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## (56) Related Art

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(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.

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MODIFIED BINDING MOLECULES  
COMPRISING CONNECTING PEPTIDESRelated 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 sulphydryl 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

5 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

10 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

15 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

20 (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

25 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

30 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

35 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 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 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.
- 10      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).
- In another embodiment, the nucleic acid molecule comprises a nucleotide sequence shown in Figure 10B (SEQ ID NO: 23).
- 20      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 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.

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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 30 in which one of the polypeptide chains are linked via at least one interchain disulfide linkage, such that treatment occurs.

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.

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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 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 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.

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 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 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.

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

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5 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,

10 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

15 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.

20 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 25 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.

25 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,

30 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.

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

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.

5 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.

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Figure 6 shows a schematic diagram of a four chain dimeric CH2 domain deleted tetravalent (N<sub>H</sub>-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 heavy chain may be changed.

Figure 7 shows a schematic diagram of a two chain dimeric tetravalent minibody (C-scFv tetravalent minibody) comprising a hinge connecting peptide (HCP). The two chain dimeric tetravalent 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)2 tetravalent (C-scFv tetravalent CH2 domain deleted) huCC49 gene. Figure 8B (SEQ ID NO:17) shows the single-stranded DNA sequence of heavy chain tetravalent CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent 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)2 tetravalent (C-scFv tetravalent CH2 domain deleted) huCC49.

Figure 9A (SEQ ID NO:19) shows the amino acid sequence of heavy chain CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) huCC49. Figure 9B (SEQ ID NO:20) shows the amino acid sequence of heavy chain CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent 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 tetravalent (N- scFv tetravalent) minibody gene. Figure 10B (SEQ ID NO:23) shows the single-stranded DNA sequence of tetravalent CH2 domain-deleted (N- scFv tetravalent) huCC49 tetravalent 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 CH2 domain-deleted (N- scFv tetravalent) huCC49 minibody. Figure 11B (SEQ ID NO:25)

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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 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 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 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

25 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.

35 Figure 17 (SEQ ID NO:33) shows the amino acid sequence of CH2 domain-deleted 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.

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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.

15 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).

20 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.

25 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.

30 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.

35 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

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

Figure 26 shows Western Blots of supernatants from representative clones producing 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 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 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] 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 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 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 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  
10 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 20 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 25 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 30 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 35 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, 10 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 15 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 20 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 25 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 30 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 35 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 5 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 10 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 15 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 20 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 25 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.

30 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 35 (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 5 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 10 "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, 15 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 20 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 25 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 30 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 35 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 15 of interest (e.g. an antigen, ligand, receptor, substrate or inhibitor). Exemplary binding 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 10 which specifically bind (e.g., immunoreact with) a given target. A polypeptide may be 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 15 molecule (e.g., antibody, minibody, domain deleted antibody, or fusion protein having 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 30 binding sites in a polypeptide. Each target binding site specifically binds one target 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
- 5 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.
- 10 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.
- 15 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;
- 20 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
- 25 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
- 30 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 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 VL domain and the N-terminus of the connecting peptide is attached to the C-terminus of the CH3 domain). 20        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 CH1 domain and the N-terminus of the connecting peptide is attached to the C-terminus of the CH3 domain). 25        In one embodiment, a synthetic connecting peptide comprises a portion of a constant region domain. For example, in one embodiment, a connecting peptide that replaces a CH2 domain can comprise a portion of the CH2 domain.

30        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 GGGSSGGGSG (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 GGGSSGGGSG (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 compliment 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 known 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 5 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 10 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 15 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 20 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 25 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 30 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, 35 camelid (e.g., from camels, llamas and related species). In another embodiment, the variable region may be constrictoid 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 5 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 10 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 15 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.

20 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 25 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 30 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 35 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

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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 G1/G3/Pro243Ala244Pro245 + [Gly/Ser] hinge connecting peptide.

5 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.

25 De-immunization can also be used to decrease the immunogenicity of an 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 5 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 10 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 15 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; 20 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.

25 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), 30 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 35 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

5 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,

10 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

15 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

20 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 or more elements or

25 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

30 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

35 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- 30 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 35 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 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).
- 15
- 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 + [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
- 25 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,
- 30 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.
- 35

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 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, 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, 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 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, 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 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 a 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).
- 25           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 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.
- 30           The target binding polypeptides of the invention are multimeric molecules. In one embodiment, the target binding polypeptides are dimers. In one 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 polypeptide chains. In another embodiment, the binding molecules comprise four polypeptide chains.
- 35           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 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 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; Decamiere, 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)); 30 CD4 (Capon et al., *Nature* 337:525-531 (1989); Traumecker et al., *Nature* 339:68-70 (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 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 Peppel 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)).

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 (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) lymphotxin 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 5 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), 10 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. 15 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 20 prorelaxin; 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 25 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).

30 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.

35 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 5 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 15 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 20 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 25 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 30 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 35 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,

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 5 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, 10 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 15 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 20 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 25 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 pd phage or Fd phagemid technology. Exemplary methods are set forth, for example, in EP 30 368 684 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, 35 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.

5 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 10 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.

15

Yet another highly efficient means for generating recombinant antibodies 20 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 25 by reference.

In another embodiment, lymphocytes can be selected by micromanipulation and the variable genes isolated. For example, peripheral blood 30 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 35 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.

10 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.

15 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.

20 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 25 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 30 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 35

- 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 Kruijff, 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 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 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 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 30 35

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

20 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 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.

30 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 NO:35) (Huston et al. 1988. Proc. Natl. Acad. Sci. USA 85:5879). Other connecting peptides are known in the art.

35 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; 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 with 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.

15 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).

20 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 30 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 35 CH3 domain.

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 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; Byrn 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 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  
25 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  
30 can convert the energy obtained upon substrate binding to distort the reaction towards a 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., 5 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.

10 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., 20 *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).

25 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, 30 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 35 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 5 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 10 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 15 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 20 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 of epitope of a molecule.

30 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 35 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<sub>γ</sub> 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<sub>γ</sub>.RI/anti-CD15, anti-p185.sup.HER2/Fc<sub>γ</sub>.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<sub>γ</sub>.RI, Fc<sub>γ</sub>.RII or Fc<sub>γ</sub>.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<sub>γ</sub>.R/anti-HIV; bispecific 30 binding molecules for tumor detection in vitro or in vivo such as anti-CEA/anti-EOTUBE, anti-CEA/anti-DPTA, anti-p185HER2/anti- -hapten); bispecific binding molecules as vaccine adjuvants (see Fanger et al., *supra*); and bispecific binding 35 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 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 5 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 10 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 15 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 20 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.

25 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 30 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, 35 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 5 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 10 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 15 library. (see, e.g., Kruij 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*, *Baculovirus*, etc. In one embodiment, the subject 20 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 25 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 30 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 35 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.

5 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).

10 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).

15 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).

20 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.

25 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 30 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., *peI*B, *pho*A, or *omp*A), 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,

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 15 ribosome entry site (IRES) to provide relatively high levels of polypeptides of the 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), HE LA (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 in 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., J. 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<sup>TM</sup>, 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 5 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, 10 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 15 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 Ba<sup>++</sup> < Ca<sup>++</sup> < Mg<sup>++</sup> < Li<sup>+</sup> < Cs<sup>+</sup> < Na<sup>+</sup> < K<sup>+</sup> < Rb<sup>+</sup> < NH<sub>4</sub><sup>+</sup>, while anions may be ranked in terms of increasing chaotropic effect as PO<sub>4</sub><sup>3-</sup> < SO<sub>4</sub><sup>2-</sup> < CH<sub>3</sub>COOO<sup>-</sup> < Cl<sup>-</sup> < Br<sup>-</sup> < NO<sub>3</sub><sup>-</sup> < ClO<sub>4</sub><sup>-</sup> < I<sup>-</sup> < SCN<sup>-</sup>.

20 In general, Na, K or NH<sub>4</sub> 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: (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> >; Na<sub>2</sub>SO<sub>4</sub> >; NaCl >; NH<sub>4</sub>Cl >; NaBr >; NaSCN. In general, salt concentrations of between about 0.75 and about 2M ammonium sulfate or between about 1 and 4M NaCl are useful.

25 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 30 coupled. The preferred HIC material comprises an agarose resin substituted with phenyl groups. Exemplary HIC material includes: phenyl SEPHAROSE<sup>TM</sup>, FAST FLOW with low or high substitution (Pharmacia LKB Biotechnology, AB, Sweden); phenyl SEPHAROSE<sup>TM</sup> High Performance column; phenyl or butyl-SEPHAROSE<sup>®</sup> CL-4B, butyl- SEPHAROSE<sup>®</sup> FF, octyl-SEPHAROSE<sup>®</sup> FF and phenyl-SEPHAROSE<sup>®</sup> FF 35 (Pharmacia LKB Biotechnology AB, Sweden); Fractogel<sup>TM</sup> EMD Propyl or FRACTOGEL<sup>TM</sup> EMC Phenyl columns (E. Merck, Germany); MACROPREP<sup>TM</sup> Methyl or MACRO-PREP<sup>TM</sup> t-Butyl Supports (Bio-Rad, California); WP HI-Propyl (C3)<sup>TM</sup> 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 5 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. Commun. 9:1466 (1964)).

The choice of a particular gel can be determined by the skilled artisan. In 10 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 15 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 pmolcs/ml gel bed. Gel capacity is a function of the particular protein in question as well as pH, 20 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 25 also be weighed against adverse effects such 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 30 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: 35 (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 hydrophobic substances, e.g., by passing the mixture through a precolumn.

- 5 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 stepwise fashion or using a salt gradient.

- 20 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.

- 25 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 CH<sub>2</sub> 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 CH<sub>2</sub> 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 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: <sup>90</sup>Y, <sup>125</sup>I, <sup>131</sup>I, <sup>123</sup>I, <sup>111</sup>In, <sup>105</sup>Rh, <sup>153</sup>Sm, <sup>67</sup>Cu, <sup>67</sup>Ga, <sup>166</sup>Ho, <sup>177</sup>Lu, <sup>186</sup>Re and <sup>188</sup>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-isothiocycamatobenzyl-3-methyldiothelene 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 <sup>111</sup>In and <sup>90</sup>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 pertechnate ( $TcO_4^-$ ) 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 pertechnate, a reducing agent such as  $SnCl_2$ , 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 <sup>131</sup>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 30 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, 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 35 metal chelators are ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DPTA), 1,4,8,11-tetraazatetradecane, 1,4,8,11-tetraazatetradecane-1,4,8,11-tetraacetic acid, 1-oxa-4,7,12,15-tetraazaheptadecane-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. "<sup>111</sup>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 Carraguillo *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 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 5 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 10 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 15 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 20 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, 25 triethyleneephosphoramide, 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 30 bleomycins, the cytotoxic nucleosides, the pteridine family of drugs, diyenes, 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, florasfur, 6- 35 mercaptopurine, cytarabine, cytosine arabinoside, podophyllotoxin, or podophyllotoxin derivatives such as etoposide or etoposide phosphate, melphalan, vinblastine, vincristine, leurosidine, 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 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
- 25 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.
- 30 Among other cytotoxins, it will be appreciated that polypeptides can also be associated with a biotoxin such as ricin subunit A, abrin, diphtheria toxin, botulinum, cyanginosins, saxitoxin, shigatoxin, tetanus, tetrodotoxin, trichothecene, verruculogen 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.

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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,

5 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

10 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

15 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

20 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, 1mg/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-

25 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

30 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

35 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  $\mu$ g/ml and in some methods 25-300  $\mu$ g/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.

5 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 10 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.

15 20 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 25 complete amelioration of symptoms of disease. Thereafter, the patient can be administered a prophylactic regime.

25 30 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  $\mu$ g to 10 mg, or 30-300  $\mu$ g DNA per patient. Doses for infectious viral vectors vary from 10-100, or more, virions per dose.

35 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<sup>TM</sup> 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 25 <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 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>87</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 30 invention. Further, in view of the instant disclosure it is submitted that one skilled in the 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 5 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 10 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 15 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 20 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.

25 However, as discussed above, selected embodiments of the invention comprise the administration of polypeptides to myelosuppressed patients or in conjunction 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, 30 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 35 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/mm<sup>3</sup> or platelet counts lower than about 150,000/mm<sup>3</sup>. 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 15 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 20 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 25 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 5 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), 10 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, 15 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 20 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 25 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 30 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 35

- 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.
- 15 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
- 20 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
- 25 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

- 30 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.
- 35 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 5 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 10 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 15 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 20 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 25 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 30 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, 35 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 primate. 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.

15 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 5 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® and Rituxan®, IDEC Pharmaceuticals Corp., San Diego), Lym 1 and Lym 2 (Technicclone), LL2 (Immunomedics Corp., New Jersey), HER2 (Herceptin®, Genentech 10 Inc., South San Francisco), B1 (Bexxar®, Coulter Pharm., San Francisco), Campath® (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- $\alpha$ ), 15 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 20 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 25 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 30 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®. Rituxan® (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 35 incorporated herein by reference). Y2B8 (90Y labeled 2B8; Zevalin®; ibritumomab tiuxetan) is the murine parent of C2B8. Rituxan® 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.* 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 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 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 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 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 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 Hodgkin's and non-Hodgkin's 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 invention are particularly effective in treating a variety of B-cell lymphomas, including low grade/ follicular non-Hodgkin's lymphoma (NHL), cell lymphoma (FCC), 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.

20 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; Drythema 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 erythema-  
tosus; Pemphigus foliaceus; Pemphigus vulgaris; Pigeon breeder's disease; Pneumonitis, hypersensitivity; Polyarteritis nodosa; Polymyalgia rheumatica; Polymyositis; Polyneuritis, 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. 44:400). In another example, an antibody which is non-toxic, but has been derivatized 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).

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

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.

10

Table 1: IgG1, IgG3 and IgG4 Hinge Regions

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

15

Table 2: Hinge Region Connecting Peptide Sequences

20

TABLE 2

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

- Nucleic acid sequences encoding the hinge region connecting peptides 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 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.
- 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 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 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 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 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 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 5 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 10 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 15 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 20 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 25 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 30 degraded and there was almost no difference in gel patterns among the samples. Under this condition, trace amount of smear 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 35 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  $A_{320}$  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	0.080	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	0.068	0.057	0.101	0.126

- 5 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
- 10 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.
- 15 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,
- 20 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
- 25 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
- 30 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 5 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.

10

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

Time (month)	pH=3	A-isoform			B-isoform			Mixture				
		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)	pH=3	A-isoform			B-isoform			Mixture				
		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

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

Time (month)	pH=3	A-isoform			B-isoform			Mixture				
		5	7	9	3	5	7	9	3	5	7	9
½	86.31	97.50	85.06	66.42	79.85	94.29	89.68	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)2 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)2 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>3</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)2 tetravalent antibody. Figure 8A shows the DNA sequence of heavy chain huCC49 domain-deleted sc(Fv)2 tetravalent antibody. Figure 8C shows the DNA sequence of light chain huCC49 domain-deleted sc(Fv)2 tetravalent antibody. Figure 9A shows the amino acid sequence of heavy chain huCC49 domain-deleted sc(Fv)2 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)2 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)2 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)2 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)2 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)2 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)2 tetravalent antibody Form A was tested in a competitive binding assay for it's ability to bind to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved fluorometric immunoassay using a Delphia fluorimeter (Wallac Inc., Gaithersburg, MD). Competitive binding curves are shown in Figure 21. 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

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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 <sup>90</sup>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 <sup>90</sup>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 mm<sup>3</sup>. Samples were harvested and processed for beta counting. Percentage injected dose (%ID) of <sup>90</sup>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
<b>HuCC49 (domain deleted)</b>					
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
<b>Tetravalent HuCC49</b>					
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 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 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 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 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. 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 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

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-

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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 fluorometric immunoassay 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- 35 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 5 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 10 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 15 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.

20 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 25 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 30 ~41 kDa doublet. 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. 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.

35 As shown in Figure 26, representative isolated minibody and 2 sc(Fv)2 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 Tetraivalent Antibody  
Comprising a Novel Connecting Peptide and Preferential Synthesis of Isoform A

5           A huCC49 2 sc(Fv)2 tetraivalent antibody (N-scFv tetraivalent 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 G1/G3/Pro243Ala244Pro245 and part of [Gly/Ser] connecting peptide. The  
15          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 tetraivalent antibody (N-scFv tetraivalent minibody) gene containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide. Figure 11B shows the amino acid sequence of huCC49 2 sc(Fv)2 tetraivalent antibody (N-scFv tetraivalent 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 tetraivalent minibody (N-scFv tetraivalent minibody) Form A and  
30          Form B isoforms. Under these conditions, huCC49 2 sc(Fv)2 tetraivalent 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 tetraivalent minibody containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser] connecting peptide was found to produce  
35          essentially all Form A tetraivalent 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 tetraivalent 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.

5       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

10      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

15      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).

20      Purified huCC49 2 sc(Fv)2 tetravalent G1/G3/Pro243Ala244Pro245 minibody was tested in a competitive binding assay for it's ability to bind to bovine submaxillary mucine, a source of the TAG-72 antigen, by time-resolved flurometric immunoassay using a Delphia fluorimeter (Wallac Inc., Gaithersburg, MD). Competitive binding curves are shown in Figure 30. HuCC49 2 sc(Fv)2 tetravalent

25      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

30      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 (Fc $\epsilon$ RII) (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 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>3</sub>Ser)<sub>3</sub> linker → VH orientation and is described in greater detail in Example 12. Correct sequences were 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 PRIMATIZED® p5E8 sc(Fv)2 tetravalent antibody.

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

20 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 antibodies is generally applicable to antibodies of varying specificities.

35

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 in the tables below:

Table 7. PCR primers used to construct CH2 domain-deleted PRIMATIZED® p5E8 VL/VH minibody containing the G1/G3/Pro243A1a244Pro245 + [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'- GGAGCCACCCCCACCGGACCCGCCACCGCCTTGATTCCACCTGGTCC-  
3'

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

15 5'-

GGGTCCGGTGGGGTGGCTCCGGGGCGGTGGCTCCGAGGTGCAGCTGGT  
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'
- 10 23-VH-1R (SEQ ID NO:42)  
5'-GGAGCCACCCCCACCGGACCCGCCACCGCCTGAGGAGACGGTGACCAGG  
AC-3'
- 15 23-VL-2F (SEQ ID NO:43)  
5'-  
15 GGGTCCGGTGGGGGTGGCTCCGGGGCGTGGCTCCGACATCCAGATGAC  
CCAGTC-3'
- 20 N-23-VL-2R (SEQ ID NO:44)  
5'- AGAGAGGTCGACTTGTATTCCACCTTGGTCC-3'
- 25 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 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)

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.

15 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 20 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 25 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 30 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

35 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.

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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.

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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.

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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.
  13. A composition comprising minibody molecules comprising two polypeptide chains, 15 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% 20 of the molecules are present in a form in which one of the polypeptide chains are linked via at least one interchain disulfide linkage.
  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.
  - 25 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.
  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.

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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 241EE 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 and at least two polypeptide chains, wherein said at least two polypeptide chains each comprise a complete Ig heavy chain, and a chimeric hinge, 25 wherein said chimeric hinge connects the CH1 and the CH2 domain of the Ig heavy chain, and

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- 5 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.
- 10 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.
- 15 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
- 20 wherein greater than 90% of the polypeptide dimers comprise polypeptide chains that are linked via at least one interchain disulfide linkage.
- 25 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 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.
- 30 32. A vector comprising the nucleic acid molecule of claim 31.

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

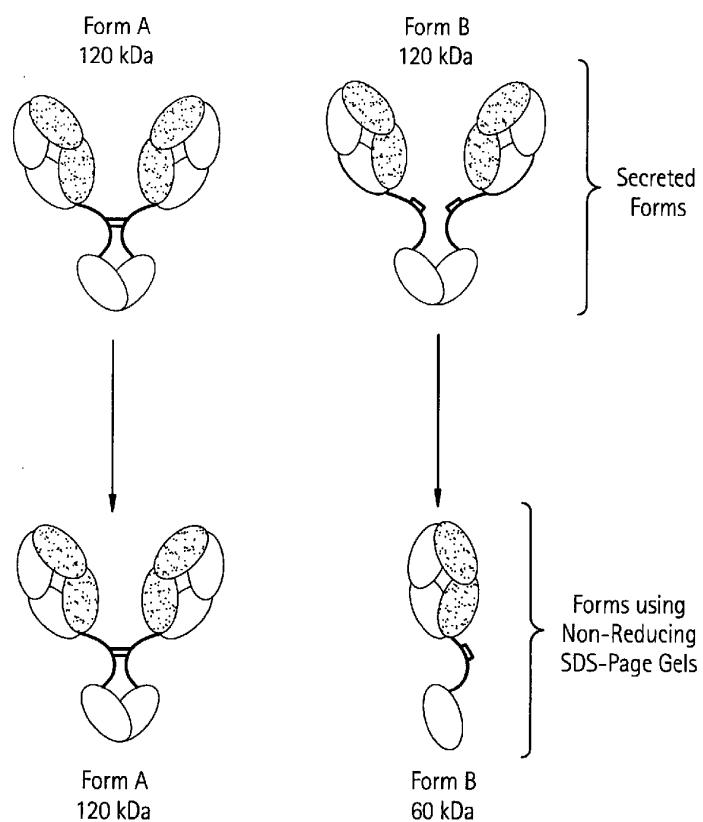
Date: 6 April 2011

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FIG. 1

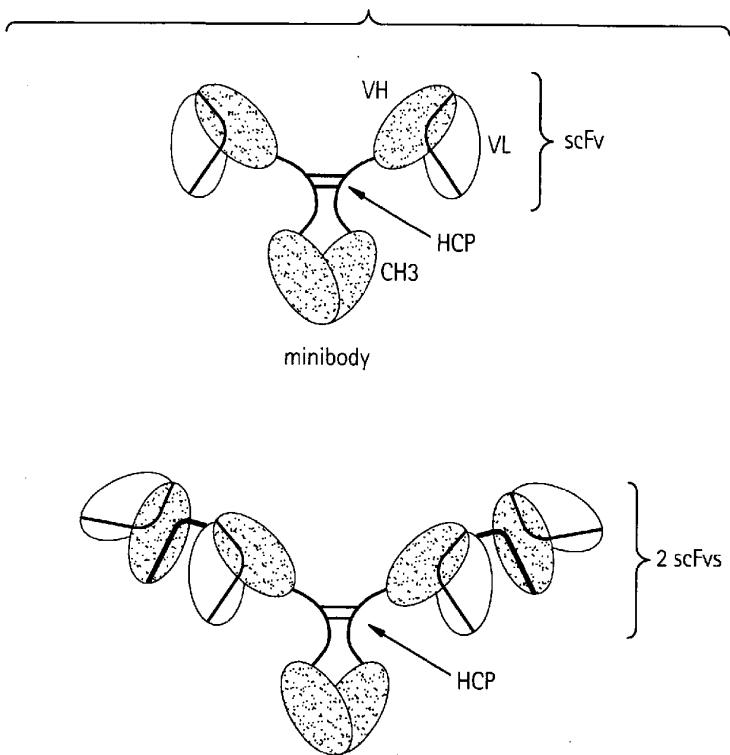
Diagrammatic Representation of the  
Two Secreted forms of the Domain Deleted Constructs



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FIG. 2



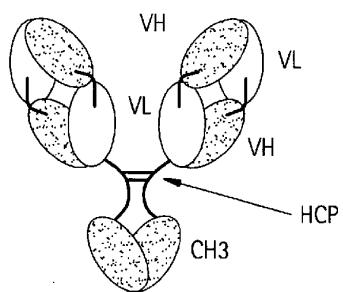
2 sc(Fv)2 Tetraivalent antibody (N-scFv tetravalent minibody)  
(can also be constructed as a bispecific N-scFv tetravalent minibody)

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
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FIG. 3



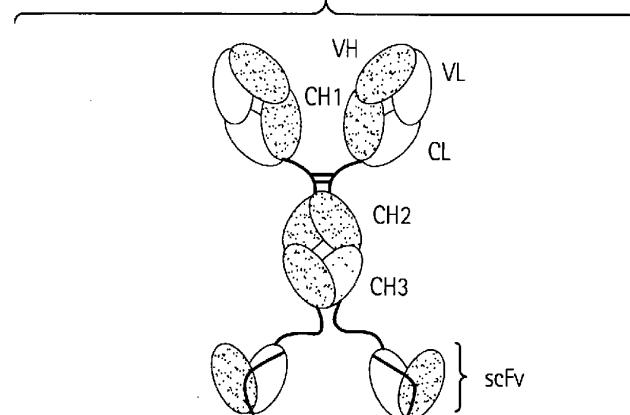
diabody

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

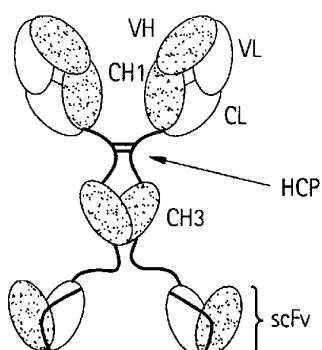
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FIG. 4



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)

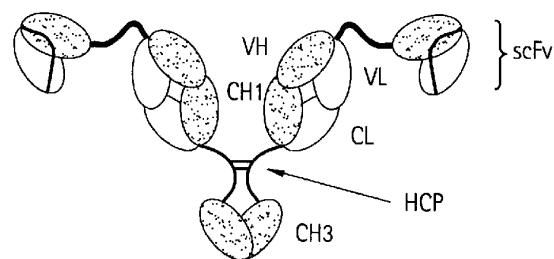
V = Variable
C = Constant
L = Light
H = Heavy
HCP = Hinge Connecting Peptide
= G4S flexible linkers

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## FIG. 5

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



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

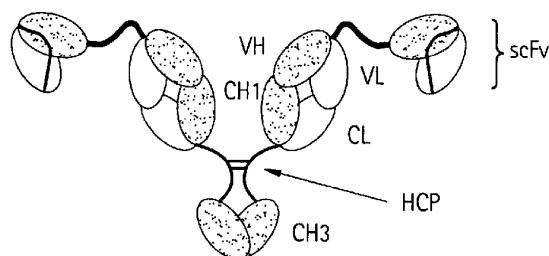
V = Variable
C = Constant
L = Light
H = Heavy
HCP = Hinge Connecting Peptide
G <sub>4</sub> S flexible linker
(G <sub>4</sub> S) <sub>4</sub> G <sub>3</sub> AS flexible linker

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## FIG. 6

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



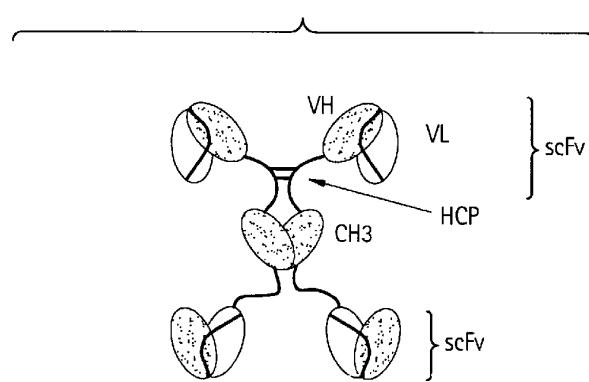
N<sub>H</sub>-scFv Tetravalent (or tetravalent bispecific) CH2 Domain Deleted Antibody

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

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FIG. 7



C - scFv tetravalent (or tetravalent bispecific) minibody

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

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## FIG. 8A

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

(SEQ ID NO:16)

```

CAGGTCCAGCTGGTGCAGTCGGCGCTGAGGTGGTGAACACCTGGGCTCCGTGA
AGATTCCTGCAGGGCAAGGGCTACACCTTCACTGATCACGCAATCCACTGGT
GAAACAGAACCTGGACAGGGCCTGGAGTGGATTGGATATTCCTCTCCCGAAC
GATGATTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACA
CATCTGCCAGCAGTGCCTACGTGGAGCTCTCCAGCTGAGATCCGAGGATACTGC
AGTGTACTTCTGCACAAGATCCCTGAATATGGCTACTGGGACAGGGAACCTG
GTCACCGTCTCCAGCGCTAGCACCAAGGGCCATCGGTCTTCCCCCTGGCACCC
CCTCCAAGAGCACCTCTGGGGCACAGCGGCCCTGGCTGCCTGGTCAAGGACTA
CTTCCCCAACCGGTGACGGTGTGACTCAGGCCTGACCAGCGCGTGC
CACACCTCCCGCTGCTCACAGTCCCTCAGGACTCTACTCCCTCAGCAGCGTGG
TGACCGTGCCTCCAGCAGCTGGGACCCAGACCTACATCTGCAACGTGAATCA
CAAGCCCAGCACACCAAGGTGGACAAGAAAGTTGAGGCCAATCTTGTGACAAA
ACTCACACATGCCAACCGTGCAGGGAGGTGGCTGAGTGGAGGCCGTTCCGGAGGGC
AGCCCCGAGAACACAGGTGTAACACCCCTGCCCATCCGGGATGAGCTGACCAA
GAACCCAGGTGACGCTGACCTGGTCAAAGGCTTCTATCCAGCGACATCGCC
GTGGAGTGGGAGAGCAATGGGAGCCGAGAACAAACTACAAGGACACGCGCTCCG
TGCTGGACTCCGACGGCTCCTCTTCTCACAGCAAGCTCAGGTGGACAAGAG
CAGGTGGCACCAAGGGAACCTCTCATGCTCCGTGATGAGGCTCTGAC
AACCACTACACCGAGAACAGCCTCTCCCTGTCTCCGGTAAATCCGGGGGGTG
GATCCGGTGGAGGGGCTCCGGCGGTGGCGGGTCCGACATCGTGTGAGCCAGTC
TCCAGACTCCCTGGCGTGTCCCTGGCGAGAGGGTACTCTGAATTGCAAGTCC
AGCCAGTCCCTGCTCTATAGCGGAAATCAGAGAACATCTGCCCTGGTATCAGC
AGAAACCAGGGAGAGCCCTAAACTGCTGATTTACTGGCATCCGTAGGGATC
CGCGTGCCTGATCGCTCAGCGCAGCGGATCTGGCACAGACTCACTTGAC
ATCAGCAGCGTGCAGGCAGAACAGCTGGCAGTCTATTATTGTCAGCAGTATTATA
GCTATCCCTCACATTGGCGTGGCACCAAGCTGGAACTTAAGGGCGGTGGCG
GTCCGGTGGGGGTGGCTCGGGGGCGGTGGCTCCCAAGGTCCAGCTGGTGCAGTCC
GGCGCTGAGGTGGTGAACCTGGGCTTCCGTGAAGATTCTGCAAGGCAAGCG
GCTACACCTTCACTGATCACGCAATCCACTGGGTAAACAGAACATCTGGACAGCG
CCTGGAGTGGATTGGATATTCTCTCCGGAAACGATGATTTAAGTACAATGAG
AGTTCAAGGGCAAGGCCACACTGACTGCAGACACATCTGCCAGCACTGCCTACG
TGGAGCTCTCCAGCCTGAGATCGAGGATACTGCACTGTACTCTGCAACAAGATC
CCTGAATATGGCCTACTGGGGACAGGGAACCTGGTACCGTCTCCAGC

```

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## FIG. 8B

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

(SEQ ID NO:17)

CAGGTCCAGCTGGTGCAGTCGGCGCTGAGGTGGTGAACCTGGGGCTTCCGTGA  
 AGATTCCTGCAAGGCAAGCGCTACACCTTCACTGATCACGCAATCCACTGGGT  
 GAAACAGAATCTGGACAGCGCTGGAGTGGATTGGATATTCCTCCGGAAAC  
 GATGATTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGAC  
 CATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGC  
 AGTGTACTTCTGCACAAGATCCCTGAATATGGCTACTGGGGACAGGGAACCTG  
 GTCACCGTCTCCAGCGCTAGCACCAAGGGCCATCGGCTTCCCGTGGCACCC  
 CCTCCAAGAGCACCTCTGGGGCACAGCGGCCCTGGGCTGCCTGGTCAAGGACTA  
 CTTCCCCGAACCGGTGACGGTGTGCTGGAACTCAGGCCTGAGCTGGGG  
 CACACCTCCCGCTGCTCACAGTCTCAGGACTCTACTCCCTCAGCAGCGTGG  
 TGACCGTGCCTCCAGCAGCTGGGACCCAGACCTACATCTGCAACGTGAATCA  
 CAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCAAATCTTGTGACAAA  
 ACTCACACATGCCACCGTGCCAGGCCAAATCTTGTGACACACCTCCCCAT  
 GCCCACGGTGCCAGGAGGTGGCTGAGTGGAGGCGTTCCGGAGGGCAGCCCC  
 AGAACCCACAGGTGTACACCCCTGCCCTGAAATCCCGGGATGAGCTGACCAAGAAC  
 GTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCAGCGACATGCCGTGGAGT  
 GGGAGAGCAATGGCAGCGGAGAACAACTACAAGACACGCCCTCCGTGCTGGA  
 CTCCGACGGCTCTTCTCACAGCAAGCTCACCGTGGACAAGAGCAGGTGG  
 CAGCAGGGGAACGTCTCATGCTCCGGTAAATCCGGGGGTGGATCCAGG  
 ACACCGAGAACGCTCTCCCTGTCTCCGGTAAATCCGGGGGTGGATCCAGG  
 TGGAGGGGGCTCCGGCGGTGGCGGATCTGATGAGCCAGTCTCCAGAC  
 TCCCTGGCGTGTCCCTGGCGAGAGGGTGAECTCTGAATTGCAAGTCCAGCAGT  
 CCCTGCTCTAGCGAAATCAGAAGAACTATCTCGCCTGGTATCAGCAGAAAC  
 AGGGCAGAGCCTAAACTGCTGTTACTGGGACAGACTCAGTCTGACAATCAGCA  
 CCTGATCGCTTACGGCAGCGGATCTGGGACAGACTCAGTCTGACAATCAGCA  
 GCGTGCAGGCAGAACGACGTGGCAGTCTATTATTGTCAGCAGTATTATAGCTATCC  
 CCTCACATTGCGCTGGCACCAAGCTGGAAACTTAAGGGGGTGGCGGGTCCGGT  
 GGGGGTGGCTCCGGGGCGGTGGCTCCAGGTCCAGCTGGTGCAGTCCGGCGCTG  
 AGGTGGTAAACCTGGGGCTTCCGTAAAGATTCTGCAAGGCAAGCGGCTACAC  
 CTTCACTGATCACGCAATCCACTGGGTAAACAGAAATCCTGGACAGCGCCTGGAG  
 TGGATTGGATATTCCTCCGGAAACGATGATTAAAGTACAATGAGAGGTCA  
 AGGGCAAGGCCACACTGACTGAGACACATCTGCCAGCACTGCCTACGTGGAGCT  
 CTCCAGCCTGAGATCCGAGGATACTGCAGTGTACTTCTGCACAAGATCCCTGAAT  
 ATGGCCTACTGGGACAGGGAAACCTGGTACCGCTCCAGC

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## 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)

```
GACATCGTGATGAGCCAGTCTCCAGACTCCCTGGCCGTGTCCTGGCGAGAGGG
TGACTCTGAATTGCAAGTCCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAA
CTATCTCGCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTAC
TGGGCATCCGCTAGGGAATCCGGCGTGCCTGATCGCTTCAGCGGCAGCGGATCTG
GGACAGACTTCACTCTGACAATCAGCAGCTGCAGGCCAGAAGACGTGGCAGTCTA
TTATTGTCAGCAGTATTATAGCTATCCCCCACATTGGCGCTGGCACCAAGCTG
GAACTGAAACGTACGGTGGCTGCACCATCTGCTTCATCTCCGCCATCTGATG
AGCAGTTGAAATCTGGAACTGCCTCTGTTGTGCCTGCTGAATAACTCTATCC
CAGAGAGGCCAAAGTACAGTGGAAAGGTGGATAACGCCCTCCAATCGGGTAACCTC
CAGGAGAGTGTACAGAGCAGGACAGCAAGGACAGCACCTACAGCCTCAGCAGCA
CCCTGACGCTGAGCAAAGCAGACTACGAGAAACACAAAGTCTACGCCCTGCGAAGT
CACCCATCAGGCCCTGAGCTCGCCCGTCACAAAGAGCTTCAACAGGGGAGAGTGT
TGA
```

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## FIG. 9A

Amino acid sequence of heavy chain huCC49 CH2 domain-deleted sc(Fv)2 tetravalent (C-scFv tetravalent CH2 domain deleted) antibody

(SEQ ID NO:19)

QVQLVQSGAEVVKGASVKISCKASGYTFTDHAIHWVKQNPQQRLEWIGYFSPGN  
DDFKYNERFKGKATLTADTSASTAYVELSLRSEDTAVYFCTRSLNMAYWGQGTL  
VTVSSASTKGPSVFLAPSSKSTSGGTAAALGCLVKDYFPEPVTVSWNSGALTSGV  
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK  
THTCPCGGGSSGGSGGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIA  
VEWESENQQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALH  
NHYTQKSLSLSPGKSGGGGGGGGGSDIVMSQSPDSLAVSLGERVTLNCKS  
SQSLLYSGNQKNYLAWYQQKPGQSPKLLIYWASARESGVPDRFSGSGGTDFLLT  
ISSVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGGGGSGGGGGSGGGSQVQLVQS  
GAEVVKGASVKISCKASGYTFTDHAIHWVKQNPQQRLEWIGYFSPGNDDFKYNE  
RFKGKATLTADTSASTAYVELSLRSEDTAVYFCTRSLNMAYWGQGTLVTVSS\*

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## FIG. 9B

Amino acid sequence of heavy chain 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.

(SEQ ID NO: 20)

QVQLVQSGAEVVKGPGASVKISCKASGYTFTDHAIHWVKQNPQQRLEWIGYFSPGN  
DDFKYNERFKGKATLTADTSASTAYVELSSLRSEDTAVYFCTRSLNMAYWGQGTL  
VTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTWSWNSGALTSGV  
HTFPAVLQSSGLYSLSSVTVPSSSLGTQTYICNVNHPNSNTKVDKVEPKSCDK  
THTCPCPEPKSCDTPPPCKRCAPGGSSGGSGQQPREPQVYTLPPSRDELT  
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYSKLTVDKS  
RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKSGGGSSGGGGSGGGSDIVMSQS  
PDSLAVSLGERVTLNCKSSQSLLYSGNQKNYLAWYQQKPGQSPKLLTYWASARES  
GVPDRFSGSGSGTDFTLTIISSVQAEDVAVYYCQQYYSYPLTFGAGTKLELKGGGG  
SGGGGSGGGGSQVQLVQSGAEVVKGPGASVKISCKASGYTFTDHAIHWVKQNPQQR  
LEWIGYFSPGNDFKYNERFKGKATLTADTSASTAYVELSSLRSEDTAVYFCTRS  
LNAYWGQGTLTVSS\*

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## 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)

GACATCGT GATGAGCCAGTCTCCAGACTCCCTGGCCGTGTCCTGGCGAGAGGG  
 TGACTCTGAATTGCAAGTCAGCCAGTCCCTGCTCTATAGCGAAATCAGAAAGAA  
 CTATCTCGCCTGGTATCAGCAGAAACCAGGGCAGAGCCCTAAACTGCTGATTAC  
 TGGGCATCCGCTAGGGATCCGGCGTGCCTGATCGCTCAGCAGCAGCGGATCTG  
 GGACAGACTTCACTCTGACAATCAGCAGCGTGCAGGAGAACAGCTGGCAGTCTA  
 TTATTGTCAGCAGTATTATAGCTATCCCTCACATTGGCGTGGCACCAAGCTG  
 GAACTTAAGGGCGGTGGCGGGTCCGGTGGGGTGGCTCCGGGGCGGTGGCTCCC  
 AGGTCCAGCTGGTGCAGTCGGCGCTGAGGTGGTGAACCTGGGCTCCGTGAA  
 GATTTCCTGCAAGGCAAGCGGCTACACCTCACTGATCACGCAATCCACTGGGTG  
 AAACAGAATCCTGGACAGCGCCTGGAGTGGATTGGATATTCTCTCCCGAAACG  
 ATGATTTAAGTACAATGAGAGGTTCAAGGGCAAGGCCACACTGACTGCAGACAC  
 ATCTGCCAGCACTGCCTACGTGGAGCTCTCCAGCCTGAGATCCGAGGATACTGCA  
 GTGTACTTCTGACAAGATCCCTGAATATGGCTACTGGGACAGGGTACCCCTGG  
 TCACCGTCTCCAGCGCGGTGGAGGGTCCGGTGGAGGGGGCTTGGAGGGGGCGG  
 TTCAGGGGGCGGTGGATCGGGCGAGGTGCTAGCGACATCGTGTAGGCAAGCT  
 CCAGACTCCCTGGCGTGTCCCTGGCGAGAGGGTGAECTCTGAATTGCAAGTCCA  
 GCCAGTCCCTGCTCTATAGCGAAATCAGAAGAACTATCTGCCGTGGTATCAGCA  
 GAAACCAGGGCAGAGCCCTAAACTGCTGATTACTGGGATCCGCTAGGGAAATCC  
 GGGGTGCTGATCGCTTCAGCGCAGCGGATCTGGGACAGACTTCACTGACAA  
 TCAGCAGCGTGCAGGCGAGACGTCAGCTATTATGTCAGCAGTATTATAG  
 CTATCCCTCACATTGGCGTGGCACCAAGCTGGAACCTAAGGGCGGTGGCGGG  
 TCCGGTGGGGCTGGCTCCGGGGCGTGGCTCCAGGTCCAGCTGGTGCAGTC  
 GCGCTGAGGTGGTGAACCTGGGCTTCCGTGAAGATTCTGCAAGGCAAGCGG  
 CTACACCTTCACTGATCACGCAATCCACTGGGTGAAACAGAATCTGGACAGCG  
 CTGGAGTGGATTGGATTTCTCTCCGGAAACAGTGAATTAAAGTACAATGAGA  
 GGTCAAGGGCAAGGCCACACTGACTGCAGACACATGCCAGCACTGCCCTACGT  
 GGAGCTCTCCAGCCTGAGATCCGAGGATACTGCACTGACTTCTGCAAGATCC  
 CTGAATATGGCTACTGGGACAGGGAAACCTGGTACCGCTCCAGCGTCGACC  
 CCAAATCTTGTGACAAAACACACATGCCAACCGTGCAGGAGGTGGCTGAGTGG  
 AGCGGATCCGGAGGGCAGCCCGAGAACACAGGTGTACACCCCTGCCCATCC  
 CGGGATGAGCTGACCAAGAACCGAGTCAGCCTGACCTGCTGGTCAAAGGCTTCT  
 ATCCCAGCGACATGCCGTGGAGTGGGAGAGCAATGGGAGCCGGAGAACAACTA  
 CAAGACCACGCCCTCCGTGCTGGACTCCGACGGCTCTTCTTCTACAGCAAG  
 CTCACCGTGGACAAGAGCAGGTGGCAGGGAAACGTTCTCATGCTCCGTGA  
 TGCATGAGGCTCTGCACAACCACTACACGAGAACAGCCTCTCCGTCTCCGGG  
 TAAATGA

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## 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
TGACTCTGAATTGCAAGTCAGCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAA
CTATCTCGCCTGGTATCAGCAGAAACCCAGGGCAGAGCCCTAAACTGCTGATTAC
TGGGCATCCGCTAGGGAAATCCGGCGTGCCTGATCGCTTCAGCGGCAGCGGATCTG
GGACAGACTTCACTCTGACAATCAGCAGCGTGCAGGGAGAAGACGTGGCAGTCTA
TTATTGTCAGCAGTATTATAGCTATCCCTCACATTGGCGCTGGCACCAAGCTG
GAACCTTAAGGGCGGTGGCGGGTCCGGTGGGGTGGCTCCGGGGCGGTGGCTCCC
AGGTCCAGCTGGTGCAGTCGGCGCTGAGGTGGTGAACACTGGGGCTTCCGTGAA
GATTTCCTGCAAGGCAAGCGGCTACACCTTCACTGATCACGCAATCCACTGGGTG
AAACAGAATCTGGACAGCGCCTGGAGTGGATTGGATATTCTCCCGAACG
ATGATTTAAGTACAATGAGAGGTTAAGGCCAACACTGACTGCAGACAC
ATCTGCCAGCACTGCCTACGTGGAGCTCCAGCCTGAGATCCGAGGATACTGCA
GTGTACTTCTGACAAGATCCCTGAATATGGCCTACTGGGGACAGGGTACCCCTGG
TCACCGTCTCCAGCGCGGTGGAGGGTCCGGTGGAGGGGGCTCTGGAGGGGGCGG
TTCAGGGGGCGGTGGATGGCGAGGGTCTAGCGACATCGTGTGAGCCAGTCT
CCAGACTCCCTGGCGTGTCCCTGGCGAGAGGGTGAECTCTGAATTGCAAGTCCA
GCCAGTCCCTGCTCTATAGCGGAAATCAGAAGAACTATCTGCCCTGGTATCAGCA
GAAACCAGGGCAGAGCCCTAAACTGCTGATTTACTGGGCATCCGCTAGGGAAATCC
GGCGTGCCTGATCGCTTCAGCGCAGCGGATCTGGGACAGACTTCACTGACAA
TCAGCAGCGTGCAGGCAGAAGACGTGGAGCTATTATTGTCAGCAGTATTATAG
CTATCCCTCACATTGGCGCTGGCACCAAGCTGGAACCTAAGGGCGGTGGCGGG
TCCGGTGGGGTGGCTCCGGGGCGGTGGCTCCAGTCCAGCTGGTGCAGTCCG
GCGCTGAGGTGGTGAACACTGGGCTTCCGTGAAGATTCTGCAAGGCAAGCGG
CTACACCTTCACTGATCACGCAATCCACTGGGTGAAACAGAATCTGGACAGCGC
CTGGAGTGGATTGGATATTCTCTCCCGAAACGATGATTTAAGTACAATGAGA
GGTTCAAGGCCAACGCCCCACTGACTGCAGACACATCTGCCAGCACTGCCCTACGT
GGAGCTCTCCAGCCTGAGATCCGAGGATACTGCACTGGTACTTCTGCACAAGATCC
CTGAATATGGCCTACTGGGACAGGGAACCTGGTCAACCGTCTCCAGCGTGCACC
CCAAATCTGTGACAAAAACTCACACATGCCAACCGTGCCAGGCCCCAAATCTTG
TGACACACCTCCCCATGCCACAGGTGCCAGGGTGGCTCGAGTGGAGGGGGA
TCCGGAGGGCAGCGGGAGAACACCACAGGTGTACACCCCTGCCCATCCGGGATG
AGCTGACCAAGAACCCAGGTCACTGACCTGCCTGGTCAAAGGCTTCTATCCCG
CGACATGCCGTGGAGTGGAGAGCAATGGCAGCCGGAGAACAACTACAAGACC
ACGCCCTCCCGTCTGGACTCCGACGGCTCCCTCTTCTACAGCAAGCTCACCG
TGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGCATGA
GGCTCTGCACAACCACATCACGCAGAAGAGCCTCTCCGTCTCCGGTAAATGA

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## 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)

```
DIVMSQSPDSLAVSLGERVTLNCKSSQSLYSGNQKNYLAWYQQKPGQSPKLLIYWA
SARESGVPDRFSGSGSGTDFTLTISSVQAEDVAVYYCQQYYSYPLTFGAGTKLELKG
GGGSGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHAIHWVKQNPQ
RLEWIGYFSPGNDFKYNERFKGKATLTADTSASTAYVELSSLRSEDTAVYFCTRSL
NMAYWGQGTLVTVSSGGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHA
IHWVKQNPQGQRLEWIGYFSPGNDFKYNERFKGKATLTADTSASTAYVELSSLRSE
DTAVYFCTRSLNMAYWGQGTLVTVSSDPKSCDKTHTCPCGGGGGGGGQPREPVYTL
PPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLYS
KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSPGK*
```

## 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)

```
DIVMSQSPDSLAVSLGERVTLNCKSSQSLYSGNQKNYLAWYQQKPGQSPKLLIYWA
SARESGVPDRFSGSGSGTDFTLTISSVQAEDVAVYYCQQYYSYPLTFGAGTKLELKG
GGGSGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHAIHWVKQNPQ
RLEWIGYFSPGNDFKYNERFKGKATLTADTSASTAYVELSSLRSEDTAVYFCTRSL
NMAYWGQGTLVTVSSGGGGSGGGGSQVQLVQSGAEVVKPGASVKISCKASGYTFTDHA
IHWVKQNPQGQRLEWIGYFSPGNDFKYNERFKGKATLTADTSASTAYVELSSLRSE
DTAVYFCTRSLNMAYWGQGTLVTVSSDPKSCDKTHTCPCPEPKSCDTPPCPRCPAP
GGGGGGGGGGQPREPVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTPPVLDSDGSFFLYSCLTVKSRWQQGNVFSCSVMHEALHNHYTQKSLSPGK*
```

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## FIG. 12A

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

(SEQ ID NO:26)

GAGGTGCAGCTGGTGGAGCTGGGGGGCTTGGCAAAGCCTGGGGGGTCCCTGA  
 GACTCTCCTGCCAGCCTCCGGGTTCAAGGTTCACCTCAATAACTACATGGAA  
 CTGGGTCCGCCAGGCTCCAGGGCAGGGCTGGAGTGGGTCACGGTATTAGTAGT  
 AGTGGTGATCCACATGGTACGCAGACTCCGTGAAGGGCAGATTACCATCTCCA  
 GAGAGAACGCCAAGAACACACTGTTCTCAATGACAGCCTGAGAGCTGAGGA  
 CACGGCTGTCTTACTGTGCGAGCTTGACTACAGGGCTGACTCCTGGGCCAG  
 GGAGTCCTGGTCAACCGTCTCCCTCAGCTAGCACCAAGGGCCATCGGTCTCCCC  
 TGGCACCCCTCCCAAGAGCACCTCTGGGGCACAGCGGCCCTGGCTGCCTGGT  
 CAAGGACTACTTCCCCGAACCGGTGACGGTGTGTTGAACTCAGGCCTGACC  
 AGCGCGTGCACACCTCCCGCTGTCTCACAGTCCTCAGGACTCTACTCCCTCA  
 GCAGCGTGGTGACCGTGCCTCCAGCAGCTGGGACCCAGACCTACATCTGCAA  
 CGTGAATACAAGCCCAGCAACACCAAGGTGGACAAGAAAGTTGAGCCAAATCT  
 TGTGACAAAATCACACATGCCACCGTGCCCAGAGCCTAACTTGTGACACAC  
 CTCCCCATGCCACGGTGCCAGCACCTGGAGGTGGCTCGAGTGGAGGCCGTT  
 CGGAGGGCAGCCCCGAGAACACAGGTGTAACCCCTGCCCATCCGGGATGAG  
 CTGACCAAGAACAGGTGACGCTGACCTGGTCAAAGGTTCTATCCAGCG  
 ACATGCCGTGGAGTGGGAGAGCAATGGCAGCCGAGAACAACTACAAGACAC  
 GCCTCCCGTGTGGACTCCGACGGCTCCCTCTCACAGCAAGCTCACCGTG  
 GACAAGAGCAGGTGGCAGCAGGGAACCTCTCATGCTCCGTGATGCATGAGG  
 CTCTGCACAAACCACTACACCGAGAACAGCCTCTCCCTGTCTCCGGTAAATCCGG  
 CGGGGGTGGATCCGGTGGAGGGGCTCCGGCGGTGGCGGGTCCGACATCCAGATG  
 ACCCAGTCTCCATCTCCCTGTCTGCATCTGTAGGGACAGAGTCACCATCACT  
 GCAGGGCAAGTCAGGACATTAGGTATTATTTAAATTGGTATCAGCAGAAACCAAGG  
 AAAAGCTCTAAGCTCTGATCTATGTCATCCAGTGTCAAAGTGGGTCCA  
 TCAAGGTTCAGCGGCAGTGGATCTGGGACAGAGTCACCTCACCGTCAGCAGCC  
 TGCAGCCTGAAGATTTCGCACTTATTACTGTCTACAGGTTATAGTACCCCTCG  
 GACGTTCGGCCAAGGGACCAAGGTGGAAATCAAAGGGTGGCAGCTGGTAGTCTGGGGCGGCT  
 GGTGGCTCCGGGGCGGTGGCTCCGAGGTGAGCTGGTAGTCTGGGGCGGCT  
 TGGCAAAGCCTGGGGGCTCTGAGACTCTCTGCAGCCTCCGGGTTCAAGGTT  
 CACCTCAATAACTACTACATGGACTGGTCCGCCAGGCTCCAGGGCAGGGCTG  
 GAGTGGGTCTCACTATTAGTAGTGTGGATCCCACATGGTACGCAGACTCCG  
 TGAAGGGCAGATTACCATCTCCAGAGAGAACGCCAGAACACACTGTTCTCA  
 AATGAACAGCCTGAGAGCTGAGGACACGGCTGTCTATTACTGTGCGAGCTTACT  
 ACAGGGTCTGACTCCTGGGCCAGGGAGTCCTGGTACCGTCTCCTCATGA

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## 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)

GACATCCAGATGACCCAGTCTCCATCTCCCTGTCTGCATCTGTAGGGGACAGAG  
TCACCATCACTTGCAGGGCAAGTCAGGACATTAGGTATTATTTAAATTGGTATCA  
GCAGAAACCAGAAAAGCTCTAAGCTCTGATCTATGTTGCATCCAGTTGCAA  
AGTGGGGTCCCCTCAAGGTCAGCGGCAAGGGATCTGGGACAGAGTTCACTCTCA  
CCGTCAAGCAGCCTGCAGCCTGAAGATTGCGACTTATTACTGTCTACAGGTTA  
TAGTACCCCTCGGACGTTGGCAAGGGACCAAGGTGGAAATCAAACGTACGGTG  
GCTGCACCCTGTTGCGCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACA  
CTGCCTCTGTTGCGCTGCTGAATAACTTCTATCCCAGAGAGGCCAAAGTACA  
GTGGAAGGTGGATAACGCCCTCCAATCGGTAACTCCCAGGAGAGTGTACAGAG  
CAGGACAGCAAGGGACAGCACCTACAGCCTCAGCAGCACCCCTGACGCTGAGCAAAG  
CAGACTACGAGAAAACACAAAGTCTACGCCCTGCGAAGTCACCCATCAGGGCCTGAG  
CTCGCCCGTCACAAAGAGCTTCAACAGGGAGAGTGTGA

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## 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  
SGDPTWYADSVKGRFTISRENAKNTLFLQMNLSRAEDTAVYYCASLTTGSDSWGQ  
GVLTVTSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVWNSGALT  
SGVHTFPAPLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKVEPKS  
CDKTHTCPPCPEPKSCDTPPPCCPRCPAPGGGSSGGSGQPREPQVYTLPPSRDE  
LTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPVLDSDGSFFLYSKLTV  
DKSRWQQGNVFSCSVMEALHNHYTQKSLSLSPGKSGGGGGGGGGSDIQM  
TQSPSSLSASVGDRVTITCRASQDIRYYLNWYQQKPGKAPKLLIYVASSLQSGVP  
SRFSGSGSGTEFTLTVSSLQPEDFATYYCLQVYSTPRTFGQGTKVEIKGGGGSGG  
GGSGGGGSEVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAPGQGL  
EWVSRISSSGDPWTWYADSVKGRFTISRENAKNTLFLQMNLSRAEDTAVYYCASLT  
TGSDSWGQGVLTVSS

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## FIG. 13B

Amino acid 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

(SEQ ID NO: 29)

DIQMTQSPSSLSASVGDRVTITCRASQDIRYYLNWYQQKPGKAPKLLIYVASSLQ  
SGVPSRFSGSGSGTEFTLTVSSLQPEDFATYYCLQVYSTPRTFGQGTKVEIKRTV  
AAPSVFIFPPSDEQLKSGTASVVCLNNFYPREAKVQWKVDNALQSGNSQESVTE  
QDSKDSTYSLSTTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC\*

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**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)

GACATCCAGATGACCCAGTCTCCATCTCCCTGTCTGCATCTGTAGGGACAGAG  
 TCACCATCACTGCAAGGCAGTCAGGACATTAGGTATTATTTAAATTGGTATCA  
 GCAGAAACCAGGAAAAGCTCTAAGCTCTGATCTATGTTGCATCCAGTTGCAA  
 AGTGGGGTCCCATCAAGGTCAGCGCAGTGGATCTGGACAGAGTTCACTCTCA  
 CCGTCAGCAGCTGCAAGATTGCGACTTATTACTGTCTACAGGTTA  
 TAGTACCCCTCGGACGTTGGCCAAGGGACCAAGGTGAAATCAAAGCGGTGGC  
 GGGTCCGGTGGGGTGGCTCCGGGGGGCTGGCTCCGAGGTGCAGCTGGTGGAGT  
 CTGGGGGGCGGCTTGGCAAAGCCTGGGGGTCCCTGAGACTCTCTGCGCAGCCTC  
 CGGGTTCAAGGTCACCTCAATAACTACATGGACTGGTCCGCCAGGCTCCA  
 GGGCAGGGGCTGGAGTGGGCTCACGTATTAGTAGTGTGGTGAATCCCACATGGT  
 ACGCAGACTCCGTGAAGGGCAGATTCAACCATCTCCAGAGAACGCCAAGAACAC  
 ACTGTTCTCAAATGAACAGCCTGAGAGCTGAGGACACGGCTGTCTATTACTGT  
 GCGAGCTTGACTACAGGGTCTGACTCCTGGGCCAGGGAGTCCTGGTCACCGCT  
 CCTCAGTCGACCCCAAATCTGTGACAAAACACATGCCACCGTGCCAG  
 GCCCAAATCTGTGACACACCTCCCCATGCCACGGTGCCAGCACCTGGAGGT  
 GGCTCGAGTGGAGGCAGATCCGGAGGGCAGCCCCGAGAACACAGGTGTACACCC  
 TGCCCCCATCCGGGATGAGCTGACCAAGAACAGGTCACTGACCTGCCTGGT  
 CAAAGGCTTCTATCCAGCGACATGCCGTGGAGTGGAGAGCAATGGCAGCCG  
 GAGAACAACTACAAGACCACGCCTCCCGTGTGGACTCCGACGGCTCTTCTCC  
 TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGAACGTCTTCTC  
 ATGCTCGTGATGCATGAGGCTCTGCACAAACACTACACGCAGAAGAGCCTCTCC  
 CTGTCTCCGGTAAATGA

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**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)

GAGGTGCAGCTGGTGGAGCTGGGGGGCCCTGGCAAAGCCTGGGGGGTCCCTGA  
 GACTCTCCTGCCAGCCTCCGGGTCAGGTCACCTCAATAACTACTACATGGA  
 CTGGGTCCGCCAGGCTCCAGGGCAGGGCTGGAGTGGGTCACGTATTAGTAGT  
 AGTGGTGATCCACATGGTACGCAGACTCCGTGAAGGGCAGATTACCCATCTCCA  
 GAGAGAACGCCAAGAACACACTGTTCTCAAATGAAACGCCCTGAGAGCTGAGGA  
 CACGGCTGTCTATTACTGTGCGAGCTTGACTACAGGGCTGACTCCTGGGCCAG  
 GGAGTCCTGGTACCGTCTCCTCAGGCGGTGGCGGGTCCGGTGGGGTGGCTCCG  
 GGGGCCTGGCTCCGACATCCAGATGACCCAGTCTCCATCTTCCCTGTCTGCATC  
 TGTAGGGGACAGAGTCACCATCACTTGCAAGGGCAAGTCAGGACATTAGGTATTAT  
 TTAAATTGGTATCAGCAGAAACCAAGGAAAGCTCTAAAGCTCCTGATCTATGTTG  
 CATCCAGTTGCAAAGTGGGGTCCCATCAAGGTTCACGGCCAGTGGATCTGGAC  
 AGAGTTCACTCTCACCGTCAGCAGCCTGCAGCCTGAAGATTTCGCACTTATTAC  
 TGTCTACAGGTTTATAGTACCCCTCGGACGTTGGCCAAGGGACCAAGGTGGAAA  
 TCAAAGTCGACCCAAATCTTGTGACAAAACCTCACACATGCCACCGTGCCAGA  
 GCCCAAATCTTGTGACACACCTCCCCATGCCACGGTGCCAGCACCTGGAGGT  
 GGCTCGAGTGGAGGCAGATCCGGAGGGCAGCCCCGAGAACACAGGTGTACACCC  
 TGCCCCCATCCGGGATGAGCTGACCAAGAACAGGTCACTGACCTGCCTGGT  
 CAAAGGCTTCTATCCCAGCGACATGCCGTGGAGTGGAGAGCAATGGCAGCCG  
 GAGAACAACTACAAGACCACGCCTCCCGTGGACTCCGACGGCTCCTCTTCC  
 TCTACAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTC  
 ATGCTCCGTGATGCATGAGGCTCTGCACAACCAACTACACGCAGAAGAGCCTCTCC  
 CTGTCCTCCGGTAAATGA

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**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  
SGVPSRFSGSGSGTEFTLTVSSLQPEDFATYYCLQVYSTPRTFGQGTKVEIKGGG  
GSGGGGSGGGSEVQLVESGGGLAKPGGLRISCAASGFRFTFNNYYMDWVRQAP  
GQGLEWVSRISSSGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYC  
ASLTTGSDSWGQGVLTWVSSVDPKSCDKTHTCPPCPEPKSCDTPPPCCPRCPAPGG  
GSSGGGSGGQPREPVYTLPPSRDELTKNQVSLTC1VKGFYPSDIAVEWESNGQP  
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS  
LSPGK

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**FIG. 17**

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

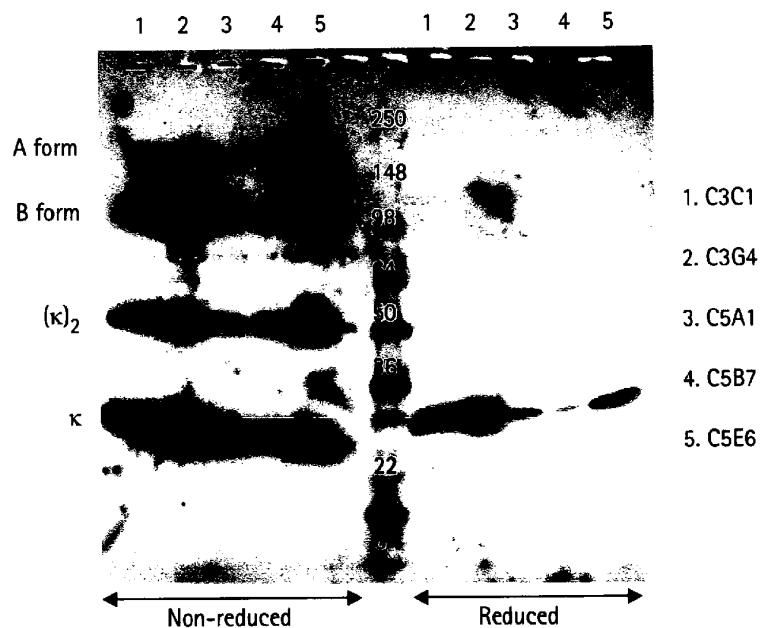
(SEQ ID NO: 33)

EVQLVESGGGLAKPGGSLRLSCAASGFRFTFNNYYMDWVRQAPGQGLEWVSRISS  
SGDPTWYADSVKGRFTISRENAKNTLFLQMNSLRAEDTAVYYCASLTGSDSWGQ  
GVLTVSSGGGGSGGGGGSDIQMTQSPSSLSASVGDRVTITCRASQDIRYY  
LNWYQQKPGKAPKLLIYVASSLQSGVPSRFSGSGSGTEFTLTVSSLQPEDFATYY  
CLQVYSTPRTFGQGTKVEIKVDPKSCDKTHTCPCPEPKSCDTPPPCCRCPAPGG  
GSSGGGSGGQPREPQVYTLPPSRDELTKNQVSLTC1VKGFYPSDIAVEWESNGQP  
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS  
LSPGK

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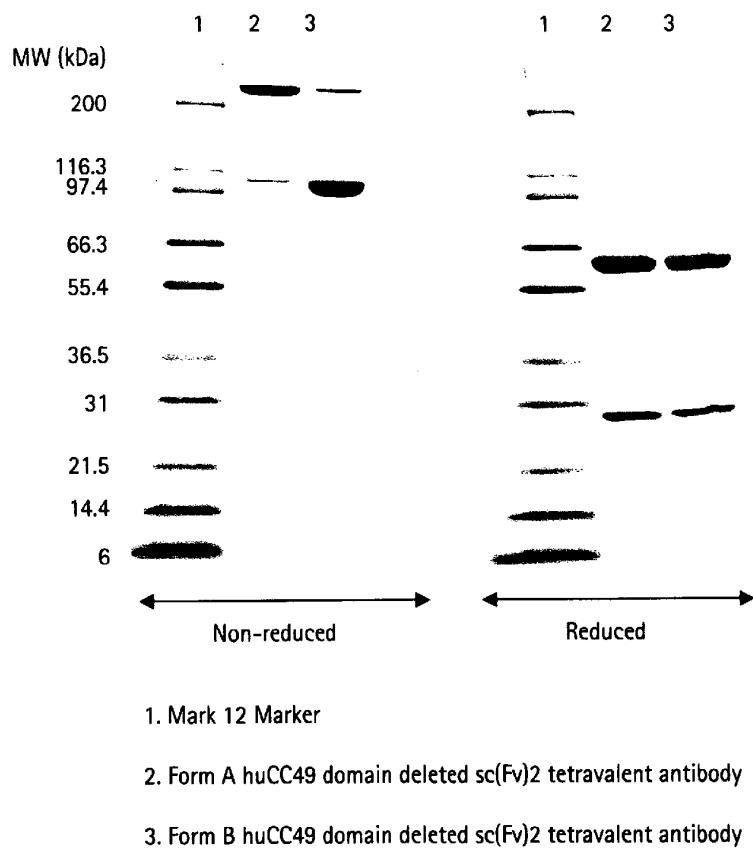
FIG. 18



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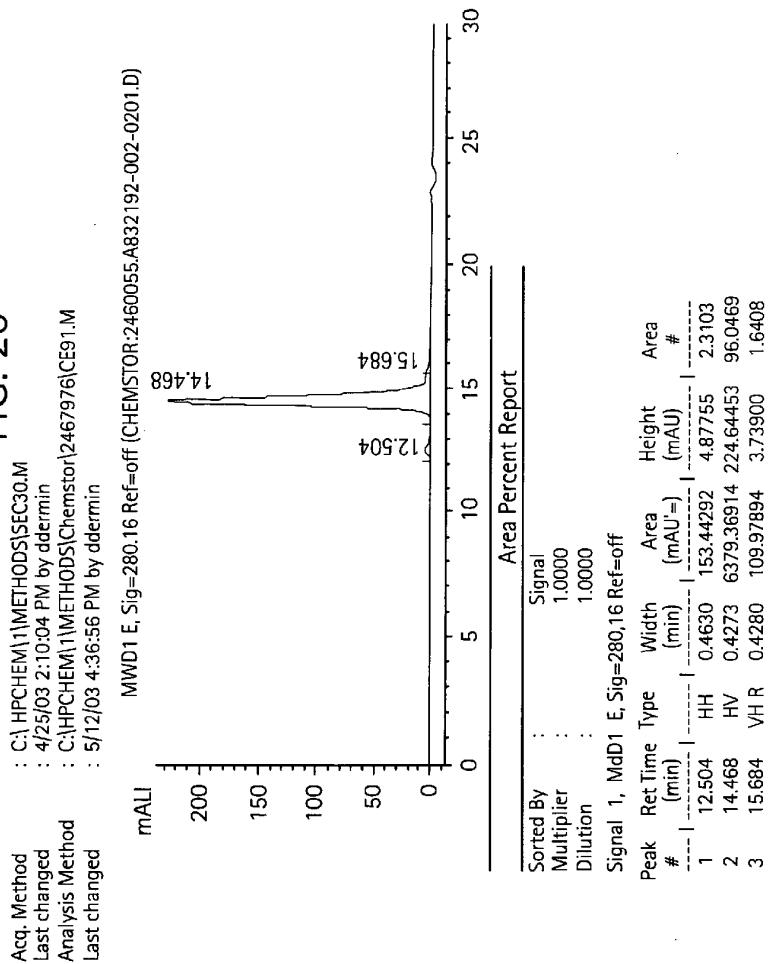
FIG. 19



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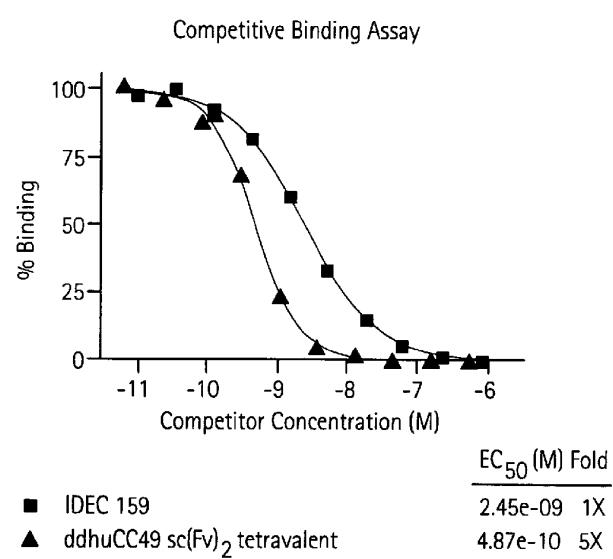
FIG. 20



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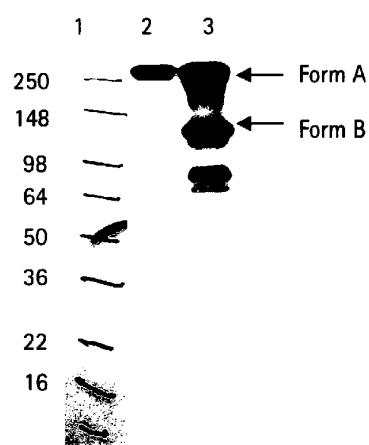
FIG. 21



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**FIG. 22**  
Western blot of huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP



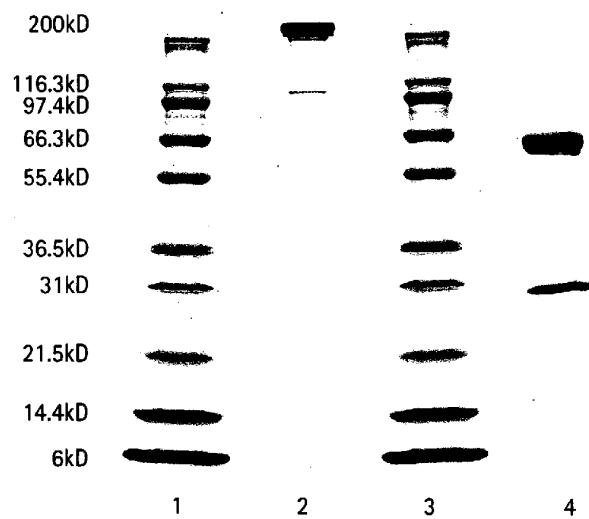
1. MW Marker
2. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent G1/G3/PAP-non-reduced
3. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent-non-reduced

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FIG. 23

huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody  
containing the G1/G3/Pro243Ala244Pro245 + [Gly/Ser]  
(SEQ ID NO:9) connecting peptide



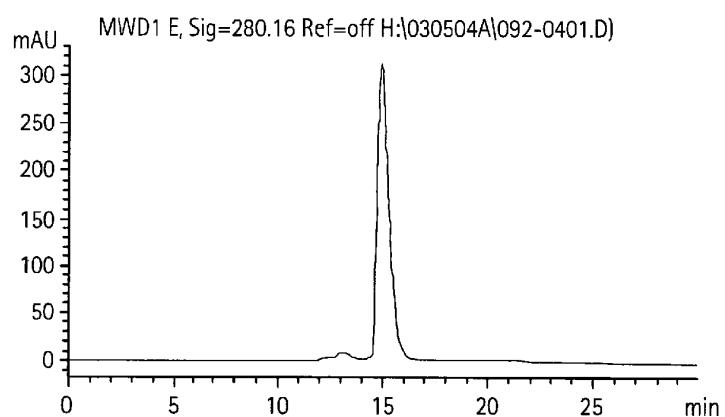
1. Mark 12 Marker
2. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody  
G1/G3/PAP non-reduced
3. Mark 12 Marker
4. huCC49 CH2 domain-deleted sc(Fv)2 tetravalent antibody  
G1/G3/PAP reduced

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FIG. 24

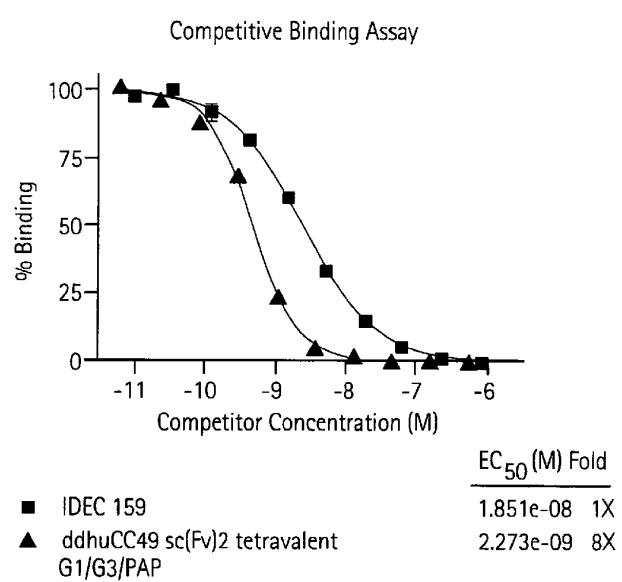
SEC analysis  
huCC49 CH2 domain-deleted sc(Fv)2 tetravalent  
G1/G3/Pro243Ala244Pro245 antibody



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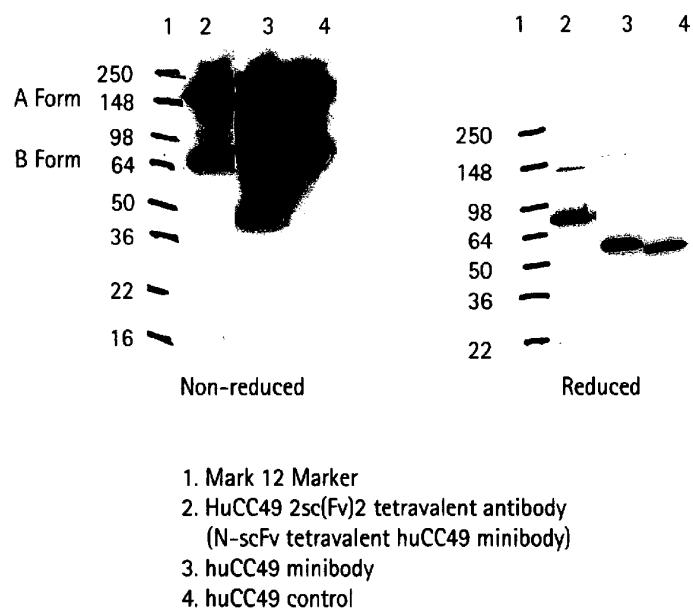
FIG. 25



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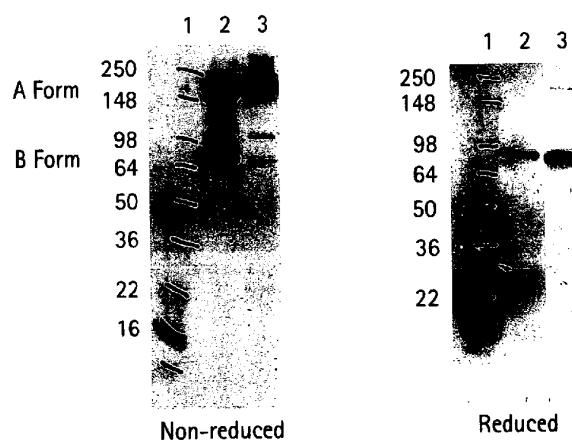
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FIG. 26



**AMENDED SHEET**

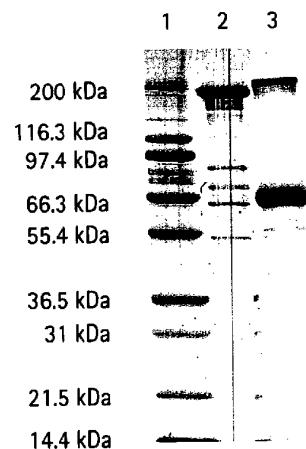
FIG. 27



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

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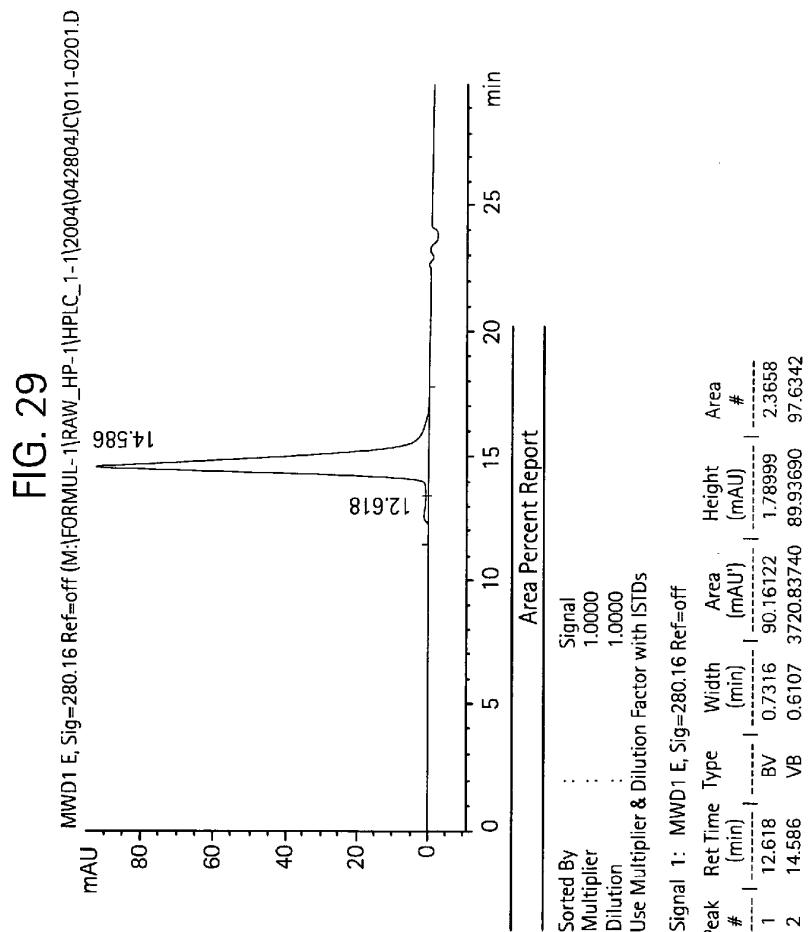
FIG. 28



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

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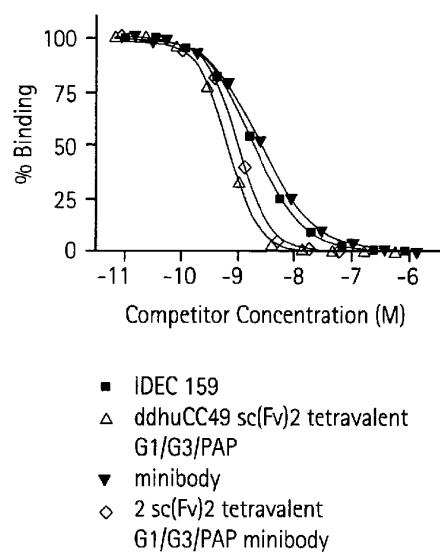


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FIG. 30

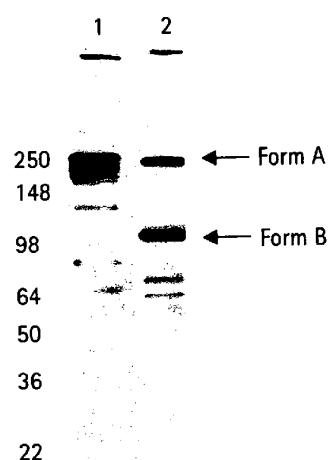
Competitive Binding Assay



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FIG. 31

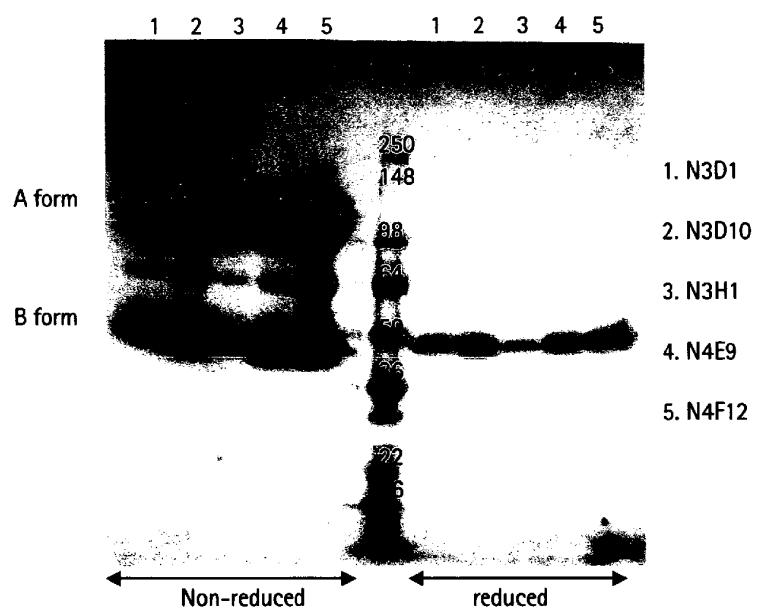


1. p5E8 + G1G3PAP hinge tetravalent Ab
2. ddCC49 tetravalent Ab

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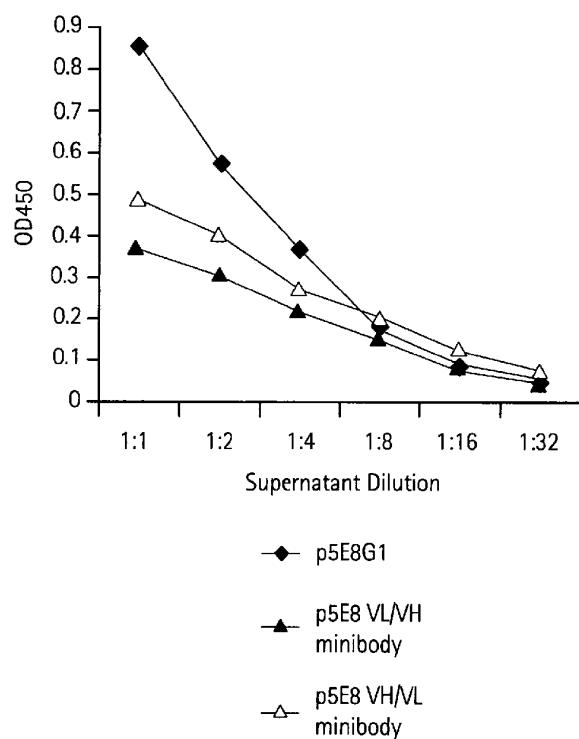
**FIG. 32**  
Western Blot of ddCC49 minibody with Gt anti-hu IgG HRPO



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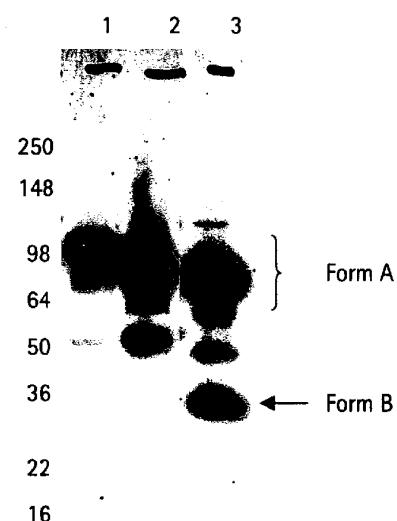
FIG. 33



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FIG. 34

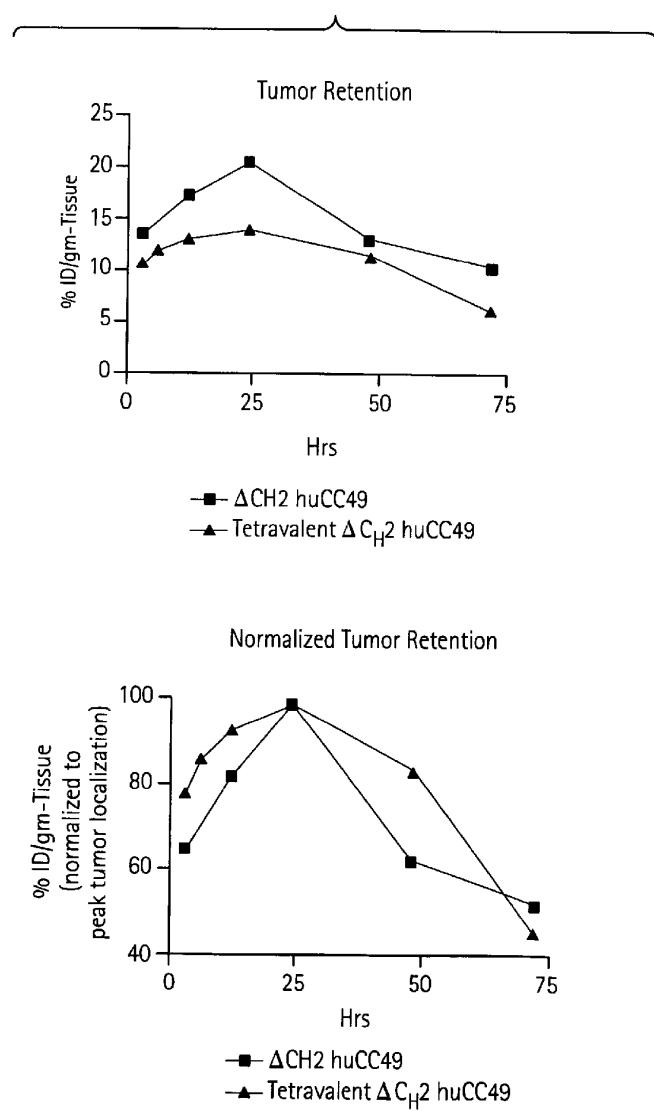


1. p5E8 VL/VH minibody+ G1G3PAP hinge
2. p5E8 VH/VL minibody+ G1G3PAP hinge
3. huCC49 minibody

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FIG. 35



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SEQUENCE LISTING

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Gly Ser Ser Gly Gly Ser Gly
 35          40

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 20          25          30
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 35          40

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 1           5           10          15
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 20          25

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<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

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1 5 10 15  
 Pro Gly Gly Gly Ser Ser Gly Gly Gly Ser Gly  
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<210> 12  
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 <213> Artificial Sequence

<220>  
 <223> synthetic construct

<400> 12  
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 <211> 27  
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 <213> Artificial Sequence

<220>  
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<400> 13  
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<210> 14  
 <211> 27  
 <212> PRT  
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<220>  
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<400> 14  
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<210> 15  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
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<400> 15  
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 aatcacaagc ccagaacac caagggtggac aagaaagtgc agccaaatc ttgtgacaaa 660  
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<210> 18

<211> 663

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 18

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 gtcttcatct tccggccatc tttatggat tttatggat tttatggat tttatggat 420  
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<210> 19

<211> 603

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 19

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 20 25 30  
 Ala Ile His Trp Val Lys Gln Asn Pro Gly Gln Arg Leu Glu Trp Ile

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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
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
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 Gly Gly Ser Ser Gly Gly Ser Gly Gly Gln Pro		
225	230	235
Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr		
245	250	255
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser		
260	265	270
Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr		
275	280	285
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr		
290	295	300
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe		
305	310	315
Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys		
325	330	335
Ser Leu Ser Leu Ser Pro Gly Lys Ser Gly Gly Ser Gly Gly		
340	345	350
Gly Gly Ser Gly Gly Ser Asp Ile Val Met Ser Gln Ser Pro		
355	360	365
Asp Ser Leu Ala Val Ser Leu Gly Glu Arg Val Thr Leu Asn Cys Lys		
370	375	380
Ser Ser Gln Ser Leu Leu Tyr Ser Gly Asn Gln Lys Asn Tyr Leu Ala		
385	390	395
Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Leu Ile Tyr Trp		
405	410	415
Ala Ser Ala Arg Glu Ser Gly Val Pro Asp Arg Phe Ser Gly Ser Gly		
420	425	430
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Ser Val Gln Ala Glu Asp		
435	440	445
Val Ala Val Tyr Tyr Cys Gln Gln Tyr Tyr Ser Tyr Pro Leu Thr Phe		
450	455	460
Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly Ser Gly Gly		
465	470	475
Gly Gly Ser Gly Gly Ser Gln Val Gln Leu Val Gln Ser Gly		
485	490	495
Ala Glu Val Val Lys Pro Gly Ala Ser Val Lys Ile Ser Cys Lys Ala		
500	505	510
Ser Gly Tyr Thr Phe Thr Asp His Ala Ile His Trp Val Lys Gln Asn		
515	520	525

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>  
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 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 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 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 Ser Ser 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 Ser 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 Gin Gin Lys Pro Gly Gin Ser Pro Lys 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 Gin Gin Tyr Tyr Ser Tyr Pro Leu  
 465 470 475 480  
 Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu Lys Gly Gly Gly Ser  
 485 490 495  
 Gly Gly Gly Ser 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  
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 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  
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 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

<220>  
<223> synthetic construct

<400> 22  
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<210> 23  
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 gtcaaaagggtt tctatcccg cggcggccgc gttggatgggg agagcaatgg gcaacccggag 1800  
 aacaatcatac aacccatccgc tccctgtgtg gactccggc gtcctttt ccttacacgc 1860  
 aagctcaccgg tggacaagag cagggtggcag cagggaaacg tcttctcatg ctccgtgtat 1920  
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 <212> PRT  
 <213> Artificial Sequence  
  
 <220>  
 <223> synthetic construct  
  
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 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 Ser Gly Gly Ser Gly Gly Ser 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 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 Ser Gly Gly Ser Gly Gly Ser Gly Gly  
 245 250 255  
 Gly Ser Gly Gly Ser 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  
 370 375 380  
 Gly Ser Gly Gly Ser Gly Gly Ser 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 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 Thr Lys 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 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 Ser Gly Gly Ser Gly Gly Ser Gly Gly Ser			
115	120	125	
Gin Val Gin 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 Ser Gly Ser Gly Gly Ser Gly Gly Ser Gly Gly			
245	250	255	
Gly Ser Gly Gly Ser 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 Gin Gin Lys Pro Gly Gin 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 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
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 Ser Gly Gly Gln Pro Arg Glu Pro		
545	550	555
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
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 tggggggagtc tggggggcgcc ttggccaaagc ctggggggtc cctgagactc 60  
 tcctgcgcag cctccgggtt caggttccacc ttcaataact actacatggc ctgggtccgc 120  
 caggctccag ggcaggggct tcacgttata gtatcccaca 180

tggtacgcag actccgtgaa gggcagattc accatctcca gagagaacgc caagaacaca 240  
ctgtttcttc aatgaacag cctgagagct gaggacacgg ctgtcttata ctgtgcgagc 300  
ttgactacag ggtctgactc ctggggccag ggatctgg tcaccgtctc ctcagctgc 360  
accaaggccc catcggttt cccctggca cccctccca agagcaccc tggggcaca 420  
gcggccctgg gctgcctgtt caaggactac ttccccgaaac cggtgacggt gtctggaaac 480  
tcaggcgcctc tgaccagcg cgtgcacacc ttcccgctgt tcctacagtc ctcagactc 540  
tactccctca gcagctgtt gaccgtgccc tccagcagct tgggcaceca gacctacatc 600  
tgcaacgtqa atcacaaggc cagcaacacc aagggtggaca agaaagtga gcccataatct 660  
tgtgacatggc ctcacacatg cccaccgtgc ccagagccca aatcttggta cacacttccc 720  
ccatgcggcc acgtggccagg acctggaggt ggtctgagtg gaggcgggtc cggaggccag 780  
ccccgagaac cacagggtgtt caccctgccc ccattccccc atgagctgac caagaaccag 840  
gtcagccctga cttggctgtt caagggttata tttcccgacg acatcgccgt ggagtgggg 900  
agcaatgggc agccggagaa caactacaag accacgcctc cctgtgttgc ctccgacggc 960  
tccttcttcc tctacagcaa gtcaccgtg gacaagagca ggttgcgacca gggaaacgctc 1020  
ttctcatgtt cctgtatgtca tgaggctctg cacaaccact acacgcagaa gagctctcc 1080  
ctgttccgg gtaatccgg cgggggttga tccgggtggat cgggctccgg cggggccgg 1140  
tccgacatcc agatgaccca gtctccatct tccctgtctg cattttaggg gtagacgatc 1200  
accatcaactt gcagggcaag tcaggacatt aggttatttt taaattggta tcagcagaaa 1260  
ccaggaaaaa ctccctaagct cttgtatctat gtttgcacca gtttgcacaa tggggteccca 1320  
tcaagggtca cggggcgtgg atctgggaca gagggttactc tcaccgtcg cagcctgcag 1380  
cctgaaggatt ttgcactta ttactgtctt ctttttttttgcgatccgcg 1440  
caagggacca aggtggaaat caaaggcggtt ggggggtccg tgggggggtgg ctccgggggc 1500  
gggtggctccg aggtggcgtt ggtggatctt gggggcgcgtt tggccaaagcc tgggggggtcc 1560  
ctgatgactctt ctggcgcggc ctccgggttc aggttcaactt tcaataacta tcatatggac 1620  
tggggccggc aggtggccgg ggggggtctg gagggttactt ctttttttttgcgatccgcg 1680  
gatcccacat ggtacgacaa ctccgtgttca ggcagattca ccatctccag agagaacgc 1740  
aagaacacacat tgggttttca aatgaacagc ctgagagctg aggacacggc tggcttattac 1800  
tggcggatgt tggactacagg tggccggcagg gaggcttgcgtt caccgtctcc 1860  
tcatgtt 1866

<210> 27

<211> 645

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 27

gacatccaga tgaccaggc tccatcttcc ctgtctgtat ctgttagggta cagagtccacc 60  
atcacttgca gggcaggta ggcatttggg tattatataa attgttatca gcagaaacca 120  
gaaaaaggctc ctaactctt gatctatgtt gcatccagg tggcaatggg ggtcccatca 180  
aggttcaggatc tgggacatggagat ttcactctca cctgtcaggatc cctgtcaggatc 240  
gaagatttt ccacttataa ctgtcttacag gtttatagtt cccttcggac gtttgcacca 300  
gggaccagg tggaaatcaa agtacgggt gtttgcacccat ctgtcttcat ctccggca 360  
tctgtatggc aggtggaaat tggaaactcc tctgttggat gctgtgttgcgaa taacttctat 420  
cccaaggagg ccaaaggatca tgggaaagggtt gataacgcctc tccaaatccggg taactcccg 480  
gagatgttca cagacggagg cagcaaggac agcaactaca gccttcaggatc caccctgac 540  
ctgagccaaatc agactacga gaaacacaaa gtttgcacccat gcaaggatc ccatcaggac 600  
ctgagctcgc cctgtcacaatc gaggatggatgttgc 645

<210> 28

<211> 621

<212> PRT

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 28

Glu Val Gln Leu Val Glu Ser 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	Gin	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	255
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	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	Ile	Tyr	Val	Ala	
420	425	430													
Ser	Ser	Leu	Gln	Ser	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	
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	Ser	Gly	Gly	Gly	
485	490	495													

Gly Ser 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 ctgttagggga cagagtacc 60  
atcaactgca gggcaagtca ggcattagg tattatttaa attggtatca gcagaaacca 120  
gaaaaagctc ctaagctctt gatctatgtt gcatccagtt tgcaaagtgg ggtcccatca 180  
aggttcagcg gcagtggatc tggacagag ttcaactctca ccgtcagcag cctgcagct 240  
gaagattttg cgaacttata ctgtctacag gtttatagta cccctcgac gttcggccaa 300  
gggaccaagg tggaaatcaa aggccgtggc ggttccgggt ggggtggctc cggggccgt 360  
ggctccgggg tgcagctgtt ggagtctggg ggccggcttg caaaggctgg ggggtccctg 420  
agactctctt cggcagccctc cgggttcagg ttacatctca ataaactata catggactgg 480  
gtcccccagg ctccaggcga ggggctggag tgggtctcac gtatttagtag tagtggat 540  
cccacatgtt acgcagactc cgtgaaggc agattcacca tctccagaga gaacgccaag 600  
aacacactgtt gaaacccatg aacacactgg agagctggg acacggctgt ctattactgt 660  
gcgactttaa ctacagggtc tgactctgg gcggccggag tccctggtaac cgttccctca 720  
gtcgacccca aatcttgtga caaaactcac acatgcccac cgtgcccaga gcccaaatct 780  
tgtgacacac ctcccccattt cccacgtgc ccagcacctg gaggtggctc gagtgagggc 840  
ggatccggag ggcagcccg agaaccacag gtgtacacccc tgcggccatc cccggatgg 900  
ctgaccaaga accaggtagc ctggacttcgc ctggtaaag gtttctatcc cagcgacatc 960  
gcgcgtggagt gggagagcaa tggccagccg gagaacaact acaagaccac gcctccctg 1020  
ctggactccg acggctctt ctccctctac agcaagctca ccgtggacaa gagcaggatgg 1080  
cagcaggggg agcttcttc atgtccctgt atgcatgagg ctctgcacaa ccactacacg 1140  
cagaagagcc ttcctctgtc tccgggtaaa tga 1173

<210> 31

<211> 1173

<212> DNA

<213> Artificial Sequence

<220>

<223> synthetic construct

<400> 31

gagggtgcagc tggtgagtc tggggccgc tttggcaaaacg ctggggggtc cctgagactc 60  
tcctgcgcag ctcgggggtt caggttccacc ttcaataact actacatgga ctgggtccgc 120  
cagggtccac ggccagggtt gggatgggtt tcacgttata gtatgtgg tgatccacca 180  
tggtacgac gactccgtaa gggcagatcc accatctcca gagagaacgc caagaacaca 240  
ctgtttcttc aaatgacacg ctggagatgt gaggacacgg ctgtcttata ctgtgcqagc 300  
ttgactacag ggtctgactc ctggggccag gggtctgtt tcaccgttcc ctcaggccgt 360  
ggccgggtccg gtgggggtgg ctccggggc ggtggctccg acatcccgat gaccggatct 420  
ccatcttccc tggacttcgc tggatggac agatgtacca tcaacttgcg ggcaagttag 480  
gacatttaggtt attttttttt tggatgtacg cagaaccacg gaaaagttcc taagctctg 540  
atctatgttgc catccagttt gaaaatgggg gtcccatca ggttcaggg cagtggatct 600  
gggacagatgttcaacttcgc ctgcagctgtt aagattttgc gacttattac 660  
tgttctacagg ttatagtttccctcgacg ttccggccaa ggaccaagggtt gggaaatcaaa 720  
gtcgacccca aatcttgc taaaactcac acatgcccac cgtgcccaga gcccaaatct 780  
tgtgacacac ctcccccattt cccacgtgc ccagcacctg gaggtggctc gagtgagggc 840  
ggatccggag ggcagcccg agaaccacag gtgtacacccc tgcggccatc cccggatgg 900  
ctgaccaaga accaggtagc ctggacttcgc ctggtaaag gtttctatcc cagcgacatc 960  
gcgcgtggagt gggagagcaa tggccagccg gagaacaact acaagaccac gcctccctg 1020  
ctggactccg acggctctt ctccctctac agcaagctca ccgtggacaa gagcaggatgg 1080  
cagcaggggg agcttcttc atgtccctgt atgcatgagg ctctgcacaa ccactacacg 1140  
cagaagagcc ttcctctgtc 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 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 Ser 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 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 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 Ser  
115 120 125  
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 Gin Gin Lys Pro Gly Lys Ala  
165 170 175  
Pro Lys 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 Gin 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 Ser Ser Gly Gly Ser Gly Gin Pro Arg Glu  
275 280 285  
Pro Gin 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 Gin 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  
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Gly Gly Gly Ser Gly Gly Ala Ser
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Cys Asp Thr Pro Pro Pro Cys Pro Arg Cys Pro Ala Pro  
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