is favored by high salt concentrations, but the actual concentrations can vary over a wide range depending on the nature of the protein and the particular HIC ligand chosen. Various ions can be arranged in a so-called soluphobic series depending on whether they promote hydrophobic interactions (salting-out effects) or disrupt the structure of water (chaotropic effect) and lead to the weakening of the hydrophobic interaction. Cations are ranked in terms of increasing salting out effect as  $\text{Ba}^{++}$ ;  $\text{Ca}^{++}$ ;  $\text{Mg}^{++}$ ;  $\text{Li}^+$ ;  $\text{Cs}^+$ ;  $\text{Na}^+$ ;  $\text{K}^+$ ;  $\text{Rb}^+$ ;  $\text{NH}_4^+$ , while anions may be ranked in terms of increasing chaotropic effect as  $\text{PO}_4^{--}$ ;  $\text{SO}_4^{--}$ ;  $\text{CH}_3\text{COOO}^-$ ;  $\text{Cl}^-$ ;  $\text{Br}^-$ ;  $\text{NO}_3^-$ ;  $\text{ClO}_4^-$ ;  $\text{I}^-$ ;  $\text{SCN}^-$ .

In general, Na, K or  $\text{NH}_4$  sulfates effectively promote ligand-protein interaction in HIC. Salts may be formulated that influence the strength of the interaction as given by the following relationship:  $(\text{NH}_4)_2\text{SO}_4$ ;  $\text{Na}_2\text{SO}_4$ ;  $\text{NaCl}$ ;  $\text{NH}_4\text{Cl}$ ;  $\text{NaBr}$ ;  $\text{NaSCN}$ . In general, salt concentrations of between about 0.75 and about 2M ammonium sulfate or between about 1 and 4M NaCl are useful.

A number of chromatographic supports may be employed in the preparation of HIC columns, the most extensively used are agarose, silica and organic polymer or co-polymer resins. The hydrophobic interaction material is generally a base matrix (e.g., a hydrophilic carbohydrate (such as cross-linked agarose) or synthetic copolymer material) to which hydrophobic ligands (e.g., alkyl or aryl groups) are coupled. The preferred HIC material comprises an agarose resin substituted with phenyl groups. Exemplary HIC material includes: phenyl SEPHAROSE™, FAST FLOW with low or high substitution (Pharmacia LKB Biotechnology, AB, Sweden); phenyl SEPHAROSE™ High Performance column; phenyl or butyl-SEPHAROSE® CL-4B, butyl-SEPHAROSE® FF, octyl-SEPHAROSE® FF and phenyl-SEPHAROSE® FF (Pharmacia LKB Biotechnology AB, Sweden); Fractogel™ EMD Propyl or FRACTOGEL™ EMC Phenyl columns (E. Merck, Germany); MACROPREP™ Methyl or MACRO-PREP™ t-Butyl Supports (Bio-Rad, California); WP HI-Propyl (C3)™ column (J.T. Baker, New Jersey). Exemplary HIC materials are also available from

Tosoh Corporation, Tokyo, Japan under the product names TOYOPEARL ether 650, phenyl 650, butyl 650 (Fractogel), ether-5PW-HR, or phenyl-5PW-HR; Miles-Yeda, Rehovot, Israel under the product name alkyl-agarose, wherein the alkyl group contains from 2-10 carbon atoms, and J.T. Baker, Phillipsburg, N.J. under the product name Bakerbond WP-HI-propyl. It is also possible to prepare the desired HIC column using conventional chemistry. (Sa: for example, Er-el. Z. gl all, Biochem. Biophys. Res. Comm. 49:383 (1972) or Ulbrich, V. rd gL Coll. Czech. Chem. Commum. 9:1466 (1964)).

The choice of a particular gel can be determined by the skilled artisan. In general the strength of the interaction of the protein and the HIC ligand increases with the chain length of the alkyl ligands but ligands having from about 4 to about 8 carbon atoms are suitable for most separations. A phenyl group has about the same hydrophobicity as a pentyl group, although the selectivity can be different owing to the possibility of pi-pi orbital interaction with aromatic groups on the protein. Selectively may also be affected by the chemistry of the supporting resin.

Ligand density is an important parameter in that it influences not only the strength of the interaction but the capacity of the column as well. The ligand density of the commercially available phenyl or octyl phenyl gels is on the order of 40 pmoles/ml gel bed. Gel capacity is a function of the particular protein in question as well as pH, temperature and salt type and concentration but generally can be expected to fall in the range of 3-20 mg/ml of gel.

In general, a decrease in temperature decreases the interaction with HIC material. However, any benefit that would accrue by increasing the temperature must also be weighed against adverse effects such as an increase may have on the stability of the protein.

In one embodiment, the polypeptides of the invention can be eluted isocratically. In isocratic elution, all compounds begin migration through the column at onset. However, each migrates at a different rate, resulting in faster or slower elution rate. For example, as described in the instant examples, form A can be eluted with the flow through of the column.

In another embodiment, one or more polypeptides of the invention can be bound to the column and eluted, e.g., using stepwise elution or gradient elution. Elution, whether stepwise or in the form of a gradient, can be accomplished in a variety of ways: (a) by changing the salt concentration, (b) by changing the polarity of the solvent or (c) by adding detergents. By decreasing salt concentration adsorbed proteins are eluted in order of increasing hydrophobicity. Changes in polarity may be affected by additions of solvents such as ethylene or propylene glycol or (iso)propanol, thereby decreasing the strength of the hydrophobic interactions. Detergents function as displacers of proteins

and have been used primarily in connection with the purification of membrane proteins

In performing the separation, the polypeptide mixture can be contacted with the HIC material e.g., using a batch purification technique or using a column. Prior to HIC purification it may be desirable to remove any chaotropic agents or very

5 hydrophobic substances, e.g., by passing the mixture through a precolumn.

For example, for batch purification, HIC material is prepared in or equilibrated to the desired starting buffer. A slurry of the HIC material is obtained. The polypeptide solution is contacted with the slurry to adsorb at least one of the polypeptides to be separated to the HIC material. The solution containing the

10 polypeptides that do not bind to the HIC material is separated from the slurry, e.g., by allowing the slurry to settle and removing the supernatant. The slurry can be subjected to one or more washing steps. If desired, the slurry can be contacted with a solution of lower conductivity to desorb polypeptides that have bound to the HIC material. In order to elute bound polypeptides, the salt concentration can be decreased.

15 In one embodiment, the HIC material can be packed in a column. A mixture comprising the polypeptides to be separated can be applied to the column allowing at least one of the polypeptides to be separated to adsorb to the column. The polypeptides that do not adsorb to the column pass through and can be collected. In order to elute bound polypeptides, the salt concentration can be decreased, e.g., in a step-

20 wise fashion or using a salt gradient.

Since form B is more hydrophobic than form A, it adsorbs irreversibly to the stationary phase using approximately 0.7 M (e.g., 0.73M) Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0 to pH 8.0 as the mobile phase. Form A binds to a lesser extent to the stationary phase under these conditions and is therefore eluted isocratically,

25 i.e. it leaves the column with the flowthrough fraction. Subsequent to the isocratic elution of form A, omitting Ammonium sulfate from the mobile phase desorbs form B.

In an exemplary purification scheme, the HIC material is equilibrated in a buffer comprising a salt concentration yielding a conductivity of from between about 160 to about 110, preferably from between about 140 to about 115, even more

30 preferably from between about 130 or about 120 to about 117 mS/cm. For example, an exemplary starting solution comprises a salt concentration of approximately 1M to 0.7M, e.g., 1M to 0.7M ammonium sulfate. In a preferred embodiment, the solution comprising the mixture of polypeptides to be separated is also brought to the same, or approximately the same conductivity (e.g., using a concentrated stock solution of salt).

35 Under these conditions, Form A is eluted from the column at a conductivity of about 120mS/cm. In order to elute Form B, a stepwise or linear gradient of reducing ammonium sulfate content can be applied to the column. Form B elutes at a conductivity of approximately 115 to approximately 100mS/cm.

In one embodiment, the subject purification method yields a composition comprising polypeptide molecules having at least two target binding sites and two heavy chain portions, wherein the heavy chain portions lack CH2 domains and wherein greater than about 50% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than about 60% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than about 70% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than about 80% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage. In another embodiment, greater than about 90% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage.

In one embodiment, the subject purification method yields a composition comprising recombinant polypeptide molecules having at least two target binding sites and two heavy chain portions, wherein greater than about 99% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage.

In one embodiment, the subject purification method yields a composition comprising polypeptide molecules having at least two target binding sites and two heavy chain portions, wherein greater than about 95% of the molecules are present in a form in which the two heavy chain portions are linked via at least one interchain disulfide linkage, and wherein the heavy chain portions of the polypeptides are derived from an antibody of the IgG4 isotype.

In one embodiment, the subject purification method yields a composition comprising polypeptide molecules having two light chain portions and two heavy chain portions, wherein the heavy chain portions lack CH2 domains and wherein greater than about 80% of the molecules are present in a form in which the two heavy chain portions are not linked via at least one interchain disulfide linkage.

In another aspect, the instant invention also provides methods for monitoring the results of purification and/or preferential biosynthesis comprising measuring the relative amounts of Form A and Form B in a composition. Form A and Form B can be measured, e.g., as described herein using non-reducing SDS polyacrylamide gel electrophoresis or mass spectrometry.



#### V. Labeling or Conjugation of Polypeptides

The polypeptide molecules of the present invention may be used in non-conjugated form or may be conjugated to at least one of a variety of molecules, e.g., to facilitate antigen detection or for imaging or therapy of the patient. The polypeptides of the invention can be labeled or conjugated either before or after purification, when purification is performed. In particular, the polypeptides of the present invention may be conjugated to cytotoxins (such as radioisotopes, cytotoxic drugs, or toxins) therapeutic agents, cytostatic agents, biological toxins, prodrugs, peptides, proteins, enzymes, viruses, lipids, biological response modifiers, pharmaceutical agents, immunologically active ligands (e.g., lymphokines or antibodies wherein the resulting molecule binds to both the neoplastic cell and an effector cell such as a T cell), or PEG. In another embodiment, a polypeptide of the invention can be conjugated to a molecule that decreases vascularization of tumors. In other embodiments, the disclosed compositions may comprise polypeptides of the invention coupled to drugs or prodrugs. Still other embodiments of the present invention comprise the use of polypeptides of the invention conjugated to specific biotoxins or their cytotoxic fragments such as ricin, gelonin, pseudomonas exotoxin or diphtheria toxin. The selection of which conjugated or unconjugated polypeptide to use will depend on the type and stage of cancer, use of adjunct treatment (e.g., chemotherapy or external radiation) and patient condition. It will be appreciated that one skilled in the art could readily make such a selection in view of the teachings herein.

It will be appreciated that, in previous studies, anti-tumor antibodies labeled with isotopes have been used successfully to destroy cells in solid tumors as well as lymphomas/leukemias in animal models, and in some cases in humans. Exemplary radioisotopes include:  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{123}\text{I}$ ,  $^{111}\text{In}$ ,  $^{105}\text{Rh}$ ,  $^{153}\text{Sm}$ ,  $^{67}\text{Cu}$ ,  $^{67}\text{Ga}$ ,  $^{166}\text{Ho}$ ,  $^{177}\text{Lu}$ ,  $^{186}\text{Re}$  and  $^{188}\text{Re}$ . The radionuclides act by producing ionizing radiation which causes multiple strand breaks in nuclear DNA, leading to cell death. The isotopes used to produce therapeutic conjugates typically produce high energy  $\alpha$ - or  $\beta$ -particles which have a short path length. Such radionuclides kill cells to which they are in close proximity, for example neoplastic cells to which the conjugate has attached or has entered. They have little or no effect on non-localized cells. Radionuclides are essentially non-immunogenic.

With respect to the use of radiolabeled conjugates in conjunction with the present invention, polypeptides of the invention may be directly labeled (such as through iodination) or may be labeled indirectly through the use of a chelating agent. As used herein, the phrases "indirect labeling" and "indirect labeling approach" both mean that a chelating agent is covalently attached to a molecule and at least one radionuclide is associated with the chelating agent. Such chelating agents are typically referred to as

bifunctional chelating agents as they bind both the polypeptide and the radioisotope. Particularly preferred chelating agents comprise 1-isothiocyanatobenzyl-3-methyldithiethylene triaminepentaacetic acid ("MX-DTPA") and cyclohexyl diethylenetriamine pentaacetic acid ("CHX-DTPA") derivatives. Other chelating agents  
 5 comprise P-DOTA and EDTA derivatives. Particularly preferred radionuclides for indirect labeling include  $^{111}\text{In}$  and  $^{90}\text{Y}$ .

As used herein, the phrases "direct labeling" and "direct labeling approach" both mean that a radionuclide is covalently attached directly to a polypeptide (typically via an amino acid residue). More specifically, these linking technologies  
 10 include random labeling and site-directed labeling. In the latter case, the labeling is directed at specific sites on the polypeptide, such as the N-linked sugar residues present only on the Fc portion of the conjugates. Further, various direct labeling techniques and protocols are compatible with the instant invention. For example, Technetium-99m labeled polypeptides may be prepared by ligand exchange processes, by reducing  
 15 pertechnetate ( $\text{TcO}_4^-$ ) with stannous ion solution, chelating the reduced technetium onto a Sephadex column and applying the polypeptides to this column, or by batch labeling techniques, e.g. by incubating pertechnetate, a reducing agent such as  $\text{SnCl}_2$ , a buffer solution such as a sodium-potassium phthalate-solution, and the molecules. In any event, preferred radionuclides for directly labeling polypeptides are well known in the  
 20 art and a particularly preferred radionuclide for direct labeling is  $^{131}\text{I}$  covalently attached via tyrosine residues. Polypeptides according to the invention may be derived, for example, with radioactive sodium or potassium iodide and a chemical oxidizing agent, such as sodium hypochlorite, chloramine T or the like, or an enzymatic oxidizing agent, such as lactoperoxidase, glucose oxidase and glucose. However, for the purposes of the  
 25 present invention, the indirect labeling approach is particularly preferred. Patents relating to chelators and chelator conjugates are known in the art. For instance, U.S. Patent No. 4,831,175 of Gansow is directed to polysubstituted diethylenetriaminepentaacetic acid chelates and protein conjugates containing the same, and methods for their preparation. U.S. Patent Nos. 5,099,069, 5,246,692, 5,286,850,  
 30 5,434,287 and 5,124,471 of Gansow also relate to polysubstituted DTPA chelates. These patents are incorporated herein in their entirety. Other examples of compatible metal chelators are ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), 1,4,8,11-tetraazatetradecane, 1,4,8,11-tetraazatetradecane-1,4,8,11-tetraacetic acid, 1-oxa-4,7,12,15-tetraazaheptadecane-  
 35 4,7,12,15-tetraacetic acid, or the like. Cyclohexyl-DTPA or CHX-DTPA is particularly preferred. Still other compatible chelators, including those yet to be discovered, may easily be discerned by a skilled artisan and are clearly within the scope of the present invention.

Compatible chelators, including the specific bifunctional chelator used to facilitate chelation in co-pending application Serial Nos. 08/475,813, 08/475,815 and 08/478,967, are preferably selected to provide high affinity for trivalent metals, exhibit increased tumor-to-non-tumor ratios and decreased bone uptake as well as greater *in vivo* retention of radionuclide at target sites, i.e., B-cell lymphoma tumor sites. However, other bifunctional chelators that may or may not possess all of these characteristics are known in the art and may also be beneficial in tumor therapy. It will also be appreciated that, in accordance with the teachings herein, polypeptides may be conjugated to different radiolabels for diagnostic and therapeutic purposes. To this end the aforementioned co-pending applications, herein incorporated by reference in their entirety, disclose radiolabeled therapeutic conjugates for diagnostic "imaging" of tumors before administration of therapeutic molecule. "In2B8" conjugate comprises a murine monoclonal antibody, 2B8, specific to human CD20 antigen, that is attached to <sup>111</sup>In via a bifunctional chelator, i.e., MX-DTPA (diethylenetriaminepentaacetic acid), which comprises a 1:1 mixture of 1-isothiocyanatobenzyl-3-methyl-DTPA and 1-methyl-3-isothiocyanatobenzyl-DTPA. <sup>111</sup>In is particularly preferred as a diagnostic radionuclide because between about 1 to about 10 mCi can be safely administered without detectable toxicity; and the imaging data is generally predictive of subsequent <sup>90</sup>Y-labeled antibody distribution. Most imaging studies utilize 5 mCi <sup>111</sup>In-labeled antibody, because this dose is both safe and has increased imaging efficiency compared with lower doses, with optimal imaging occurring at three to six days after antibody administration. See, for example, Murray, *J. Nuc. Med.* 26: 3328 (1985) and Carragullo *et al.*, *J. Nuc. Med.* 26: 67 (1985).

As indicated above, a variety of radionuclides are applicable to the present invention and those skilled in the art can readily determine which radionuclide is most appropriate under various circumstances. For example, <sup>131</sup>I is a well known radionuclide used for targeted immunotherapy. However, the clinical usefulness of <sup>131</sup>I can be limited by several factors including: eight-day physical half-life; dehalogenation of iodinated antibody both in the blood and at tumor sites; and emission characteristics (*e.g.*, large gamma component) which can be suboptimal for localized dose deposition in tumor. With the advent of superior chelating agents, the opportunity for attaching metal chelating groups to proteins has increased the opportunities to utilize other radionuclides such as <sup>111</sup>In and <sup>90</sup>Y. <sup>90</sup>Y provides several benefits for utilization in radioimmunotherapeutic applications: the 64 hour half-life of <sup>90</sup>Y is long enough to allow accumulation by tumor and, unlike *e.g.*, <sup>131</sup>I, <sup>90</sup>Y is a pure beta emitter of high energy with no accompanying gamma irradiation in its decay, with a range in tissue of 100 to 1,000 cell diameters. Furthermore, the minimal amount of penetrating radiation allows for outpatient administration of <sup>90</sup>Y-labeled molecules. Additionally, internalization of labeled polypeptides is not required for cell

killing, and the local emission of ionizing radiation should be lethal for adjacent tumor cells lacking the target antigen.

Those skilled in the art will appreciate that these non-radioactive conjugates may also be assembled using a variety of techniques depending on the selected agent to be conjugated. For example, conjugates with biotin are prepared e.g. by reacting the polypeptides with an activated ester of biotin such as the biotin N-hydroxysuccinimide ester. Similarly, conjugates with a fluorescent marker may be prepared in the presence of a coupling agent, e.g. those listed above, or by reaction with an isothiocyanate, preferably fluorescein-isothiocyanate. Conjugates of the polypeptides of the invention with cytostatic/cytotoxic substances and metal chelates are prepared in an analogous manner.

Preferred agents for use in the present invention are cytotoxic drugs, particularly those which are used for cancer therapy. As used herein, "a cytotoxin or cytotoxic agent" means any agent that is detrimental to the growth and proliferation of cells and may act to reduce, inhibit or destroy a cell or malignancy. Exemplary cytotoxins include, but are not limited to, radionuclides, biotoxins, enzymatically active toxins, cytostatic or cytotoxic therapeutic agents, prodrugs, immunologically active ligands and biological response modifiers such as cytokines. Any cytotoxin that acts to retard or slow the growth of immunoreactive cells or malignant cells is within the scope of the present invention.

Exemplary cytotoxins include, in general, cytostatic agents, alkylating agents, antimetabolites, anti-proliferative agents, tubulin binding agents, hormones and hormone antagonists, and the like. Exemplary cytostatics that are compatible with the present invention include alkylating substances, such as mechlorethamine, triethylenephosphoramide, cyclophosphamide, ifosfamide, chlorambucil, busulfan, melphalan or triaziquone, also nitrosourea compounds, such as carmustine, lomustine, or semustine. Other preferred classes of cytotoxic agents include, for example, the maytansinoid family of drugs. Other preferred classes of cytotoxic agents include, for example, the anthracycline family of drugs, the vinca drugs, the mitomycins, the bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diynenes, and the podophyllotoxins. Particularly useful members of those classes include, for example, adriamycin, carminomycin, daunorubicin (daunomycin), doxorubicin, aminopterin, methotrexate, methopterin, mithramycin, streptonigrin, dichloromethotrexate, mitomycin C, actinomycin-D, porfiromycin, 5-fluorouracil, floxuridine, florafur, 6-mercaptopurine, cytarabine, cytosine arabinoside, podophyllotoxin, or podophyllotoxin derivatives such as etoposide or etoposide phosphate, melphalan, vinblastine, vincristine, leurosine, vindesine, leurosine and the like. Still other cytotoxins that are compatible with the teachings herein include taxol, taxane, cytochalasin B, gramicidin D, ethidium

bromide, emetine, tenoposide, colchicin, dihydroxy anthracin dione, mitoxantrone, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Hormones and hormone antagonists, such as corticosteroids, e.g. prednisone, progestins, e.g. hydroxyprogesterone or medroprogesterone, estrogens, e.g.

5 diethylstilbestrol, antiestrogens, e.g. tamoxifen, androgens, e.g. testosterone, and aromatase inhibitors, e.g. aminoglutethimide are also compatible with the teachings herein. As noted previously, one skilled in the art may make chemical modifications to the desired compound in order to make reactions of that compound more convenient for purposes of preparing conjugates of the invention.

10 One example of particularly preferred cytotoxins comprise members or derivatives of the enediyne family of anti-tumor antibiotics, including calicheamicin, esperamicins or dynemicins. These toxins are extremely potent and act by cleaving nuclear DNA, leading to cell death. Unlike protein toxins which can be cleaved *in vivo* to give many inactive but immunogenic polypeptide fragments, toxins such as  
15 calicheamicin, esperamicins and other enediynes are small molecules which are essentially non-immunogenic. These non-peptide toxins are chemically-linked to the dimers or tetramers by techniques which have been previously used to label monoclonal antibodies and other molecules. These linking technologies include site-specific linkage via the N-linked sugar residues present only on the Fc portion of the constructs. Such  
20 site-directed linking methods have the advantage of reducing the possible effects of linkage on the binding properties of the constructs.

As previously alluded to, compatible cytotoxins may comprise a prodrug. As used herein, the term "prodrug" refers to a precursor or derivative form of a pharmaceutically active substance that is less cytotoxic to tumor cells compared to the  
25 parent drug and is capable of being enzymatically activated or converted into the more active parent form. Prodrugs compatible with the invention include, but are not limited to, phosphate-containing prodrugs, thiophosphate-containing prodrugs, sulfate containing prodrugs, peptide containing prodrugs,  $\beta$ -lactam-containing prodrugs, optionally substituted phenoxyacetamide-containing prodrugs or optionally substituted  
30 phenylacetamide-containing prodrugs, 5-fluorocytosine and other 5-fluorouridine prodrugs that can be converted to the more active cytotoxic free drug. Further examples of cytotoxic drugs that can be derivatized into a prodrug form for use in the present invention comprise those chemotherapeutic agents described above.

Among other cytotoxins, it will be appreciated that polypeptides can also be  
35 associated with a biotoxin such as ricin subunit A, abrin, diphtheria toxin, botulinum, cyanginosins, saxitoxin, shigatoxin, tetanus, tetrodotoxin, trichothecene, verrucologen or a toxic enzyme. Preferably, such constructs will be made using genetic engineering techniques that allow for direct expression of the binding molecule-toxin construct. Other

biological response modifiers that may be associated with the polypeptides of the invention of the present invention comprise cytokines such as lymphokines and interferons. In view of the instant disclosure it is submitted that one skilled in the art could readily form such constructs using conventional techniques.

5 Another class of compatible cytotoxins that may be used in conjunction with the disclosed polypeptides are radiosensitizing drugs that may be effectively directed to tumor or immunoreactive cells. Such drugs enhance the sensitivity to ionizing radiation, thereby increasing the efficacy of radiotherapy. An conjugate internalized by the tumor cell would deliver the radiosensitizer nearer the nucleus where radiosensitization would be  
10 maximal. The unbound radiosensitizer linked polypeptides of the invention would be cleared quickly from the blood, localizing the remaining radiosensitization agent in the target tumor and providing minimal uptake in normal tissues. After rapid clearance from the blood, adjunct radiotherapy would be administered in one of three ways: 1.) external beam radiation directed specifically to the tumor, 2.) radioactivity directly implanted in the  
15 tumor or 3.) systemic radioimmunotherapy with the same targeting molecule. A potentially attractive variation of this approach would be the attachment of a therapeutic radioisotope to the radiosensitized immunoconjugate, thereby providing the convenience of administering to the patient a single drug.

In one embodiment, a moiety that enhances the stability or efficacy of the  
20 polypeptide can be conjugated. For example, in one embodiment, PEG can be conjugated to the polypeptides of the invention to increase their half-life in vivo. Leong, S.R., et al. 2001. *Cytokine* 16:106; 2002; *Adv. in Drug Deliv. Rev.* 54:531; or Weir et al. 2002. *Biochem. Soc. Transactions* 30:512.

25

#### VI. Administration of Polypeptides

Methods of preparing and administering polypeptides of the invention to a subject are well known to or are readily determined by those skilled in the art. The  
30 route of administration of the polypeptide of the invention may be oral, parenteral, by inhalation or topical. The term parenteral as used herein includes intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, rectal or vaginal administration. The intravenous, intraarterial, subcutaneous and intramuscular forms of parenteral administration are generally preferred. While all these forms of administration  
35 are clearly contemplated as being within the scope of the invention, a form for administration would be a solution for injection, in particular for intravenous or intraarterial injection or drip. Usually, a suitable pharmaceutical composition for injection may comprise a buffer (e.g. acetate, phosphate or citrate buffer), a surfactant

(e.g. polysorbate), optionally a stabilizer agent (e.g. human albumin), etc. However, in other methods compatible with the teachings herein, the polypeptides can be delivered directly to the site of the adverse cellular population thereby increasing the exposure of the diseased tissue to the therapeutic agent.

5           Preparations for parenteral administration includes sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. In the subject  
10 invention, pharmaceutically acceptable carriers include, but are not limited to, 0.01-0.1M and preferably 0.05M phosphate buffer or 0.8% saline. Other common parenteral vehicles include sodium phosphate solutions, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's, or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers, such as those based on Ringer's dextrose, and the  
15 like. Preservatives and other additives may also be present such as for example, antimicrobials, antioxidants, chelating agents, and inert gases and the like. More particularly, pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In such cases,  
20 the composition must be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and will preferably be preserved against the contaminating action of microorganisms, such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (e.g., glycerol, propylene glycol, and liquid polyethylene  
25 glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid,  
30 thimerosal and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols, such as mannitol, sorbitol, or sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

35           In any case, sterile injectable solutions can be prepared by incorporating an active compound (e.g., a polypeptide by itself or in combination with other active agents) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated herein, as required, followed by filtered sterilization. Generally,

dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying, which yields a powder of an active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof. The preparations for injections are processed, filled into containers such as ampoules, bags, bottles, syringes or vials, and sealed under aseptic conditions according to methods known in the art. Further, the preparations may be packaged and sold in the form of a kit such as those described in co-pending U.S.S.N. 09/259,337 and U.S.S.N. 09/259,338 each of which is incorporated herein by reference. Such articles of manufacture will preferably have labels or package inserts indicating that the associated compositions are useful for treating a subject suffering from, or predisposed to autoimmune or neoplastic disorders.

Effective doses of the compositions of the present invention, for the treatment of the above described conditions vary depending upon many different factors, including means of administration, target site, physiological state of the patient, whether the patient is human or an animal, other medications administered, and whether treatment is prophylactic or therapeutic. Usually, the patient is a human but non-human mammals including transgenic mammals can also be treated. Treatment dosages may be titrated using routine methods known to those of skill in the art to optimize safety and efficacy. For passive immunization with an antibody, the dosage can range, e.g., from about 0.0001 to 100 mg/kg, and more usually 0.01 to 5 mg/kg (e.g., 0.02 mg/kg, 0.25 mg/kg, 0.5 mg/kg, 0.75 mg/kg, 1 mg/kg, 2 mg/kg, etc.), of the host body weight. For example dosages can be 1 mg/kg body weight or 10 mg/kg body weight or within the range of 1-10 mg/kg, preferably at least 1 mg/kg. Doses intermediate in the above ranges are also intended to be within the scope of the invention. Subjects can be administered such doses daily, on alternative days, weekly or according to any other schedule determined by empirical analysis. An exemplary treatment entails administration in multiple dosages over a prolonged period, for example, of at least six months. Additional exemplary treatment regimes entail administration once per every two weeks or once a month or once every 3 to 6 months. Exemplary dosage schedules include 1-10 mg/kg or 15 mg/kg on consecutive days, 30 mg/kg on alternate days or 60 mg/kg weekly. In some methods, two or more monoclonal antibodies with different binding specificities are administered simultaneously, in which case the dosage of each antibody administered falls within the ranges indicated.

Polypeptides of the invention can be administered on multiple occasions. Intervals between single dosages can be weekly, monthly or yearly. Intervals can also be irregular as indicated by measuring blood levels of polypeptide or antigen in the patient.



In some methods, dosage is adjusted to achieve a plasma polypeptide concentration of 1-1000 µg/ml and in some methods 25-300 µg/ml. Alternatively, binding molecules can be administered as a sustained release formulation, in which case less frequent administration is required. Dosage and frequency vary depending on the half-life of the molecule in the patient. In general, humanized antibodies show the longest half-life, followed by chimeric antibodies and nonhuman antibodies. In one embodiment, the binding molecules of the invention can be administered in unconjugated form, In another embodiment, the polypeptides of the invention can be administered multiple times in conjugated form. In still another embodiment, the binding molecules of the invention can be administered in unconjugated form, then in conjugated form, or vice versa.

The dosage and frequency of administration can vary depending on whether the treatment is prophylactic or therapeutic. In prophylactic applications, compositions containing the present polypeptides or a cocktail thereof are administered to a patient not already in the disease state to enhance the patient's resistance. Such an amount is defined to be a "prophylactic effective dose." In this use, the precise amounts again depend upon the patient's state of health and general immunity, but generally range from 0.1 to 25 mg per dose, especially 0.5 to 2.5 mg per dose. A relatively low dosage is administered at relatively infrequent intervals over a long period of time. Some patients continue to receive treatment for the rest of their lives.

In therapeutic applications, a relatively high dosage (e.g., from about 1 to 400 mg/kg of antibody per dose, with dosages of from 5 to 25 mg being more commonly used for radioimmunoconjugates and higher doses for cytotoxin-drug conjugated molecules) at relatively short intervals is sometimes required until progression of the disease is reduced or terminated, and preferably until the patient shows partial or complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

In one embodiment, a subject can be treated with a nucleic acid molecule encoding a polypeptide of the invention (e.g., in a vector). Doses for nucleic acids encoding polypeptides range from about 10 ng to 1 g, 100 ng to 100 mg, 1 µg to 10 mg, or 30-300 µg DNA per patient. Doses for infectious viral vectors vary from 10-100, or more, virions per dose.

Therapeutic agents can be administered by parenteral, topical, intravenous, oral, subcutaneous, intraarterial, intracranial, intraperitoneal, intranasal or intramuscular means for prophylactic and/or therapeutic treatment. The most typical route of administration of an immunogenic agent is subcutaneous although other routes can be equally effective. The next most common route is intramuscular injection. This type of injection is most typically performed in the arm or leg muscles. In some methods, agents are injected directly into a particular tissue where deposits have

accumulated, for example intracranial injection. Intramuscular injection or intravenous infusion are preferred for administration of molecules. In some methods, particular therapeutic molecules are injected directly into the cranium. In some methods, molecules are administered as a sustained release composition or device, such as a  
 5 Medipad™ device.

Agents of the invention can optionally be administered in combination with other agents that are effective in treating the disorder or condition in need of treatment (e.g., prophylactic or therapeutic).

Effective single treatment dosages (i.e., therapeutically effective  
 10 amounts) of <sup>90</sup>Y-labeled polypeptides of the invention range from between about 5 and about 75 mCi, more preferably between about 10 and about 40 mCi. Effective single treatment non-marrow ablative dosages of <sup>131</sup>I-labeled antibodies range from between about 5 and about 70 mCi, more preferably between about 5 and about 40 mCi. Effective single treatment ablative dosages (i.e., may require autologous bone marrow  
 15 transplantation) of <sup>131</sup>I-labeled antibodies range from between about 30 and about 600 mCi, more preferably between about 50 and less than about 500 mCi. In conjunction with a chimeric antibody, owing to the longer circulating half life vis-à-vis murine antibodies, an effective single treatment non-marrow ablative dosages of iodine-131 labeled chimeric antibodies range from between about 5 and about 40 mCi, more  
 20 preferably less than about 30 mCi. Imaging criteria for, e.g., the <sup>111</sup>In label, are typically less than about 5 mCi.

While a great deal of clinical experience has been gained with <sup>131</sup>I and <sup>90</sup>Y, other radiolabels are known in the art and have been used for similar purposes. Still other radioisotopes are used for imaging. For example, additional radioisotopes which  
 25 are compatible with the scope of the instant invention include, but are not limited to, <sup>123</sup>I, <sup>125</sup>I, <sup>32</sup>P, <sup>57</sup>Co, <sup>64</sup>Cu, <sup>67</sup>Cu, <sup>77</sup>Br, <sup>81</sup>Rb, <sup>81</sup>Kr, <sup>87</sup>Sr, <sup>113</sup>In, <sup>127</sup>Cs, <sup>129</sup>Cs, <sup>132</sup>I, <sup>197</sup>Hg, <sup>203</sup>Pb, <sup>206</sup>Bi, <sup>177</sup>Lu, <sup>186</sup>Re, <sup>212</sup>Pb, <sup>212</sup>Bi, <sup>47</sup>Sc, <sup>105</sup>Rh, <sup>109</sup>Pd, <sup>153</sup>Sm, <sup>188</sup>Re, <sup>199</sup>Au, <sup>225</sup>Ac, <sup>211</sup>At, and <sup>213</sup>Bi. In this respect alpha, gamma and beta emitters are all compatible with in the instant invention. Further, in view of the instant disclosure it is submitted that one skilled in the  
 30 art could readily determine which radionuclides are compatible with a selected course of treatment without undue experimentation. To this end, additional radionuclides which have already been used in clinical diagnosis include <sup>125</sup>I, <sup>123</sup>I, <sup>99</sup>Tc, <sup>43</sup>K, <sup>52</sup>Fe, <sup>67</sup>Ga, <sup>68</sup>Ga, as well as <sup>111</sup>In. Antibodies have also been labeled with a variety of radionuclides for potential use in targeted immunotherapy (Peirersz et al. *Immunol. Cell Biol.* 65: 111-125  
 35 (1987)). These radionuclides include <sup>188</sup>Re and <sup>186</sup>Re as well as <sup>199</sup>Au and <sup>67</sup>Cu to a lesser extent. U.S. Patent No. 5,460,785 provides additional data regarding such radioisotopes and is incorporated herein by reference.

Whether or not the polypeptides of the invention are used in a conjugated or unconjugated form, it will be appreciated that a major advantage of the present invention is the ability to use these polypeptides in myelosuppressed patients, especially those who are undergoing, or have undergone, adjunct therapies such as radiotherapy or chemotherapy. That is, the beneficial delivery profile (i.e. relatively short serum dwell time, high binding affinity and enhanced localization) of the polypeptides makes them particularly useful for treating patients that have reduced red marrow reserves and are sensitive to myelotoxicity. In this regard, the unique delivery profile of the polypeptides make them very effective for the administration of radiolabeled conjugates to myelosuppressed cancer patients. As such, the polypeptides of the invention are useful in a conjugated or unconjugated form in patients that have previously undergone adjunct therapies such as external beam radiation or chemotherapy. In other preferred embodiments, the polypeptides (again in a conjugated or unconjugated form) may be used in a combined therapeutic regimen with chemotherapeutic agents. Those skilled in the art will appreciate that such therapeutic regimens may comprise the sequential, simultaneous, concurrent or coextensive administration of the disclosed molecules and one or more chemotherapeutic agents. Particularly preferred embodiments of this aspect of the invention will comprise the administration of a radiolabeled polypeptide.

While the polypeptides may be administered as described immediately above, it must be emphasized that in other embodiments conjugated and unconjugated polypeptides may be administered to otherwise healthy patients as a first line therapeutic agent. In such embodiments the polypeptides may be administered to patients having normal or average red marrow reserves and/or to patients that have not, and are not, undergoing adjunct therapies such as external beam radiation or chemotherapy.

However, as discussed above, selected embodiments of the invention comprise the administration of polypeptides to myelosuppressed patients or in combination or conjunction with one or more adjunct therapies such as radiotherapy or chemotherapy (i.e. a combined therapeutic regimen). As used herein, the administration of polypeptides in conjunction or combination with an adjunct therapy means the sequential, simultaneous, coextensive, concurrent, concomitant or contemporaneous administration or application of the therapy and the disclosed molecules. Those skilled in the art will appreciate that the administration or application of the various components of the combined therapeutic regimen may be timed to enhance the overall effectiveness of the treatment. For example, chemotherapeutic agents could be administered in standard, well known courses of treatment followed within a few weeks by radioimmunoconjugates of the present invention. Conversely, cytotoxin associated polypeptides could be administered intravenously followed by tumor localized external beam radiation. In yet other embodiments, the polypeptide may be administered concurrently with one or more selected chemotherapeutic

agents in a single office visit. A skilled artisan (e.g. an experienced oncologist) would be readily be able to discern effective combined therapeutic regimens without undue experimentation based on the selected adjunct therapy and the teachings of the instant specification.

5 In this regard it will be appreciated that the combination of the polypeptide (with or without cytotoxin) and the chemotherapeutic agent may be administered in any order and within any time frame that provides a therapeutic benefit to the patient. That is, the chemotherapeutic agent and polypeptide may be administered in any order or concurrently. In selected embodiments the polypeptides of the present invention will be  
10 administered to patients that have previously undergone chemotherapy. In yet other embodiments, the polypeptides and the chemotherapeutic treatment will be administered substantially simultaneously or concurrently. For example, the patient may be given the binding molecule while undergoing a course of chemotherapy. In preferred embodiments the binding molecule will be administered within 1 year of any chemotherapeutic agent or  
15 treatment. In other preferred embodiments the polypeptide will be administered within 10, 8, 6, 4, or 2 months of any chemotherapeutic agent or treatment. In still other preferred embodiments the polypeptide will be administered within 4, 3, 2 or 1 week of any chemotherapeutic agent or treatment. In yet other embodiments the polypeptide will be administered within 5, 4, 3, 2 or 1 days of the selected chemotherapeutic agent or treatment.  
20 It will further be appreciated that the two agents or treatments may be administered to the patient within a matter of hours or minutes (i.e. substantially simultaneously).

Moreover, in accordance with the present invention a myelosuppressed patient shall be held to mean any patient exhibiting lowered blood counts. Those skilled in the art will appreciate that there are several blood count parameters conventionally used as  
25 clinical indicators of myelosuppression and one can easily measure the extent to which myelosuppression is occurring in a patient. Examples of art accepted myelosuppression measurements are the Absolute Neutrophil Count (ANC) or platelet count. Such myelosuppression or partial myeloablation may be a result of various biochemical disorders or diseases or, more likely, as the result of prior chemotherapy or radiotherapy. In this  
30 respect, those skilled in the art will appreciate that patients who have undergone traditional chemotherapy typically exhibit reduced red marrow reserves. As discussed above, such subjects often cannot be treated using optimal levels of cytotoxin (i.e. radionuclides) due to unacceptable side effects such as anemia or immunosuppression that result in increased mortality or morbidity.

35 More specifically conjugated or unconjugated polypeptides of the present invention may be used to effectively treat patients having ANCs lower than about  $2000/\text{mm}^3$  or platelet counts lower than about  $150,000/\text{mm}^3$ . More preferably the polypeptides of the present invention may be used to treat patients having ANCs of less

than about 1500/mm<sup>3</sup>, less than about 1000/mm<sup>3</sup> or even more preferably less than about 500/mm<sup>3</sup>. Similarly, the polypeptides of the present invention may be used to treat patients having a platelet count of less than about 75,000/mm<sup>3</sup>, less than about 50,000/mm<sup>3</sup> or even less than about 10,000/mm<sup>3</sup>. In a more general sense, those skilled in the art will  
5 easily be able to determine when a patient is myelosuppressed using government implemented guidelines and procedures.

As indicated above, many myelosuppressed patients have undergone courses of treatment including chemotherapy, implant radiotherapy or external beam radiotherapy. In the case of the latter, an external radiation source is for local irradiation of  
10 a malignancy. For radiotherapy implantation methods, radioactive reagents are surgically located within the malignancy, thereby selectively irradiating the site of the disease. In any event, the disclosed polypeptides may be used to treat disorders in patients exhibiting myelosuppression regardless of the cause.

In this regard it will further be appreciated that the polypeptides of the instant invention may be used in conjunction or combination with any chemotherapeutic agent or agents (e.g. to provide a combined therapeutic regimen) that eliminates, reduces, inhibits or controls the growth of neoplastic cells *in vivo*. As discussed, such agents often result in the reduction of red marrow reserves. This reduction may be offset, in whole or in part, by the diminished myelotoxicity of the compounds of the  
15 present invention that advantageously allow for the aggressive treatment of neoplasias in such patients. In other preferred embodiments the radiolabeled immunoconjugates disclosed herein may be effectively used with radiosensitizers that increase the susceptibility of the neoplastic cells to radionuclides. For example, radiosensitizing compounds may be administered after the radiolabeled binding molecule has been  
20 largely cleared from the bloodstream but still remains at therapeutically effective levels at the site of the tumor or tumors.

With respect to these aspects of the invention, exemplary chemotherapeutic agents that are compatible with the instant invention include alkylating agents, vinca alkaloids (e.g., vincristine and vinblastine), procarbazine,  
30 methotrexate and prednisone. The four-drug combination MOPP (mechlethamine (nitrogen mustard), vincristine (Oncovin), procarbazine and prednisone) is very effective in treating various types of lymphoma and comprises a preferred embodiment of the present invention. In MOPP-resistant patients, ABVD (e.g., adriamycin, bleomycin, vinblastine and dacarbazine), ChlVPP (chlorambucil, vinblastine, procarbazine and  
35 prednisone), CABS (lomustine, doxorubicin, bleomycin and streptozotocin), MOPP plus ABVD, MOPP plus ABV (doxorubicin, bleomycin and vinblastine) or BCVPP (carmustine, cyclophosphamide, vinblastine, procarbazine and prednisone) combinations can be used. Arnold S. Freedman and Lee M. Nadler, *Malignant Lymphomas*, in

HARRISON'S PRINCIPLES OF INTERNAL MEDICINE 1774-1788 (Kurt J. Isselbacher *et al.*, eds., 13<sup>th</sup> ed. 1994) and V. T. DeVita *et al.*, (1997) and the references cited therein for standard dosing and scheduling. These therapies can be used unchanged, or altered as needed for a particular patient, in combination with one or more polypeptides of the invention as described herein.

Additional regimens that are useful in the context of the present invention include use of single alkylating agents such as cyclophosphamide or chlorambucil, or combinations such as CVP (cyclophosphamide, vincristine and prednisone), CHOP (CVP and doxorubicin), C-MOPP (cyclophosphamide, vincristine, prednisone and procarbazine), CAP-BOP (CHOP plus procarbazine and bleomycin), m-BACOD (CHOP plus methotrexate, bleomycin and leucovorin), ProMACE-MOPP (prednisone, methotrexate, doxorubicin, cyclophosphamide, etoposide and leucovorin plus standard MOPP), ProMACE-CytaBOM (prednisone, doxorubicin, cyclophosphamide, etoposide, cytarabine, bleomycin, vincristine, methotrexate and leucovorin) and MACOP-B (methotrexate, doxorubicin, cyclophosphamide, vincristine, fixed dose prednisone, bleomycin and leucovorin). Those skilled in the art will readily be able to determine standard dosages and scheduling for each of these regimens. CHOP has also been combined with bleomycin, methotrexate, procarbazine, nitrogen mustard, cytosine arabinoside and etoposide. Other compatible chemotherapeutic agents include, but are not limited to, 2-chlorodeoxyadenosine (2-CDA), 2'-deoxycoformycin and fludarabine.

For patients with intermediate- and high-grade NHL, who fail to achieve remission or relapse, salvage therapy is used. Salvage therapies employ drugs such as cytosine arabinoside, cisplatin, etoposide and ifosfamide given alone or in combination. In relapsed or aggressive forms of certain neoplastic disorders the following protocols are often used: IMVP-16 (ifosfamide, methotrexate and etoposide), MIME (methyl-gag, ifosfamide, methotrexate and etoposide), DHAP (dexamethasone, high dose cytarabine and cisplatin), ESHAP (etoposide, methylprednisolone, HD cytarabine, cisplatin), CEPP(B) (cyclophosphamide, etoposide, procarbazine, prednisone and bleomycin) and CAMP (lomustine, mitoxantrone, cytarabine and prednisone) each with well known dosing rates and schedules.

The amount of chemotherapeutic agent to be used in combination with the polypeptides of the instant invention may vary by subject or may be administered according to what is known in the art. See for example, Bruce A Chabner *et al.*, *Antineoplastic Agents*, in GOODMAN & GILMAN'S THE PHARMACOLOGICAL BASIS OF THERAPEUTICS 1233-1287 (Joel G. Hardman *et al.*, eds., 9<sup>th</sup> ed. 1996).

As previously discussed, the polypeptides of the present invention, immunoreactive fragments or recombinants thereof may be administered in a pharmaceutically effective amount for the *in vivo* treatment of mammalian disorders. In this regard, it will be

- appreciated that the disclosed binding molecules will be formulated so as to facilitate administration and promote stability of the active agent. Preferably, pharmaceutical compositions in accordance with the present invention comprise a pharmaceutically acceptable, non-toxic, sterile carrier such as physiological saline, non-toxic buffers, preservatives and the like. For the purposes of the instant application, a pharmaceutically effective amount of the polypeptide, immunoreactive fragment or recombinant thereof, conjugated or unconjugated to a therapeutic agent, shall be held to mean an amount sufficient to achieve effective binding to an antigen and to achieve a benefit, e.g., to ameliorate symptoms of a disease or disorder or to detect a substance or a cell. In the case of tumor cells, the polypeptide will be preferably be capable of interacting with selected immunoreactive antigens on neoplastic or immunoreactive cells and provide for an increase in the death of those cells. Of course, the pharmaceutical compositions of the present invention may be administered in single or multiple doses to provide for a pharmaceutically effective amount of the polypeptide.
- In keeping with the scope of the present disclosure, the polypeptides of the invention may be administered to a human or other animal in accordance with the aforementioned methods of treatment in an amount sufficient to produce a therapeutic or prophylactic effect. The binding molecules of the invention can be administered to such human or other animal in a conventional dosage form prepared by combining the binding molecule of the invention with a conventional pharmaceutically acceptable carrier or diluent according to known techniques. It will be recognized by one of skill in the art that the form and character of the pharmaceutically acceptable carrier or diluent is dictated by the amount of active ingredient with which it is to be combined, the route of administration and other well-known variables. Those skilled in the art will further appreciate that a cocktail comprising one or more species of polypeptides according to the present invention may prove to be particularly effective.

#### VII. Methods of Use

- The polypeptides of the invention can be used for diagnostic or therapeutic purposes. Preferred embodiments of the present invention provide compounds, compositions, kits and methods for the diagnosis and/or treatment of disorders, e.g., neoplastic disorders in a mammalian subject in need of such treatment. Preferably, the subject is a human.
- The polypeptides of the instant invention will be useful in a number of different applications. For example, in one embodiment, the subject binding molecules should be useful for reducing or eliminating cells bearing an epitope recognized by a binding molecule of the invention. In another embodiment, the subject binding

molecules are effective in reducing the concentration of or eliminating soluble antigen in the circulation

In one embodiment, tumor size, inhibiting tumor growth and/or prolonging the survival time of tumor-bearing animals. Accordingly, this invention also relates to a method of treating tumors in a human or other animal by administering to such human or animal an effective, non-toxic amount of polypeptide. One skilled in the art would be able, by routine experimentation, to determine what an effective, non-toxic amount of polypeptide would be for the purpose of treating malignancies. For example, a therapeutically active amount of a polypeptide may vary according to factors such as the disease stage (e.g., stage I versus stage IV), age, sex, medical complications (e.g., immunosuppressed conditions or diseases) and weight of the subject, and the ability of the molecule to elicit a desired response in the subject. The dosage regimen may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily, or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. Generally, however, an effective dosage is expected to be in the range of about 0.05 to 100 milligrams per kilogram body weight per day and more preferably from about 0.5 to 10, milligrams per kilogram body weight per day.

For purposes of clarification "mammal" refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, sports, or pet animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human. "Treatment" refers to both therapeutic treatment and prophylactic or preventative measures. Those in need of treatment include those already with the disease or disorder as well as those in which the disease or disorder is to be prevented. Hence, the mammal may have been diagnosed as having the disease or disorder or may be predisposed or susceptible to the disease.

As discussed above, the polypeptides of the present invention may be immunoreactive with one or more tumor antigens or antigens associated with immune disorders. For example, for neoplastic disorders, the antigen binding site (i.e. the variable region or immunoreactive fragment or recombinant thereof) of the disclosed polypeptides binds to a selected tumor associated antigen at the site of the malignancy. Similarly, in immune (including autoimmune) disorders the disclosed polypeptides will bind to selected markers on the offending cells. Given the number of reported antigens associated with neoplasias and immune disorders, and the number of related antibodies, those skilled in the art will appreciate that the presently disclosed polypeptides may therefore be derived from any one of a number of whole antibodies. More generally, polypeptides useful in the present invention may be obtained or derived from any antibody (including those previously reported in the literature) that reacts with a



molecule or marker associated with the selected condition. Further, the parent or precursor antibody, or fragment thereof, used to generate the disclosed polypeptides may be murine, human, chimeric, humanized, non-human primate or primatized. In other preferred embodiments the polypeptides of the present invention may comprise single chain antibody constructs (such as that disclosed in U.S. Pat. No. 5,892,019 which is incorporated herein by reference) having altered constant domains as described herein. Consequently, any of these types of antibodies modified in accordance with the teachings herein is compatible with the instant invention.

As used herein, "tumor associated antigens" means any antigen which is generally associated with tumor cells, i.e., occurring at the same or to a greater extent as compared with normal cells. More generally, tumor associated antigens comprise any antigen that provides for the localization of immunoreactive antibodies at a neoplastic cell irrespective of its expression on non-malignant cells. Such antigens may be relatively tumor specific and limited in their expression to the surface of malignant cells. Alternatively, such antigens may be found on both malignant and non-malignant cells. For example, CD20 is a pan B antigen that is found on the surface of both malignant and non-malignant B cells that has proved to be an extremely effective target for immunotherapeutic antibodies for the treatment of non-Hodgkin's lymphoma. In this respect, pan T cell antigens such as CD2, CD3, CD5, CD6 and CD7 also comprise tumor associated antigens within the meaning of the present invention. Still other exemplary tumor associated antigens comprise but not limited to MAGE-1, MAGE-3, MUC-1, HPV 16, HPV E6 & E7, TAG-72, CEA, L6-Antigen, CD19, CD22, CD37, CD52, HLA-DR, EGF Receptor and HER2 Receptor. In many cases immunoreactive antibodies for each of these antigens have been reported in the literature. Those skilled in the art will appreciate that each of these antibodies may serve as a precursor for polypeptides of the invention in accordance with the present invention.

The polypeptides of the present invention preferably associate with, and bind to, tumor or immune associated antigens as described above. Accordingly, as will be discussed in some detail below the polypeptides of the present invention may be derived, generated or fabricated from any one of a number of antibodies that react with tumor associated antigens. In preferred embodiments the polypeptides are modified or domain deleted antibodies that are derived using common genetic engineering techniques whereby at least a portion of one or more constant region domains are deleted or altered so as to provide the desired biochemical characteristics such as reduced serum half-life. More particularly, one skilled in the art may readily isolate the genetic sequence corresponding to the variable and/or constant regions of the subject antibody and delete or alter the appropriate nucleotides to provide polypeptides of the invention for use as monomeric subunits in accordance with the instant invention. It

will further be appreciated that compatible polypeptides of the invention may be expressed and produced on a clinical or commercial scale using well-established protocols.

Previously reported antibodies that react with tumor associated antigens may be altered as described herein to provide the polypeptides of the present invention. Exemplary antibodies that may be used to provide antigen binding regions for, generate or derive the disclosed polypeptides include, but are not limited to 2B8 and C2B8 (Zevalin<sup>®</sup> and Rituxan<sup>®</sup>, IDEC Pharmaceuticals Corp., San Diego), Lym 1 and Lym 2 (Techniclone), LL2 (Immunomedics Corp., New Jersey), HER2 (Herceptin<sup>®</sup>, Genentech Inc., South San Francisco), B1 (Bexxar<sup>®</sup>, Coulter Pharm., San Francisco), Campath<sup>®</sup> (Millennium Pharmaceuticals, Cambridge) MB1, BH3, B4, B72.3 (Cytogen Corp.), CC49 (National Cancer Institute) and 5E10 (University of Iowa). Other antibody binding sites that can be incorporated into the subject binding molecules include: Orthoclone OKT3 (CD3), ReoPro (GpIIb/IIIa), Zenapax (C25), Remicade (TNF- $\alpha$ ), Simulect (CD25), Synagis (RSV), Mylotarg (CD33), and Campath (CD52). In preferred embodiments, the polypeptides of the present invention will bind to the same tumor associated antigens as the antibodies enumerated immediately above. In particularly preferred embodiments, the polypeptides will be derived from or bind the same antigens as 2B8, C2B8, CC49 and C5E10 and, even more preferably, will lack all or part of a CH2 domain.

In one embodiment, a binding molecule of the invention binds to the CD23 (U.S. patent 6,011,138). In a preferred embodiment, a binding molecule of the invention binds to the same epitope as the 5E8 antibody. In another embodiment, a binding molecule of the invention comprises at least one CDR from an anti-CD23 antibody, e.g., the 5E8 antibody.

In one embodiment, a binding molecule of the invention binds to the CRIPTO-I antigen (WO02/088170A2 or WO03/083041A2). In a preferred embodiment, a binding molecule of the invention binds to the same epitope as the B3F6 antibody. In another embodiment, a binding molecule of the invention comprises at least one CDR from an anti-CRIPTO-I antibody, e.g., the B3F6 antibody.

In a first preferred embodiment, the polypeptide will bind to the same tumor associated antigen as Rituxan<sup>®</sup>. Rituxan<sup>®</sup> (also known as, rituximab, IDEC-C2B8 and C2B8) was the first FDA-approved monoclonal antibody for treatment of human B-cell lymphoma (see U.S. Patent Nos. 5,843,439; 5,776,456 and 5,736,137 each of which is incorporated herein by reference). Y2B8 (90Y labeled 2B8; Zevalin<sup>®</sup>; ibritumomab tiuxetan) is the murine parent of C2B8. Rituxan<sup>®</sup> is a chimeric, anti-CD20 monoclonal antibody which is growth inhibitory and reportedly sensitizes certain lymphoma cell lines for apoptosis by chemotherapeutic agents *in vitro*. The antibody efficiently binds human

complement, has strong FcR binding, and can effectively kill human lymphocytes *in vitro* via both complement dependent (CDC) and antibody-dependent (ADCC) mechanisms (Reff *et al.*, *Blood* 83: 435-445 (1994)). Those skilled in the art will appreciate that dimeric variants (homodimers or heterodimers) of C2B8 or 2B8, modified according to the instant disclosure, may be used in conjugated or unconjugated forms to effectively treat patients presenting with CD20+ malignancies. More generally, it must be reiterated that the polypeptides disclosed herein may be used in either a "naked" or unconjugated state or conjugated to a cytotoxic agent to effectively treat any one of a number of disorders. In other preferred embodiments of the present invention, the polypeptide of the invention will be derived from, or bind to, the same tumor associated antigen as CC49. As previously alluded to, CC49 binds human tumor associated antigen TAG-72 which is associated with the surface of certain tumor cells of human origin, specifically the LS174T tumor cell line. LS174T [American Type Culture Collection (herein ATCC) No. CL 188] is a variant of the LS180 (ATCC No. CL 187) colon adenocarcinoma line. It will further be appreciated that numerous murine monoclonal antibodies have been developed which have binding specificity for TAG-72. One of these monoclonal antibodies, designated B72.3, is a murine IgG1 produced by hybridoma B72.3 (ATCC No. HB-8108). B72.3 is a first generation monoclonal antibody developed using a human breast carcinoma extract as the immunogen (see Colcher *et al.*, *Proc. Natl. Acad. Sci. (USA)*, 78:3199-3203 (1981); and U.S. Pat. Nos. 4,522,918 and 4,612,282 each of which is incorporated herein by reference). Other monoclonal antibodies directed against TAG-72 are designated "CC" (for colon cancer). As described by Schlom *et al.* (U.S. Pat. No. 5,512,443 which is incorporated herein by reference) CC monoclonal antibodies are a family of second generation murine monoclonal antibodies that were prepared using TAG-72 purified with B72.3. Because of their relatively good binding affinities to TAG-72, the following CC antibodies have been deposited at the ATCC, with restricted access having been requested: CC49 (ATCC No. HB 9459); CC 83 (ATCC No. HB 9453); CC46 (ATCC No. HB 9458); CC92 (ATCC No. HB 9454); CC30 (ATCC No. HB 9457); CC11 (ATCC No. 9455); and CC15 (ATCC No. HB 9460). U.S.P.N. 5,512,443 further teaches that the disclosed antibodies may be altered into their chimeric form by substituting, e.g., human constant regions (Fc) domains for mouse constant regions by recombinant DNA techniques known in the art. Besides disclosing murine and chimeric anti-TAG-72 antibodies, Schlom *et al.* have also produced variants of a humanized CC49 antibody as disclosed in PCT/US99/25552 and single chain constructs as disclosed in U.S. Pat. No. 5,892,019 each of which is also incorporated herein by reference. Those skilled in the art will appreciate that each of the foregoing antibodies, constructs or recombinants, and variations thereof, may be modified and used to provide polypeptides in accordance with the present invention.

In addition to the anti-TAG-72 antibodies discussed above, various groups have also reported the construction and partial characterization of domain-deleted CC49 and B72.3 antibodies (e.g., Calvo et al. *Cancer Biotherapy*, 8(1):95-109 (1993), Slavin-Chiorini et al. *Int. J. Cancer* 53:97-103 (1993) and Slavin-Chiorini et al. *Cancer. Res.*

5 55:5957-5967 (1995)

Still other preferred embodiments of the present invention comprise modified antibodies that are derived from or bind to the same tumor associated antigen as C5E10. As set forth in co-pending application 09/104,717, C5E10 is an antibody that recognizes a glycoprotein determinant of approximately 115 kDa that appears to be specific  
10 to prostate tumor cell lines (e.g. DU145, PC3, or ND1). Thus, in conjunction with the present invention, polypeptides (e.g. CH2 domain-deleted antibodies) that specifically bind to the same tumor associated antigen recognized by C5E10 antibodies could be produced and used in a conjugated or unconjugated form for the treatment of neoplastic disorders. In particularly preferred embodiments, the modified antibody will be derived or comprise all  
15 or part of the antigen binding region of the C5E10 antibody as secreted from the hybridoma cell line having ATCC accession No. PTA-865. The resulting modified antibody could then be conjugated to a radionuclide as described below and administered to a patient suffering from prostate cancer in accordance with the methods herein.

In general, the disclosed invention may be used to prophylactically or  
20 therapeutically treat any neoplasm comprising an antigenic marker that allows for the targeting of the cancerous cells by the binding molecule. Exemplary cancers that may be treated include, but are not limited to, prostate, gastric carcinomas such as colon, skin, breast, ovarian, lung and pancreatic. More particularly, the binding molecules of the instant invention may be used to treat Kaposi's sarcoma, CNS neoplasias (capillary  
25 hemangioblastomas, meningiomas and cerebral metastases), melanoma, gastrointestinal and renal sarcomas, rhabdomyosarcoma, glioblastoma (preferably glioblastoma multiforme), leiomyosarcoma, retinoblastoma, papillary cystadenocarcinoma of the ovary, Wilm's tumor or small cell lung carcinoma. It will be appreciated that appropriate polypeptides may be derived for tumor associated antigens related to each of  
30 the forgoing neoplasias without undue experimentation in view of the instant disclosure. Exemplary hematologic malignancies that are amenable to treatment with the disclosed invention include Hodgkins and non-Hodgkins lymphoma as well as leukemias, including ALL-L3 (Burkitt's type leukemia), chronic lymphocytic leukemia (CLL) and monocytic cell leukemias. It will be appreciated that the compounds and methods of the present  
35 invention are particularly effective in treating a variety of B-cell lymphomas, including low grade/ follicular non-Hodgkin's lymphoma (NHL), cell lymphoma (FCL), mantle cell lymphoma (MCL), diffuse large cell lymphoma (DLCL), small lymphocytic (SL) NHL, intermediate grade/ follicular NHL, intermediate grade diffuse NHL, high grade

immunoblastic NHL, high grade lymphoblastic NHL, high grade small non-cleaved cell NHL, bulky disease NHL and Waldenstrom's Macroglobulinemia. It should be clear to those of skill in the art that these lymphomas will often have different names due to changing systems of classification, and that patients having lymphomas classified under  
 5 different names may also benefit from the combined therapeutic regimens of the present invention. In addition to the aforementioned neoplastic disorders, it will be appreciated that the disclosed invention may advantageously be used to treat additional malignancies bearing compatible tumor associated antigens.

Besides neoplastic disorders, the polypeptides of the instant invention are  
 10 particularly effective in the treatment of autoimmune disorders or abnormal immune responses. In this regard, it will be appreciated that the polypeptide of the present invention may be used to control, suppress, modulate or eliminate unwanted immune responses to both external and autoantigens. For example, in one embodiment, the antigen is an autoantigen. In another embodiment, the antigen is an allergen. In yet other embodiments,  
 15 the antigen is an alloantigen or xenoantigen. Use of the disclosed polypeptides to reduce an immune response to alloantigens and xenoantigens is of particular use in transplantation, for example to inhibit rejection by a transplant recipient of a donor graft, e.g. a tissue or organ graft or bone marrow transplant. Additionally, suppression or elimination of donor T cells within a bone marrow graft is useful for inhibiting graft versus host disease.

In yet other embodiments the polypeptides of the present invention may be used to treat immune disorders that include, but are not limited to, allergic bronchopulmonary aspergillosis; Allergic rhinitis Autoimmune hemolytic anemia; Acanthosis nigricans; Allergic contact dermatitis; Addison's disease; Atopic dermatitis; Alopecia areata; Alopecia universalis; Amyloidosis; Anaphylactoid purpura;  
 25 Anaphylactoid reaction; Aplastic anemia; Angioedema, hereditary; Angioedema, idiopathic; Ankylosing spondylitis; Arteritis, cranial; Arteritis, giant cell; Arteritis, Takayasu's; Arteritis, temporal; Asthma; Ataxia-telangiectasia; Autoimmune oophoritis; Autoimmune orchitis; Autoimmune polyendocrine failure; Behcet's disease; Berger's disease; Buerger's disease; bronchitis; Bullous pemphigus; Candidiasis, chronic  
 30 mucocutaneous; Caplan's syndrome; Post-myocardial infarction syndrome; Post-pericardiotomy syndrome; Carditis; Celiac sprue; Chagas's disease; Chediak-Higashi syndrome; Churg-Strauss disease; Cogan's syndrome; Cold agglutinin disease; CREST syndrome; Crohn's disease; Cryoglobulinemia; Cryptogenic fibrosing alveolitis; Dermatitis herpetiformis; Dermatomyositis; Diabetes mellitus; Diamond-Blackfan syndrome;  
 35 DiGeorge syndrome; Discoid lupus erythematosus; Eosinophilic fasciitis; Episcleritis; Erythema elevatum diutinum; Erythema marginatum; Erythema multiforme; Erythema nodosum; Familial Mediterranean fever; Felty's syndrome; Fibrosis pulmonary; Glomerulonephritis, anaphylactoid; Glomerulonephritis, autoimmune; Glomerulonephritis,

- post-streptococcal; Glomerulonephritis, post-transplantation; Glomerulopathy, membranous; Goodpasture's syndrome; Granulocytopenia, immune-mediated; Granuloma annulare; Granulomatosis, allergic; Granulomatous myositis; Grave's disease; Hashimoto's thyroiditis; Hemolytic disease of the newborn; Hemochromatosis, idiopathic; Henoch-  
 5 Schoenlein purpura; Hepatitis, chronic active and chronic progressive; Histiocytosis X; Hypereosinophilic syndrome; Idiopathic thrombocytopenic purpura; Job's syndrome; Juvenile dermatomyositis; Juvenile rheumatoid arthritis (Juvenile chronic arthritis); Kawasaki's disease; Keratitis; Keratoconjunctivitis sicca; Landry-Guillain-Barre-Strohl syndrome; Leprosy, lepromatous; Loeffler's syndrome; lupus; Lyell's syndrome; Lyme  
 10 disease; Lymphomatoid granulomatosis; Mastocytosis, systemic; Mixed connective tissue disease; Mononeuritis multiplex; Muckle-Wells syndrome; Mucocutaneous lymph node syndrome; Mucocutaneous lymph node syndrome; Multicentric reticulohistiocytosis; Multiple sclerosis; Myasthenia gravis; Mycosis fungoides; Necrotizing vasculitis, systemic; Nephrotic syndrome; Overlap syndrome; Panniculitis; Paroxysmal cold hemoglobinuria;  
 15 Paroxysmal nocturnal hemoglobinuria; Pemphigoid; Pemphigus; Pemphigus erythematous; Pemphigus foliaceus; Pemphigus vulgaris; Pigeon breeder's disease; Pneumonitis, hypersensitivity; Polyarteritis nodosa; Polymyalgia rheumatic; Polymyositis; Polyncuritis, idiopathic; Portuguese familial polyneuropathies; Pre-eclampsia/eclampsia; Primary biliary cirrhosis; Progressive systemic sclerosis (Scleroderma); Psoriasis; Psoriatic arthritis;  
 20 Pulmonary alveolar proteinosis; Pulmonary fibrosis, Raynaud's phenomenon/syndrome; Reidel's thyroiditis; Reiter's syndrome, Relapsing polychondritis; Rheumatic fever; Rheumatoid arthritis; Sarcoidosis; Scleritis; Sclerosing cholangitis; Serum sickness; Sezary syndrome; Sjogren's syndrome; Stevens-Johnson syndrome; Still's disease; Subacute sclerosing panencephalitis; Sympathetic ophthalmia; Systemic lupus erythematosus;  
 25 Transplant rejection; Ulcerative colitis; Undifferentiated connective tissue disease; Urticaria, chronic; Urticaria, cold; Uveitis; Vitiligo; Weber-Christian disease; Wegener's granulomatosis and Wiskott-Aldrich syndrome.

In another embodiment, the binding molecules of the invention can be used for pretargeting applications. For example, the same advantages will be apparent in  
 30 pretargeting applications for chemotherapeutic drug delivery.

For example, in pretargeting a tumor is pretargeted with a binding construct that has affinity for the tumor-associated antigen on the one hand and for, e.g., a radiolabeled hapten on the other. The radiolabeled hapten is administered later, preferably after the binding molecule has cleared (see, e.g., Boerman et al. 2003. J. Nuclear Med.  
 35 44:400). In another example, an antibody which is non-toxic, but has been derivitized to react with a drug or prodrug that is toxic only when bound by the binding molecule. Given the biodistribution data in the instant examples, the binding molecules of the invention are

well suited to use in pretargeting applications. In one embodiment, a clearing agent could be eliminated from the pretargeting methodology by using the instant binding molecules.

This invention is further illustrated by the following examples which  
5 should not be construed as limiting. The contents of all references, patents and published patent applications cited throughout this application are incorporated herein by reference.

## 10 Examples

### Example 1: Identification of A and B Isoforms

Solutions of antibody molecules comprise two different isoforms. One form, Form A comprises heavy chain molecules that are linked via at least one disulfide  
15 linkage. The other form, Form B, comprises heavy chain molecules that are not linked via at least one disulfide linkage. Form B does not appear or appears at a very low frequency in with intact gamma 1 MAbs, such as Rituxan®. However with domain deleted (dd) constructs having a similar hinge, the frequency of Form B is much higher. These forms can be distinguished using denaturing, non-reducing SDS page. In domain  
20 deleted antibody preparations, Form A appears as a 120kDa dimer while Form B appears as a 60kDa monomer (Figure 1).

### 25 Example 2: Identification of Hinge Region Heterogeneity in CH2 Domain Deleted MAb Fragments

Hinge domains can be subdivided into three distinct regions: upper, middle, and lower hinge regions (Roux et al. J. Immunol. 1998 161:4083). Polypeptide sequences encompassing these regions for IgG1 and IgG3 hinges are shown  
30 in Table 1. The IgG3 hinge middle region contains, in addition to the two conserved cysteine residues, a 15 amino acid motif that repeats three times. Amino acid sequences from these regions were used to design synthetic IgG1/IgG3 connecting peptides. These consisted of IgG1 upper hinge residues corresponding to positions 226 through 238, an IgG1 middle hinge corresponding to positions 239 through 241, and a single IgG3  
35 middle hinge repeat motif corresponding to positions 241EE through 242 combined with either an added proline at position 243 or an added proline, alanine, proline at positions 243, 244, and 245, respectively (Kabat numbering system), followed by a flexible Gly/Ser spacer (Table 2). In addition, novel connecting peptides were designed

- 90 -

consisting of a serine amino acid residue substituted for the cysteine at positions 239 or 242 combined with either an added proline at position 243 or an added proline, alanine, proline at positions 243, 244, and 245, respectively (Kabat numbering system). Pro243Ala244Pro245 and Pro 243 connecting peptides were also made. The amino acid sequence of the parent CH2 domain deleted humanized CC49 connecting peptide beginning at the first residue of the IgG1 hinge (position 226, Kabat numbering system) to the last residue of the hinge/GlySer connecting peptide is shown in Table 2. Also shown are the various connecting peptide designs by alignment to CC49 with positions of the cysteine residues indicated in Kabat numbering system.

Table 1: IgG1, IgG3 and IgG4 Hinge Regions

<b>IgG</b>	<b>Upper Hinge</b>	<b>Middle Hinge</b>	<b>Lower Hinge</b>
<b>IgG1</b>	EPKSCDKTHT (SEQ ID NO:2 )	CPPCP (SEQ ID NO:3)	APELLGGP (SEQ ID NO:4 )
<b>IgG3</b>	ELKIPLGDTTHT (SEQ ID NO:5 )	CPRCPP (EPKSCDTPPPCPRCP) <sub>3</sub> (SEQ ID NO:6)	APELLGGP (SEQ ID NO:4)
<b>IgG4</b>	ESKYGPP (SEQ ID NO:45 )	CPSCP (SEQ ID NO:46)	APEFLGGP (SEQ ID NO:47)

Table 2: Hinge Region Connecting Peptide Sequences



TABLE 2	
Kabat hinge position:	226 227 228 229 230 232 233 236 237 238 239 240 241 241EB 241FF 241GG 241HH 241II 241JJ 241KK 241LL 241MM 241NN 241OO 241PP 241QQ 241RR 241SS 242 C P A P 243 C P A P 244 C P A P 245
IgG1 hinge sequence	E P K S C D K T H T C P P
IgG4 hinge sequence	E S K Y G P P C P S
IgG3 middle hinge sequence	C P E P K S C D T P P C P R
Connecting peptide:	Connecting peptide sequences
G1	E P K S C D K T H T C P P (Seq. ID NO:7) C GGSSSSGGSG
G1/G3/Pro243	E P K S C D K T H T C P P C P E P K S C D T P P C P R C P GGSSSSGGSG
G1/G3/Pro243/Ala244/Pro245	E P K S C D K T H T C P P C P E P K S C D T P P C P R C P A P GGSSSSGGSG
G1/Cys239Ser:Pro243	E P K S C D K T H T S P P (Seq. ID NO:10) C P GGSSSSGGSG
G1/Cys239Ser:Pro243/Ala244/Pro245	E P K S C D K T H T S P P (Seq. ID NO:11) C P A P GGSSSSGGSG
G1/Cys242Ser:Pro243	E P K S C D K T H T C P P (Seq. ID NO:12) S P GGSSSSGGSG
G1/Cys242Ser:Pro243/Ala244/Pro245	E P K S C D K T H T C P P (Seq. ID NO:13) S P A P GGSSSSGGSG
G1/Pro243/Ala244/Pro245	E P K S C D K T H T C P P (Seq. ID NO:14) C P A P GGSSSSGGSG
G1/Pro243	E P K S C D K T H T C P P (Seq. ID NO:15) C P GGSSSSGGSG
G4/G3/Pro243/Ala244/Pro245	E S K Y G P P C P S C P E P K S C D T P P C P R C P A P (Seq. ID NO:48)

Example 3. Construction of Connecting Polypeptides and Preferential Synthesis of Isoforms

Nucleic acid sequences encoding the hinge region connecting peptides  
5 shown in Table 2 were introduced into CH2 domain deleted huCC49 gene sequences  
using the Splicing by Overlap Extension (SOE) method (Horton, R.M. 1993 Methods  
in Molecular Biology, Vol 15:PCR Protocols: Current Methods and applications. Ed.  
B.A. White). Correct modifications to the hinge region were confirmed by DNA  
sequence analysis. Plasmid DNA was used to transform CHO DG44 cells for stable  
10 production of antibody protein.

CH2 domain deleted huCC49 antibodies containing the eight designed  
synthetic connecting peptides indicated in Table 2 were constructed and antibody  
produced in CHO DG44 cells. Supernatants were collected from isolated cell lines and  
concentration of antibody in the culture supernatants determined by immunoassay.  
15 Supernatants containing antibody ranging from 0 to 30 ng of total antibody protein from  
each cell line was analyzed by non-reducing SDS-PAGE electrophoresis followed by  
Western Blot with anti-human kappa HRP conjugated antibody to detect CH2 domain  
deleted huCC49 Form A and Form B isoforms. Under these conditions, Form A  
migrates as a single 120 kDa homodimer and Form B as a 60 kDa doublet. Also visible  
20 are kappa chain monomer and dimers. Connecting peptides shown in SEQ ID NOs: 8,  
9, 14, and 15 were all found to increase the proportion of Form A produced.

Table 3. The percentage of Form A antibody after affinity chromatography (Protein G) and after HIC purification

CH2 domain deleted Antibody	% Form A Antibody	
	After Protein G	After HIC purification
HuCC49 (connecting peptide SEQ ID NO:7)	60	98
HuCC49 PAP (connecting peptide SEQ ID NO:14)	83	98
HuCC49 V2 PAP (connecting peptide SEQ ID NO:14)	90	99
HuCC49 G1/G3/PAP (connecting peptide SEQ ID NO:9)	98	Not done
HuCC49 V2 G1/G3/PAP (connecting peptide SEQ ID NO:9)	96	Not done

5                    These data show that novel, engineered synthetic hinge region connecting peptides can be used to preferentially favor the formation of the A or B isoform. These studies also reveal the importance of the cysteine residues at position 242 (Kabat numbering system) in synthesizing the CH2 domain-deleted antibody Form A isoform. Substituting the cysteine at either position 239 or 242 with serine (e.g., using connecting

10 peptides shown in SEQ ID NOs:10, 11, 12, or 13) shifts CH2 domain-deleted antibody biosynthesis to the Form B isoform. Accordingly, in one embodiment, a connecting peptide of the invention comprises a cysteine at at least one of position 239 or 242. The use of connecting peptides which increase the proportion of Form A produced will lead to a beneficial improvement in process, yield and/or stability. These synthetic hinge

15 region connecting peptides are useful for favoring synthesis of CH2 domain deleted antibody Form A isoform for any antibody isotype, e.g., IgG1, IgG2, IgG3, or IgG4, based on the extremely high degree of homology among the CH3 domains for all four human isotypes. Including identical and conserved amino acid residues, IgG1 CH3 domain is 98.13% homologous to IgG2 CH3, 97.20% homologous to IgG3 CH3, and

20 96.26% homologous to IgG4 CH3.

Example 4: Purification of Form A and Form B from a Monoclonal Antibody Mixture Containing Both Isoforms

5 10 mL of ddCC49 supernatant was titrated with 1M Tris pH 9.0 to a final pH of 7.5. This material was filtered through a series of Sol-Vac 0.8  $\mu$  and 0.4  $\mu$  membranes. A 100 mL XK50 Protein G column was pre-equilibrated with 1xPBS at a flow rate of 80 ml/min. The titrated, filtered supernatant was loaded onto the column at 80 ml/min. Bound protein was washed with the equilibrium buffer for 2 column  
10 volumes and then eluted with 100 mM Glycine at pH 3.0. The fractions containing the ddCC49 peak were collected and immediately titrated with 1 M Tris pH 9.0 to a final pH of 7.0.

A Toso Biosep Phenyl 5PW-HR column was pre-equilibrated with 20 mM Phosphate pH 7.2; 1 M Ammonium Sulfate. The Protein G eluate was titrated to 1  
15 M Ammonium Sulfate using a 3.5 M Ammonium Sulfate pH 7.2 stock and loaded at a concentration of 2 mg/ml of gel bed. Bound protein was washed with a 20mM Phosphate pH 4 or 7.2 Ammonium Sulfate to adjust the conductivity to 116.4 mS/cm. The material eluted from this condition has an apparent molecular weight about 120 kD (Form A) on a non-reducing SDS-PAGE. The remaining bound antibody was further  
20 eluted with a linear gradient of reducing Ammonium Sulfate content in the Phosphate buffer. This method separates Forms A and B in two separate peaks. The latter eluted antibody apparently lacks the disulfide linkage between the heavy chains and its molecular weight is about 60 kDa (Form B).

Both of the above purified materials can be recaptured by bringing the  
25 ammonium sulfate concentration to 1M and reloading it onto the cleaned Phenyl 5PW-HR column. Bound protein is eluted with 20mM Phosphate pH 7.2 and dialyzed into 1 x PBS.

30 Example 5. Comparison of Stability of Form A and Form B

The biologic activity of Forms A and B (as measured in preliminary experiments e.g., using direct binding or competition studies) revealed that Forms A and B have similar biologic activity.

The stability of Forms A and B was also compared. Purified ddCC49  
35 molecules were concentrated to about 5 mg/ml by Amicon concentrator fitted with YM30 membrane (Millipore). The concentrated materials were equally divided into four portions for each isoforms and each fraction was put into 10K dialysis cassette (Pierce, cat# 66410) for 16 h dialysis in the following buffers: 1) 10 mM Sodium Phosphate,

pH3; 2) 10 mM Sodium acetate, pH 5; 3) 10 mM Sodium Phosphate, pH 7; and 4) 10 mM Sodium Borate, pH 9. After dialysis, the protein concentration of each solution was adjusted to 3 mg/ml. In addition to the pure A and B isoform solution, a portion of A and B solutions from each pH were mixed to create a mixture containing 50% each isoform. Total of 12 formulations were created (four pH levels times 3 antibody solutions). The solutions were filtered and filled in 3 ml Type-1 glass serum vials (West Pharmaceuticals) with gray butyl stopper.

Three temperatures, 2-8°C, 20-25°C, and 38-42°C were chosen to store the protein solutions for stability testing. Prior to storage, 500 µl samples were drawn from each formulation for physical and chemical analyses, these zero-time point data were referred to as control. Once in storage, samples were drawn at the following schedule, 2 weeks, 1 month, 2 months and 3 months and submitted for testing immediately.

To evaluate the physical and chemical stability of the two isoforms, the following methods were used: turbidity measured at OD<sub>320</sub>, non-reducing SDS-PAGE, and size-exclusion chromatography.

Non-reducing SDS-PAGE was performed on for samples stored at 2-8°C, 20-25°C and 38-42°C for various time points. Both A and B form are relatively stable at pH 5 when stored at 2-8°C. However, when formulated at pH 7 and 9, both A and B forms showed degradation as indicated by increasing in number of bands that were smaller than the original major bands (120 kDa for form A and 60 kDa for form B). It was noticed that, particularly for pH 7 and 9 samples stored at low and intermediate temperatures, the intensity and number of bands that were less than 55 kDa were higher in B-isoform than A. This indicated that under these conditions the A-isoform is more stable than B-isoform. However, this seems not to be the case for A-isoform in pH 5 and stored at 20-25°C. This sample seemed to have more fragments than B-isoform. This appears to have been an artifact due to microbial contamination (discussed in more detail below). At high storage temperature, both forms at pH 9 were significantly degraded and there was almost no difference in gel patterns among the samples. Under this condition, trace amount of smearing bands showed up at top of the gel which indicated the formation of aggregates. Because aggregates could be dissolved by SDS, the aggregation was investigated using the methods described in the following sections.

Table 4A through Table 4C list the turbidity data for ddCC49 stored at three different temperatures. The turbidity measures both the soluble and non-soluble aggregates and it is based on the amount of light scattered by these particles. When present, aggregates will scatter light and result in an increase in A<sub>320</sub>. As showed in Table 4A-C, the turbidity of ddCC49 molecules stored at 2-8°C increases as pH

increased for both A and B isoforms, with the former being less turbid than the latter. This trend held true for samples stored for less than a month at higher temperatures (20-25°C and 38-40°C). As storage time reached 3 months, the turbidity increased significantly for samples at high pH and temperature, and the difference between A and B forms diminished. These results parallel those of SDS-PAGE and indicate that both isoforms are relatively stable (in terms of not forming aggregates) at pH 3 and 5, and that A-isoform is less susceptible to aggregation than the B isoform.

Table 4A. Turbidity measured at  $A_{320}$  for ddCC49 samples stored at 2-8°C.

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
0	0.030	0.038	0.044	0.056	0.034	0.042	0.046	0.066	0.036	0.042	0.051	0.061
½	0.029	0.029	0.046	0.045	0.030	0.038	0.048	0.058	0.034	0.033	0.043	0.055
1	0.033	0.039	0.035	0.055	0.033	0.035	0.044	0.059	0.032	0.040	0.039	0.066
2	0.042	0.022	0.042	0.044	0.039	0.037	0.055	0.067	0.042	0.024	0.040	0.058
3	0.035	0.047	0.051	0.050	0.038	0.041	0.066	0.081	0.027	0.048	0.051	0.065

10

Table 4B. Turbidity measured at  $A_{320}$  for ddCC49 samples stored at 20-25°C

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
½	0.031	0.032	0.056	0.066	0.039	0.034	0.064	0.083	0.034	0.039	0.060	0.071
1	0.025	0.043	0.055	0.090	0.034	0.042	0.070	0.084	0.028	0.039	0.055	0.094
2	0.034	0.053	0.077	0.113	0.046	0.032	0.090	0.087	0.037	0.038	0.066	0.108
3	0.036	0.056	0.156	0.143	0.029	0.060	0.121	0.125	0.044	0.050	0.101	0.142

Table 4C. Turbidity measured at A<sub>320</sub> for ddCC49 samples stored at 38-42°C

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
½	0.041	0.042	0.068	0.063	0.041	0.044	0.080	0.067	0.041	0.039	0.070	0.064
1	0.041	0.043	0.071	0.065	0.036	0.040	0.079	0.069	0.032	0.048	0.078	0.070
2	0.047	0.030	0.066	0.060	0.046	0.045	0.087	0.082	0.051	0.034	0.078	0.079
3	0.058	0.051	0.098	0.105	0.046	0.057	0.101	0.157	0.056	0.057	0.101	0.126

Size exclusion chromatography (SEC) is a powerful method for revealing the percent of intact molecules and the degraded products (both fragments and soluble aggregates) and is highly reproducible. In Table 4A-C the percent of intact monomer of A-isoform, B-isoform and the mixture stored at different temperatures are listed. For samples stored at 2-8°C, it is clear that Form A has a higher percentage of monomer as compared to Form B, and the mixture of Form A and Form B was somewhere in between. At this storage temperature, both forms were relatively stable at pH 3, 5 and 7 (with pH 5 being the most stable condition) for about three months. However, at pH 9 there was a significant decrease in percentage of monomer for Form B but only a slight decrease for Form A.

At elevated temperatures, all samples showed a significant decrease in percent of monomer as storage time increased; the A-isoform outperformed the B-isoform. However there was an exception, the sample of A-isoform in pH 5 stored at room temperature exhibited much more degradation than the B-isoform or the mixture under similar storage conditions. A close examination of this particular A-isoform vial, the data from SDS-PAGE, and SEC of the sample suggested that microbial contamination might have caused this unexpected result. First, both the SEC and SDS-PAGE results indicated that the degradation for this sample was primarily accounted for by a increase in fragmentation, presumably resulting from microbial digestion, otherwise some degree of increase in aggregation would have been expected. Second, the fact that the mixture sample, which contained 50% each of A and B-isoform, showed a better stability profile than B-isoform indicating that a more stable A-isoform must have contributed to the higher percent of monomer. Finally, A-isoform in pH 5 stored at 2-8°C and 38-42°C both showed higher percent of monomer than B-isoform under similar conditions. Therefore, intermediate storage temperature should have yielded similar results. Due to the limited amount of sample, an assay for microbial contamination could not be performed.

It was also noted that for both isoforms of IDEC-159 stored in high pH (9) and at 40°C, the percent of monomer reduced to about 30%. Under these severe conditions, the stability differences between the two isoforms disappeared. This SEC result mirrors of the results found using SDS-PAGE. Both results indicate that, although some chemical and physical characteristics differ between the two isoforms, the mechanism and by-products of degradation for both isoforms are similar, if not identical.

In summary, the SEC results indicate that both A and B-isoforms have optimal pH at about 5, and that A-isoform is more stable than B-isoform in terms of retaining higher percent of intact monomer at similar storage conditions.

Table 5A. Percent of monomer for ddCC49 samples stored at 2-8°C.

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
0	98.81	99.13	98.16	97.93	97.02	97.70	96.88	93.51	97.83	98.27	97.44	95.81
½	98.98	99.16	98.25	98.00	97.15	97.87	96.96	91.95	98.15	98.49	97.68	95.59
1	98.80	99.20	97.99	97.11	97.02	97.81	96.62	88.99	98.04	98.45	97.41	94.45
2	98.74	99.01	98.00	95.67	97.15	97.69	95.50	84.84	98.06	98.34	96.81	92.17
3	98.28	98.89	97.88	95.31	96.69	98.14	95.37	85.98	97.61	98.15	96.65	89.90

Table 5B. Percent of monomer for ddCC49 samples stored at 20-25°C

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
½	97.83	99.04	97.12	93.65	95.84	97.62	93.71	79.61	96.75	98.30	95.37	87.67
1	96.60	96.63	95.65	88.09	94.38	97.23	90.69	72.26	95.36	97.99	93.05	80.92
2	93.62	92.79	93.17	80.06	91.71	96.96	85.51	66.53	92.78	97.51	89.33	73.91
3	92.81	89.56	x	74.31	89.30	96.04	82.57	63.25	90.46	97.02	86.80	69.36

Table 5C. Percent of monomer for ddCC49 samples stored at 38-42°C

Time (month)	A-isoform				B-isoform				Mixture			
	pH=3	5	7	9	3	5	7	9	3	5	7	9
½	86.31	97.50	85.06	66.42	79.85	94.29	69.68	63.64	82.09	95.70	76.24	63.95
1	78.71	95.19	73.77	51.55	66.73	89.37	54.70	50.10	68.53	92.02	62.93	49.28
2	66.64	91.63	60.45	38.43	60.29	81.08	42.98	37.09	61.33	85.81	51.08	36.68
3	57.87	86.99	52.82	30.81	43.61	74.23	36.68	29.73	46.75	80.93	44.35	30.18



## Example 7. Preparative Purification of Forms A and B

IDEC-159 (ddCC49) is a CH2 domain deleted monoclonal antibody directed against TAG-72 antigen, which is expressed on the surface of tumors. IDEC-159 contains two isoforms of the antibody, called Form A and Form B. The current cell culture process for IDEC-159 produces an approximate 50:50 ratio of Form A to Form B. The Form A isoform is an antibody with a deleted CH2 region in the F<sub>C</sub> portion of the heavy chain. In addition to having a deleted CH2 region, Form B also lacks the disulfide bond linkage across the F<sub>C</sub> region and is only held together by hydrophobic interactions and salt bridges.

The third and final chromatography step in the IDEC-159 purification process was developed to separate the two isoforms of IDEC-159. The separation is achieved by hydrophobic interaction chromatography (HIC), using a Phenyl TSKgel 5PW-HR adsorbent. Since form B is more hydrophobic than form A, it adsorbs irreversibly to the stationary phase using approximately 0.73 M Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0 – pH 7.0 as the mobile phase. Form A binds to a lesser extent to the stationary phase under these conditions and is therefore eluted isocratically, i.e. it leaves the column with the flowthrough fraction. Subsequent to the isocratic elution of Form A, omitting Ammonium sulfate from the mobile phase desorbs Form B. The following method was used to separate the two isoforms of IDEC-159:

- The column was sanitized using  $\geq 3$  CVs of 0.5 N NaOH, at  $\leq 150$  cm/hr.
- The column was equilibrated using  $\geq 5$  CVs of 0.73 M Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0, at  $\leq 150$  cm/hr.
- The column was loaded with room temperature TMAE Flowthrough that has been adjusted to include 0.43 volumes of 2.5 M Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0 liquid stock solution, at 5 mg per ml of resin.
- The antibody was loaded onto the column at pH 4.0, at  $\leq 100$  cm/hr.
- Collection of the antibody started when the outlet O.D. at 280 nm reaches 10 mAU.
- The column was washed using 15 CVs of 0.73 M Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0, at  $\leq 100$  cm/hr.
- Continue antibody collection throughout the 15 CV wash, then the outlet was diverted back to waste.
- The column was stripped using  $\geq 5$  CVs of 20 mM Sodium Phosphate, pH 4.0, at  $\leq 100$  cm/hr. 6. The column was cleaned with  $\geq 3$  CVs 0.5 N NaOH, at  $\leq 150$  cm/hr.
- The column was equilibrated with  $\geq 3$  CVs of 0.73 M Ammonium Sulfate / 20 mM Sodium Phosphate, pH 4.0, at  $\leq 150$  cm/hr.
- The column was stored in  $\geq 3$  CVs of 20% Ethanol, at  $\geq 150$  cm/hr.

The separation of the two forms at a preparative scale (5L column volume, total IDEC-159 load approximately 20 g) is shown in Figures 13 (A and B). The first two peaks comprise the isocratic elution of Form A, the second peak shows the  
 5 eluted Form B, while the third peak contains impurities, which are removed from the stationary phase during cleaning.

The capability of this method to separate Forms A and B at preparative scale was also demonstrated by SDS PAGE.

#### 10 Example 8. Preparation of a CH2 Domain-Deleted Tetravalent Antibody

HuCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody design was based on appending a huCC49 single chain Fv (scFv) to the carboxyl terminus of the HuCC49 CH2 domain-deleted antibody CH3 domain. A schematic diagram of HuCC49  
 15 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody is shown in Figure 4. An equivalent reference to this design is C-scFv Tetravalent CH2 Domain Deleted antibody. The preparation of single polypeptide chain binding molecules of the Fv region, single-chain Fv molecules, is described in U.S. Pat. No. 4,946,778. A CC49 scFv immunoglobulin-like antibody is described in U.S. Pat. No. 5,892,019. The huCC49 scFv is comprised  
 20 of a VL and a VH region sequence tethered by a short synthetic linker (VL → (Gly<sub>4</sub>Ser)<sub>3</sub> linker → VH orientation) and was synthesized by PCR amplification. The 5' VL PCR primer included a Bam HI restriction endonuclease site followed by sequence encoding a (Gly<sub>4</sub>Ser)<sub>2</sub> linker peptide. The 3' VL PCR primer included sequence partially encoding a (Gly<sub>4</sub>Ser)<sub>3</sub> linker peptide used to connect the two VL and VH regions. The 5' VH PCR  
 25 primer also included sequence partially encoding a (Gly<sub>4</sub>Ser)<sub>3</sub> linker peptide used to connect the VL and VH regions. Finally, the 3' VH PCR primer included a stop codon followed by a Bam HI site. The two V regions were amplified with the two sets of PCR primers from plasmid DNA substrate containing huCC49 CH2 domain-deleted antibody and assembly of the scFv was accomplished in a second PCR reaction through the  
 30 common overlapping sequences encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker. The huCC49 scFv gene fragment was gel isolated, digested with Bam HI restriction endonuclease and cloned into a single Bam HI site previously introduced into the huCC49 CH2 domain-deleted antibody Polycistronic expression vector. (See US 2003 0157641 A1.). Briefly, the vector was modified by removing an existing stop codon at the 3' end of the gene coding  
 35 for the CH3 domain and replacing with nucleotides coding for the amino acid sequence Ser-Gly-Gly-Gly immediately followed by a Bam HI restriction endonuclease site (coding for Gly-Ser). The Bam HI digested huCC49 scFv fragment was cloned into the Bam HI site of the vector resulting in a fusion product of the huCC49 scFv to the

carboxyl terminus of the huCC49 CH2 domain-deleted antibody CH3 domain through a 16 amino acid Ser(Gly<sub>4</sub>Ser)<sub>3</sub> linker. Correct sequences were confirmed by DNA sequence analysis. Plasmid DNA was used to transform CHO DG44 cells for stable production of antibody protein. The engineered antibody was designated huCC49 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody. Figure 8A shows the DNA sequence of heavy chain huCC49 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody. Figure 8C shows the DNA sequence of light chain huCC49 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody. Figure 9A shows the amino acid sequence of heavy chain huCC49 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody.

Supernatants were collected from isolated cell lines and concentration of antibody in the culture supernatants determined by immunoassay. Supernatants were analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with anti-human kappa- HRP conjugated antibody to detect huCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody Form A and Form B isoforms. Under these conditions, Form A was observed to migrate as a single ~170 kDa homodimer and Form B as a ~85 kDa doublet in approximately a 50:50 ratio of A to B isoform. As shown in Figure 18, five individually isolated clones were all found to produce an A and B CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody isoform. One of the clones was selected for antibody production and purification using HIC chromatography as described in Example 4. Purified huCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody Forms A and B were analyzed by non-reducing and reducing SDS-PAGE and the results are shown in Figure 19. Form A huCC49 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody was effectively separated from Form B in excess of 95% purity. As expected, both purified Forms behaved identically under reducing SDS-PAGE conditions. HuCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody Form A was examined by size exclusion chromatography and found to elute primarily as a single peak (96%) indicating that there was no significant aggregation or decomposition of antibody product (Figure 20).

#### Example 9. Biodistribution Profile of HuCC49 CH2 Domain-Deleted Tetravalent Antibody.

HuCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody Form A was tested in a competitive binding assay for its ability to bind to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved fluometric immunoassay using a Delphia fluorimeter (Wallac Inc., Gaithersburg, MD). Competitive binding curves are shown in Figure 21. HuCC49 CH2 domain-deleted sc(Fv)<sub>2</sub> tetravalent antibody Form A and control parent CH2 domain-deleted huCC49 (referred to as HuCC49 or IDEC 159) antibodies were evaluated. Relative binding activity for the

SUBSTITUTE SHEET 102

AMENDED SHEET

tetravalent antibody was found to be 5-fold more avid than the control parent CC49 antibody (GraphPad Prism 4.0 for Windows, GraphPad, Software, San Diego California USA. [www.graphpad.com](http://www.graphpad.com)), consistent with an expected increase in the number of antigen binding sites.

- 5 Biodistribution of  $^{90}\text{Y}$ - radiolabeled huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody was compared to results previously generated with control parent huCC49 antibody in athymic nude mice bearing LS-174T human tumor xenografts. HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody was conjugated with the chelator Chx-DTPA and binding activity assessed. Conjugated huCC49 CH2
- 10 domain-deleted sc(Fv)2 tetravalent antibody showed a 5-6 fold higher avidity than the conjugated control parent huCC49 demonstrating that the tetravalent antibody could be derivatized without losing significant binding activity. HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody was labeled with  $^{90}\text{Y}$  and a single dose of radiolabeled antibody was administered by i.v. tail vein injection to mice presenting pre-established
- 15 tumors of approximately  $250\text{ mm}^3$ . Samples were harvested and processed for beta counting. Percentage injected dose (%ID) of  $^{90}\text{Y}$  radiolabeled antibody per gram of tumor or normal tissue was determined from 3 to 72 hours and is shown in Table 6.

Table 6.

5-7 mice/group					
% ID/gm-tissue					
	Blood	Spleen	Kidney	Liver	Tumor
HuCC49 (domain deleted)					
3-5 hrs	18.24	5.39	9.15	8.83	13.66
12 hrs	3.21	5.01	15.50	14.20	17.33
24 hrs	1.03	6.21	14.09	10.84	20.53
48 hrs	0.17	6.92	11.15	11.85	13.17
72 hrs	0.10	6.22	9.01	10.59	10.55
Tetravalent HuCC49					
3 hrs	20.3	12.60	7.60	9.60	10.80
6 hrs	15.4	12.20	6.70	12.40	12.10
12 hrs	7.20	15.10	5.10	13.90	13.10
24 hrs	2.40	9.50	3.90	12.10	14.00
48 hrs	0.50	10.00	2.60	11.90	11.50
72 hrs	0.10	**	1.90	12.70	6.40

Data represent mean values +/- standard errors.

\*\* technical problem with sample

HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody showed lower accumulation in the kidney than control huCC49 antibody, likely due to the increased molecular mass of the antibody. Tumor accumulation of huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody was similar  
 5 to that achieved with the control parent huCC49 (Table 6). However, as mentioned, these two biodistribution studies were not conducted simultaneously and are subject to experimental variation. A figure showing tumor retention for control parent huCC49 and huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody as measured by %ID/gm is shown in Figure 35. Figure 35 also shows the same tumor retention data normalized to  
 10 peak accumulation of radioactivity. AUC analysis of the two normalized retention curves in Figure 35 was calculated using GraphPad Prism 4.0 for Windows, GraphPad, Software, San Diego California USA. [www.graphpad.com](http://www.graphpad.com). Control parent huCC49 had a Total Peak Area of 2037 units and huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody had a Total Peak Area of 2562 units representing a 25.8% increase in area for  
 15 the tetravalent protein. Representation of the data in this format suggests that there is improved retention of tetravalent antibody in the tumor compartment compared to the parent divalent molecule.

The tetrameric HuCC49 construct affords certain advantages for pretargeting, e.g., radioimmunotherapeutic (RIT) pretargeting. This advantage is  
 20 defined by three observations derived from Table 6. First, the tetrameric HuCC49 CH2 domain-deleted construct has a blood clearance rate comparable to that of the current CH2 domain-deleted HuCC49 construct. This fast blood clearance may obviate the need for a "clearing agent" to expedite removal of the antibody from the blood prior to administration of the radiolabeled ligand that localizes to the tumor-bound antibody.  
 25 Elimination of the clearing agent significantly reduces the complexity of this modality of treatment in the clinical setting. Second, tumor retention for the tetrameric construct is comparable to the current dimeric construct. Therefore, radiation dose delivered will be comparable for the two constructs ((Table 6). Third, the lower kidney and liver uptake compared to the CH2 domain-deleted HuCC49 may enable larger input doses of the  
 30 radiolabeled ligand before reaching organ-limiting toxicity.

#### Example 10. Preparation of a HuCC49 CH2 Domain-Deleted Tetravalent Antibody Comprising a Novel Connecting Peptide and Preferential Synthesis of Isoform A

35 A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody similar to the design shown in the bottom half of Figure 4 but containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] synthetic connecting peptide indicated in Table 2 was constructed. Briefly, gene sequences coding for partial

G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide was synthesized by PCR amplification using a 5' connecting peptide PCR primer encoding a Sal I restriction endonuclease site and a 3' connecting peptide PCR primer encoding an Xho I site. Plasmid DNA containing the gene sequence coding for the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide was used as substrate. The PCR product encodes all of G1/G3/Pro243Ala244Pro245 and part of [Gly/Ser] connecting peptide. The G1/G3/Pro243Ala244Pro245 hinge fragment was gel isolated, digested with Sal I and Xho I restriction endonucleases and cloned into the Sal I and Xho I vector sites reconstituting the full length G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Correct sequences were confirmed by DNA sequence analysis. Plasmid DNA was used to transform CHO DG44 cells for stable production of antibody protein. Figure 8B shows the DNA sequence of heavy chain huCC49 domain-deleted sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 8C shows the DNA sequence of light chain huCC49 domain-deleted sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 9B shows the amino acid sequence of heavy chain huCC49 domain-deleted sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide.

Supernatants were collected from isolated cell lines and concentration of antibody in the culture supernatants determined by immunoassay. Supernatants were analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with anti-IgG- HRP conjugated antibody to detect huCC49 CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain-deleted) antibody Form A and Form B isoforms. Under these conditions, Form A is expected to migrate as a single ~170 kDa homodimer and Form B as a ~85 kDa doublet in approximately a 50:50 ratio of A to B isoform. As shown in Figure 22, a representative CH2 domain-deleted sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide was found to produce all Form A tetravalent antibody isoform.

A cell line containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] (SEQ ID NO:9) connecting peptide introduced into the huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody sequence was used for antibody production. Antibody was produced and purified using methods described in Example 4 above. HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] (SEQ ID NO:9) connecting peptide was purified using only the Protein G column, eluted essentially as a single peak at 96% purity without further HIC purification. Reduced and non-reduced purified protein samples were analyzed by SDS-

SUBSTITUTE SHEET 105

AMENDED SHEET

PAGE electrophoresis. Under non-reducing conditions, Form A is expected to migrate as a single 170 kDa homodimer and Form B as a 85 kDa doublet. The connecting peptide shown in SEQ ID NO: 9 was found to substantially increase the proportion of Form A produced. Exemplary results are shown in Figure 23. This result shows that the

5 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge (SEQ ID NO:9) resulted in the production of essentially all Form A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody with little or no detectable Form B, demonstrating that the utility of this hinge for producing the Form A isoform is generally applicable to complex antibodies such as multivalent antibodies. It is clear that

10 this invention is also applicable to bispecific tetravalent antibody formats as well. HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 antibody was examined by size exclusion chromatography and found to essentially elute as a single peak containing 84% monomer. The fraction containing the residual aggregates was removed by preparative size exclusion chromatography with the

15 resulting preparation containing essentially 95% monomer (Figure 24) HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 antibody was tested in a competitive binding assay for its ability to bind to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved fluoroimmunoassay using a Delphia fluorimeter (Wallac Inc., Gaithersburg, MD). Competitive

20 binding curves are shown in Figure 25. HuCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody Form A and control parent CH2 domain-deleted huCC49 (referred to as HuCC49 or IDEC 159) antibodies were evaluated. Relative binding activity for the tetravalent antibody was found to be 8-fold more avid than the control parent HuCC49 antibody (GraphPad Prism 4.0 for Windows, GraphPad, Software, San Diego California

25 USA. [www.graphpad.com](http://www.graphpad.com)), consistent with an expected increase in the number of antigen binding sites.

These results show that the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide (SEQ ID NO:9) resulted in the production of essentially all Form A huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody with no detectable Form B.

30 The purified antibody demonstrated an increase in avidity towards antigen.

#### Example 11. Preparation of a CH2 Domain-Deleted Tetravalent Antibody

A huCC49 minibody and a huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody) as shown in Figure 2 were both constructed. Briefly, huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody) design was based on inserting a huCC49 single chain Fv (scFv) between the carboxyl end of the first scFv domain and the amino-end of the hinge connecting peptide in the huCC49 minibody.

The preparation of single polypeptide chain binding molecules of the Fv region, single-chain Fv molecules, is described in U.S. Pat. No. 4,946,778. A CC49 scFv immunoglobulin-like antibody is described in U.S. Pat. No. 5,892,019. The preparation of a minibody construct is described in U.S. Pat. No. 5,837,821. The huCC49 scFv is comprised of a VL and a VH region sequence tethered by a short synthetic linker (VL → (Gly<sub>4</sub>Ser)<sub>3</sub> linker → VH orientation) and was synthesized by PCR amplification and used to construct a huCC49 minibody. Second, the minibody vector was modified by adding a modified (Gly<sub>4</sub>Ser)<sub>5</sub> linker to the carboxyl end of the amino-terminal scFv domain of the huCC49 minibody by PCR amplification, replacing nucleotides coding for the last two amino acids residues of the linker with an Nhe I restriction endonuclease site. This results in a (Gly<sub>4</sub>Ser)<sub>4</sub>-Gly<sub>3</sub>-Ala-Ser linker followed by a Sal I restriction endonuclease site separated by several nucleotides. A second huCC49 scFv was amplified using PCR from plasmid DNA substrate containing huCC49 scFv gene with the 5' VL PCR primer encoding an Nhe I restriction endonuclease site and the 3' VH PCR primer encoding a Sal I site. The huCC49 scFv fragment was gel isolated, digested with Nhe I and Sal I restriction endonucleases and cloned into Nhe I and Sal I sites between the first scFv and the hinge connecting peptide. This results in a fusion product consisting of a leader peptide to two serial huCC49 scFvs to a hinge connecting peptide to a CH3 domain. Correct sequences were confirmed by DNA sequence analysis.

Plasmid DNA was used to transform CHO DG44 cells for stable production of antibody protein. Figure 10A shows the DNA sequence of huCC49 tetravalent (N-scFv tetravalent) minibody gene. Figure 11A shows the amino acid sequence of huCC49 tetravalent (N-scFv tetravalent) minibody.

Supernatants were collected from isolated cell lines and concentration of antibody in the culture supernatants determined by immunoassay. Supernatants were analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with anti-IgG- HRP conjugated antibody to detect heavy chain constant sequences in the Form A and Form B isoforms. Under these conditions, huCC49 minibody Form A is expected to migrate as a single ~82 kDa homodimer and half molecules or Form B as a ~41 kDa doublet. HuCC49 2 sc(Fv)<sub>2</sub> tetravalent minibody Form A is expected to migrate as a single ~138 kDa homodimer and Form B as a ~69 kDa doublet in approximately a 50:50 ratio of A to B isoform. By comparison, huCC49 CH2 domain deleted antibody (expected to migrate as a single ~120 kDa homodimer and Form B as a ~60 kDa doublet in approximately a 50:50 ratio of A to B isoform) was used as control.

As shown in Figure 26, representative isolated minibody and 2 sc(Fv)<sub>2</sub> tetravalent minibody clones were found to secrete both A and B isoforms consistent with the expected molecular masses under both non-reducing and reducing SDS-PAGE conditions.



Example 12. Preparation of a huCC49 CH2 Domain-Deleted Tetravalent Antibody  
Comprising a Novel Connecting Peptide and Preferential Synthesis of Isoform A

- 5                   A huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv tetravalent minibody)  
similar to the design shown in the bottom of Figure 2 but containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide indicated in Table 2 was  
constructed. Briefly, gene sequences coding for partial G1/G3/Pro243Ala244Pro245 +  
[Gly/Ser] connecting peptide was synthesized by PCR amplification using a 5'  
10 connecting peptide PCR primer encoding a Sal I restriction endonuclease site and a 3'  
connecting peptide PCR primer encoding an Xho I site. Plasmid DNA containing the  
gene sequence coding for the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting  
peptide was used as substrate. The PCR product encodes all of  
15 G1/G3/Pro243Ala244Pro245 and part of [Gly/Ser] connecting peptide. The  
G1/G3/Pro243Ala244Pro245 hinge fragment was gel isolated, digested with Sal I and  
Xho I restriction endonucleases and cloned into the Sal I and Xho I vector sites  
reconstituting the full length G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting  
peptide. Correct sequences were confirmed by DNA sequence analysis. Plasmid DNA  
was used to transform CHO DG44 cells for stable production of antibody protein. Figure  
20 10B shows the DNA sequence of huCC49 2 sc(Fv)2 tetravalent antibody (N-scFv  
tetravalent minibody) gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser]  
connecting peptide. Figure 11B shows the amino acid sequence of huCC49 2 sc(Fv)2  
tetravalent antibody (N-scFv tetravalent minibody) gene containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide.  
25                   Supernatants were collected from isolated cell lines and concentration of  
antibody in the culture supernatants determined by immunoassay. Supernatants were  
analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with  
anti-IgG- HRP conjugated antibody to anti-IgG- HRP conjugated antibody to detect  
huCC49 2 sc(Fv)2 tetravalent minibody (N-scFv tetravalent minibody) Form A and  
30 Form B isoforms. Under these conditions, huCC49 2 sc(Fv)2 tetravalent minibody  
Form A is expected to migrate as a single ~138 kDa homodimer and Form B as a ~69  
kDa doublet in approximately a 50:50 ratio of A to B isoform. As shown in Figure 27, a  
representative huCC49 2 sc(Fv)2 tetravalent minibody containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide was found to produce  
35 essentially all Form A tetravalent minibody isoform.

A cell line containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] (SEQ  
ID NO:9) connecting peptide introduced into the huCC49 2 sc(Fv)2 tetravalent  
minibody sequence was used for antibody production. Because huCC49 2 sc(Fv)2

tetravalent minibody lacks a CH1 domain, protein cannot be purified using Protein G chromatography. Antibody was subsequently purified using a combination of anion exchange, hydrophobic interaction, and size exclusion chromatography methods. HuCC49 2 sc(Fv)2 tetravalent minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] (SEQ ID NO:9) connecting peptide was purified essentially as a single peak at 97.6 % purity. Reduced and non-reduced purified protein samples were analyzed by SDS-PAGE electrophoresis. Under non-reducing conditions, Form A is expected to migrate as a single ~138 kDa homodimer and Form B as a ~69 kDa doublet. The connecting peptide shown in SEQ ID NO: 9 was found to substantially increase the proportion of Form A produced. Exemplary results are shown in Figure 28. This result shows that the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge (SEQ ID NO:9) resulted in the production of essentially all Form A huCC49 2 sc(Fv)2 tetravalent minibody (N-scFv tetravalent minibody) with little or no detectable Form B, demonstrating that the utility of this hinge for producing the Form A isoform is generally applicable to complex antibodies such as multivalent antibodies. It is clear that this invention is also applicable to bispecific tetravalent antibody formats as well. Purified huCC49 2 sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 minibody was examined by size exclusion chromatography and found to essentially elute as a single peak containing 97.6% monomer (Figure 29).

Purified huCC49 2 sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 minibody was tested in a competitive binding assay for its ability to bind to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved fluoroimetric immunoassay using a Delphia fluorimeter (Wallac Inc., Gaithersburg, MD). Competitive binding curves are shown in Figure 30. HuCC49 2 sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 minibody, huCC49 minibody, huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 antibody, and control parent CH2 domain-deleted huCC49 (referred to as HuCC49 or IDEC 159) antibodies were evaluated. Relative binding activities for both tetravalent antibodies were found to be more avid than the control parent huCC49 antibody or minibody (GraphPad Prism 4.0 for Windows, GraphPad, Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)), consistent with an expected increase in the number of antigen binding sites.

Example 13. Preparation of a PRIMATIZED® p5E8 CH2 Domain-Deleted Tetravalent Antibody Comprising a Novel Connecting Peptide and Preferential Synthesis of Isoform A

PRIMATIZED® p5E8G1 is a chimeric macaque/human (PRIMATIZED®) monoclonal antibody containing macaque heavy and light variable regions fused to human gamma 1 and kappa constant regions, respectively.

PRIMATIZED® p5E8G1 binds to human CD23, the low affinity receptor for IgE (FcεRII) (Mavromatis and Cheson. 2003. J. Clin. Oncol. 21:1874; US Patent Application 20030059424). CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] synthetic  
 5 connecting peptide indicated in Table 2 (SEQ ID NO:9) was constructed using a similar strategy as that described in Example 10. The PRIMATIZED® p5E8 scFv used for constructing the sc(Fv)2 tetravalent antibody is comprised of a p5E8 VL and a VH region sequence tethered by a short synthetic linker in the VL → (Gly<sub>4</sub>Ser)<sub>3</sub> linker → VH orientation and is described in greater detail in Example 12. Correct sequences were  
 10 confirmed by DNA sequence analysis. Plasmid DNA was used to transform CHO DG44 cells for stable production of antibody protein. Figure 12A shows the DNA sequence of heavy chain CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 12B shows the DNA sequence of light chain CH2 domain-deleted  
 15 PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody. Figure 13A shows the amino acid sequence of heavy chain CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 13B shows the amino sequence of light chain CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody containing the  
 20 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide.

Supernatant was collected from a stably transfected cell line and concentration of antibody in the culture supernatant determined by immunoassay. Supernatant was analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with anti-human IgG- HRP conjugated antibody to detect Form A and  
 25 Form B isoforms of CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Under these conditions, Form A is expected to migrate as a single ~170 kDa homodimer and Form B as a ~85 kDa doublet. The connecting peptide shown in SEQ ID NO: 9 was found to substantially increase the proportion of Form A tetravalent antibody produced.  
 30 Exemplary results are shown in Figure 31. These results show that the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide (SEQ ID NO:9) resulted in the production of essentially all Form A CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody with no detectable Form B, demonstrating that the utility of this hinge for producing the Form A isoform CH2 domain-deleted sc(Fv)2 tetravalent  
 35 antibodies is generally applicable to antibodies of varying specificities.

Example 14. Preparation of PRIMATIZED® p5E8 Minibodies Comprising a Novel Connecting Peptide and Preferential Synthesis of Isoform A

Minibodies are single chain polypeptides consisting of an scFv fused to immunoglobulin hinge region and CH3 domain. The preparation of minibody single polypeptide chain binding molecules is described in U.S. Pat. No. 5,837,821. Normally, in intact IgG molecules the cysteine residue at position 230 in the hinge region forms a covalent disulfide bond with the carboxyl-terminal light chain constant domain cysteine residue at position 214, joining heavy and light chain polypeptides (Kabat numbering system). In the minibody design the light chain constant domain is absent, eliminating one of the two cysteines that participate in forming the disulfide bond. The single remaining cysteine in the hinge region at position 230, however, has been proposed to participate in a homotypic interchain disulfide bond possibly contributing to hinge region structure and therefore was left in place.

Minibodies, which lack a CH2 domain, are secreted from cells and accumulate in culture supernatant as a mixture containing Form A and Form B isoforms and perhaps unassembled half molecules as shown in Figure 32. The ratio of these forms is highly variable and presents a challenge for preparing a reproducibly pure product. The absence of the immunoglobulin CH1/CL domains precludes isolation of total minibody using Protein G immunoaffinity matrices. While the unassembled half molecules (MW ~ 40-45 kD) can be separated from the homodimers (MW ~ 80-90 kD) using a number of chromatographic strategies, the remaining intact A and B isoforms cannot be efficiently separated from one another using techniques such as HIC chromatography (described herein) also due to the absence of CH1/CL domains. These isoforms also cannot be separated by size exclusion chromatography because the molecular masses of the two isoforms are nearly identical, prohibiting separation based on this characteristic. Thus the composition of minibody preparations may actually be complex, consisting of a mixture of A and B isoforms. The production of CH2 domain-deleted PRIMATIZED® p5E8 minibodies engineered to contain the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide indicated in Table 2 (SEQ ID NO:9) was examined.

PRIMATIZED® p5E8 scFvs were constructed by PCR amplification. ScFvs were built in both orientations (VL → (Gly<sub>4</sub>Ser)<sub>3</sub> linker → VH (VL/VH) and VH → (Gly<sub>4</sub>Ser)<sub>3</sub> linker → VL (VH/VL)). Oligonucleotides used in the construction are shown the tables below:

Table 7. PCR primers used to construct CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. In SEQ ID NO:37 the BspLU11 I restriction endonuclease site is underlined. In SEQ ID NO:40 the Sal I restriction endonuclease site is underlined.

5

N-23VL-1F (SEQ ID NO:37)

5'- AGAGAGACATGTGGCGACATCCAGATGACCCAGTC-3'

10 23-VL-1R (SEQ ID NO:38)

5'- GGAGCCACCCCAACCGACCCGCCACCGCCTTTGATTCCACCTTGGTCC-  
3'

23-VH-2F (SEQ ID NO:39)

15 5'-

GGGTCCGGTGGGGGTGGCTCCGGGGGCGGTGGCTCCGAGGTGCAGCTGGT  
GGAGTC-3'

N-23-VH-2R (SEQ ID NO:40)

20 5'- AGAGAGGTCGACTGAGGAGACGGTGACCAGGAC-3'

Table 8. PCR primers used to construct CH2 domain-deleted PRIMATIZED® p5E8 VH/VL minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. In SEQ ID NO:41 the BspLU11 I restriction endonuclease site is underlined. In SEQ ID NO:44 the Sal I restriction endonuclease site is underlined.

5

N-23VH-1F (SEQ ID NO:41)

5'- AGAGAGACATGTGGCGAGGTGCAGCTGGTGGAGTC-3'

23-VH-1R (SEQ ID NO:42)

10 5'-GGAGCCACCCCAACCGGACCCGCCACCGCCTGAGGAGACGGTGACCAGG  
AC-3'

23-VL-2F (SEQ ID NO:43)

5'-

15 GGGTCCGGTGGGGGTGGCTCCGGGGGCGGTGGCTCCGACATCCAGATGAC  
CCAGTC-3'

N-23-VL-2R (SEQ ID NO:44)

5'- AGAGAGGTCGACTTTGATTCCACCTTGGTCC-3

20

The VL/VH scFv was constructed in two steps. The 5' VL PCR forward primer N-23VL-1F (SEQ ID NO:37) included a BspLU11 I restriction endonuclease site to permit joining of the scFv to the immunoglobulin signal peptide in the expression vector. The 3' VL PCR reverse primer 23-VL-1R (SEQ ID NO:38) included sequence  
25 partially encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker peptide used to join the VL and VH regions. The 5' forward VH PCR primer 23-VH-2F (SEQ ID NO:30) also included sequence partially encoding a (Gly<sub>4</sub>Ser)<sub>3</sub> linker peptide used to connect the VL and VH regions and the 3' VH PCR reverse primer N-23-VH-2R (SEQ ID NO:40) included a Sal I restriction endonuclease site to join the scFv to the hinge region. The two V regions were amplified  
30 with the two sets of PCR primers from plasmid DNA substrate containing PRIMATIZED® p5E8G1 antibody and assembly of the scFv was accomplished in a second PCR reaction through the common overlapping sequences encoding the (Gly<sub>4</sub>Ser)<sub>3</sub> linker. The PRIMATIZED® p5E8 scFv gene fragment was gel isolated, digested with BspLU11 I and Sal I restriction endonucleases and cloned into the  
35 BspLU11 I and Sal I double-digested CH2 domain-deleted antibody Polycistronic expression vector containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. The PRIMATIZED® p5E8 VH/VL scFv was constructed in a similar manner using PCR primer pairs N-23VH-1F (SEQ ID NO:41) and 23-VH-1R (SEQ ID NO:36)

- 113 -

and 23-VL-2F (SEQ ID NO:43) and N-23-VL-2R (SEQ ID NO:44) shown in Tables 7 and 8. Correct sequences of both completed constructs were confirmed by DNA sequence analysis. Plasmid DNA was used to transform CHO DG44 cells for transient production of antibody protein. Figure 14 shows the DNA sequence of CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide in the VL → VH orientation (VL/VH). Figure 15 shows the DNA sequence of CH2 domain-deleted PRIMATIZED® p5E8 VH/VL minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide in the VH → VL orientation (VH/VL). Figure 16 shows the amino acid sequence of CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 17 shows the amino acid sequence of CH2 domain-deleted PRIMATIZED® p5E8 VH/VL minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide.

Supernatant was collected from the transfected cell lines and concentration of antibody in the culture supernatant determined by immunoassay. Minibody molecules present in culture supernatant were analyzed for binding to soluble CD23 antigen immobilized on plastic microtiter plates by ELISA. Results shown in Figure 33 demonstrate that both PRIMATIZED® p5E8 VH/VL and VL/VH minibodies containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide are equally capable of binding to the CD23 antigen in a dose-responsive manner.

Supernatants were analyzed by non-reducing SDS-PAGE electrophoresis followed by Western Blot with anti-human IgG- HRP conjugated antibody to detect Form A and Form B isoforms of CH2 domain-deleted PRIMATIZED® p5E8 minibodies containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Under these conditions, Form A is expected to migrate as a single ~80-90 kDa homodimer and Form B as a ~40-45 kDa doublet. The connecting peptide shown in SEQ ID NO: 9 was found to substantially increase the proportion of Form A for both minibodies produced. Exemplary results are shown in Figure 34. These results show that the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide (SEQ ID NO:9) resulted in the production of essentially all Form A CH2 domain-deleted PRIMATIZED® p5E8 minibody with no detectable Form B.

#### Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

2004252171 30 Mar 2011

The term "comprise" and variants of the term such as "comprises" or "comprising" are used herein to denote the inclusion of a stated integer or stated integers but not to exclude any other integer or any other integers, unless in the context or usage an exclusive interpretation of the term is required.

5

Any reference to publications cited in this specification is not an admission that the disclosures constitute common general knowledge in Australia.



2004252171 30 Mar 2011

The claims defining the invention are as follows:

1. A composition comprising polypeptide dimers comprising at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion and a synthetic connecting peptide comprising a chimeric hinge, wherein said synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system) and at least a portion of an IgG3 hinge region, and wherein greater than 90% of the dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.
2. A composition comprising polypeptide dimers comprising at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion from an IgG1 antibody isotype and a synthetic connecting peptide comprising a chimeric hinge, and wherein greater than 90% of the dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.
3. The composition of claim 1 or claim 2, wherein the polypeptide dimers comprise at least one binding site specific for a soluble ligand.
4. The composition of claim 1 or claim 2, wherein the polypeptide dimers comprise at least one binding site specific for a cell surface molecule.
5. The composition of claim 1 or claim 2, wherein the polypeptide dimers comprise two binding sites specific for a tumor cell antigen and two binding sites specific for a prodrug.
6. The composition of claim 5, wherein the binding sites specific for the prodrug are catalytic.
7. The composition of claim 1 or claim 2, wherein the polypeptide dimers comprise four polypeptide chains and wherein two of the polypeptide chains comprise at least one immunoglobulin heavy chain portion and a synthetic connecting peptide comprising a chimeric hinge.
8. The composition of claim 1 or claim 2, wherein the polypeptide chains have at least one binding site derived from an antibody selected from the group consisting of: 2B8, Lym 1, Lym 2, LL2, Her2, B1, MB1, BH3, B4, B72.3, CC49, 5E8, B3F6, and 5E10.

2004252171 30 Mar 2011

9. The composition of claim 1 or claim 2, wherein the polypeptide dimers are tetravalent minibody molecules.
10. The composition of claim 1 or claim 2, wherein the polypeptide dimers are tetravalent domain deleted antibody molecules.
- 5 11. The composition of claim 1 or claim 2, wherein the polypeptide dimers are diabodies.
12. A composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise an immunoglobulin heavy chain portion and a synthetic connecting peptide comprising a chimeric hinge, wherein said synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system) and at least a portion of an IgG3 hinge region and at least four binding sites,, wherein the polypeptide chains lack all or part of a CH2 domain, and wherein greater than 90% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.
- 10
13. A composition comprising minibody molecules comprising two polypeptide chains, wherein the polypeptide chains comprise at least one immunoglobulin heavy chain portion from an IgG1 antibody isotype and a synthetic connecting peptide comprising a chimeric hinge, wherein said synthetic connecting peptide comprises at least middle hinge portion of an IgG1 hinge region corresponding to positions 239 through 241 (Kabat numbering system), at least four binding sites, and at least a portion of an IgG3 hinge region, and wherein greater than 90% of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.
- 15
14. The composition of any one of claims 1, 2, 12 or 13, wherein at least one of the polypeptide chains comprises a CH3 domain directly linked to a VL, VH or CH1 domain via the connecting peptide.
- 20
15. The composition of any one of claims 1, 2, 12 or 13, wherein the polypeptide chains lack all or part of a CH2 domain.
- 25
16. The composition of any one of claims 1, 2, 12 or 13, wherein the dimers of claims 1 or 2, or the molecules of claims 12 or 13, are linked via two or more interchain disulfide linkages.

2004252171 30 Mar 2011

17. The composition of claim 1 or claim 12, wherein the heavy chain portion is derived from an antibody of an IgG1 isotype.
18. The composition of any one of claims 1, 2, 12 or 13, comprising IgG1 upper hinge residues corresponding to positions 226 through 238 (Kabat numbering system).
- 5 19. The composition of any one of claims 1, 2, 12 or 13, wherein the dimers of claims 1 or 2, or the molecules of claims 12 or 13, are bispecific.
20. The composition of any one of claims 1, 2, 12 or 13, wherein the synthetic connecting peptide comprises a proline residue at position 243, Kabat numbering system.
- 10 21. The composition of any one of claims 1, 2, 12 or 13, wherein the synthetic connecting peptide further comprises an alanine residue at position 244 and a proline residue at position 245, Kabat numbering system.
22. The composition of any one of claims 1, 2, 12 or 13, wherein the synthetic connecting peptide comprises at least a portion of an IgG1 hinge domain and, at least a portion of an IgG3 hinge domain.
- 15 23. The composition of any one of claims 1, 2, 12, 13 or 22, wherein the chimeric hinge comprises a single IgG3 middle hinge repeat motif corresponding to positions 241E1 through 242 (Kabat numbering system).
24. The composition of any one of claims 1, 2, 12 or 13, wherein the connecting peptide comprises an amino acid sequence selected from the group consisting of: SEQ ID NOs: 8 and
- 20 9.
25. The composition of any one of claims 1, 2, 12 or 13, wherein said binding sites are individually selected from the group consisting of: an antigen binding site, a ligand binding portion of a receptor, and a receptor binding portion of a ligand.
26. A composition comprising polypeptide dimers having at least four antigen binding sites
- 25 and at least two polypeptide chains, wherein said at least two polypeptide chains each comprise a complete Ig heavy chain, and a chimeric hinge,
- wherein said chimeric hinge connects the CH1 and the CH2 domain of the Ig heavy chain, and

2004252171 06 Apr 2011

wherein greater than about 90% of the dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage, and

wherein amino acids at positions 226-242 (Kabat numbering) comprise: (i) the human IgG1 upper hinge region sequence EPKSCDKTHT (SEQ ID NO:2) or the human IgG4 upper hinge region sequence ESKYGPP (SEQ ID NO:45) at Kabat hinge positions 226-238; (ii) a cysteine residue (C) at Kabat hinge position 239; (iii) a proline residue (P) at Kabat hinge position 240; (iv) a proline (P) or serine (S) residue at Kabat hinge position 241; (v) the human IgG3 middle hinge sequence CPEPKSCDTPPPCPR (SEQ ID NO:49) at Kabat hinge positions 241EE-241SS; and (vi) a cysteine residue (C) at Kabat hinge position 242.

27. A composition comprising polypeptide dimers having at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise at least one immunoglobulin heavy chain portion and lack all or part of a CH2 domain, wherein greater than 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

28. A composition comprising polypeptide dimers having at least four binding sites and at least two polypeptide chains, wherein said at least two polypeptide chains comprise (i) an Ig heavy chain lacking a CH2 domain, and (ii) a chimeric hinge,

wherein said chimeric hinge comprises an amino acid sequence selected from the group consisting of SEQ ID NOs: 8-15 and wherein said chimeric hinge connects said heavy chain to at least one of said binding sites, and

wherein greater than 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

29. A method of treating a subject that would benefit from treatment with an antigen binding molecule, the method comprising administering a composition of any one of claims 1 to 28 to the subject such that treatment occurs.

30. The method of claim 29, wherein the subject is suffering from cancer, lymphoma, an autoimmune disease or disorder, or an inflammatory disease or disorder.

31. A nucleic acid molecule comprising a nucleotide sequence encoding a polypeptide chain of the composition of any one of claims 1 to 28.

32. A vector comprising the nucleic acid molecule of claim 31.

2004252171 06 Apr 2011

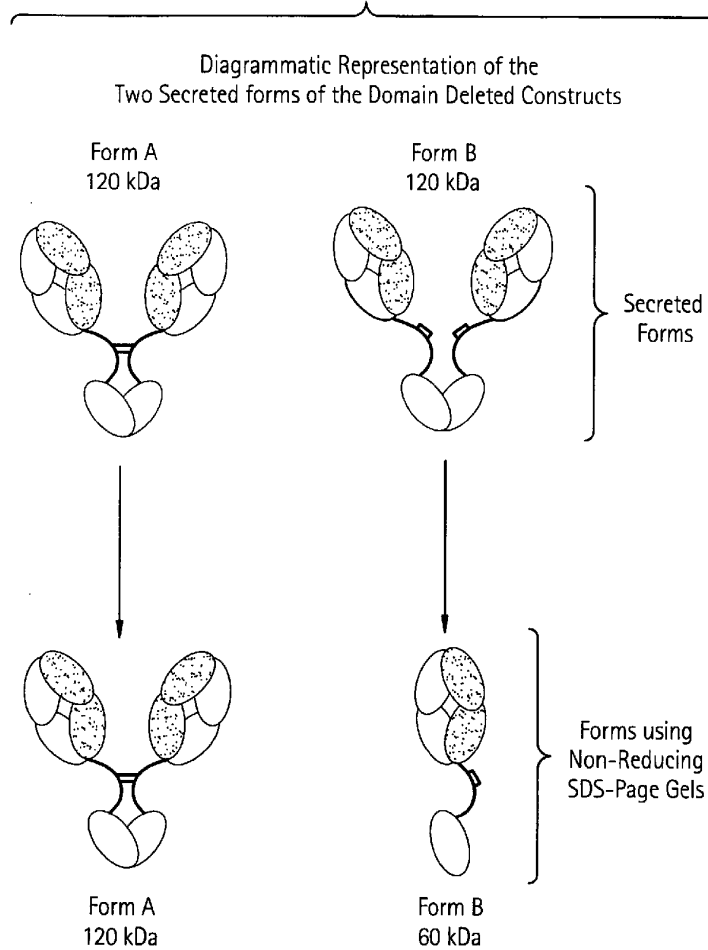
33. A host cell comprising the vector of claim 32.

Date: 6 April 2011

- 120 -

1/41

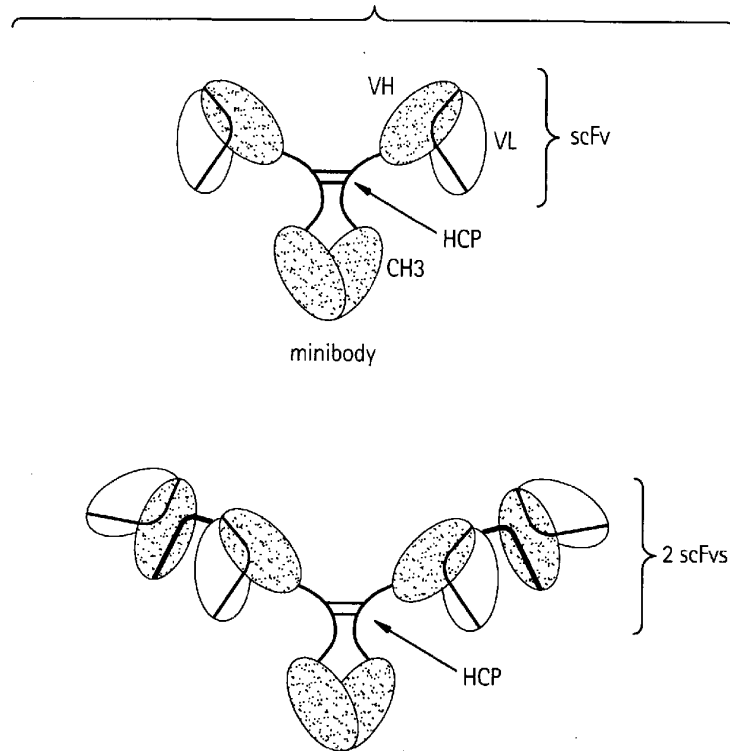
FIG. 1





AMENDED SHEET

2/41

FIG. 2



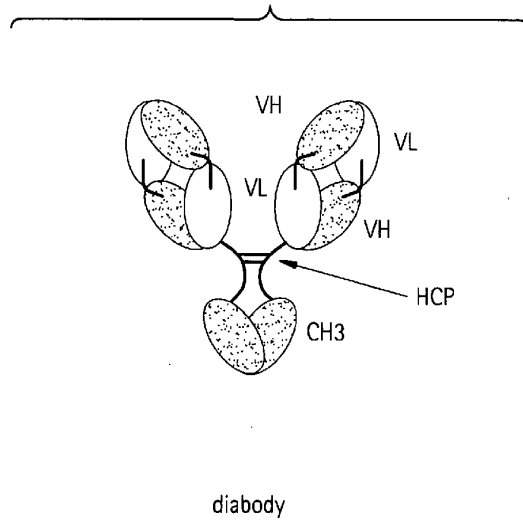
2 sc(Fv)2 Tetravalent antibody (N-scFv tetravalent minibody)  
 (can also be constructed as a bispecific N-scFv tetravalent minibody)

V=Variable
C=Constant
L=Light
H=Heavy
HCP=Hinge Connecting Peptide
 =G <sub>4</sub> S flexible linker
 =(G <sub>4</sub> S) <sub>4</sub> G <sub>3</sub> AS flexible linker

AMENDED SHEET

3/41

FIG. 3



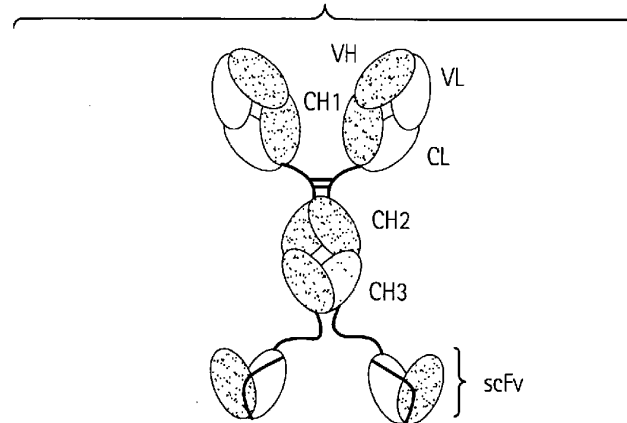
V = Variable  
C = Constant  
L = Light  
H = Heavy  
HCP = Hinge Connecting Peptide  
J = Short linker

AMENDED SHEET

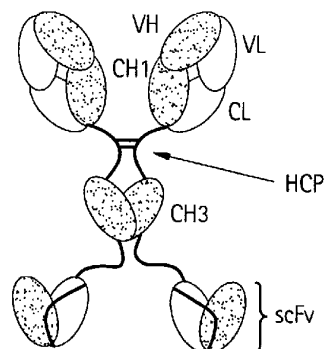


4/41

FIG. 4



sc(Fv)2 Tetraivalent (C-scFv Tetraivalent) Antibody  
(can also be constructed as a bispecific C-scFv Tetraivalent antibody)



sc(Fv)2 Tetraivalent CH2 Domain Deleted (C-scFv Tetraivalent CH2  
Domain Deleted) Antibody (can also be constructed as a bispecific  
C-scFv Tetraivalent CH2 Domain Deleted antibody)

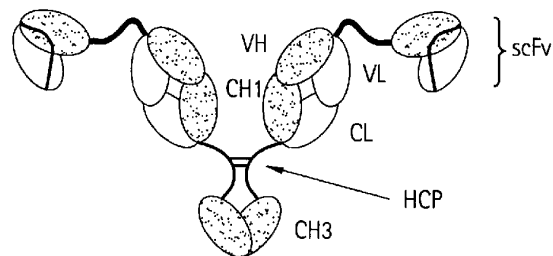
V = Variable  
C = Constant  
L = Light  
H = Heavy  
HCP = Hinge Connecting Peptide  
G<sub>4</sub>S = G<sub>4</sub>S flexible linkers

AMENDED SHEET



5/41

FIG. 5

Tetravalent or bispecific tetravalent CH2 domain-deleted antibody with  
a scFv appended to the N-terminus of the light chain



N<sub>L</sub>-scFv Tetravalent (or tetravalent bispecific) CH2 Domain Deleted Antibody

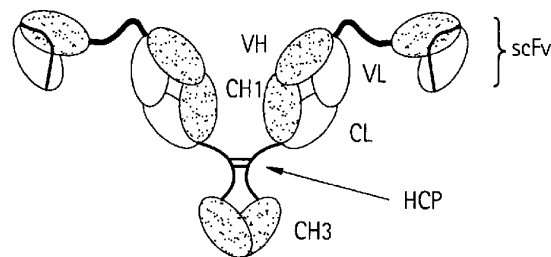
V = Variable
C = Constant
L = Light
H = Heavy
HCP = Hinge Connecting Peptide
 G <sub>4</sub> S flexible linker
 (G <sub>4</sub> S) <sub>4</sub> G <sub>3</sub> AS flexible linker

AMENDED SHEET



6/41

FIG. 6

Tetravalent or bispecific tetravalent CH2 domain-deleted antibody with a scFv appended to the N-terminus of the heavy chain.



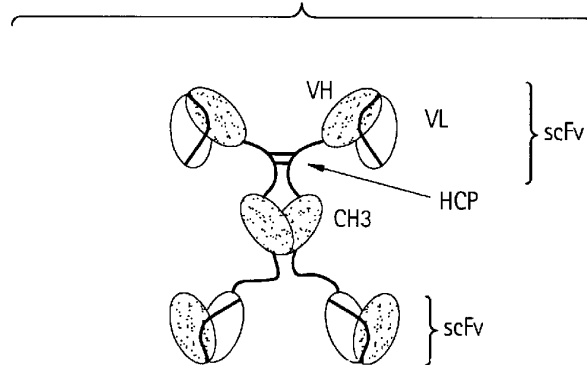
N<sub>H</sub>-scFv Tetravalent (or tetravalent bispecific) CH2 Domain Deleted Antibody

V	= Variable
C	= Constant
L	= Light
H	= Heavy
HCP	= Hinge Connecting Peptide
	G <sub>4</sub> S flexible linker
	(G <sub>4</sub> S) <sub>4</sub> G <sub>3</sub> AS flexible linker

AMENDED SHEET

7/41

FIG. 7



C - scFv tetraivalent (or tetraivalent bispecific) minibody

V = Variable
C = Constant
L = Light
H = Heavy
HCP = Hinge Connecting Peptide
(G <sub>4</sub> S) <sub>3</sub> flexible linkers

AMENDED SHEET

## FIG. 8A

Single-stranded DNA sequence of heavy chain huCC49 CH2 domain-deleted sc(Fv)2 tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody gene

(SEQ ID NO:16)

CAGGTCCAGCTGGTGCAGTCCGGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGA  
AGATTTCTTGCAAGGCAAGCGGCTACACCTTCACTGATCACGCAATCCACTGGGT  
GAAACAGAATCCTGGACAGCGCTGGAGTGGATTGGATATTTCTCTCCCGAAAC  
GATGATTTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACA  
CATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGC  
AGTGTACTTCTGCACAAGATCCCTGAATATGGCCTACTGGGGACAGGGAACCCTG  
GTCACCGTCTCCAGCGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCCCT  
CCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTA  
CTTCCCGAACCAGGTGACGGTGTCTGGAAGTCAAGCGCCCTGACCAGCGGCGTG  
CACACCTTCCCGGCTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGG  
TGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCA  
CAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA  
ACTCACACATGCCACCGTGCAGGAGGTGGCTCGAGTGGAGGCGGTTCCGGAGGGC  
AGCCCCGAGAACCACAGGTGTACACCTGCCCCATCCCGGGATGAGCTGACCAA  
GAACAGGTGAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCCAGCGACATCGCC  
GTGGAGTGGGAGAGCAATGGGCAGCCGAGAACAACTACAAGACCACGCTCCCG  
TGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAG  
CAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCAC  
AACCCTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATCCGGCGGGGGTG  
GATCCGGTGGAGGGGGCTCCGGCGGTGGCGGGTCCGACATCGTGATGAGCCAGTC  
TCCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGGTGACTCTGAATTGCAAGTCC  
AGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAACTATCTCGCCTGGTATCAGC  
AGAAACCAGGGCAGAGCCCTAAACTGCTGATTTACTGGGCATCCGCTAGGGAATC  
CGGCGTGCCTGATCGCTTCAAGCGCAGCGGATCTGGGACAGACTTCACTCTGACA  
ATCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTATTATTGTCAGCAGTATTATA  
GCTATCCCCCTCACATTCGGCGCTGGCACCAGCTGGAACCTTAAGGGCGGTGGCGG  
GTCCGGTGGGGGTGGCTCCGGGGGCGGTGGCTCCCAGGTCCAGCTGGTGCAGTCC  
GGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGAAGATTTCTGCAAGGCAAGCG  
GCTACACCTTCACTGATCACGCAATCCACTGGGTGAAACAGAATCCTGGACAGCG  
CCTGGAGTGGATTGGATATTTCTCTCCCGGAAACGATGATTTTAAGTACAATGAG  
AGGTTCAAGGGCAAGGCCACACTGACTGCAGACACATCTGCCAGCACTGCCTACG  
TGGAGCTCTCCAGCCTGAGATCCGAGGATACTGCAGTGTACTTCTGCACAAGATC  
CCTGAATATGGCCTACTGGGGACAGGGAACCCTGGTCAACGCTCTCCAGC

AMENDED SHEET

## FIG. 8B

Single-stranded DNA sequence of heavy chain huCC49 CH2 domain-deleted sc(Fv)2 tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

(SEQ ID NO:17)

CAGGTCCAGCTGGTGCAGTCCGGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGA  
AGATTTCTGCAAGGCAAGCGGCTACACCTTCACTGATCACGCAATCCACTGGGT  
GAAACAGAATCCTGGACAGCGCTGGAGTGGATTGGATATTTCTCTCCCGGAAAC  
GATGATTTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACA  
CATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGC  
AGTGTACTTCTGCACAAGATCCCTGAATATGGCCTACTGGGGACAGGGAACCTG  
GTCACCGTCTCCAGCGCTAGCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCT  
CCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTA  
CTTCCCGAACC GGTTGACGGTGTCTGGAACCTCAGGCGCCCTGACCAGCGGCGTG  
CACACCTTCCCGGCTGTCTACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGG  
TGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAATCA  
CAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAAATCTTGTGACAAA  
ACTCACACATGCCACCGTGGCCAGAGCCCAAATCTTGTGACACACCTCCCCAT  
GCCCACGGTGCCAGGAGGTGGCTCGAGTGGAGGCGGTTCGGGAGGGCAGCCCCG  
AGAACCACAGGTGTACACCTGCCCCCATCCCGGGATGAGCTGACCAAGAACCAG  
GTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATCGCCGTGGAGT  
GGGAGAGCAATGGGCAGCCGAGAGCAACTACAAGACCACGCCTCCCGTGTGGA  
CTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGG  
CAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGGCTCTGCACAACCACT  
ACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATCCGGCGGGGGTGGATCCGG  
TGGAGGGGGCTCCGGCGGTGGCGGGTCCGACATCGTGATGAGCCAGTCTCCAGAC  
TCCCTGGCCGTGTCCCTGGGCGAGAGGCTGACTCTGAATTGCAAGTCCAGCCAGT  
CCCTGCTCTATAGCGGAAATCAGAAGAACTATCTCGCCTGGTATCAGCAGAAACC  
AGGGCAGAGCCCTAAACTGCTGATTTACTGGGCATCCGCTAGGGAATCCGGCGTG  
CCTGATCGCTTACAGCGGCAGCGGATCTGGGACAGACTTCACTCTGACAATCAGCA  
GCGTGACAGGCAGAAGACGTGGCAGTCTATTATTGTCAGCAGTATTATAGCTATCC  
CCTCACATTCCGGCGCTGGCACCAAGCTGGAACCTAAGGGCGGTGGCGGGTCCGGT  
GGGGGTGGCTCCGGGGGGCGGTGGCTCCCAGGTCCAGCTGGTGCAGTCCGGCGCTG  
AGGTGGTGAACCTGGGGCTTCCGTGAAGATTTCTGCAAGGCAAGCGGCTACAC  
CTTCACTGATCACGCAATCCACTGGGTGAAACAGAATCCTGGACAGCGCCTGGAG  
TGGATTGGATATTTCTCTCCCGGAAACGATGATTTAAGTACAATGAGAGGTTCA  
AGGGCAAGGCCACACTGACTGCAGACACATCTGCCAGCACTGCCTACGTGGAGCT  
CTCCAGCCTGAGATCCGAGGATACTGCAGTGTACTTCTGCACAAGATCCCTGAAT  
ATGGCCTACTGGGGACAGGGAACCTGGTCACCGTCTCCAGC

AMENDED SHEET

10/41

## FIG. 8C

Single-stranded DNA sequence of light chain huCC49 CH2 domain-deleted sc(Fv)2  
tetravalent (C-scFv tetravalent CH2 domain deleted) antibody gene

(SEQ ID NO: 18)

GACATCGTGATGAGCCAGTCTCCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGG  
TGA CTCTGAATTGCAAGTCCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAA  
CTATCTCGCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTTAC  
TGGGCATCCGCTAGGGAATCCGGCGTGCCTGATCGCTTCAGCGGCAGCGGATCTG  
GGACAGACTTCACTCTGACAATCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTA  
TTATTGTCAGCAGTATTATAGCTATCCCCTCACATTCGGCGCTGGCACCAAGCTG  
GAACTGAAACGTACGGTGGCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATG  
AGCAGTTGAAATCTGGAAGTGCCTCTGTGTGTGCCTGCTGAATAACTTCTATCC  
CAGAGAGGCCAAAGTACAGTGGAAGGTGGATAACGCCCTCCAATCGGGTAACTCC  
CAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCA  
CCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGT  
CACCCATCAGGGCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGT  
TGA

AMENDED SHEET

11/41

## FIG. 9A

Amino acid sequence of heavy chain huCC49 CH2 domain-deleted sc(Fv)<sub>2</sub>  
tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody

(SEQ ID NO:19)

QVQLVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPGRLEWIGYFSPGN  
DDFKYNERFKGKATLTADTSASTAYVELSSLRSED TAVYFCTRSLNMAYWGQGT  
LVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV  
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK  
THTCPPCGGGSSGGSGGGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA  
VEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALH  
NHYTQKSLSLSPGKSGGGSGGGSGGGSDIVMSQSPDSLAVSLGERVTLNCKS  
SQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWASARESGVPDRFSGSGSGTDFTLT  
ISSVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGGGSGGGSGGGGSQVQLVQS  
GAEVVKPGASVKISCKASGYTFTDHAHVKQNPGRLEWIGYFSPGNDDFKYNE  
RFGKATLTADTSASTAYVELSSLRSED TAVYFCTRSLNMAYWGQGT LVTVSS\*

AMENDED SHEET



12/41

## FIG. 9B

Amino acid sequence of heavy chain huCC49 CH2 domain-deleted sc(Fv)<sub>2</sub>  
tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

(SEQ ID NO:20)

QVQLVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPGRLEWIGYFSPGN  
DDFKYNERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTRSLNMAYWGQGT  
LVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGV  
HTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK  
THTCPPCPPEPKSCDTPPPCPRCPAPGGGSSGGGSGGQPREPQVYTLPPSRDELTK  
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKS  
RWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKSGGGGSGGGGSGGGGSDIVMSQS  
PDSLAVSLGERVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWASARES  
GVPDRFSGSGSGTDFTLTISVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGSGG  
SGGGGSGGGGSGVQLVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPGR  
LEWIGYFSPGNDDFKYNERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTR  
SLNMAYWGQGT LVTSS\*

AMENDED SHEET

13/41

## FIG. 10A

Single-stranded DNA sequence of CH2 domain-deleted huCC49 tetravalent minibody  
(N-scFv tetravalent minibody or 2sc(Fv)2 Tetravalent antibody) gene.

(SEQ ID NO: 22)

```
GACATCGTGATGAGCCAGTCTCCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGG
TGACTCTGAATTGCAAGTCCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAA
CTATCTCGCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTAC
TGGGCATCCGCTAGGGAATCCGGCGTGCTGATCGCTTCAGCGGCAGCGGATCTG
GGACAGACTTCACTCTGACAATCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTA
TTATTGTCAGCAGTATTATAGCTATCCCTCACATTCGGCGCTGGCACCAGCTG
GAACTTAAGGGCGGTGGCGGGTCCGGTGGGGGTGGCTCCGGGGGCGGTGGCTCCC
AGGTCCAGCTGGTGCAGTCCGGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGAA
GATTTCTGCAAGGCAAGCGGTACACCTTCACTGATCAGCAATCCACTGGGTG
AAACAGAATCCTGGACAGCGCTGGAGTGGATTGGATATTTCTCTCCCGGAAACG
ATGATTTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACAC
ATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGCA
GTGTACTTCTGCACAAGATCCCTGAATATGGCCTACTGGGGACAGGGTACCCTGG
TCACCGTCTCCAGCGGCGGTGGAGGGTCCGGTGGAGGGGGCTCTGGAGGGGGCGG
TTCAGGGGGCGGTGGATCGGGCGGAGGTGCTAGCGACATCGTGATGAGCCAGTCT
CCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGGTGACTCTGAATTGCAAGTCCA
GCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAACTATCTCGCCTGGTATCAGCA
GAAACCAGGGCAGAGCCCTAAACTGCTGATTTACTGGGCATCCGCTAGGGAATCC
GGCGTGCCTGATCGCTTCAGCGGCAGCGGATCTGGGACAGACTTCACTCTGACAA
TCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTATTATTGTGACAGTATTATAG
CTATCCCTCACATTCGGCGCTGGCACCAGCTGGAACCTAAGGGCGGTGGCGGG
TCCGGTGGGGGTGGCTCCGGGGGCGGTGGCTCCCAGGTCCAGCTGGTGCAGTCCG
GCGCTGAGGTGGTGAACCTGGGGCTTCCGTGAAGATTTCTGCAAGGCAAGCGG
CTACACCTTCACTGATCAGCAATCCACTGGGTGAAACAGAATCCTGGACAGCGC
CTGGAGTGGATTGGATATTTCTCTCCCGGAAACGATGATTTTAAGTACAATGAGA
GGTTCAAGGGCAAGGCCACACTGACTGCAGACACATCTGCCAGCACTGCCTACGT
GGAGCTCTCCAGCCTGAGATCCGAGGATACTGCAGTGTACTTCTGCACAAGATCC
CTGAATATGGCCTACTGGGGACAGGGAACCCTGGTCACCGTCTCCAGCGTCGACC
CCAAATCTTGTGACAAAACCTCACACATGCCACCGTGCGGAGGTGGCTCGAGTGG
AGGCGGATCCGGAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCATCC
CGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCT
ATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACATA
CAAGACCACGCCTCCCGTGTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAG
CTCACCCTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGA
TGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGG
TAAATGA
```

AMENDED SHEET

14/41

## FIG. 10B

Single-stranded DNA sequence of CH2 domain-deleted huCC49 tetravalent minibody (N-scFv tetravalent minibody or 2sc(Fv)2 Tetravalent antibody) gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

(SEQ ID NO:23)

GACATCGTGATGAGCCAGTCTCCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGG  
TGACTCTGAATTGCAAGTCCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAA  
CTATCTCGCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTAC  
TGGGCATCCGCTAGGGAATCCGGCGTGCCGTGATCGCTTCAGCGGCAGCGGATCTG  
GGACAGACTTCACTCTGACAATCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTA  
TTATTGTGAGCAGTATTATAGCTATCCCTCACATTGCGCGCTGGCACCAGCTG  
GAACTTAAGGGCGGTGGCGGGTCCGGTGGGGGTGGCTCCGGGGCGGTGGCTCCC  
AGGTCCAGCTGGTGCAGTCCGGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGAA  
GATTTCTGCAAGGCAAGCGGTACACCTTCACTGATCACGCAATCCACTGGGTG  
AAACAGAATCCTGGACAGCGCTGGAGTGGATTGGATATTTCTCTCCCGGAAACG  
ATGATTTTAAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACAC  
ATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGCA  
GTGTACTTCTGCACAAGATCCCTGAATATGGCCTACTGGGGACAGGGTACCCTGG  
TCACCGTCTCCAGCGCGGTGGAGGGTCCGGTGGAGGGGGCTCTGGAGGGGGCGG  
TTCAGGGGGCGGTGGATCGGGCGGAGGTGCTAGCGACATCGTGATGAGCCAGTCT  
CCAGACTCCCTGGCCGTGTCCCTGGGCGAGAGGGTGAATCTGAATTGCAAGTCCA  
GCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAACTATCTCGCCTGGTATCAGCA  
GAAACCAGGGCAGAGCCCTAAACTGCTGATTTACTGGGCATCCGCTAGGGAATCC  
GGCGTGCCTGATCGCTTCAGCGGCAGCGGATCTGGGACAGACTTCACTCTGACAA  
TCAGCAGCGTGCAGGCAGAAGACGTGGCAGTCTATTATTGTCAGCAGTATTATAG  
CTATCCCTCACATTGCGCGCTGGCACCAGCTGGAACCTTAAGGGCGGTGGCGGG  
TCCGGTGGGGGTGGCTCCGGGGCGGTGGCTCCAGGTCCAGCTGGTGCAGTCCG  
GCGCTGAGGTGGTGAACCTGGGGCTTCCGTGAAGATTTCTTGCAAGGCAAGCGG  
CTACACCTTCACTGATCACGCAATCCACTGGGTGAAACAGAATCCTGGACAGCGC  
CTGGAGTGGATTGGATATTTCTCTCCCGGAAACGATGATTTTAAAGTACAATGAGA  
GGTTCAAGGGCAAGGCCACACTGACTGCAGACACATCTGCCAGCACTGCCTACGT  
GGAGCTCTCCAGCCTGAGATCCGAGGATACTGCAGTGTACTTCTGCACAAGATCC  
CTGAATATGGCCTACTGGGGACAGGGAACCCTGGTCACCGTCTCCAGCGTCGACC  
CCAAATCTTGTGACAAAACCTCACACATGCCCACCGTGCCCAGAGGCCAAATCTTG  
TGACACACCTCCCCATGCCACGGTGCCCAGGAGGTGGCTCGAGTGGAGGCGGA  
TCCGGAGGGCAGCCCCGAGAACCACAGGTGTACACCTGCCCCATCCCGGGATG  
AGCTGACCAAGAACCAGGTACGCTGACCTGCCTGGTCAAAGGCTTCTATCCCAG  
CGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACACTACAGACC  
ACGCTCCCGTGGTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCG  
TGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGTCCGTGATGCATGA  
GGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATGA

AMENDED SHEET

15/41

## FIG. 11A

Amino acid sequence of CH2 domain-deleted huCC49 tetravalent minibody (N-scFv tetravalent minibody or 2sc(Fv)2 Tetravalent antibody).

(SEQ ID NO:24)

DIVMSQSPDSLAVSLGERVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWA  
SARESGVPDRFSGSGSGTDFTLTISVQAEDVAVYYCQQYYSYPLTFGAGTKLELKG  
GGGSGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPQG  
RLEWIGYFSPGNDDFKYNERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTRSL  
NMAYWGQGTLLTVSSGGGSGGGSGGGSGGGSGGGSGGGASDIVMSQSPDSLAVSLGE  
RVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWASARESGVPDRFSGSGSG  
TDFTLTISVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGGGGSGGGSGGGGSQVQ  
LVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPQGRLEWIGYFSPGNDDFKY  
NERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTRSLNMAYWGQGTLLTVSSVD  
PKSCDKTHTCPPCGGSGGGSGGGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYP  
SDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMEHA  
LHNHYTQKSLSLSPGK\*

## FIG. 11B

Amino acid sequence of CH2 domain-deleted huCC49 tetravalent minibody (N-scFv tetravalent minibody or 2sc(Fv)2 Tetravalent antibody) containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

(SEQ ID NO:25)

DIVMSQSPDSLAVSLGERVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWA  
SARESGVPDRFSGSGSGTDFTLTISVQAEDVAVYYCQQYYSYPLTFGAGTKLELKG  
GGGSGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPQG  
RLEWIGYFSPGNDDFKYNERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTRSL  
NMAYWGQGTLLTVSSGGGSGGGSGGGSGGGSGGGSGGGASDIVMSQSPDSLAVSLGE  
RVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWASARESGVPDRFSGSGSG  
TDFTLTISVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGGGGSGGGSGGGGSQVQ  
LVQSGAEVVKPGASVKISCKASGYTFTDHAHVKQNPQGRLEWIGYFSPGNDDFKY  
NERFKGKATLTADTSASTAYVELSSLRSEDVAVYFCTRSLNMAYWGQGTLLTVSSVD  
PKSCDKTHTCPPCPEPKSCDTPPPCPRCPAPGGGSGGGSGGGQPREPQVYTLPPSRD  
ELTKNQVSLTCLVKGFYPSPDIAVEWESNGQPENNYKTTTPVLDSDGSFFLYSKLTV  
DKSRWQQGNVFSQSVMEHALHNHYTQKSLSLSPGK\*

AMENDED SHEET

16/41

## FIG. 12A

Single-stranded DNA sequence of heavy chain CH2 domain-deleted PRIMATIZED®  
p5E8 sc(Fv)2 tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody gene  
containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO:26)

GAGGTGCAGCTGGTGGAGTCTGGGGGCGGCTTGGCAAAGCCTGGGGGGTCCCTGA  
GACTCTCCTGCGCAGCCTCCGGGTTTACGTTTACCTTCAATACTACTACATGGA  
CTGGGTCCGCCAGGCTCCAGGGCAGGGGCTGGAGTGGGTCTCACGTATTAGTAGT  
AGTGGTGATCCCATGGTACGCAGACTCCGTGAAGGGCAGATTACCATCTCCA  
GAGAGAACGCCAAGAACACACTGTTTCTTCAAATGAACAGCCTGAGAGCTGAGGA  
CACGGCTGTCTATTACTGTGCGAGCTTGACTACAGGGTCTGACTCCTGGGGCCAG  
GGAGTCTGGTCACCGTCTCCTCAGCTAGCACCAAGGGCCCATCGGTCTTCCCC  
TGGCACCCCTCCTCCAAGAGCACCTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGT  
CAAGGACTACTTCCCCGAACCGGTGACGGTGTCTGGAAGTCAAGGCGCCCTGACC  
AGCGGGCTGCACACCTTCCCGGCTGTCTACAGTCTCAGGACTCTACTCCCTCA  
GCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAA  
CGTGAATCACAAGCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCCAATCT  
TGTGACAAAACCTCACACATGCCACCGTGGCCAGAGCCCAATCTTGTGACACAC  
CTCCCCCATGCCACGGTGCCAGCACCTGGAGGTGGCTCGAGTGGAGGCGGTTC  
CGGAGGGCAGCCCCGAGAACCACAGGTGTACCCCTGCCCCCATCCCGGGATGAG  
CTGACCAAGAACCAGGTGACCTGACCTGGTCAAAGGCTTCTATCCAGCG  
ACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGAGAACAACCTACAAGACCAC  
GCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCTCTACAGCAAGCTCACCGTG  
GACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGAGG  
CTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTAAATCCGG  
CGGGGGTGGATCCGGTGGAGGGGGCTCCGGCGGTGGCGGGTCCGACATCCAGATG  
ACCCAGTCTCCATCTTCCCTGTCTGCATCTGTAGGGGACAGAGTCACCATCACTT  
GCAGGGCAAGTCAGGACATTAGGTATTATTTAAATTTGGTATCAGCAGAAACCAGG  
AAAAGCTCCTAAGCTCCTGATCTATGTTGCATCCAGTTTGCAAAGTGGGGTCCCA  
TCAAGGTTTCAAGGTCAGTGGATCTGGGACAGAGTTCACCTCTACCGTCAGCAGCC  
TGCAGCCTGAAGATTTTGCGACTTATTACTGTCTACAGGTTTATAGTACCCTCG  
GACGTTTCGGCCAAAGGACCAAGGTGGAATCAAAGGCGGTGGCGGGTCCGGTGGG  
GGTGGCTCCGGGGGCGGTGGCTCCGAGGTGCAGCTGGTGGAGTCTGGGGGCGGCT  
TGGCAAAGCCTGGGGGGTCCCTGAGACTCTCCTGCGCAGCCTCCGGGTTTCAAGTT  
CACCTTCAATACTACTACATGGACTGGGTCCGCCAGGCTCCAGGGCAGGGGCTG  
GAGTGGGTCTCACGTATTAGTAGTAGTGGTGATCCCATGGTACGCAGACTCCG  
TGAAGGGCAGATTACCATCTCCAGAGAGAACGCCAAGAACACACTGTTTCTTCA  
AATGAACAGCCTGAGAGCTGAGGACACGGCTGTCTATTACTGTGCGAGCTTGACT  
ACAGGGTCTGACTCCTGGGGCCAGGGAGTCTGGTCAACGTCTCCTCATGA

AMENDED SHEET

17/41

## FIG. 12B

Single-stranded DNA sequence of light chain CH2 domain-deleted PRIMATIZED®  
p5E8 sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody  
containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide gene

(SEQ ID NO:27)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCATCTGTAGGGGACAGAG  
TCACCATCACTTGCAGGGCAAGTCAGGACATTAGGTATTATTTAAATTGGTATCA  
GCAGAAACCAGGAAAAGCTCCTAAGCTCCTGATCTATGTTGCATCCAGTTTGCAA  
AGTGGGGTCCCATCAAGGTTCAAGCGGCAGTGGATCTGGGACAGAGTTCACCTCTCA  
CCGTCAGCAGCCTGCAGCCTGAAGATTTGCGACTTATTACTGTCTACAGGTTTA  
TAGTACCCCTCGGACGTTCCGGCCAAGGGACCAAGGTGGAAATCAAACGTACGGTG  
GCTGCACCATCTGTCTTCATCTTCCCGCCATCTGATGAGCAGTTGAAATCTGGAA  
CTGCCTCTGTTGTGTGCCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACA  
GTGGAAGGTGGATAACGCCCTCCAATCGGGTAACCTCCAGGAGAGTGTACACAGAG  
CAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAG  
CAGACTACGAGAAACACAAAGTCTACGCCTGCGAAGTCACCCATCAGGGCCTGAG  
CTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGTGA

AMENDED SHEET

18/41

## FIG. 13A

Amino acid sequence of heavy chain CH2 domain-deleted PRIMATIZED® p5E8  
sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO:28)

EVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAPGQGLEWVSRISS  
SGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYCASLTGSDSWGQ  
GVLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALT  
SGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKS  
CDKTHTCPPCPEPKSCDTPPPCPRCPAPGGGSSGGGSGGQPREPQVYTLPPSRDE  
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSGSEFFLYSKLTV  
DKSRWQQGNVFCFSVMHEALHNHYTQKSLSLSPGKSGGGGSGGGGSGGGGSDIQM  
TQSPSSLSASVGDRVTITCRASQDIRYYLNWYQQKPKAPKLLIYVASSLQSGVP  
SRFSGSGSGTEFTLTVSSSLQPEDFATYYCLQVYSTPRTFGQGTKVEIKGGGSGG  
GGSGGGGSEVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAPGQGL  
EWVSRISSSGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYCASLT  
TGSDSWGQGVLTIVSS

AMENDED SHEET

19/41

## FIG. 13B

Amino acid sequence of light chain CH2 domain-deleted PRIMATIZED® p5E8 sc(Fv)2  
tetraivalent (C-scFv tetraivalent CH2 domain deleted) antibody containing the  
G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO:29)

DIQMTQSPSSLSASVGDRVTITCRASQDIRYYLNWYQQKPGKAPKLLIYVASSLQ  
SGVPSRFSGSGSGTEFTLTVSSLPEDFATYYCLQVYSTPRTFGQGTKVEIKRTV  
AAPSVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTE  
QDSKDYSLSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC\*

AMENDED SHEET



20/41

**FIG. 14**

Single-stranded DNA sequence of PRIMATIZED® p5E8 minibody gene (VL → VH orientation) containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO: 30)

GACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCATCTGTAGGGGACAGAG  
TCACCATCACTTGCAGGGCAAGTCAGGACATTAGGTATTATTTAAATTGGTATCA  
GCAGAAACCAGGAAAAGCTCCTAAGCTCCTGATCTATGTTGCATCCAGTTTGCAA  
AGTGGGGTCCCATCAAGGTTCAAGCGGCAGTGGATCTGGGACAGAGTTCACTCTCA  
CCGTCAGCAGCCTGCAGCCTGAAGATTTTGCAGCTTATTACTGTCTACAGGTTTA  
TAGTACCCCTCGGACGTTTCGGCCAAGGGACCAAGGTGGAAATCAAAGGCGGTGGC  
GGGTCCGGTGGGGTGGCTCCGGGGGCGGTGGCTCCGAGGTGCAGCTGGTGGAGT  
CTGGGGGCGGCTTGGCAAAGCCTGGGGGGTCCCTGAGACTCTCCTGCGCAGCCTC  
CGGGTTTCAGGTTACCTTCAATAACTACTACATGGACTGGGTCCGCCAGGCTCCA  
GGGCAGGGGCTGGAGTGGGTCTCACGTATTAGTAGTAGTGGTGATCCACATGGT  
ACGCAGACTCCGTGAAGGGCAGATTACCATCTCCAGAGAGAACGCCAAGAACAC  
ACTGTTTCTTCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTCTATTACTGT  
GCGAGCTTGACTACAGGGTCTGACTCCTGGGGCCAGGGAGTCCCTGGTCACCGTCT  
CCTCAGTCGACCCCAAATCTTGTGACAAAACCTCACACATGCCCACCGTGCCCA  
GCCCAAATCTTGTGACACACCTCCCCATGCCACGGTGCCCAGCACCTGGAGGT  
GGCTCGAGTGGAGGCGGATCCGGAGGGCAGCCCCGAGAACCACAGGTGTACACCC  
TGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCCTGGT  
CAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG  
GAGAACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCC  
TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTC  
ATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC  
CTGTCTCCGGTAAATGA

AMENDED SHEET

21/41

**FIG. 15**

Single-stranded DNA sequence of PRIMATIZED® p5E8 minibody gene (VH → VL orientation) containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO: 31)

```
GAGGTGCAGCTGGTGGAGTCTGGGGGCGGCTTGGCAAAGCCTGGGGGGTCCCTGA
GACTCTCCTGCGCAGCCTCCGGGTTTCAAGTTTACCTTCAATACTACTACATGGA
CTGGGTCCGCCAGGCTCCAGGGCAGGGGCTGGAGTGGGTCTCACGTATTAGTAGT
AGTGGTGATCCCACATGGTACGCAGACTCCGTGAAGGGCAGATTACCATCTCCA
GAGAGAACGCCAAGAACACACTGTTTCTTCAAATGAACAGCCTGAGAGCTGAGGA
CACGGCTGTCTATTACTGTGCGAGCTTGACTACAGGGTCTGACTCCTGGGGCCAG
GGAGTCTGGTCACCGTCTCCTCAGGCGGTGGCGGGTCCGGTGGGGGTGGCTCCG
GGGGCGGTGGCTCCGACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCATC
TGTAGGGGACAGAGTCACCATCACTTGCAGGGCAAGTCAGGACATTAGGTATTAT
TTAAATTGGTATCAGCAGAAACCAGGAAAAGCTCCTAAGCTCCTGATCTATGTTG
CATCCAGTTTGCAAAGTGGGGTCCCATCAAGGTTTCAAGGTCAGCGGCAGTGGATCTGGGAC
AGAGTTCACTCTCACCGTCAGCAGCCTGCAGCCTGAAGATTTTGC GACTTATTAC
TGTCTACAGGTTTATAGTACCCCTCGGACGTTCCGGCCAAGGGACCAAGGTGGAAA
TCAAAGTCGACCCCAAATCTTGTGACAAAACCTCACACATGCCCACCGTGCCGAGA
GCCCCAATCTTGTGACACACCTCCCCCATGCCCACGGTGCCCAGCACCTGGAGGT
GGCTCGAGTGGAGGCGGATCCGGAGGGCAGCCCCGAGAACCACAGGTGTACACCC
TGCCCCCATCCCGGGATGAGCTGACCAAGAACCAGGTGAGCCTGACCTGCCTGGT
CAAAGGCTTCTATCCCAGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCG
GAGAACAACCTACAAGACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCC
TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTC
ATGCTCCGTGATGCATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCC
CTGTCTCCGGGTAAATGA
```

AMENDED SHEET

22/41

**FIG. 16**

Amino acid sequence of PRIMATIZED® p5E8 minibody (VL → VH orientation)  
containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO: 32)

DIQMTQSPSSLSASVGDRVTITCRASQDIRYYLNWYQQKPGKAPKLLIYVASSLQ  
SGVPSRFSGSGSGTEFTLTVSSLPEDFATYYCLQVYSTPRTFGQGTKVEIKGGG  
GSGGGGSGGGGSEVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAP  
GQGLEWVSRISSSGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYC  
ASLTTGSDSWGQGVLTVSSVDPKSCDKTHTCPPCPEPKSCDTPPPCPRCPAPGG  
GSSGGGSGGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP  
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS  
LSPGK

AMENDED SHEET

23/41

**FIG. 17**

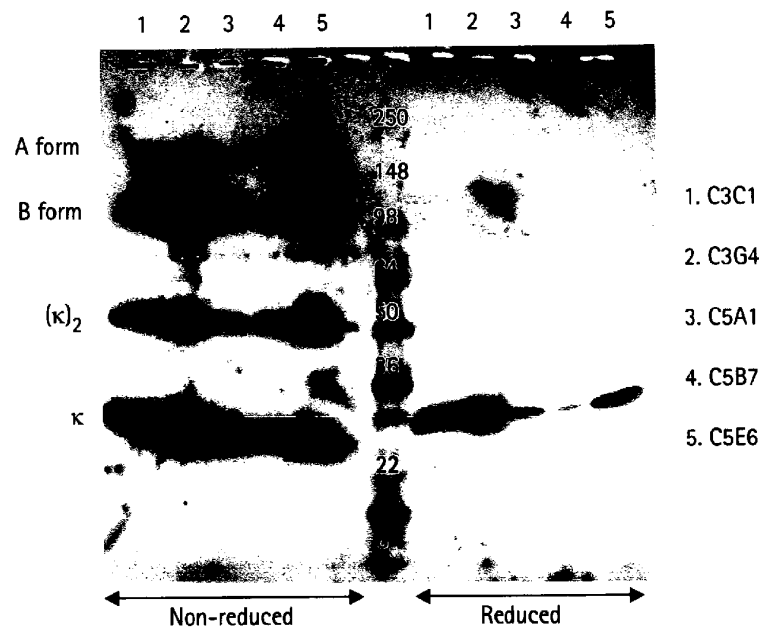
Amino acid sequence of PRIMATIZED® p5E8 minibody (VH → VL orientation)  
containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide

(SEQ ID NO: 33)

EVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAPGQGLEWVSRISS  
SGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYCASLTGSDSWGQ  
GVLVTVSSGGGSGGGGSGGGSDIQMTQSPSSLSASVGDRTITCRASQDIRYY  
LNWYQQKPGKAPKLLIYVASSLQSGVPSRFSGSGSGTEFTLTVSSLPEDFATYY  
CLQVYSTPRTFGQGTKVEIKVDPKSCDKTHTCPPCPPEPKSCDTPPFCPRCPAPGG  
GSSGGGSGGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP  
ENNYKTTTPVLDSDGSFFLYSKLTVDKSRWQQGNVFCFSVMHEALHNHYTQKSLS  
LSPGK

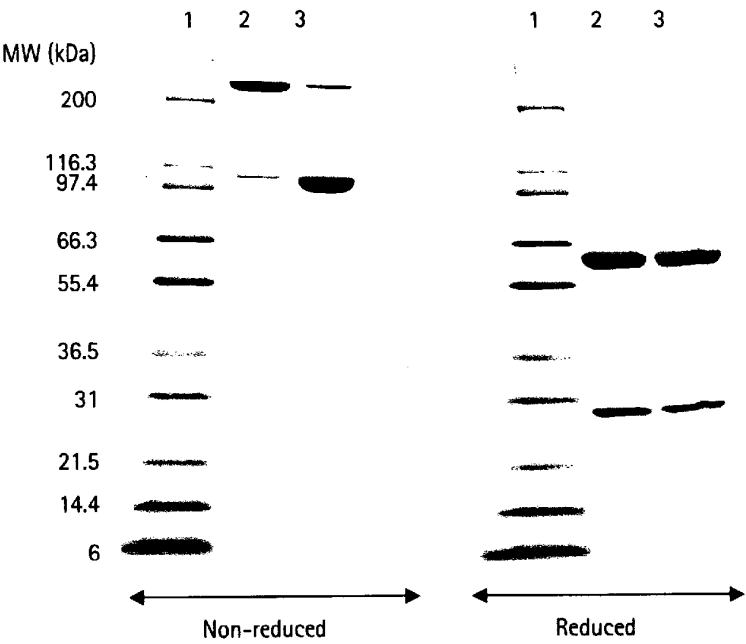
AMENDED SHEET

FIG. 18



AMENDED SHEET

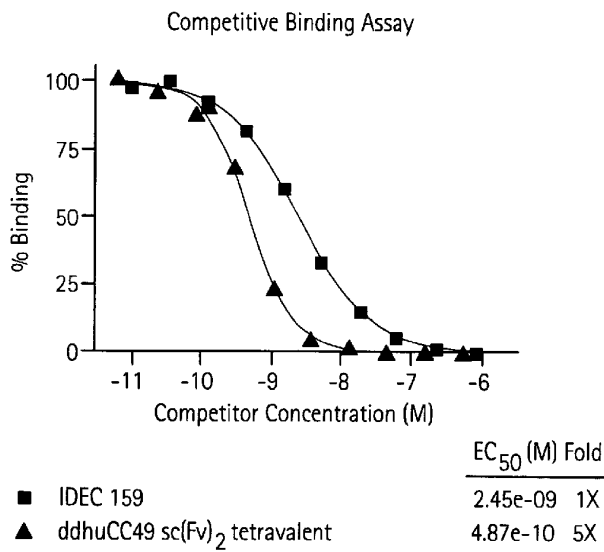
FIG. 19



- 1. Mark 12 Marker
- 2. Form A huCC49 domain deleted sc(Fv)2 tetravalent antibody
- 3. Form B huCC49 domain deleted sc(Fv)2 tetravalent antibody



FIG. 21



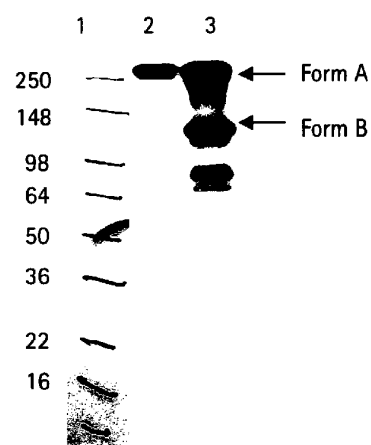
AMENDED SHEET



28/41

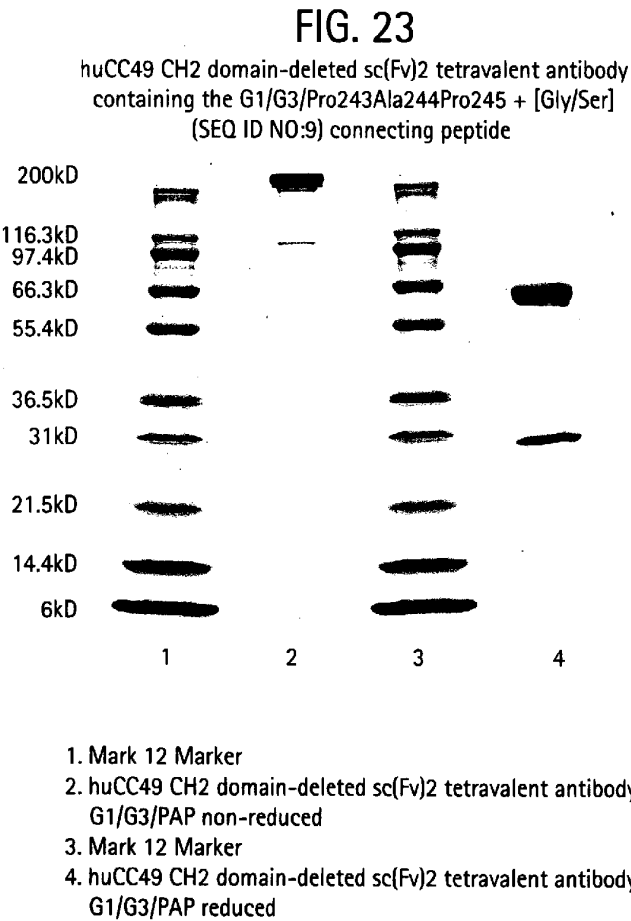
**FIG. 22**

Western blot of huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP



1. MW Marker
2. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP-non-reduced
3. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent-non-reduced

AMENDED SHEET

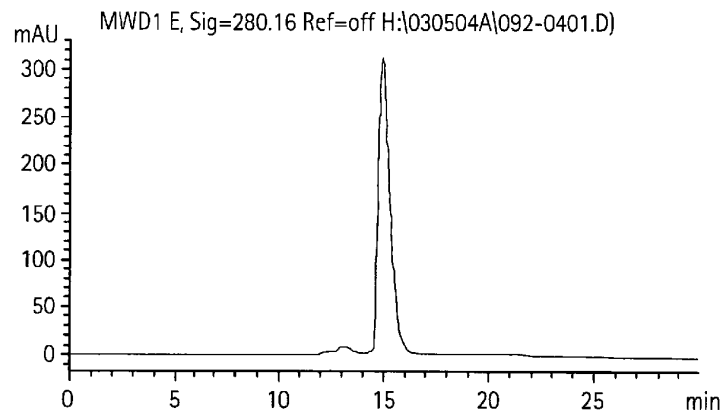


AMENDED SHEET

30/41

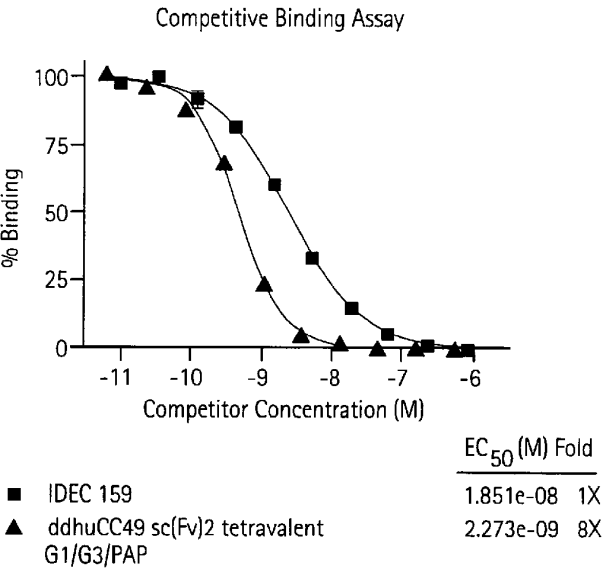
## FIG. 24

SEC analysis  
huCC49 CH2 domain-deleted sc(Fv)2 tetravalent  
G1/G3/Pro243Ala244Pro245 antibody



AMENDED SHEET

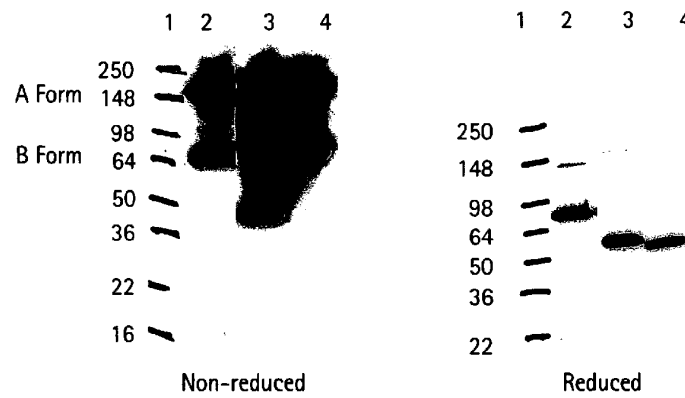
FIG. 25



AMENDED SHEET

32/41

FIG. 26

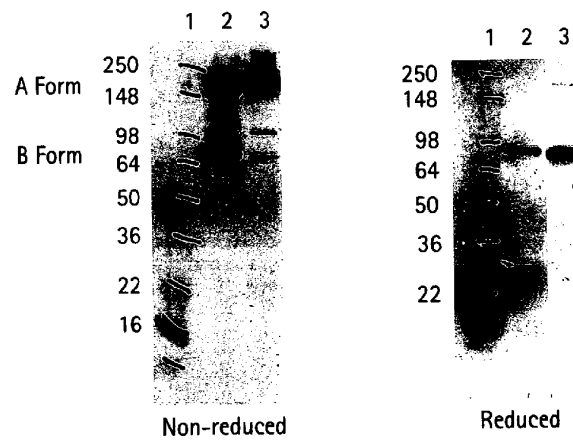


1. Mark 12 Marker
2. HuCC49 2sc(Fv)2 tetravalent antibody  
(N-scFv tetravalent huCC49 minibody)
3. huCC49 minibody
4. huCC49 control

AMENDED SHEET

33/41

FIG. 27

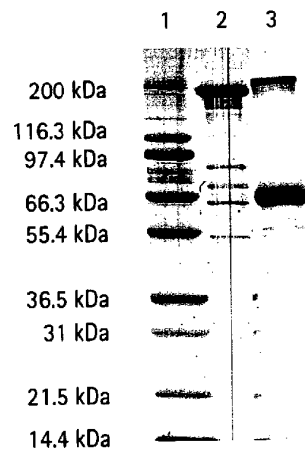


1. Mark 12 Marker
2. HuCC49 2sc(Fv)2 tetravalent antibody (N-scFv tetravalent huCC49 minibody)
3. HuCC49 2sc(Fv)2 tetravalent antibody (N-scFv tetravalent huCC49 minibody) G1/G3/PAP

AMENDED SHEET

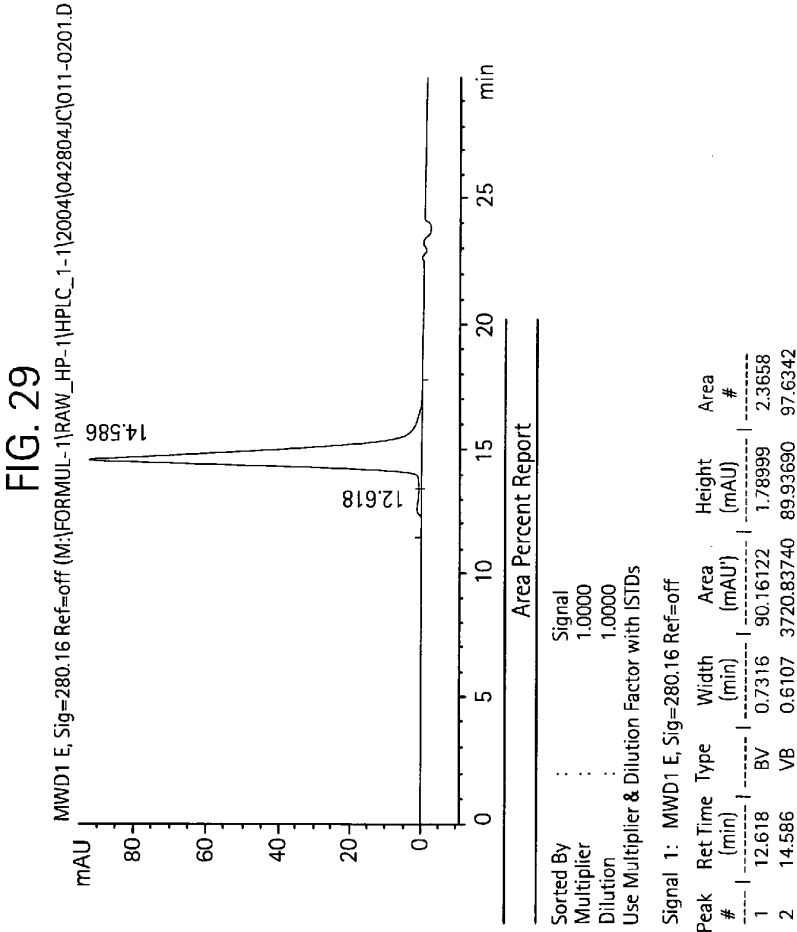
34/41

FIG. 28



Lane 1 Marker  
Lane 2 huCC49 2 sc(Fv)<sub>2</sub> tetravalent antibody  
(N-scFv tetravalent huCC49 minibody)  
G1/G3/PAP - non-reduced  
Lane 3 huCC49 2 sc(Fv)<sub>2</sub> tetravalent antibody  
(N-scFv tetravalent huCC49 minibody)  
G1/G3/PAP - reduced

AMENDED SHEET

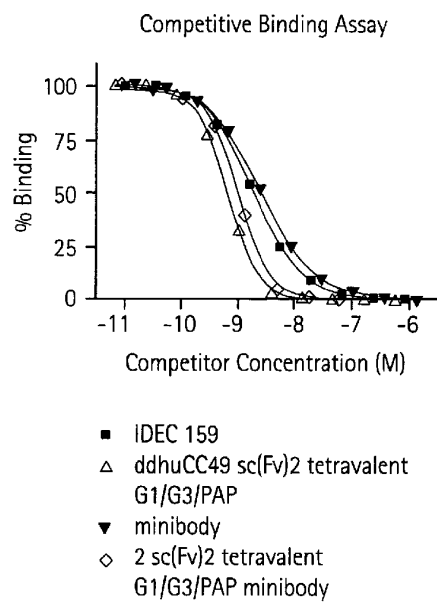


AMENDED SHEET



36/41

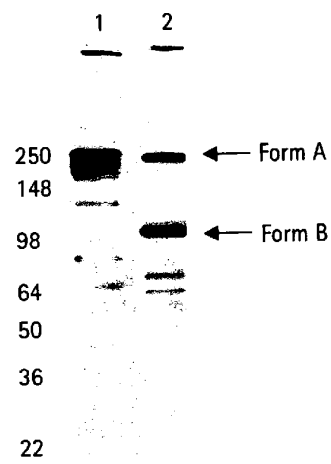
FIG. 30



AMENDED SHEET

37/41

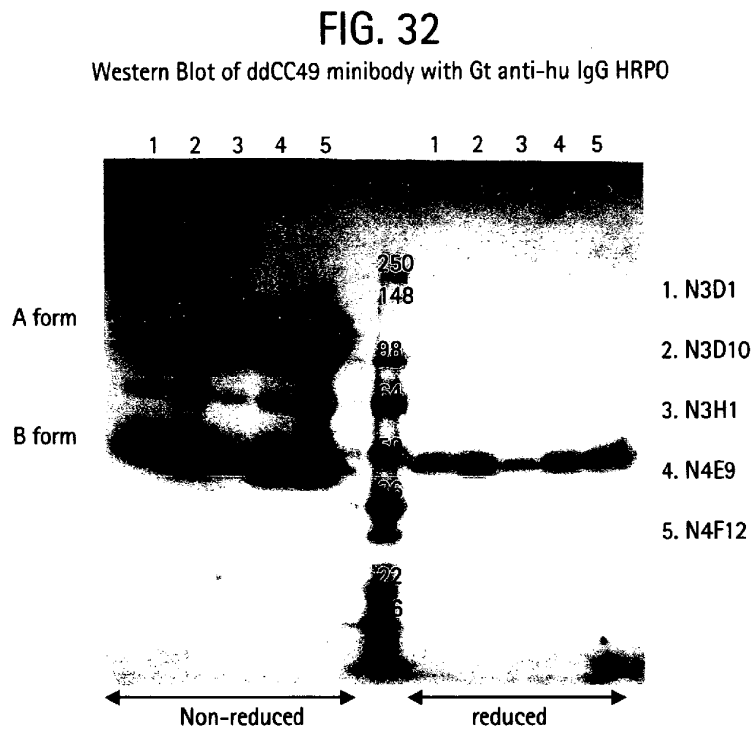
FIG. 31



1. p5E8 + G1G3PAP hinge tetravalent Ab
2. ddCC49 tetravalent Ab

AMENDED SHEET

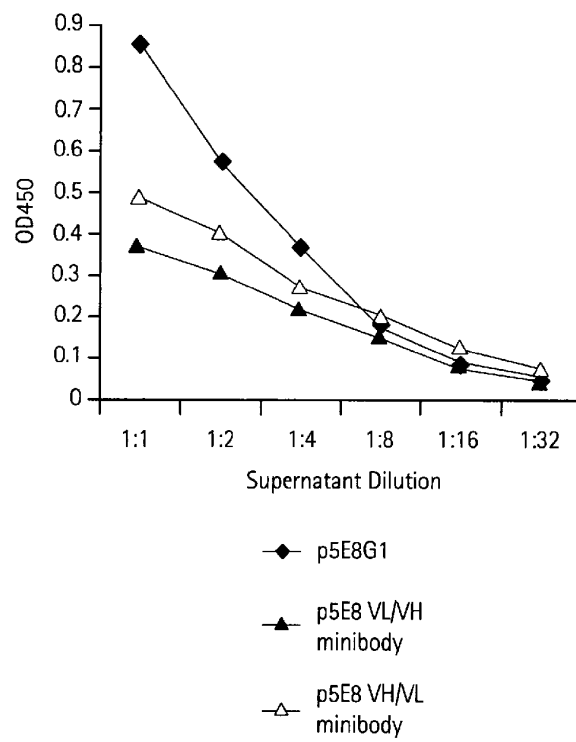
38/41



AMENDED SHEET

39/41

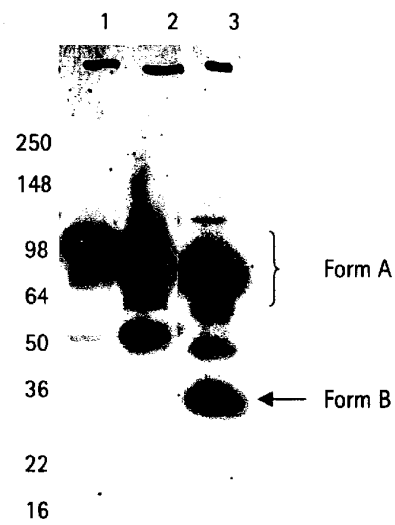
FIG. 33



AMENDED SHEET

40/41

FIG. 34

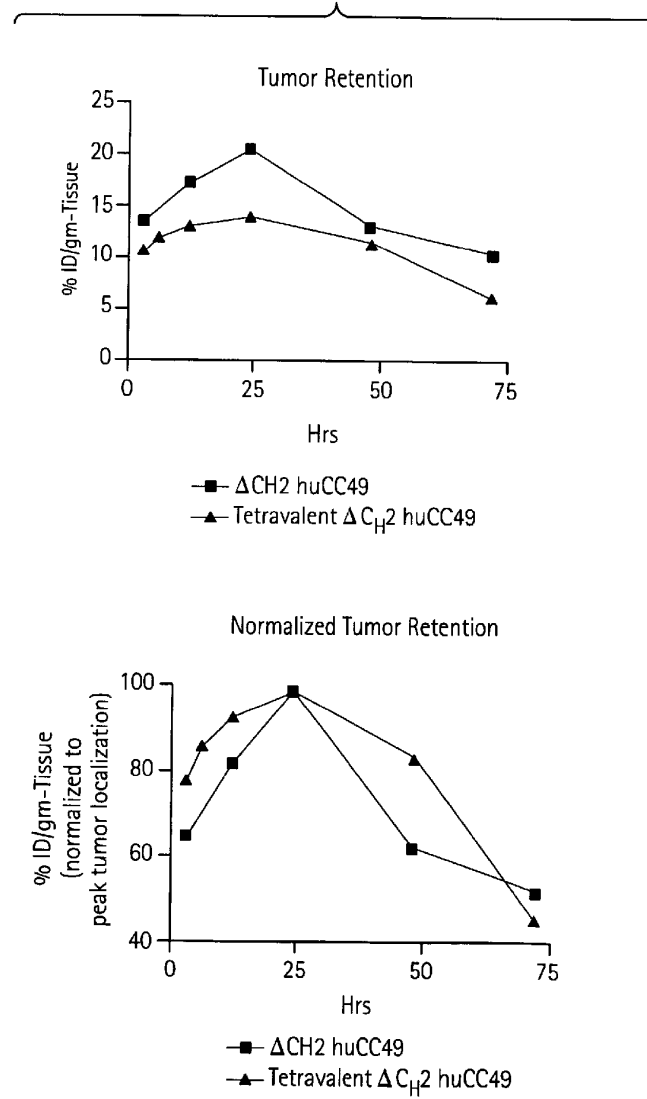


1. p5E8 VL/VH minibody+ G1G3PAP hinge
2. p5E8 VH/VL minibody+ G1G3PAP hinge
3. huCC49 minibody

AMENDED SHEET

41/41

FIG. 35



AMENDED SHEET

# SEQUENCE LISTING

<110> BIOGEN IDEC MA INC. et al.

<120> MODIFIED BINDING MOLECULES COMPRISING  
CONNECTING PEPTIDES

<130> IDV-005PC

<140> PCT/US2004/020945

<141> 2004-06-28

<150> 60/483877

<151> 2003-06-27

<150> 60/508810

<151> 2003-10-03

<150> 60/515351

<151> 2003-10-28

<150> 60/516030

<151> 2003-10-30

<160> 49

<170> FastSEQ for Windows Version 4.0

<210> 1

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 1

Gly	Gly	Gly	Ser	Ser	Gly	Gly	Gly	Ser	Gly
1				5					10

<210> 2

<211> 10

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 2

Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr
1				5					10

<210> 3

<211> 5

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 3

Cys Pro Pro Cys Pro  
1 5

<210> 4

<211> 8

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 4

Ala Pro Glu Leu Leu Gly Gly Pro  
1 5

<210> 5

<211> 12

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 5

Glu Leu Lys Thr Pro Leu Gly Asp Thr Thr His Thr  
1 5 10

<210> 6

<211> 20

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 6

Cys Pro Arg Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys  
1 5 10 15  
Pro Arg Cys Pro  
20

<210> 7

<211> 24

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 7

Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Gly Gly  
1 5 10 15  
Gly Ser Ser Gly Gly Gly Ser Gly  
20



<210> 8  
 <211> 40  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
 <400> 8  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Glu  
 1 5 10 15  
 Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Gly Gly  
 20 25 30  
 Gly Ser Ser Gly Gly Gly Ser Gly  
 35 40

<210> 9  
 <211> 42  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 9  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Glu  
 1 5 10 15  
 Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala Pro  
 20 25 30  
 Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly  
 35 40

<210> 10  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 10  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Ser Pro Pro Cys Pro Gly  
 1 5 10 15  
 Gly Gly Ser Ser Gly Gly Gly Ser Gly  
 20 25

<210> 11  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 11  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Ser Pro Pro Cys Pro Ala

1                    5                    10                    15  
 Pro Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly  
                   20                    25

<210> 12  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 12  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Ser Pro Gly  
   1                    5                    10                    15  
 Gly Gly Ser Ser Gly Gly Gly Ser Gly  
                   20                    25

<210> 13  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 13  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Ser Pro Ala  
   1                    5                    10                    15  
 Pro Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly  
                   20                    25

<210> 14  
 <211> 27  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 14  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala  
   1                    5                    10                    15  
 Pro Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly  
                   20                    25

<210> 15  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 15  
 Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Gly  
   1                    5                    10                    15

Gly Gly Ser Ser Gly Gly Gly Ser Gly  
20 25

<210> 16  
<211> 1809  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 16  
caggtccagc tgggtgcagtc cggcgctgag gtggtgaaac ctggggcttc cgtgaagatt 60  
tcctgcaagg caagcggcta caccttcact gatcacgcaa tccactgggt gaaacagaat 120  
cctggacagc gcctggagtg gattggatat ttctctcccg gaaacgatga ttttaagtac 180  
aatgagaggt tcaagggcaa ggccacactg actgcagaca catctgccag cactgcctac 240  
gtggagctct ccagcctgag atccgaggat actgcagtgt acttctgcac aagatccctg 300  
aatatggcct actggggaca gggaaccctg gtcaccgtct ccagcgctag caccaagggc 360  
ccatcggtct tccccctggc accctcctcc aagagcacct ctgggggcac agcggccctg 420  
ggctgccttg tcaaggacta cttccccgaa ccggtgacgg tgtcgtggaa ctcaggcgcc 480  
ctgaccagcg gcgtgcacac cttcccggt gtccctacagt cctcaggact ctactccctc 540  
agcagcgtgg tgaccgtgcc cttccagcagc ttgggcaccc agacctacat ctgcaacgtg 600  
aatcacaagc ccagcaacac caaggtggac aagaaagttg agcccaaatc ttgtgacaaa 660  
actcacacat gccaccctg cggaggtggc tctagtgtag gcggttccgg agggcagccc 720  
cgagaaccac aggtgtacac cctgccccca tcccggtatg agctgaccaa gaaccaggtc 780  
agcctgacct gcctggtaaa aggtttctat cccagcgaca tgcctgtgga gtgggagagc 840  
aatgggcagc cggagaacaa ctacaagacc acgctctccg tgcctggactc cgacggctcc 900  
ttcttctctc acagcaagct caccgtggac aagagcaggt gccagcaggg gaacgtcttc 960  
tcattgctcc tgatgcattg ggtctctgac aaccactaca cgcagaagag cctctccctg 1020  
tctccgggta aatccggcgg ggtgggatac ggtggagggg gctccggcgg tggcgggtcc 1080  
gacatcgtga tgaagcagtc tccagactcc ctggccgtgt cctggggcga gagggtgact 1140  
ctgaattgca agtccagcca gtccctgctc tatagcggaa atcagaagaa ctatctcgcc 1200  
tggtatcagc agaaaccagg gcagagccct aaactgctga ttactgggc atccgctagg 1260  
gaatccggcg tgcctgacg cttcagcggc agcggatctg ggacagactt cactctgaca 1320  
atcagcagcg tgcagcgaga agacgtggca gtctattatt gtcagcagta ttatagctat 1380  
cccctcacat tcggcgctgg caccagctg gaacttaagg gcggtggcgg gtccggtggg 1440  
ggtggtccg gggcggtgg cttccaggtc cagctggtgc agtccggcgc tgaggtggtg 1500  
aaacctgggg cttccgtgaa gatttctgac aaggcaagcg gctacacctt cactgatcac 1560  
gcaatccact ggggtgaaaca gaatcctgga cagcgctcgg agtggattgg atatttctct 1620  
cccggaaacg atgattttta gtacaatgag aggttcaagg gcaaggccac actgactgca 1680  
gacacatctg ccagcactgc ctacgtggag ctctccagcc tgagatccga ggatactgca 1740  
gtgtacttct gcacaagatc cctgaatatg gcctactggg gacagggaac cctgggtcac 1800  
gtctccagc 1809

<210> 17  
<211> 185/  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 17  
caggtccagc tgggtgcagtc cggcgctgag gtggtgaaac ctggggcttc cgtgaagatt 60  
tcctgcaagg caagcggcta caccttcact gatcacgcaa tccactgggt gaaacagaat 120  
cctggacagc gcctggagtg gattggatat ttctctcccg gaaacgatga ttttaagtac 180  
aatgagaggt tcaagggcaa ggccacactg actgcagaca catctgccag cactgcctac 240  
gtggagctct ccagcctgag atccgaggat actgcagtgt acttctgcac aagatccctg 300  
aatatggcct actggggaca gggaaccctg gtcaccgtct ccagcgctag caccaagggc 360  
ccatcggtct tccccctggc accctcctcc aagagcacct ctgggggcac agcggccctg 420

```

ggctgcctgg tcaaggacta cttccccgaa cgggtgacgg tgtcgtggaa ctccaggcgc 480
ctgaccagcg gcgtgcacac cttccccgct gtccctacagt cctcaggact ctactccctc 540
agcagecgtg tgacctgtgc cttccagcagc ttgggcaccc agacctacat ctgcaacgtg 600
aatcacaaag ccagcaacac caaggtggac aagaaagtgt agcccaaatc ttgtgacaaa 660
actcacacat gccaccctgt cccagagccc aaatcttgtg acacacctcc cccatgccca 720
cggtgccccag gaggtggctc gagtggaggg ggttccggag ggcagccccg agaaccacag 780
gtgtacaccc tgcctccatc cggggatgag ctgaccaaga accagggtcag cctgacctgc 840
ctggtcaaaag gcttctatcc cagcgacatc gccgtggagt gggagagcaa tgggcagccg 900
gagaacaact acaagaccac gcctcccgtg ctggactccg acggctcctt cttcctctac 960
agcaagctca ccgtggacaa gagcaggtgg cagcagggga acgtcttctc atgctccgtg 1020
atgcatgagg ctctgcacaa ccactacacg cagaagagcc tctccctgtc tccgggtaaa 1080
tccggcgggg gtggatccgg tggagggggc tccggcgggt gcgggtccga catcgtgatg 1140
agccagtctc cagactccct ggccgtgtcc ctgggcgaga ggggtactct gaattgcaag 1200
tccagccagt cctctgtcta tagcggaaat cagaagaact atctcgctgt gtatcagcag 1260
aaaccagggc agagccctaa actgctgatt tactgggcat ccgtaggga atccggcgtg 1320
cctgatcgct tcagcggcag cggatctggg acagacttca ctctgacaat cagcagcgtg 1380
caggcagaag acgtggcagt ctattattgt cagcagtatt atagctatcc cctcacattc 1440
ggcgctggca ccaagctgga acttaagggc ggtggcgggt ccggtggggg tggctccggg 1500
ggcggtggct cccaggtcca gctggtgcag tccggcgtgt agtggtgtaa acctggggct 1560
tccgtgaaga tttcctgcaa ggcaagcggc tacaccttca ctgacacgc aatccactgg 1620
gtgaacacaga atcctggaca gcgcctggag tggattggat atttctctcc cggaaacgat 1680
gattttaagt acaatgagag gttcaagggc aagccacac tgactgcaga cacatctgcc 1740
agcaactgct acgtggagct cttcagcctg agatccgagg ataactgcagt gtacttctgc 1800
acaagatccc tgaatatggc ctactgggga cagggaaccc tggtcaccgt ctccagc 1857

```

<210> 18  
 <211> 663  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

```

<400> 18
gacatcgtga tgagccagtc tccagactcc ctggccgtgt ccttgggcga gaggggtgact 60
ctgaattgca agtccagcca gtccctgtct tatagcggaa atcagaagaa ctatctcgcc 120
tggtatcagc agaaccaggg gcagagccct aaactgctga ttacttgggc atccgctagg 180
gaatccggcg tgccgtgatcg cttcagcggc agcggatctg ggacagactt cactctgaca 240
atcagcagcg tgcaggcaga agacgtggca gtctattatt gtcagcagta ttatagctat 300
ccctccacat tcggcgctgg caccagctg gaactgaaac gtacgggtggc tgcaccatct 360
gtcttcatct tcccgcctc tgatgagcag ttgaaatctg gaactgcctc tgttgtgtgc 420
ctgctgaata acttctatcc cagagaggcc aaagtacagt ggaagggtgga taacgccttc 480
caatcgggta actcccagga gagtgtcaca gagcaggaca gcaaggacag cactacacg 540
ctcagcagca ccttgacgct gagcaagca gactacgaga aacacaaagt ctacgcctgc 600
gaagtcaacc atcagggcct gagctcgccc gtcacaaaga gcttcaacag gggagagtgt 660
tga
663

```

<210> 19  
 <211> 603  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

```

<400> 19
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala
1           5           10          15
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His
20          25          30
Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile

```

-173-

Pro Gly Gln Arg Leu Glu Trp Ile Gly Tyr Phe Ser Pro Gly Asn Asp  
530 535 540  
Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala Thr Leu Thr Ala  
545 550 555 560  
Asp Thr Ser Ala Ser Thr Ala Tyr Val Glu Leu Ser Ser Leu Arg Ser  
565 570 575  
Glu Asp Thr Ala Val Tyr Phe Cys Thr Arg Ser Leu Asn Met Ala Tyr  
580 585 590  
Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
595 600

<210> 20  
<211> 621  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 20  
Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala  
1 5 10 15  
Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His  
20 25 30  
Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile  
35 40 45  
Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe  
50 55 60  
Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr  
65 70 75 80  
Val Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
85 90 95  
Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr  
100 105 110  
Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu Ala Pro  
115 120 125  
Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys Leu Val  
130 135 140  
Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser Gly Ala  
145 150 155 160  
Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser Ser Gly  
165 170 175  
Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser Leu Gly  
180 185 190  
Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn Thr Lys  
195 200 205  
Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His Thr Cys  
210 215 220  
Pro Pro Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro  
225 230 235 240  
Arg Cys Pro Ala Pro Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly Gly  
245 250 255  
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu  
260 265 270  
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr  
275 280 285  
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn  
290 295 300  
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe  
305 310 315 320

Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn  
 325 330 335  
 Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr  
 340 345 350  
 Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Gly Gly Gly Ser  
 355 360 365  
 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met Ser Gln  
 370 375 380  
 Ser Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Val Thr Leu Asn  
 385 390 395 400  
 Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr  
 405 410 415  
 Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Leu Ile  
 420 425 430  
 Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly  
 435 440 445  
 Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala  
 450 455 460  
 Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu  
 465 470 475 480  
 Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly Gly Gly Ser  
 485 490 495  
 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln Leu Val Gln  
 500 505 510  
 Ser Gly Ala Glu Val Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys  
 515 520 525  
 Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp Val Lys  
 530 535 540  
 Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile Gly Tyr Phe Ser Pro Gly  
 545 550 555 560  
 Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala Thr Leu  
 565 570 575  
 Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr Val Glu Leu Ser Ser Leu  
 580 585 590  
 Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys Thr Arg Ser Leu Asn Met  
 595 600 605  
 Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser  
 610 615 620

<210> 21  
 <211> 220  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 21  
 Asp Ile Val Met Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly  
 1 5 10 15  
 Glu Arg Val Thr Leu Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser  
 20 25 30  
 Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln  
 35 40 45  
 Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val  
 50 55 60  
 Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
 65 70 75 80  
 Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln  
 85 90 95

Tyr	Tyr	Ser	Tyr	Pro	Leu	Thr	Phe	Gly	Ala	Gly	Thr	Lys	Leu	Glu	Leu
			100					105					110		
Lys	Arg	Thr	Val	Ala	Ala	Pro	Ser	Val	Phe	Ile	Phe	Pro	Pro	Ser	Asp
		115					120					125			
Glu	Gln	Leu	Lys	Ser	Gly	Thr	Ala	Ser	Val	Val	Cys	Leu	Leu	Asn	Asn
	130				135						140				
Phe	Tyr	Pro	Arg	Glu	Ala	Lys	Val	Gln	Trp	Lys	Val	Asp	Asn	Ala	Leu
	145			150						155				160	
Gln	Ser	Gly	Asn	Ser	Gln	Glu	Ser	Val	Thr	Glu	Gln	Asp	Ser	Lys	Asp
			165					170					175		
Ser	Thr	Tyr	Ser	Leu	Ser	Ser	Thr	Leu	Thr	Leu	Ser	Lys	Ala	Asp	Tyr
			180					185				190			
Glu	Lys	His	Lys	Val	Tyr	Ala	Cys	Glu	Val	Thr	His	Gln	Gly	Leu	Ser
	195					200						205			
Ser	Pro	Val	Thr	Lys	Ser	Phe	Asn	Arg	Gly	Glu	Cys				
	210					215					220				

<210> 22  
 <211> 1932  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 22  
 gacatcgtga tgagccagtc tccagactcc ctggccgtgt cctggggcga gaggggtgact 60  
 ctgaattgca agtccagcca gtccctgctc tatagcggaa atcagaagaa ctatctcgcc 120  
 tgggtatcagc agaaccagcg gcagagccct aaactgctga ttactgggc atccgctagg 180  
 gaatccggcg tgctgatcg ctccagcggc agcggatctg ggacagactt cactctgaca 240  
 atcagcagcg tgcaggcaga agacgtggca gtctattatt gtcagcagta ttatagctat 300  
 cccctcacat tcggcgctcg caccaagctg gaacttaagg gcggtggcgg gtccggtggg 360  
 ggtggtccg gggcggtgg ctcccaggtc cagctggtgc agtccggcgc tgaggtggtg 420  
 aaacctgggg ctccgtgaa gatttctgc aaggcaagcg gctacacctt cactgacac 480  
 gcaatccact ggggtgaaaca gaatcctgga cagcgctcgg agtggattgg atatttctct 540  
 cccggaaacg atgattttta gtacaatgag aggttcaagg gcaaggccac actgactgca 600  
 gacacatctg ccagcaactgc ctacgtggag ctctccagcc tgagatccga ggatactgca 660  
 gtgtacttct gcacaagatc cctgaatatg gctactcggg gacagggtac cctggtcacc 720  
 gtctccagcg gcggtggagg gtccggtgga gggggtctcg gaggggcgcg ttcagggggc 780  
 ggtggatcgg gcggaggtgc tagcgacatc gtgatgagcc agtctccaga ctccctggcc 840  
 gtgtccctgg gcgagagggg gactctgaat tgcaagtcca gccagtcctt gctctatagc 900  
 ggaaatcaga agaactatct cgcttggtat cagcagaaac cagggcagag ccctaaactg 960  
 ctgatttact gggcatccgc tagggaatcc gggtgcctg atcgcttcag cggcagcgga 1020  
 tctgggacag acttcaactc gacaatcagc agcgtgcagg cagaagacgt ggcagtcctat 1080  
 tattgtcagc agtattatag ctatcccctc acattcggcg ctggcaccaa gctggaactt 1140  
 aaggggcggtg gcgggtccgg tgggggtggt tccggggcggt gtggctccca ggtccagctg 1200  
 gtgcagtcgg gcgctgaggt ggtgaaacct ggggcttccg tgaagatttc ctgcaaggca 1260  
 agcggctaca ccttcaactg tcacgcaatc cactgggtga aacagaatcc tggacagcgc 1320  
 ctggagtggg ttggatatct ctctcccgga aacgatgatt ttaagtacaa tgagaggttc 1380  
 aaggggcaagg ccacactgac tgcagacaca tctgccagca ctgectacgt ggagctctcc 1440  
 agcctgagat ccgaggatgc tgcagtgtac ttctgcacaa gatccctgaa tatggcctac 1500  
 tggggacagc gaacctggt caccgtctcc agcgtcgacc ccaaatcttg tgacaaaact 1560  
 cacacatgcc caccgtgcgg aggtggctcg agtggaggcg gatccggagg gcagcccccga 1620  
 gaaccacagc tgtacacctt gccccatcc cgggatgagc tgaccaagaa ccagggtcagc 1680  
 ctgacctgcc tgggtcaaagg ctctatccc agcgacatcg ccgtggagtg ggagagcaat 1740  
 gggcagcccg agaacaacta caagaccacg cctcccgtgc tggactccga cggctccttc 1800  
 ttctctaca gcaagctcac cgtggacaag agcaggtggc agcaggggaa cgtcttctca 1860  
 tgctccgtga tgcattgagg tctgcacaac cactacacgc agaagagcct ctccctgtct 1920  
 ccgggtaaat ga 1932



<210> 23  
 <211> 1980  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 23  
 gacatcgtga tgagccagtc tccagactcc ctggccgtgt ccctgggcca gaggggtgact 60  
 ctgaattgca agtccagcca gtccctgctc tatagcggaa atcagaagaa ctatctcggc 120  
 tggatcagc agaaccagg gcagagccct aaactgctga ttacttgggc atccgctagg 180  
 gaatccggcg tgcctgatcg ctccagcggc agcggatctg ggacagactt cactctgaca 240  
 atcagcagcg tgcaggcaga agcgtggcca gtctattatt gtcagcagta ttatagctat 300  
 cccctcacat tcggcgctgg caccaagctg gaacttaagg gcggtggcgg gtccggtggg 360  
 ggtggctccg gggcggtgg ctcccaggtc cagctgggtc agtccggcgc tgagggtggtg 420  
 aaacctgggg ctccgtgaa gatttctgc aaggcaagcg gctacacctt cactgatcac 480  
 gcaatccact ggggtgaaaca gaatccctgga cagcgccctg agtggattgg atatttctct 540  
 cccggaaacg atgattttta gtacaatgag aggttcaagg gcaaggccac actgactgca 600  
 gacacatctg ccagcactgc ctacgtggag ctctccagcc tgagatccga ggatactgca 660  
 gtgtacttct gcacaagatc cctgaatatg gcctactggg gacagggtac cctggtcacc 720  
 gtctccagcg cgggtggagg gtccgggtgga gggggtctcg gagggggcgg ttccaggggc 780  
 ggtggatcgg gcggaggtgc tagcgacatc gtgatgagcc agtctccaga ctccctggcc 840  
 gtgtccctgg gcgagagggg gactctgaat tgcaagtcca gccagtcctt gctctatagc 900  
 ggaatccaga agaactatct cgcctgtgat cagcagaaac cagggcagag ccctaaactg 960  
 ctgatttact gggcatccgc tagggaatcc ggcgtgcctg atcgcttcag oggcagcgga 1020  
 tctgggacag acttactctt gacaatcagc agcgtgcagg cagaagacgt ggcagtctat 1080  
 tattgtcagc agtattatag ctatcccttc acattcggcg ctggcaccac gctggaactt 1140  
 aaggggcggtg gcgggtcccg tgggggtggc tcggggggcg gtggctccca ggtccagctg 1200  
 gtgcagtcgg gcgctgaggt ggtgaaacct ggggcttcgg tgaagatttc ctgcaaggca 1260  
 agcgggtaca ccttactgta tcacgcaatc cactgggtga aacagaatcc tggacagcgc 1320  
 ctggagtgga ttggatatct ctctcccgga aacgatgatt ttaagtacaa tgagagggtc 1380  
 aagggcaagg ccacactgac tgcagacaca tctgccagca ctgcctacgt ggagctctcc 1440  
 agcctgagat ccgaggatac tgcagtgtac ttctgcacaa gatccctgaa tatggcctac 1500  
 tggggacagg gaacctgggt caccgtctcc agcgtcgacc ccaaatcttg tgacaaaact 1560  
 cacacatgcc caccgtgccc agagcccaaa tcttgtgaca cactccccc atgcccacgg 1620  
 tgcccaggag gtggctcgag tggaggcgga tcgggagggc agccccgaga accacaggtg 1680  
 tacacctgct ccccatcccg ggtgagctg accaagaacc aggtcagcct gacctgcctg 1740  
 gtcaaaaggct tctatcccag cgacatcgcc gtggagtggg agagcaatgg gcagccggag 1800  
 aacaactaca agaccacgcc tcccgtgctg gactccgacg gctccttctt cctctacagc 1860  
 aagctcaccg tggacaagag caggtggcag caggggaacg tcttctcatg ctccgtgatg 1920  
 catgaggctc tgcacaacca ctacacgcag aagagcctct cctgtctcc gggtaaatga 1980

<210> 24  
 <211> 643  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 24  
 Asp Ile Val Met Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly  
 1 5 10 15  
 Glu Arg Val Thr Leu Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser  
 20 25 30  
 Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln  
 35 40 45  
 Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val  
 50 55 60

Pro Asp Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
 65 70 75 80  
 Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln  
 85 90 95  
 Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu  
 100 105 110  
 Lys Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser  
 115 120 125  
 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala  
 130 135 140  
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His  
 145 150 155 160  
 Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile  
 165 170 175  
 Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe  
 180 185 190  
 Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr  
 195 200 205  
 Val Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
 210 215 220  
 Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr  
 225 230 235 240  
 Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly  
 245 250 255  
 Gly Ser Gly Gly Gly Ser Gly Gly Gly Ala Ser Asp Ile Val Met  
 260 265 270  
 Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Val Thr  
 275 280 285  
 Leu Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys  
 290 295 300  
 Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu  
 305 310 315 320  
 Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe  
 325 330 335  
 Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val  
 340 345 350  
 Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr  
 355 360 365  
 Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly Gly  
 370 375 380  
 Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gln Val Gln Leu  
 385 390 395 400  
 Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala Ser Val Lys Ile  
 405 410 415  
 Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp  
 420 425 430  
 Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile Gly Tyr Phe Ser  
 435 440 445  
 Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala  
 450 455 460  
 Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr Val Glu Leu Ser  
 465 470 475 480  
 Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys Thr Arg Ser Leu  
 485 490 495  
 Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Val  
 500 505 510  
 Asp Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Gly Gly  
 515 520 525  
 Gly Ser Ser Gly Gly Gly Ser Gly Gly Gln Pro Arg Glu Pro Gln Val  
 530 535 540  
 Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser

545                      550                      555                      560  
 Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu  
                                  565                      570                      575  
 Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro  
                                  580                      585                      590  
 Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val  
                                  595                      600                      605  
 Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met  
                                  610                      615                      620  
 His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser  
 625                      630                      635                      640  
 Pro Gly Lys

<210> 25  
 <211> 661  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 25  
 Asp Ile Val Met Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly  
 1                      5                      10                      15  
 Glu Arg Val Thr Leu Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser  
                                  20                      25                      30  
 Gly Asn Gln Lys Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln  
                                  35                      40                      45  
 Ser Pro Lys Leu Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val  
                                  50                      55                      60  
 Pro Asp Arg Phe Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr  
 65                      70                      75                      80  
 Ile Ser Ser Val Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln  
                                  85                      90                      95  
 Tyr Tyr Ser Tyr Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu  
                                  100                      105                      110  
 Lys Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser  
                                  115                      120                      125  
 Gln Val Gln Leu Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala  
                                  130                      135                      140  
 Ser Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His  
 145                      150                      155                      160  
 Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile  
                                  165                      170                      175  
 Gly Tyr Phe Ser Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe  
                                  180                      185                      190  
 Lys Gly Lys Ala Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr  
                                  195                      200                      205  
 Val Glu Leu Ser Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys  
                                  210                      215                      220  
 Thr Arg Ser Leu Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr  
 225                      230                      235                      240  
 Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly  
                                  245                      250                      255  
 Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ala Ser Asp Ile Val Met  
                                  260                      265                      270  
 Ser Gln Ser Pro Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Val Thr  
                                  275                      280                      285  
 Leu Asn Cys Lys Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys

```

      290              295              300
Asn Tyr Leu Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu
305              310              315              320
Leu Ile Tyr Trp Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe
      325              330              335
Ser Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val
      340              345              350
Gln Ala Glu Asp Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr
      355              360              365
Pro Leu Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly Gly
      370              375              380
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Ser Gln Val Gln Leu
385              390              395              400
Val Gln Ser Gly Ala Glu Val Val Lys Pro Gly Ala Ser Val Lys Ile
      405              410              415
Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp
      420              425              430
Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile Gly Tyr Phe Ser
      435              440              445
Pro Gly Asn Asp Asp Phe Lys Tyr Asn Glu Arg Phe Lys Gly Lys Ala
      450              455              460
Thr Leu Thr Ala Asp Thr Ser Ala Ser Thr Ala Tyr Val Glu Leu Ser
465              470              475              480
Ser Leu Arg Ser Glu Asp Thr Ala Val Tyr Phe Cys Thr Arg Ser Leu
      485              490              495
Asn Met Ala Tyr Trp Gly Gln Gly Thr Leu Val Thr Val Ser Ser Val
      500              505              510
Asp Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Glu
      515              520              525
Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala Pro
      530              535              540
Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly Gly Gln Pro Arg Glu Pro
      545              550              555              560
Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln
      565              570              575
Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala
      580              585              590
Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr
      595              600              605
Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu
      610              615              620
Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser
625              630              635              640
Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser
      645              650              655
Leu Ser Pro Gly Lys
      660

```

```

<210> 26
<211> 1866
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> synthetic construct

```

```

<400> 26
gaggtgcagc tggtggagtc tgggggaggc ttggcaaaagc ctgggggggtc cctgagactc 60
tctgcgcag cctcggggtt caggttcacc ttcaataact actacatgga ctgggtccgc 120
caggctccag ggcaggggct ggagtgggtc tcacgtatta gtagtagtgg tgatcccaca 180

```

```

tggtacgcag actccgtgaa gggcagattc accatctcca gagagaacgc caagaacaca 240
ctgtttcttc aaatgaacag cctgagagct gaggacacgg ctgtctatta ctgtgcgagc 300
ttgactacag ggtctgactc ctggggccag ggagtcctgg tcaccgtctc ctgagctagc 360
accaagggcc catcggtctt cccctggca cctcctcca agagcacctc tgggggcaca 420
gcggccctgg gctgcctggt caaggactac ttcccgaac cgtgacggt gtcgtggaac 480
tcaggcgccc tgaccagcgg cgtgcacacc ttcccggctg tcctacagtc ctgaggactc 540
tactcctca gcagcgtggt gaccgtgccc tccagcagct tgggcaccca gacctacatc 600
tgcaacgtga atcacaagcc cagcaacacc aaggtaggaca agaaagtga gcccaaatct 660
tgtgacaaaa ctcacacatg cccaccgtgc ccagagccca aatcttgtga cacacctccc 720
ccatgcccac ggtgcccagc acctggaggt ggctcgagtg gaggcggttc cggaggggcag 780
ccccgagaac cacaggtgta caccctgccc ccatcccggt atgagctgac caagaaccag 840
gtcagcctga cctgcctggt caaaggcttc tatcccagcg acatcgccgt ggagtgggag 900
agcaatgggc agccggagaa caactacaag accacgcctc ccgtgctgga ctccgacggc 960
tcctttcttc tctacagcaa gctcaccgtg gacaagagca ggtggcagca ggggaacgtc 1020
ttctcatgct ccgtgatgca tgaggctctg cacaaccact acacgcagaa gacccctctc 1080
ctgtctcccg gtaaatccgg cgggggtgga tccggtggag ggggctccgg cgttggcggg 1140
tccgacatcc agatgaccca gtctccatct tccctgtctg catctgtagg ggacagagtc 1200
accatcactt gcagggcaag tcaggacatt aggtattatt taaatttgta tcagcagaaa 1260
ccagggaaaag ctctaaagct cctgatctat gttgcatcca gtttgcaaaag tggggtccca 1320
tcaaggttca cgggcagtggt atctgggaca gagtccactc tcaccgtcag cagcctgcag 1380
cctgaagatt ttgcgactta ttactgtcta caggtttata gtaccctcg gacgttcggc 1440
caagggacca aggtggaat caaaggcggg ggcgggtcgg gtgggggtgg ctccgggggc 1500
ggtggctccg aggtgcagct ggtggagctt gggggcggtc tggcaaaagc tggggggtcc 1560
ctgagactct cctgcgcagc ctccgggttc aggttcacct tcaataacta ctacatggac 1620
tgggtccgcc aggtccagc gcaggggctg gagtgggtct cactattag tagtagtggt 1680
gatccacat ggtaacgaga ctccgtgaag ggcagattca ccatctccag agagaacgcc 1740
aagaacacac tgtttcttca aatgaacagc ctgagagctg aggacacggc tgtctattac 1800
tgtgcgagct tgactacagc gtctgactcc tggggccagg gagtctctgt caccgtctcc 1860
tcatga

```

<210> 27  
 <211> 645  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

```

<400> 27
gacatccaga tgacccagtc tccatcttcc ctgtctgcat ctgtagggga cagagtcacc 60
atcacttgca gggcaagtca ggacattagg tattatttaa attggtatca gcagaaacca 120
ggaaaagctc ctaagctcct gatctatgtt gcattccagt tgcaaaagtg ggtcccatca 180
aggttcagcg gcagtgatc tgggacagag ttcactctca ccgtcagcag cctgcagcct 240
gaagattttg cgacttatta ctgtctacag gtttatagta cccctcggac gttcggccaa 300
gggaccaagg tggaaatcaa acgtacggtg gctgcacat ctgtcttcat ctcccgcca 360
tctgatgagc agttgaaatc tggaaactgcc tctgttgtgt gctgtgtgaa taacttctat 420
cccagagagg ccaaaagtaca gtggaagggt gataacgccc tccaatcggg taactcccag 480
gagagtgtca cagagcagga cagcaaggac agcacctaca gcctcagcag caccctgacg 540
ctgagcaaaag cagactacga gaaacacaaa gtctacgctc gcgaagtcac ccacagggc 600
ctgagctcgc ccgtcacaaa gagcttcaac aggggagagt gttga

```

<210> 28  
 <211> 621  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 28  
 Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Ala Lys Pro Gly Gly

1	5	10	15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Arg Phe Thr Phe Asn			
20	25	30	
Asn Tyr Tyr Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu			
35	40	45	
Trp Val Ser Arg Ile Ser Ser Ser Gly Asp Pro Thr Trp Tyr Ala Asp			
50	55	60	
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Glu Asn Ala Lys Asn Thr			
65	70	75	80
Leu Phe Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr			
85	90	95	
Tyr Cys Ala Ser Leu Thr Thr Gly Ser Asp Ser Trp Gly Gln Gly Val			
100	105	110	
Leu Val Thr Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val Phe Pro			
115	120	125	
Leu Ala Pro Ser Ser Lys Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly			
130	135	140	
Cys Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn			
145	150	155	160
Ser Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln			
165	170	175	
Ser Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser			
180	185	190	
Ser Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser			
195	200	205	
Asn Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr			
210	215	220	
His Thr Cys Pro Pro Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro			
225	230	235	240
Pro Cys Pro Arg Cys Pro Ala Pro Gly Gly Gly Ser Ser Gly Gly Gly			
245	250	255	
Ser Gly Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser			
260	265	270	
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys			
275	280	285	
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln			
290	295	300	
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly			
305	310	315	320
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln			
325	330	335	
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn			
340	345	350	
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Ser Gly Gly			
355	360	365	
Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln			
370	375	380	
Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly Asp Arg Val			
385	390	395	400
Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg Tyr Tyr Leu Asn Trp			
405	410	415	
Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile Tyr Val Ala			
420	425	430	
Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser			
435	440	445	
Gly Thr Glu Phe Thr Leu Thr Val Ser Ser Leu Gln Pro Glu Asp Phe			
450	455	460	
Ala Thr Tyr Tyr Cys Leu Gln Val Tyr Ser Thr Pro Arg Thr Phe Gly			
465	470	475	480
Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Gly Ser Gly Gly Gly			
485	490	495	

Gly Ser Gly Gly Gly Gly Ser Glu Val Gln Leu Val Glu Ser Gly Gly  
                   500                  505                  510  
 Gly Leu Ala Lys Pro Gly Gly Ser Leu Arg Leu Ser Cys Ala Ala Ser  
                   515                  520                  525  
 Gly Phe Arg Phe Thr Phe Asn Asn Tyr Tyr Met Asp Trp Val Arg Gln  
                   530                  535                  540  
 Ala Pro Gly Gln Gly Leu Glu Trp Val Ser Arg Ile Ser Ser Ser Gly  
                   545                  550                  555                  560  
 Asp Pro Thr Trp Tyr Ala Asp Ser Val Lys Gly Arg Phe Thr Ile Ser  
                   565                  570                  575  
 Arg Glu Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Asn Ser Leu Arg  
                   580                  585                  590  
 Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Ser Leu Thr Thr Gly Ser  
                   595                  600                  605  
 Asp Ser Trp Gly Gln Gly Val Leu Val Thr Val Ser Ser  
                   610                  615                  620

<210> 29  
 <211> 214  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 29  
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
   1                  5                  10                  15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg Tyr Tyr  
                   20                  25                  30  
 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
                   35                  40                  45  
 Tyr Val Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
                   50                  55                  60  
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Val Ser Ser Leu Gln Pro  
                   65                  70                  75                  80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln Val Tyr Ser Thr Pro Arg  
                   85                  90                  95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Arg Thr Val Ala Ala  
                   100                  105                  110  
 Pro Ser Val Phe Ile Phe Pro Pro Ser Asp Glu Gln Leu Lys Ser Gly  
                   115                  120                  125  
 Thr Ala Ser Val Val Cys Leu Leu Asn Asn Phe Tyr Pro Arg Glu Ala  
                   130                  135                  140  
 Lys Val Gln Trp Lys Val Asp Asn Ala Leu Gln Ser Gly Asn Ser Gln  
                   145                  150                  155                  160  
 Glu Ser Val Thr Glu Gln Asp Ser Lys Asp Ser Thr Tyr Ser Leu Ser  
                   165                  170                  175  
 Ser Thr Leu Thr Leu Ser Lys Ala Asp Tyr Glu Lys His Lys Val Tyr  
                   180                  185                  190  
 Ala Cys Glu Val Thr His Gln Gly Leu Ser Ser Pro Val Thr Lys Ser  
                   195                  200                  205  
 Phe Asn Arg Gly Glu Cys  
                   210

<210> 30  
 <211> 1173  
 <212> DNA  
 <213> Artificial Sequence

```

<220>
<223> synthetic construct

<400> 30
gacatccaga tgacccagtc tccatcttcc ctgtctgcat ctgtagggga cagagtcacc 60
atcacttgca gggcaagtcg ggacattagg tattatttaa attgggtatca gcagaaacca 120
ggaaaagctc ctaagctcct gatctatgtt gcattccagt tgcaaaagtg ggtcccatca 180
aggtttcagc gcagtggtgc tgggacagag ttcactctca ccgtcagcag cctgcagcct 240
gaagattttg cgacttatta ctgtctacag gtttatagta cccctcggac gttcggccaa 300
gggaccaagg tggaaatcaa aggcgggtgg ggtcccggtg ggggtggctc cggggcggtg 360
ggctccgagg tgcagctggt ggagtcgtgg ggcggttg caaagcctgg ggggtccctg 420
agactctcct gcgcagcctc cgggttcagg ttcacctca ataactacta catggactgg 480
gtccgcagag ctccagggca ggggtctgag tgggtctcac gtattagtag tagtgggtat 540
cccacatggt acgcagactc cgtgaagggc agattcacca tctccagaga gaacgccaag 600
aacacactgt ttcttcaaat gaacagcctg agagctgagg acacggctgt ctattactgt 660
gcgagcttga ctacagggtc tgactcctgg ggcaggggag tectgggtcac cgtctcctca 720
gtcgacccca aatcttctga caaaactcac acatgcccac cgtgccaga gcccaaatct 780
tgtgacacac ctcccccatg cccacggtgc ccagcacctg gagtggtgctc gaggtaggagc 840
ggatccggag ggcagccccc agaaccacag gtgtacaccc tgcccccatc ccgggatgag 900
ctgaccaaga accaggtcag cctgacctgc ctggtcaaa gcttctatcc cagcgacatc 960
gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gctcccgtg 1020
ctggactccg acggctcctt ctctctctac agcaagctca ccgtggacaa gagcaggtgg 1080
cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1140
cagaagagcc tctccctgtc tccgggtaaa tga 1173

<210> 31
<211> 1173
<212> DNA
<213> Artificial Sequence

<220>
<223> synthetic construct

<400> 31
gaggtgcagc tgggtggagtc tgggggcggc ttggcaaacg ctgggggggc cctgagactc 60
tcctgcgcag cctccgggtt caggttcacc ttcaataact actacatgga ctgggtccgc 120
caggtccagc ggcaggggct ggagtggttc tcacgtatta gtagtagtgg tgatccacac 180
tggtacgcag actccgtgaa gggcagattc accatctcca gagagaacgc caagaacaca 240
ctgtttcttc aaatgaacag cctgagagct gaggacacgg ctgtctatta ctgtgcgagc 300
ttgactacag ggtctgactc ctggggccag ggagtcctgg tcaccgtctc ctccagcggt 360
ggcgggtccg gtgggggtgg ctccgggggc ggtggctccg acatccagat gaccagctc 420
ccatcttccc tgtctgcac tgtaggggac agagtcacca tcacttgagc ggcaagtca 480
gacattaggt attattttaa ttggtatcag cagaaccagc gaaaagctcc taagtcctc 540
atctatgttg catccagttt gcaaaagtgg gtcccatcaa ggttcagcgg cagtggtatc 600
gggacagagt tcactctcac cgtcagcagc ctgcagcctg aagattttgc gacttattac 660
tgtctacagg tttatagtag cctcggacg ttccggccag ggaccaaggt ggaatcaaa 720
gtcgacccca aatcttctga caaaactcac acatgcccac cgtgccaga gcccaaatct 780
tgtgacacac ctcccccatg cccacggtgc ccagcacctg gaggtggctc gaggtaggagc 840
ggatccggag ggcagccccc agaaccacag gtgtacaccc tgcccccatc ccgggatgag 900
ctgaccaaga accaggtcag cctgacctgc ctggtcaaa gcttctatcc cagcgacatc 960
gccgtggagt gggagagcaa tgggcagccg gagaacaact acaagaccac gctcccgtg 1020
ctggactccg acggctcctt ctctctctac agcaagctca ccgtggacaa gagcaggtgg 1080
cagcagggga acgtcttctc atgctccgtg atgcatgagg ctctgcacaa ccactacacg 1140
cagaagagcc tctccctgtc tccgggtaaa tga 1173

<210> 32
<211> 390
<212> PRT
<213> Artificial Sequence

```



<220>  
 <223> synthetic construct

<400> 32  
 Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu Ser Ala Ser Val Gly  
 1 5 10 15  
 Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln Asp Ile Arg Tyr Tyr  
 20 25 30  
 Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala Pro Lys Leu Leu Ile  
 35 40 45  
 Tyr Val Ala Ser Ser Leu Gln Ser Gly Val Pro Ser Arg Phe Ser Gly  
 50 55 60  
 Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Val Ser Ser Leu Gln Pro  
 65 70 75 80  
 Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln Val Tyr Ser Thr Pro Arg  
 85 90 95  
 Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys Gly Gly Gly Ser  
 100 105 110  
 Gly Gly Gly Gly Ser Gly Gly Gly Ser Glu Val Gln Leu Val Glu  
 115 120 125  
 Ser Gly Gly Gly Leu Ala Lys Pro Gly Gly Ser Leu Arg Leu Ser Cys  
 130 135 140  
 Ala Ala Ser Gly Phe Arg Phe Thr Phe Asn Asn Tyr Tyr Met Asp Trp  
 145 150 155 160  
 Val Arg Gln Ala Pro Gly Gln Gly Leu Glu Trp Val Ser Arg Ile Ser  
 165 170 175  
 Ser Ser Gly Asp Pro Thr Trp Tyr Ala Asp Ser Val Lys Gly Arg Phe  
 180 185 190  
 Thr Ile Ser Arg Glu Asn Ala Lys Asn Thr Leu Phe Leu Gln Met Asn  
 195 200 205  
 Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr Tyr Cys Ala Ser Leu Thr  
 210 215 220  
 Thr Gly Ser Asp Ser Trp Gly Gln Gly Val Leu Val Thr Val Ser Ser  
 225 230 235 240  
 Val Asp Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro  
 245 250 255  
 Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala  
 260 265 270  
 Pro Gly Gly Gly Ser Ser Gly Gly Ser Gly Gly Gln Pro Arg Glu  
 275 280 285  
 Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn  
 290 295 300  
 Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile  
 305 310 315 320  
 Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr  
 325 330 335  
 Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys  
 340 345 350  
 Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys  
 355 360 365  
 Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu  
 370 375 380  
 Ser Leu Ser Pro Gly Lys  
 385 390

<210> 33  
 <211> 390  
 <212> PRT  
 <213> Artificial Sequence

```

<220>
<223> synthetic construct

<400> 33
Glu Val Gln Leu Val Glu Ser Gly Gly Gly Leu Ala Lys Pro Gly Gly
 1          5          10          15
Ser Leu Arg Leu Ser Cys Ala Ala Ser Gly Phe Arg Phe Thr Phe Asn
 20          25          30
Asn Tyr Tyr Met Asp Trp Val Arg Gln Ala Pro Gly Gln Gly Leu Glu
 35          40          45
Trp Val Ser Arg Ile Ser Ser Gly Asp Pro Thr Trp Tyr Ala Asp
 50          55          60
Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Glu Asn Ala Lys Asn Thr
 65          70          75          80
Leu Phe Leu Gln Met Asn Ser Leu Arg Ala Glu Asp Thr Ala Val Tyr
 85          90          95
Tyr Cys Ala Ser Leu Thr Thr Gly Ser Asp Ser Trp Gly Gln Gly Val
100          105          110
Leu Val Thr Val Ser Ser Gly Gly Gly Ser Gly Gly Gly Gly Ser
115          120          125
Gly Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Ser Pro Ser Ser Leu
130          135          140
Ser Ala Ser Val Gly Asp Arg Val Thr Ile Thr Cys Arg Ala Ser Gln
145          150          155          160
Asp Ile Arg Tyr Tyr Leu Asn Trp Tyr Gln Gln Lys Pro Gly Lys Ala
165          170          175
Pro Lys Leu Leu Ile Tyr Val Ala Ser Ser Leu Gln Ser Gly Val Pro
180          185          190
Ser Arg Phe Ser Gly Ser Gly Thr Glu Phe Thr Leu Thr Val
195          200          205
Ser Ser Leu Gln Pro Glu Asp Phe Ala Thr Tyr Tyr Cys Leu Gln Val
210          215          220
Tyr Ser Thr Pro Arg Thr Phe Gly Gln Gly Thr Lys Val Glu Ile Lys
225          230          235          240
Val Asp Pro Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro
245          250          255
Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala
260          265          270
Pro Gly Gly Gly Ser Ser Gly Gly Ser Gly Gly Gln Pro Arg Glu
275          280          285
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn
290          295          300
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
305          310          315          320
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
325          330          335
Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
340          345          350
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
355          360          365
Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser Leu
370          375          380
Ser Leu Ser Pro Gly Lys
385          390

<210> 34
<211> 10
<212> PRT
<213> Artificial Sequence

```

<220>  
 <223> synthetic construct  
  
 <400> 34  
 Ile Gly Lys Thr Ile Ser Lys Lys Ala Lys  
 1 5 10  
  
 <210> 35  
 <211> 15  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
 <400> 35  
 ggggsggggs ggggs 15  
  
 <210> 36  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
 <400> 36  
 Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly  
 1 5 10 15  
 Gly Gly Gly Ser Gly Gly Gly Ala Ser  
 20 25  
  
 <210> 37  
 <211> 35  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
 <400> 37  
 agagagacat gtggcgacat ccagatgacc cagtc 35  
  
 <210> 38  
 <211> 50  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
 <400> 38  
 ggagccaccc ccaccggacc cgccaccgcc ttgatttcc accttggtcc 50  
  
 <210> 39  
 <211> 56  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 39  
 ggggtccggtg ggggtggctc cgggggcggt ggctccgagg tgcagctggt ggagtc 56

<210> 40  
 <211> 33  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 40  
 agagaggtcg actgaggaga cggtgaccag gac 33

<210> 41  
 <211> 86  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 41  
 agagagacat gtggcgaggt gcagctgggt gagtcggagc cccccccacc ggaccgcga 60  
 ccgctgagg agacggtgac caggac 86

<210> 42  
 <211> 51  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 42  
 ggagccaccc ccaccggacc cgccaccgcc tgaggagacg gtgaccagga c 51

<210> 43  
 <211> 56  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 43  
 ggggtccggtg ggggtggctc cgggggcggt ggctccgaca tccagatgac ccagtc 56

<210> 44  
 <211> 32  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 44  
 agagaggtcg actttgattt ccacottggt cc 32

<210> 45  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 45  
Glu Ser Lys Tyr Gly Pro Pro  
1 5

<210> 46  
<211> 5  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 46  
Cys Pro Ser Cys Pro  
1 5

<210> 47  
<211> 8  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 47  
Ala Pro Glu Phe Leu Gly Gly Pro  
1 5

<210> 48  
<211> 29  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> synthetic construct

<400> 48  
Glu Ser Lys Tyr Gly Pro Pro Cys Pro Ser Cys Pro Glu Pro Lys Ser  
1 5 10 15  
Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala Pro  
20 25

<210> 49  
<211> 15  
<212> PRT  
<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 49

Cys	Pro	Glu	Pro	Lys	Ser	Cys	Asp	Thr	Pro	Pro	Pro	Cys	Pro	Arg
1				5					10					15