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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 September 2005 (09.09.2005)

PCT

(10) International Publication Number
WO 2005/082004 A2

(51) International Patent Classification: Not classified

(21) International Application Number:
PCT/US2005/005879

(22) International Filing Date: 22 February 2005 (22.02.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/547,133 24 February 2004 (24.02.2004) US

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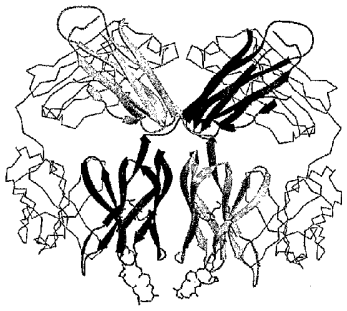
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,

[Continued on next page]

(54) Title: RATIONALLY DESIGNED ANTIBODIES HAVING A DOMAIN-EXCHANGED SCAFFOLD



The 2G12 monomer spontaneously forms a dimer resulting in a cross heavy chain dimer motif. TPO peptides grafted into the HC-CDR2 regions would result in peptides in close proximity to one another in an anti-parallel fashion.

(57) Abstract: Domain-exchanged antibodies having CDR regions replaced or fused with biologically active peptides are described. Flanking sequences may optionally be attached at one or both the carboxy-terminal and amino-terminal ends of the peptide in covalent association with adjacent framework regions. Compositions containing such modified domain-exchanged antibodies are useful in therapeutic and diagnostic modalities.

WO 2005/082004 A2

SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

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RATIONALLY DESIGNED ANTIBODIES HAVING A DOMAIN-EXCHANGED SCAFFOLD

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application Serial No. 60/547,133 filed February 24, 2004, the disclosure of which is incorporated herein by this reference.

TECHNICAL FIELD

The present disclosure relates to antibodies and biologically active peptides as diagnostic and therapeutic reagents. More specifically, the present disclosure relates to recombinant antibodies that include a biologically active peptide engineered into a domain-exchanged antibody scaffold.

BACKGROUND OF RELATED ART

Antibodies are produced by B lymphocytes and defend against infection. Antibodies are produced in millions of forms, each with a different amino acid sequence. Antibody molecules are composed of two identical light chains and two identical heavy chains. When digested by the enzyme papain, two identical Fab fragments are produced along with one Fc fragment. When digested with the enzyme pepsin one $F(ab')_2$ fragment is produced. Light and heavy chains consist of constant and variable regions. Within the variable regions are hypervariable regions (aka

complementarity determining regions (CDRs)) which form the antigen binding site. The remaining parts of the variable regions are referred to as framework regions.

Important biological functions, such as receptor binding, activation and enzymatic activity, are often attributable to discrete regions of larger protein molecules, comprising a limited number of amino acid residues. Peptides displaying binding, activation or enzymatic activity have also been discovered by screening libraries of peptides generated by the random linking of amino acid residues. These peptides may not correspond to a linear arrangement of amino acids in a larger protein molecule exhibiting similar biological activity and are referred to as discontinuous peptide epitopes or mimotopes. Certain peptide mimetics have been described and cloned. See, e.g., U.S. Pat. NO: 6,083,913 (thrombopoietin (TPO) mimetic), U.S. Pat. NO: 5,835,382 (erythropoietin (EPO) mimetic), U.S. Pat. NO: 5,830,851 (EPO mimetic) and Wrighton et al, Science, (1996) 273:458-63. Peptide epitopes and mimotopes due to their small size are potentially advantageous over large protein molecules for use as therapeutic reagents. However, the results with these peptides as therapeutics may often be unsatisfactory. One drawback to the use of peptides as therapeutic reagents is that they are generally unstable *in vivo*, i.e., their clearance rates from serum may be quite rapid. In addition, it is difficult to predict the activity, therapeutic or otherwise, of a peptide if it is fused into a larger molecule since conformational changes and other molecular forces may interfere with or totally negate the activity of the peptide. Attempts have been made to introduce certain polypeptides into CDR regions of antibodies. See, e.g., PCT Appln. WO 94/18221. However, as mentioned previously, due to conformational changes which may be caused by

surrounding amino acids, the biological activity of active polypeptides may be diminished or negated.

Thrombopoietin (TPO) is a cytokine active in many stages in the development of megakaryocytic precursors that ultimately lead to platelet production. Small peptides mimicking the active portion of TPO have previously been synthesized and tested for activity against the thrombopoietin cell surface receptor, c- Mpl. (Cwirla et al., Science, Vol. 276, pages 1696-1699 (1997)). The most active peptides were shown to be significantly more potent by simply linking the C-termini of two monomeric peptides creating a pseudosymmetric dimer, prompting the hypothesis that the TPO peptide dimer possesses increased activity at least partially through promotion of c-Mpl receptor dimerization.

Grafting two TPO mimetic peptides into a typical antibody Fab framework creates a c- Mpl receptor agonist which promotes receptor dimerization and activation as described in published international application WO 02/46238 A2. A technical hurdle of this system involves presentation of the grafted peptides in an optimal conformation on the Fab scaffold. Multiple combinations are described in WO 02/46238 A2, including grafting two TPO peptides into different positions chosen from LC-CDR1, LC-CDR2, HC-CDR2, and HC-CDR3 on the Fab framework. Improving how any grafted peptide is displayed on the surface of the Fab requires optimal spacing between the two peptides grafted into different CDR loops and correct directionality of the grafted peptides relative to one another. The spacing can be adjusted to some

degree within the confines of the typical Fab scaffold, however the distances are limited by the dimensions of the Fab fragment. Moreover, the directionality of the peptides is invariant in a typical antibody Fab framework in that there is no way to shift the orientation of the two peptides relative to each other from a parallel to an antiparallel mode.

SUMMARY

Provided herein are biologically active recombinant antibodies and fragments thereof that mimic the activity of biologically active molecules, methods of making such antibodies and methods for their use in therapy and diagnosis. Recombinant antibodies in accordance with this disclosure include a domain-exchanged antibody or fragment thereof having a peptide of interest inserted into a complementarity determining region (CDR) of the domain-exchanged antibody. The domain-exchanged antibody serves as a scaffold for presentation of the peptide. The peptide optionally replaces all or part of the amino acids of a CDR region, or may be added to an existing CDR as defined by either of the two accepted schemes (see, Kabat et al., Sequences of Proteins of Immunologics Interest, 5th ed (1991), NIH Publication 91-3242 and Chothia et al., J. Mol. Bio., volume 227, pages 776-98 (1992).). One suitable antibody scaffold is the domain-exchanged antibody 2G12.

In certain embodiments, the biologically active peptide, when substituted for all or part of a CDR region or added into a CDR region, can have one, two or more additional flanking amino acid residues proximate to the amino and/or the carboxyl termini of the peptide. Methods for screening of libraries of domain-exchanged

antibodies having a common peptide substituted for or into a CDR region but different flanking amino acid residues are also described herein.

Further provided are nucleic acid molecules encoding the present recombinant antibody molecules or fragments thereof in which a biologically active peptide is substituted for or into one or more CDR regions of a domain-exchanged antibody. These nucleic acid molecules can be present in an expression vector, which can be introduced (e.g., transfected) into a recombinant host cell for expression of these molecules. Also provided are methods of producing an antibody molecule or fragment thereof containing a biologically active peptide, comprising culturing a recombinant host cell under conditions such that the nucleic acid contained within the cell is expressed.

Also provided are compositions that contain 1) a domain-exchanged antibody or fragment thereof which has a biologically active peptide substituted for or into one or more CDR regions and 2) a pharmaceutically acceptable carrier.

Further provided are methods of engineering antibody molecules or fragments thereof to exhibit an activity (property) of a biologically active molecule in which a biologically active peptide is substituted for or into one or more CDR regions of light and/or heavy chains of a domain-exchanged antibody. The methods encompass inserting a nucleic acid molecule encoding a biologically active peptide in place of at least a portion of a CDR region of a nucleic acid molecule encoding a domain-exchanged antibody heavy or light chain or adding the molecule to the native CDR sequence; and expressing the nucleic acid molecule encoding the antibody heavy or light chain variable domain.

Also provided are methods of preparing domain-exchanged antibody molecules or fragments thereof by providing an antibody or fragment thereof that has a biologically active peptide substituted for or into one or more CDR regions and modifying the antibody to produce a domain-exchanged antibody.

In yet another aspect, this disclosure provides methods for creation of a library of monoclonal antibodies that can be screened for a desired activity. These methods of making a library include the steps of inserting a nucleic acid molecule encoding a biologically active peptide into, or in place of at least a portion of, one or more CDR regions of a nucleic acid molecule encoding a domain-exchanged antibody heavy or light chain, providing one or more randomizing trinucleotides or sets of randomizing trinucleotides on either side of the inserted nucleic acid molecule, and expressing a library of monoclonal antibodies. The library of monoclonal antibodies thus produced can then be screened for a desired activity.

Also provided are libraries of different antibody molecules or fragments thereof wherein a biologically active peptide is substituted for or into one or more complementarity determining regions (CDRs) of a domain-exchanged antibody and which have at least one additional amino acid residue at the amino or the carboxyl terminus or both termini of the biologically active peptide, and wherein the antibody molecules or fragments of the library differ by the additional amino acid residue(s) of the peptide.

DESCRIPTION OF THE DRAWINGS

Figure 1 shows a typical structure of an antibody Fab domain.

Figures 2A and B show a model of a 2G12 antibody with TPO peptides grafted into HC-CDR2.

Figure 3 is a model of a 2G12 dimer with TPO peptides grafted into the HC-CDR3 regions.

Figure 4 shows the nucleic acid sequence (SEQ. ID NO: 11) encoding a 2G12 Light Chain codon optimized variant referred to herein as 2G12-LCwt and the encoded amino acid sequence (SEQ. ID NO: 12).

Figure 5 shows the nucleic acid sequence (SEQ. ID NO: 13) encoding a 2G12 Heavy Chain codon optimized variant referred to herein as 2G12-HCwt and the encoded amino acid sequence (SEQ. ID NO: 14).

Figure 6 shows the nucleic acid sequence (SEQ. ID NO: 16) encoding a peptide grafted 2G12 heavy chain codon optimized variant (2G12HCvarH2) and the encoded amino acid sequence (SEQ. ID NO: 17).

Figure 7 shows the nucleic acid sequence (SEQ. ID NO: 22) encoding a peptide grafted 2G12 heavy chain codon optimized variant (2G12HCvarH3) and the encoded amino acid sequence (SEQ. ID NO: 23).

Figure 8 shows the nucleic acid sequence (SEQ. ID NO: 37) encoding the heavy chain of Fab clone X4b and the encoded amino acid sequence (SEQ. ID NO: 38).

Figure 9 shows the nucleic acid sequence (SEQ. ID NO: 39) encoding the light chain of Fab clone X4b and the encoded amino acid sequence (SEQ. ID NO: 40).

DETAILED DESCRIPTION

Recombinant antibodies in accordance with this disclosure include a domain-exchanged antibody or fragment thereof having a peptide of interest inserted into a complementarity determining region (CDR) of the domain-exchanged antibody. The domain-exchanged antibody serves as a scaffold for presentation of the peptide.

As used herein, "antibody" refers to an entire immunoglobulin molecule or molecules that contain immunologically active portions of whole immunoglobulin molecules and includes Fab, $F(ab')_2$, scFv, Fv, heavy chain variable regions and light chain variable regions.

Any peptide that exhibits a useful property is suitable for insertion in a domain-exchanged antibody framework. Peptide activities and uses include, but are not limited to, binding a receptor, binding a membrane bound surface molecule, binding a ligand, binding an enzyme or structural protein, activating or inhibiting a receptor, targeted drug delivery, or any enzymatic activity. Those peptides whose utility can be increased from the enhanced stability conferred upon them when presented in the context of a domain-exchanged antibody are usually selected. It should be understood that "biological activity" as used herein includes any activity associated with a molecule having activity in a biological system, including, but not limited to, the stimulatory or inhibitory activity triggered by protein-protein interactions as well as the kinetics surrounding such interactions including the stability of a protein-protein complex. Enhancing or increasing "biological activity" herein is meant to include an increase in overall activity or an increase in any component of overall activity. It should be understood that a peptide may exhibit one biological activity (such as, e.g.,

simply binding to a target) before insertion into the domain-exchanged antibody framework, and a different or enhanced biological activity (such as, e.g., agonist activity) after insertion into the antibody framework.

Many peptides which could benefit from display in the context of an antibody have been identified and are known to those who practice the art, e.g., EPO and TPO mimetic peptides. Other examples include peptides that bind to receptors which are activated by ligand-induced homo-dimerization including active fragments displaying G-CSF activity, GHR activity and prolactin activity as described in Whitty and Borysenko, *Chem Biol.*, (1999) Apr 6(4):R107-18; other examples of suitable peptides include a nerve growth factor mimetic from the CD loop as described in Zaccaro et al., *Med. Chem.* (2000) 43(19); 3530-40; an IL-2 mimetic as described in Eckenberg, et al., *J. Immunol.* (2000) 165(8):4312-8; glucagon-like peptide-1 as described in Evans et al., *Drugs R.D.* (1999) 2(2): 75-94; tetrapeptide I (D-lysine-L-asparaginyl-L-prolyl-L-tyrosine) which stimulates mitogen activated B cell proliferation as described in Gagnon et al., *Vaccine* (2000) 18(18):1886-92. Peptides which exhibit receptor antagonistic activity are also contemplated. For example, N-terminal peptide of vMIP-II as an antagonist of CXCR4 for HIV therapy as described in Luo et al., *Biochemistry* (2000) 39(44):13545-50; antagonist peptide ligand (AFLARAA (SEQ ID NO:)) of the thrombin receptor for antithrombotic therapy as described in Pakala et al., *Thromb. Res.* (2000) 100(1): 89-96; peptide CGRP receptor antagonist CGRP (8-37) for attenuating tolerance to narcotics as described in Powell et al., *Br. J. Pharmacol.* (2000) 131(5): 875-84; parathyroid hormone (PTH)-1 receptor antagonist known as tuberoinfundibular peptide (7-39) as described in Hoare et al., *J. Pharmacol. Exp.*

Ther. (2000) 295(2):761-70; opioid growth factor as described in Zagon et al., *Int. J. Oncol.* (2000) 17(5): 1053-61; high affinity type I interleukin 1 receptor antagonists as disclosed in Yanofsky, et al., *Proc. Natl. Acad. Sci. USA*, Vol. 93, pp. 7381-7386, July 1996 and Vigers, et al., *J. Biol. Chem.*, Vol 275, No 47, pages 36927-36933, 2000; and acid fibroblast growth factor binding peptide as described in Fan et al., *IUBMB Life* (2000) 49 (6) 545-48. Further examples of biologically active peptide which can be incorporated into antibodies or antibody fragments in accordance with this disclosure include proteins secreted by the heart as part of the body's response to congestive heart failure, such as, for example, human brain natriuretic peptide (hBNP) as described in Mukoyama, et al., *J. Clin. Invest.* 87(4): 1402-12 (1991) and Clemens, et al., *J. Pharmacol. Exp. Ther.* 287(1): 67-71(1998). Additional examples of biologically active peptide which can be used in accordance with this disclosure include proteins which have the potential to preserve or improve beta-cell function (e.g., by inducing glucose-dependent insulinotropic effect), such as, for example, exendin-4, GLP-1 (7-36), GLP-2 (1-34), glucagon or PACAP-38 (see, Raufman, et al., *J. Biol. Chem.* 267(30): 21432-7 (1992).)

In a particularly useful embodiment, the peptide replacing the amino acids of a domain-exchanged antibody CDR is a TPO mimetic peptide, such as a peptide having at least the sequence IEGPTLRQWLAARA (SEQ. ID. NO: 1). Another biologically active peptide that can replace the amino acid residues of a domain-exchanged antibody CDR is an EPO mimetic peptide, such as a peptide having at least the amino acid sequence DYHCRMGPLTWVCKPLGG (SEQ. ID. NO: 2). In further particular embodiments, the peptide replacing the amino acids of at least one domain-

exchanged antibody CDR is a human brain natriuretic peptide (hBNP). One such peptide is hBNP-32 which has at least the sequence FGRKMDRISSSSGLG (SEQ. ID. NO: 3). In further particular embodiments, the peptide replacing the amino acids of at least one domain-exchanged antibody CDR is a peptide involved in insulin production. Such peptides include exendin -4, GLP-1 (7-36), GLP-2 (1-34), glucagon and PACAP-38 which have at least the following sequences:

Exendin-4: (SEQ. ID NO: 4)
HGEGRFTSDLSKQMEEEAVRLFIEWLKNGGPSSGAPPPS

GLP-1: (SEQ. ID NO: 5)
HAEGTFTSDVSSYLEGQAAKEFIAWLVKGR

GLP-2: (SEQ. ID NO: 6)
HADGSFSDEMNTILDNLAARDFINWLIQTKITDR

Glucagon: (SEQ. ID NO: 7)
HSQGTFTSDYSKYLDSRRAQDRVQWLMNT

PACAP-38: (SEQ. ID NO: 8)
HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNK

In further particular embodiments, the peptide replacing the amino acids of at least one domain-exchanged antibody CDR is an adipocyte-specific secretory protein. One such peptide is Adiponectin (Acrp30). The globular region of Acrp30 has at least a functional portion of the sequence:

FSVGLETYVTIPNMPIRFTKIFYNQQNHYDGSTGKFHCNIPGLYYFAYHITVYM
KDVKVSLFKKDKAMLFTYDQYQENNVDQASGSVLLHLEVGDQVWLQVYGEGE
RNGLYADNDNDSTFTGFLLYHDTN (SEQ. ID. NO: 9).

Another example of a peptide that can be incorporated into an antibody scaffold is a peptide that represents a ligand-binding domain of liver/lymph node-specific

intercellular adhesion molecule-3 grabbing integrin ("L-SIGN", also sometimes referred to as "DC-SIGN-R," "DC-SIGN2" or "CD209L"), such as a peptide having the amino acid sequence: RYWNSGEPNNSGNEDCAEFSGSGWNDNRCDVDN (SEQ ID NO: 10).

A domain-exchanged antibody serves as a scaffold sequence into which the peptide of interest is inserted. As used herein, the phrase "domain-exchanged antibody" means an antibody wherein at least one domain of the heavy chain variable region is positioned away from the light chain variable region. As those skilled in the art will appreciate, a "typical" antibody is composed of two identical light chains and two identical heavy chains, with the hypervariable regions (aka complementarity determining regions (CDRs)) of the light and heavy chains forming the antigen binding site. In a "typical" antibody, the heavy chain variable region is closely associated with the variable region of the light chain. In a domain-exchanged antibody, the variable region of the heavy chain is positioned away from the light chain, as shown in Fig. 2A.

The domain-exchanged antibody into which the peptide of interest is inserted can be a complete domain-exchanged antibody or any fragment thereof containing a CDR, such as, for example, an Fab or $F(ab')_2$ fragment or other portion thereof containing at least one hypervariable region of either a heavy chain or a light chain.

The structure of a domain-exchanged antibody spontaneously forms a dimer, for example where the heavy chains of two Fab fragments swap with each other forming a heavy chain cross-dimerization motif. Using molecular modeling, the distance and the orientation of the grafted peptides can be approximated. For example, analysis of modeling a peptide into the CDR2

regions of a domain-exchanged antibody shows that the peptides can be closer to each other when the domain-exchanged antibody dimerizes than is obtainable in a typical prior art antibody scaffold at an equal distance from the center of the molecule. Thus, a domain-exchanged antibody allows for more optimal receptor dimerization.

Additionally, the directionality of the peptides relative to each other can be advantageous with a domain-exchanged antibody scaffold compared to prior art antibody scaffolds as the symmetry within the dimer can generate peptides in an anti-parallel orientation relative to each other when they are grafted into the same CDR loop. For example, if the peptides are grafted into HC-CDR2, they are presented in a bidentate fashion with a 2-fold symmetrical presentation generated by the cross-dimerization of domain-exchanged antibody Fab fragments.

The domain-exchanged antibody can be naturally occurring (as is the case with the 2G12 antibody discussed below) or can be artificially created by making one or more modifications to a "typical" antibody thereby producing a domain-exchanged antibody.

As disclosed in Calarese, et al. (Science, volume 300, pages 2065-2071 (2003), the entire contents of which are incorporated herein by this reference), the modifications that will convert a "typical" antibody into a domain-exchanged antibody can include one or more: 1) modifications that weaken the V_H/V_L interface contacts (closed interface); 2) modifications that provide an unusual sequence and structure of the elbow region connecting the V_H and C_H1 domains (hinge loop); and 3)

modifications that create a favorable V_H/V_H' interface (open interface). The modifications can be made as specific point mutations or as more general randomization (e.g. within the hinge loop of the starting, "typical" antibody). Techniques for making such modifications are within the purview of one skilled in the art.

The modifications to convert a "typical" antibody into a domain-exchanged antibody can be made before, after or simultaneously with the introduction of a biologically active peptide in accordance with this disclosure. Thus, in a particularly useful embodiment, a "typical" antibody which has previously had a biologically active peptide inserted into or in place of at least a portion of at least one CDR is converted into a domain-exchanged antibody.

To determine whether a domain-exchange has been achieved, the existence of a domain-swapped dimer is confirmed. Any known technique for detecting the presence of the dimer can be used. For example, sedimentation equilibrium analytical ultracentrifugation, gel filtration, native gel electrophoresis, sedimentation coefficients and/or negative-stain electron microscopy can be employed to confirm the formation of a domain-swapped dimer consistent with the presence of a domain-exchanged antibody. These processes are well known to those skilled in the art.

One such domain-exchanged antibody is the human monoclonal antibody 2G12 deposited under ECACC Acc. Nr. 93091517 or a variation of a 2G12 antibody. As used herein, "variation of a 2G12 antibody" or "2G12 variant" means an antibody having at least 80% identity, and preferably at least 90% identity to the aforementioned 2G12 antibody deposited under ECACC Acc. Nr. 93091517. The structure of 2G12 is

disclosed in Calarese, et al., *supra*. A particularly useful variation of a 2G12 antibody, is a 2G12 antibody that has been codon optimized for bacterial expression, such as the 2G12 variant having a light chain and heavy chain having the sequence shown in Figures 4 and 5.

The 2G12 antibody reportedly contains point mutations that cause one domain of the heavy chain to swing away from the light chain (see, Figures 2A and 2B). This structure spontaneously forms a 2G12 dimer where the heavy chains of two Fab fragments swap with each other forming a heavy chain cross-dimerization motif. As seen in Fig. 2B, where peptides are grafted into HC-CDR2, they are presented in a bidentate fashion with a 2-fold symmetrical presentation generated by the cross-dimerization of the 2G12 Fab fragments. Specifically, as seen in Figure 2A, point mutations in a 2G12 monomer antibody scaffold cause one domain of the heavy chain to move away from the antibody light chain. However, as seen in Figure 2B the 2G12 monomer spontaneously forms a dimer resulting in a cross heavy chain dimer motif. Thus, TPO peptides grafted into the HC-CDR2 regions would result in peptides in close proximity to one another in an anti-parallel fashion. This orientation more closely approximates the C-terminus to C-terminus peptide fusion of a TPO mimetic that was shown to be 4000 fold more potent than its respective monomer by Cwirla et al. in Science, Vol. 276 pages 1696-1699 (1997).

Alternatively, a library of domain-exchanged antibodies can have one or more heavy and/or light chain CDRs replaced with a desired peptide. The resulting library can then be screened to identify antibodies having a desired activity. It should be

understood that randomization within the substituted peptide can also be provided to generate an antibody library.

Grafting of the DNA sequence of the peptide of choice into a domain-exchanged antibody so as to replace the CDR(s) of an antibody with the peptide sequence is carried out using recombinant DNA techniques within the purview of those skilled in the art.

Examples of methods which can be utilized to graft a desired peptide having biological activity in place of a CDR region include, but are not limited to, PCR overlap, restriction enzyme site cloning, site specific mutagenesis and completely synthetic means. For a description of techniques involving overlap PCR, see, e.g., Example 1 herein. Site specific mutagenesis can be accomplished in several ways. One is based on dut/ung Kunkel mutagenesis (Kunkel, T.A., *Proc. Natl. Acad. Sci.* (1985) vol. 82, pp. 488-92). The Muta-Gene *in Vitro* Mutagenesis kit is available from BioRad based on this methodology (cat. # 170-3581 or 170-3580). Several PCR amplification based mutagenesis approaches are also commercially available such as Stratagene's QuickChange Site-Directed Mutagenesis Kit and the ExSite PCR-based Site-Directed Mutagenesis Kit. Another non-PCR method is available from Promega as the GeneEditor *in vitro* Site-Directed Mutagenesis System. Completely synthetic means are also well-known and described, e.g., in Deng, et al., *Methods Mol. Biol.* (1995) 51:329-42; Kutemeler et al., *Biotechniques*, (1994) 17(2): 242-246; Shi et al., *PCR Methods Appl.*, (1993) 3(1): 46-53 and Knuppik et al., *J. Mol. Biol.*, (2000) 11:296(1): 571-86 each incorporated herein by reference. In addition, the above methods used for replacing all or a portion of at least one CDR sequence can be utilized to graft a

desired peptide into or adjacent to at least one native CDR sequence without replacing the original CDR sequence. In this manner, a CDR/biologically active peptide mimetic fusion construct is formed.

It is contemplated that flanking sequences may be added to the carboxyl and/or amino terminal ends of the biologically active peptide. Flanking sequences can be useful to reduce structural constraints on the grafted peptide to allow it to more easily adopt a conformation necessary for biological activity.

In one embodiment, a flanking region can be generated by randomizing amino acid positions on each side of the peptide graft in order to determine the best sequence. In this manner, a library having members with multiple varied sequences can be generated. The resulting constructs are then tested for biological activity as described below by, e.g., panning techniques. Recombinant proteins can be generated that have random amino acids at specific positions. This can be accomplished by modifying the encoding DNA. When introducing randomization at a specific amino acid's codon position, a preferable deoxyribonucleotide "doping strategy" is $(NNK)_x$ in order to cover all 20 amino acids and to minimize the number of encoded stop codons. Accordingly, N may be A, C, G, or T (nominally equimolar), K is G or T (nominally equimolar), and x is typically 1 to 3 but can be as high as 8 or more, thereby producing antibody libraries with randomized flanking sequences of any desired number of amino acids. The third position may also be G or C, designated "S". Thus, NNK or NNS (i) code for all the amino acids, (ii) code for only one stop codon, and (iii) reduce the range of codon bias from 6:1 to 3:1. There are 32 possible codons resulting from the NNK motif: 1 for each of 12 amino acids, 2 for each of 5

amino acids, 3 for each of 3 amino acids, and only one of the three stop codons. Other alternatives include, but are not limited to:

$(NNN)_x$ which would provide all possible amino acids and all stops;

$(NNY)_x$ eliminates all stops and still covers 14 of 20 amino acids;

$(NNR)_x$ covers 14 of 20 amino acids; and

$(NNS)_x$ covers all 20 amino acids and only one stop.

The third nucleotide position in the codon can be custom engineered using any of the known degenerate mixtures. However, the group NNK, NNN, NNY, NNR, NNS cover the most commonly used doping strategies and the ones used herein.

The collection of engineered domain-exchanged antibodies that are created during this process can be surveyed for those that exhibit desirable properties. Suitable techniques for screening antibodies are within the purview of one skilled in the art, including but not limited to the use of phage displayed antibodies, essentially as has been described in Barbas, C.F., III, Kang, A.S., Lerner R.A., and Benkovic, S.J., Assembly of combinatorial antibody libraries on phage surfaces: the gene III site, *Proc. Natl. Acad. Sci. USA*, 88, 1991, pages 7978-7982 incorporated herein by reference. This technology allows recombinant antibodies (as complete antibodies, Fab, $F(ab')_2$, or scFv) to be expressed on the surface of a filamentous bacteriophage. That same phage will have within it the genes encoding that specific antibody. An alternative technique for screening antibodies involves high throughput screening of solutions containing antibodies expressed by bacterial or mammalian cells as Fabs or whole IgG for biological activity.

It is contemplated that any other known method of introducing randomization into a sequence may be utilized herein. For example, error prone PCR can introduce random mutations into nucleic acid sequences (See, e.g., Hawkins et al., J. Mol. Biol, (1992) 226(3): 889-96). Briefly, PCR is run under conditions which compromise the fidelity of replication, thus introducing random mutations in sequences as those skilled in the art would accomplish. After generation of such random mutants, they can be placed into phage display formats, panned and thus evaluated for activity. Likewise, particular bacteria known to provide random mutations of genes, such as Epicurian Coli® XL1-Red Competent cells (commercially available from Stratagene, La Jolla, CA.), which do so during plasmid replication can be utilized to provide random mutants which are then screened for biological activity in accordance with the present disclosure.

It is also contemplated that randomization may be introduced at any point in the nucleotide sequence after incorporation of an active peptide into the antibody or fragment thereof to alter the overall biological activity of the antibody. In this manner, not only can alterations be made in the biological activity of a peptide mimetic by causing mutations within the peptide's sequence, but mutations in the surrounding domain-exchanged antibody scaffold can be incorporated with the resulting constructs being assayed for alterations in biological activity or expression. More specifically, the presentation of the peptide can be altered by point mutations and randomization strategies, which affect how the peptide is presented. For example, if the peptide is grafted into HC-CDR3, modeling of the structure

shows that the histidine residue at position 32 comes into close contact with the peptide (Figure 3). Randomly mutating this position in conjunction with randomly mutating the flanking positions of the peptide can lead to an optimized presentation of the peptides. Indeed, it is contemplated that libraries having repertoires of multiple constructs resulting from such randomization can be generated and assayed.

Selection involves isolating from the library the best candidates that specifically bind to the peptide's target molecule and display biological activity. Methods for whole cell panning have been described previously (Siegel, D.L., Chang, T.Y., Russell, S.L., and Bunya, V.Y. 1997. *J. Immunol. Methods* 206:73-85 incorporated herein by reference). Other techniques for selection which can be applied include fluorescent activated cell sorting (FACs). Alternative methods for selection using libraries include, but are not limited to, ribosome display and plaque hybridization to a labeled antigen. Following panning to isolate high affinity antibody binders, bioassays for functional screens of agonist antibodies can be carried out.

Once binding antibodies are identified by panning or another selection method, the individual clones, each expressing a unique antibody on the phage surface are tested for proliferation, differentiation, activation or survival effects on target cells. In addition, activity of the selected antibodies is examined in bioassays, such as the assays described below:

Biological Assays for Screening for TPO-like Activity

1. Colony formation assays - Megakaryocytic colonies from bone marrow (Megacult C Kit from Stem Cell Technologies Inc., Vancouver BC, Canada).

2. Proliferation assays - proliferation of Ba/F3 cells (Cwirla et al. 1997, Science, Vol. 276 pages 1696-1699). The Ba/F3-mpl cell line was established (F. de Sauvage *et al.,* Nature, 369:533 (1994)) by introduction of the cDNA encoding the entire cMpl receptor into the IL-3 dependent murine lymphoblastoid cell line Ba/F3. Stimulation of proliferation of Ba/F3-mpl cells in response to various concentrations of antibodies or TPO was measured by the amount of incorporation of ^{3}H-thymidine as previously described (F. de Sauvage *et al., supra).*

3. Phosphorylation assays - phosphorylation of JAK2 (Drachman et al., J. Biol. Chem., (1999), Vol. 274, pages 13480-13484).

4. Transcriptional based assays - Transiently co-transfect full length cMpl receptor with c-Fos promoter luciferase reporter construct. 24 hour post transfection starve the cells in 0.5% FCS for 24 hours. Stimulate the cells, harvest after 6 hours and take luciferase readings.

Biological Assays for Screening for EPO-like Activity

1. Bone marrow erthroid colony formation in Methylcellulose (Wrighton et al., Science, 1996, Vol. 273 pages 458-463).

2. TF-1 cell (Human erythroleukemia cell line) proliferation. TF-1 cells express both full length and a truncated form of the Epo-R. (J.Cell Physiol., 1989, Vol 140, pages 323-334).

3. The EPO receptor couples directly to JAK2 kinase to induce tyrosine phosphorylation. EPO induces cFos in TF-1 cells. c-Fos transcriptional activation. (Witthuhn et al., Cell, (1993), Vol. 74, pages 227-236).

Biological Assays for Screening for hBNP-like Activity

Functional screening of isolated clones is conducted using a cell based assay system for the evaluation of human brain natriuretic peptide (hBNP) activity on natriuretic peptide receptor type A (NPRA)-bearing cells (e.g., neuroblastoma cell line, SK-N-SH).

Biological Assays for Screening for GLP-1-like or Exendin-like Activity

Functional screening of insulinotropic activity of isolated clones can be conducted by:

1. Rat pancreas perfusion experiments or stimulation of cyclic AMP production evaluated using cultured RINm5F insulinoma cells using the methods described in Watanabe, et al., J Endocrinol. Jan;140(1):45-52 (1994); Gallwitz et al., J Mol Endocrinol. Jun;10(3):259-68 (1993); Richter , et al., J Endocrinol., Sep;126(3):445-50 (1990); Gallwitz et al., J Mol Endocrinol., Aug;5(1):33-9 (1990); Flatt et al., Diabetes Res., Feb;13(2):55-9 (1990); and/or Goke, J Endocrinol., Mar;116(3):357-62 (1988).

2. Evaluating binding affinity to GLP-1 receptor using techniques known to those skilled in the art.

Biological Assays for Screening for Adiponectin-like Activity

1. Oleate oxidation in isolated muscles and in mouse C2C12 skeletal muscle cells and Hepa-1-6 hepatocytes as described in Fruebis, et al., *Proc. Natl. Acad. Sci. USA* 98(4): 2005-10 (2001) .

2. Cell proliferation assay using human aortic smooth muscle cells (HASMCs) (Clonetics) and human aortic endothelial cells (HAECs) (Clonetics) with or without

PDGF-BB, HB-EGF, FGF, and EGF as described by Matsuda, et al., *J. Biol. Chem.* 277(40): 37487-91 (2002).

3. Inhibition of TNF-α-induced THP-1 adhesion and expression of VCAM-1, E-selectin, and ICAM-1 on HAECs as described by Ouchi, et al., *Circulation* 100 (25): 2473-6 (1999).

4. Binding to PDGF-BB and suppression of PDGF-BB-induced proliferation of HASMCs as disclosed by Arita, et al., *Circulation* 105 (24): 2893-8 (2002).

A number of bioassays can be used in high-throughput screening. Those of ordinary skill in the art are familiar with these and other suitable bioassays. Several non-radioactive assays have been developed in which either DNA synthesis or enzyme activity can be analyzed. A variety of useful assays are described in published US patent application US20030049683A1, the disclosure of which is incorporated herein in its entirety.

For conversion of antibody clones into full domain-exchanged antibodies, the coding regions for both the light and heavy chains, or fragments thereof, can be separately cloned out of a bacterial vector and into mammalian vector(s). A single vector system can be used to clone both light and heavy chain cassettes into the same plasmid. Alternatively, dual expression vectors where heavy and light chains are produced by separate plasmids can be used. Mammalian signal sequences need to be either already present in the final vector(s) or appended to the 5' end of the light and heavy chain DNA inserts. This can be accomplished by initial transfer of the chains into a shuttle vector(s) containing the proper mammalian leader sequences. Following restriction enzyme digestion, the light chain and heavy chain regions, or fragments

thereof, are introduced into final vector(s) where the remaining constant regions for IgG1 are provided either with or without introns. In some cases where introns are used, primer design for PCR amplifying the light and heavy chain variable regions out of expression vectors may need to include exon splice donor sites in order to get proper splicing and production of the antibodies in mammalian cells.

With either vector expression system (single or dual plasmid), the production of antibody heavy and light chains can be driven by promoters that work in mammalian cells such as, but not limited to, CMV, SV40, or IgG promoters. Additionally, the vector(s) will contain a selectable marker for growth in bacteria (such as, but not limited to, ampicillin, chloramphenicol, kanamycin, or zeocin resistance). Selectable markers for mammalian cells (such as, but not limited to, DHFR, GS, gpt, Neomyocin, or hygromyocin resistance) may also be present in the IgG vector(s), or could be provided on a separate plasmid by co-transfection.

Those of ordinary skill in the art using known techniques would be able to synthesize antibodies in other organisms such as yeast, mammalian, insect, and plants (Carlson, J.R. and Weissman, I.L., *Mol. Cell. Biol.,* 8:2647-2650, 1988; Trill, J.J., Shatzman, A.R., Ganguly, S. *Curr. Opin. Biotechnol.* 6:553-560, 1995; Hiatt, A., Cafferkey, R. Bowdish, K. *Nature* 342: 76-78, 1989).

The molecules encompassed by the claims can be used in diagnostics where the antibodies or fragments thereof are conjugated to detectable markers or used as primary antibodies with secondary antibodies that are conjugated to detectable markers. Detectable markers include radioactive and non-radioactive labels and are well-known to those with skill in the art. Common non-radioactive labels include

detectable enzymes such as horseradish peroxidase, alkaline phosphatase and fluorescent molecules. Fluorescent molecules absorb light at one wavelength and emit it at another, thus allowing visualization with, e.g., a fluorescent microscope. Spectrophotometers, fluorescence microscopes, fluorescent plate readers and flow sorters are well-known and are often used to detect specific molecules which have been made fluorescent by coupling them covalently to a fluorescent dye. Fluorochromes such as green flurorescent protein, red shifted mutants of green fluorescent protein, amino coumarin acetic acid (AMCA), fluorescein isothiocyanate (FITC), tetramethylrhodamine isothiocyanate (TRITC), Texas Red, Cy3.0 and Cy5.0 are examples of useful labels.

The molecules can be used in cell isolation strategies such as fluorescence-activated cell sorting (FACS) if fluorescent markers are used. In fluorescence-activated cell sorting, cells tagged with fluorescent molecules are sorted electronically on a flow cytometer such as a Becton-Dickinson (San Jose, California) FACS IV cytometer or equivalent instrument. The fluorescent molecules are antibodies that recognize specific cell surface antigens. The antibodies are conjugated to fluorescent markers such as fluorescein isothiocyanate (FITC) or Phycoerythrin (PE).

Alternatively, radiolabeled antibodies can be used for diagnostic purposes.

Antibodies and fragments thereof disclosed herein are useful for the amplification of a variety of clinically relevant cell types. Treatment can be *in vivo* or *ex vivo*. For example, agonist antibodies are useful to treat patients suffering from a deficiency in a cell population caused by disease, disorder or treatment related to for example suppression of hematopoiesis where less than the normal number of cells of

a given lineage or lineages are present in a patient. Those who practice the art will be able to identify other diseases and conditions that can be treated with the antibodies containing biologically active peptides disclosed herein.

The molecules encompassed by the present disclosure can also be used for *ex vivo* proliferation and differentiation of cells. This is useful for gene therapy purposes, for example for traditional viral vector approaches, and for autologous bone marrow transplants.

In addition, certain antibodies in accordance with the present disclosure can be radiolabeled for radioimmunotherapy or conjugated to toxins to deliver such toxins to specific cell types and result in the killing of those cells.

A biologically active *c-mpl* agonist antibody capable of stimulating proliferation, differentiation and maturation of hematopoietic cells may be used in a sterile pharmaceutical preparation or formulation to stimulate megakaryocytopoietic or thrombopoietic activity in patients suffering from thrombocytopenia due to impaired production, sequestration, or increased destruction of platelets. Thrombocytopenia-associated bone marrow hypoplasia *(e.g.,* aplastic anemia following chemotherapy or bone marrow transplant) may be effectively treated with the disclosed antibodies as well as disorders such as disseminated intravascular coagulation (DIC), immune thrombocytopenia (including HIV-induced ITP and non HIV-induced ITP), chronic idiopathic thrombocytopenia, congenital thrombocytopenia, myelodysplasia, thrombotic thrombocytoenia, chronic liver disease, and lupus. In addition, c-Mpl agonist antibodies may be used to stimulate platelet production prior to platelet harvesting by apheresis for autologous or allogeneic transfusion, or for the

mobilization of Peripheral Blood Progenitor Cells for autologous or allogeneic transplantation.

The antibodies disclosed herein containing the TPO mimetic peptide may be used in the same way and for the same indications as thrombopoietin (TPO). Thrombopoietin (TPO) stimulates megakaryocytopoiesis and platelet production.

EPO mimetic antibodies herein stimulate hematopoiesis in a manner similar to naturally occurring EPO. Such therapy is useful in treating conditions where red blood cell production is compromised such as in chronic renal failure.

In another aspect, this disclosure contemplates the treatment of congestive heart failure (CHF), either prophylactically or during CHF. Domain-exchanged antibody antibodies having hBNP incorporated therein in accordance with this disclosure can be administered intravenously into acutely decompensated CHF patients, to exert diuresis, natriuresis, and vasodilatation in a dose dependent manner.

In yet another aspect, the present disclosure contemplates methods of treating diabetes by administering antibodies having biologically active peptides incorporated therein. Thus, for example, in one embodiment, the domain-exchanged antibody is engineered to contain the glucagon-like peptide (GLP)-1, a potent insulinotropic hormone. In yet another aspect, the present disclosure contemplates methods to halt or delay the progressive deterioration of the diabetic state associated with type 2 diabetes by administering a domain-exchanged antibody containing GLP-1 to a patient afflicted with type 2 diabetes.

The Adiponectin- domain-exchanged antibody described herein can also be used as a diabetes treatment. Methods of increasing systemic insulin sensitivity by

administering adiponectin antibodies are also contemplated. In another aspect, methods of increasing glucose uptake by muscle cells by administering domain-exchanged antibodies having adiponectin incorporated therein are provided. In yet another aspect, administration of adiponectin antibodies prepared in accordance with the present disclosure decrease hepatic glucose output. In addition, reduction of insulin resistance and hyperinsulinemia can be treated by administering the present adiponectin-containing antibodies, thereby reducing obesity and the development of diabetes. In particularly useful embodiments Acrp30(C39S) or wild-type Acrp30 are engineered into domain-exchanged antibodies in accordance with the present disclosure.

The route of antibody administration is in accord with known methods, *e.g.,* injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, subcutaneous, intraocular, intraarterial, intrathecal, inhalation or intralesional routes, topical or by sustained release systems as noted below. The antibody is preferably administered continuously by infusion or by bolus injection. One may administer the antibodies in a local or systemic manner.

The antibodies in accordance with this disclosure may be prepared in a mixture with a pharmaceutically acceptable carrier. Techniques for formulation and administration of the compounds of the instant application may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition. This therapeutic composition can be administered intravenously or through the nose or lung, preferably as a liquid or powder aerosol (lyophilized). The composition may also be administered parenterally or subcutaneously as desired. When

administered systematically, the therapeutic composition should be sterile, pyrogen-free and in a parenterally acceptable solution having due regard for pH, isotonicity, and stability. When used for *in vivo* administration, the antibody formulation must be sterile and can be formulated according to conventional pharmaceutical practice. These conditions are known to those skilled in the art.

Pharmaceutical compositions suitable for use include compositions wherein one or more recombinant domain-exchanged antibodies are contained in an amount effective to achieve their intended purpose. More specifically, a therapeutically effective amount means an amount of antibody effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated. Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein. Therapeutically effective dosages may be determined by using *in vitro* and *in vivo* methods. A typical daily dosage might range from about 1μg/kg to up to 100 mg/kg or more, depending on the factors mentioned above. Typically, the clinician will administer the molecule until a dosage is reached that achieves the desired effect. The progress of this therapy is easily monitored by conventional assays.

The present antibodies can also be used in diagnostic assays, e.g., for detecting expression of certain proteins in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases (Zola, *Monoclonal Antibodies: A Manual of Techniques,* CRC Press, Inc. (1987) pp. 147-158). The

antibodies used in the diagnostic assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ^{3}H, ^{14}C, ^{32}P, ^{35}S, or ^{125}I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin, or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter et al., *Nature,* 144:945 (1962); David et al., *Biochemistry,* 13:1014 (1974); Pain et al., *J. Immunol. Meth.,* 40:219 (1981); and Nygren, *J. Histochem. and Cytochem.,* 30:407 (1982).

The present antibodies also are useful for the affinity purification of proteins from recombinant cell culture or natural sources. In this process, the antibodies are immobilized on a suitable support, such a Sephadex resin or filter paper, using methods well known in the art. The immobilized antibody then is contacted with a sample containing the protein to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the protein, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the protein from the antibody.

EXAMPLES

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

Example 1

Library Construction Of TPO Mimetic Sequences
Grafted Into The Heavy Chain CDR2 of a Domain-Exchanged Antibody

An agonist TPO mimetic-peptide IEGPTLRQWLAARA (SEQ. ID. NO: 1) was grafted into the heavy chain CDR2 (HCDR2) position (Kabat *et. al.*, 1991, U.S. Department of Health, NIH, Publication number 91-3242) of a 2G12 Fab (namely, 2G12-HCwt a codon-optimized heavy chain synthesized by Blue Heron Biotechnology, Bothell, WA, USA ; see Fig. 5), maintaining the first two amino acids (SI) of the HCDR2 and replacing the entire balance of the HCDR2 sequence. The agonist peptide is inserted into the HCDR2 region with two amino acid positions on each side of the peptide graft randomized in order that the best presentation of the peptide could be achieved. Thus, the final library will have the following in the CDR2-postions: SIXXIEGPTLRQWLAARAXX (SEQ. ID. NO: 15), where the X's represent the randomized amino acids flanking the TPO mimetic. See Fig. 6.

The 2G12 Fab is amplified as two fragments. Fragment A is amplified using the forward primer lead VH having the sequence 5' GCT GCC CAA CCA GCC ATG GCC 3' (SEQ. ID. NO: 18) and the reverse primer 2G12R2TPOR (5' CGC CAG CCA CTG GCG CAG GGT CGG GCC TTC AAT MNN MNN GAT CGA TGC TAC CCA TTC CAG 3') (SEQ. ID. NO: 19) which anneals to the end of framework 2 and the first two amino acids (SI) of the CDR2 of the heavy chain. Fragment B is created using the forward primer 2G12R2TPOF (5' CCG ACC CTG CGC CAG TGG CTG GCG GCG CGC GCG NNK NNK CTG TTT ACC GTC TCC CGT GAT 3') (SEQ. ID. NO: 20) which anneals to the beginning of framework 3, and the reverse primer Ndp-bsiWI (5' AGC GTA GTC CGG AAC GTC ATA C 3') (SEQ. ID. NO: 21) which binds to the HA epitope tag

portion of the vector downstream of the HC constant region. After the fragments are generated they are gel isolated and combined for overlap extension PCR. The new CDR coding primers contain a complementary 24 base overlap region. The vector specific primers Lead VH and Ndp-bsiWI are used for the overlap PCR protocol to generate the full Fab DNA product. Fragments A and B were combined in the absence of primers. The Expand High Fidelity PCR System from Roche Diagnostics GmbH (Indianapolis, IN) was used for the extension PCR, following the PCR program: 94° 4', then 10 cycles of 94° 30", 56° 30", and 72° 2', followed by an extension period of 10' at 72° and then a 4° hold. The Lead VH and Ndp-bsiWI primers were then added and the above program run for an additional 30 cycles to amplify the overlap extension PCR product. After generation, the fragment is gel isolated and digested with Xho I and Apa I. The insert is again gel purified and ligated into the similarly digested pRL5 –Bsi WI vector which already contains the 2G12 light chain (namely, the light chain codon optimized variant "2G12-LCwt" shown in FIG. 4). The DNA is precipitated and transformed into bacteria for expression of the 2G12 CDR2 library.

Example 2

Library Construction of TPO Mimetic Sequences
Grafted Into the Heavy Chain CDR3 of a 2G12 Antibody

A library of Fabs having a TPO mimetic peptide (SEQ. ID NO: 1) placed in the HC CDR3 position is prepared. See, Fig. 7. This peptide replaces the entire original CDR3 (Kabat *et. al.*, 1991, U.S. Department of Health, NIH, Publication number 91-3242) of a 2G12 heavy chain (namely the heavy chain of 2G12-HCwt (synthesized by Blue Heron Biotechnology, Bothell, WA, USA) shown in Fig. 5). The TPO mimetic

peptide is again flanked by two random amino acids on either side to create a library from which to select for optimal presentation of the peptide. In addition, the histidine residue (H32) in the 2G12 HC CDR 1 is randomized. This HC CDR1 histidine residue position is randomized in order to select for any amino acid that interacts with and stabilizes the TPO mimetic peptide in the HC CDR3 position.

The library is made step-wise using overlap extension PCR. In the first step the histidine within the HC CDR1 is randomized using overlap extension PCR. Fragment A is generated using the forward primer lead VH (SEQ. ID NO: 18) and the reverse primer 2G12HtoXR (5' ACG ACG TAC CCA GTT CAT AGT MNN TGC AGA AAT ACG GAA ATT AGA 3') (SEQ. ID. NO: 24) which anneals to the region encoding the last amino acid of framework 1, the HC CDR1 and the first four amino acids of framework 2 of the heavy chain. Fragment B is created using the forward primer 2G12HtoXF (5' ACT ATG AAC TGG GTA CGT CGT GTA CCA 3') (SEQ. ID. NO: 25) which anneals to the region encoding the last three amino acids of the HC CDR1 and the first six amino acids region of framework 2, and the reverse primer Ndp-bsiWI (SEQ. ID NO: 21). After the fragments are generated, they are gel isolated and combined for overlap extension PCR. The new CDR coding primers contain a complementary 21 base overlap region. The vector specific primers Lead VH and Ndp-bsiWI are used for the overlap PCR protocol (see Example 1). After generation, the fragment is gel isolated and digested with Xho I and Apa I. The insert is again gel purified and ligated into the similarly digested pRL5 –Bsi WI vector which already contains a 2G12 light chain (See, Fig. 4).

The DNA is precipitated and transformed into TOP 10 F' bacteria for the growth for plasmid isolation as a library pool for continuation into step 2. In step 2, the TPO mimetic peptide is placed into the HC CDR 3 using overlap extension PCR similar to above, with the exception that fragment A is generated using the reverse primer 2G12H3TPOR (5' CGC CAG CCA CTG GCG CAG GGT CGG GCC TTC AAT MNN MNN ACG TGC GCA GTA GTA AAT AGC 3') (SEQ. ID. NO: 26), which binds to the end of framework 3 of the 2G12 heavy chain. Fragment B is created using the forward primer 2G12H3TPOF (5' CCG ACC CTG CGC CAG TGG CTG GCG GCG CGC GCG NNK NNK TGG GGT CCA GGT ACC GTA GTA 3') (SEQ. ID. NO: 27), which binds to the beginning of framework 4. The fragments are gel isolated and combined for overlap extension PCR as detailed above. The fragment is again gel isolated and then digested with BsmBI and Apa I. This fragment is gel isolated and ligated into the pooled library vector plasmid prep in which the H32 was randomized (as described above), that has been similarly digested with BsmBI and Apa I. The DNA is precipitated and transformed into bacteria for expression of the 2G12 CDR3 library.

Example 3

Library Construction of hBNP Mimetic Sequences
Grafted Into the Heavy Chain CDR3 of a 2G12 Antibody

A library of Fabs having BNP mimetic peptides placed in the HC CDR3 position is prepared. This peptide replaces the entire original CDR3 (Kabat *et. al.*, 1991, U.S. Department of Health, NIH, Publication number 91-3242) of the 2G12 heavy chain. The BNP mimetic peptide (SEQ. ID NO: 3) is flanked by two random amino acids on either side to create a library from which to select for optimal presentation of the

peptide. In addition the histidine residue (H32) in the 2G12 HC CDR 1 is randomized in order to select for any amino acid that interacts with and stabilizes the BNP mimetic peptide in the HC CDR3 position.

The library is made step-wise using overlap extension PCR as follows. In the first step, the histidine within the HC CDR1 is randomized using overlap extension PCR. Fragment A is generated using the forward primer lead VH (SEQ. ID NO: 18) and the reverse primer 2g12HtoXR (5' ACG ACG TAC CCA GTT CAT AGT MNN TGC AGA AAT ACG GAA ATT AGA 3') (SEQ. ID. NO: 24) which anneals to the region encoding the last amino acid of framework 1, the HC CDR1 and the first four amino acids of framework 2 of the heavy chain. Fragment B is created using the forward primer 2G12HtoXF (5' ACT ATG AAC TGG GTA CGT CGT GTA CCA 3') (SEQ. ID. NO: 25) which anneals to the region encoding the last three amino acids of the HC CDR1 and the first six amino acids region of framework 2, and the reverse primer Ndp-bsiWI (SEQ. ID NO: 21). After the fragments are generated they are gel isolated and combined for overlap extension PCR. The new CDR coding primers contain a complementary 21 base overlap region. The vector specific primers Lead VH and Ndp-bsiWI are used for the overlap PCR protocol (as detailed previously). After generation, the fragment is gel isolated and digested with Xho I and Apa I. The insert is again gel purified and ligated into the similarly digested pRL5 –Bsi WI vector which already contains the 2G12 light chain (see Fig. 4). The DNA is precipitated and transformed into TOP 10 F' bacteria for the growth for plasmid isolation as a library pool for continuation into step 2. In step 2, the BNP mimetic peptide is placed into the HC CDR 3 using overlap extension PCR similar to above, with the exception that fragment A is

generated using the reverse primer 2G12H3BNPR (5' CTG GAG GAG CTG ATC CGG TCC ATC TTC CTC CCA AAG CAM NNM NNA CGT GCG CAG TAG TAA ATA GC 3') (SEQ. ID. NO: 28), which binds to the end of framework 3 of the2G12 heavy chain. Fragment B is created using the forward primer 2G12H3BNPF (5' AGA TGG ACC GGA TCA GCT CCT CCA GTG GCC TGG GCT GCN NKN NKT GGG GTC CAG GTA CCG TAG TA 3') (SEQ. ID. NO: 29), which binds to the beginning of framework 4. The fragments are gel isolated and combined for overlap extension PCR as detailed above. The fragment is again gel isolated and then digested with BsmBI and Apa I. This fragment is gel isolated and ligated into the pooled library vector plasmid prep in which the H32 was randomized (as described above), that has been similarly digested with BsmBI and Apa I. The DNA is precipitated and transformed into bacteria for expression of the 2G12 CDR3 library.

Example 4

Library Construction of ANP Mimetic Sequences
Grafted Into the Heavy Chain CDR3 of a 2G12 Antibody

A library is created wherein ANP mimetic peptide is placed in the HC CDR3 position of a 2G12 Antibody. This peptide replaces the entire original CDR3 (Kabat et. al., 1991) of the heavy chain of the 2G12 antibody shown in Figure 5. The ANP mimetic peptide (CFGGRMDRIGAQSGLGC (SEQ. ID NO: 30)) is flanked by two random amino acids on either side to create a library from which to select for optimal presentation of the peptide. In addition the histidine residue (H32) in the 2G12 HC CDR 1 is randomized in order to select for any amino acid that interacts with and stabilizes the ANP mimetic peptide in the HC CDR3 position.

The library is made step-wise using overlap extension PCR. In the first step the histidine within the HC CDR1 is randomized using overlap extension PCR. Fragment A is generated using the forward primer lead VH (SEQ. ID NO: 18) and the reverse primer 2G12HtoXR (5' ACG ACG TAC CCA GTT CAT AGT MNN TGC AGA AAT ACG GAA ATT AGA 3') (SEQ. ID. NO: 24) which anneals to the region encoding the last amino acid of framework 1, the HC CDR1 and the first four amino acids of framework 2 of the heavy chain. Fragment B is created using the forward primer 2G12HtoXF (5' ACT ATG AAC TGG GTA CGT CGT GTA CCA 3') (SEQ. ID. NO: 25) which anneals to the region encoding the last three amino acids of the HC CDR1 and the first six amino acids region of framework 2, and the reverse primer Ndp-bsiWI (SEQ. ID NO: 21). After the fragments are generated they are gel isolated and combined for overlap extension PCR. The new CDR coding primers contain a complementary 21 base overlap region. The vector specific primers Lead VH and Ndp-bsiWI are used for the overlap PCR protocol (as detailed previously). After generation, the fragment is gel isolated and digested with Xho I and Apa I. The insert is again gel purified and ligated into the similarly digested pRL5 –Bsi WI vector which already contains the 2G12 light chain shown in Fig. 4. The DNA is precipitated and transformed into TOP 10 F' bacteria for the growth for plasmid isolation as a library pool for continuation into step 2. In step 2, the ANP mimetic peptide is placed into the HC CDR 3 using overlap extension PCR similar to above, with the exception that fragment A is generated using the reverse primer 2G12H3ANPR (5' CTC TGG GCT CCA ATC CTG TCC ATC CTG CCC CCG AAG CAM NNM NNA CGT GCG CAG TAG TAA ATA GC 3') (SEQ. ID. NO: 31), which binds to the end of framework 3 of the 2G12 heavy chain. Fragment B

is created using the forward primer 2G12H3ANPF (5' GGA TGG ACA GGA TTG GAG CCC AGA GCG GAC TGG GCT GTN NKN NKT GGG GTC CAG GTA CCG TAG TA 3') (SEQ. ID. NO: 32), which binds to the beginning of framework 4. The fragments are gel isolated and combined for overlap extension PCR as detailed above. The fragment is again gel isolated and then digested with BsmBI and Apa I. This fragment is gel isolated and ligated into the pooled library vector plasmid prep in which the H32 was randomized (as described above), that has been similarly digested with BsmBI and Apa I. The DNA is precipitated and transformed into bacteria for expression of the 2G12 CDR3 library.

Example 5

Construction of Library Having GLP-1 Mimetic Peptides
Grafted Into the Heavy Chain CDR3 of a 2G12 Antibody

A library is made wherein GLP-1 mimetic peptides are placed in the HC CDR3 position. This peptide replaces the entire original CDR3 (Kabat et. al., 1991) of the 2G12 heavy chain shown in Fig. 5. The GLP-1 mimetic peptide (SEQ. ID NO: 5) is flanked by two random amino acids on either side to create a library from which to select for optimal presentation of the peptide. In addition the histidine residue (H32) in the 2G12 HC CDR 1 is randomized in order to select for any amino acid that interacts with and/or stabilizes the GLP-1 mimetic peptide in the HC CDR3 position.

The library is made step-wise using overlap extension PCR. In the first step, the histidine within the HC CDR1 is randomized using overlap extension PCR. Fragment A is generated using the forward primer lead VH (SEQ. ID NO: 18) and the reverse primer 2G12HtoXR (5' ACG ACG TAC CCA GTT CAT AGT MNN TGC AGA

AAT ACG GAA ATT AGA 3') (SEQ. ID. NO: 24) which anneals to the region encoding the last amino acid of framework 1, the HC CDR1 and the first four amino acids of framework 2 of the heavy chain. Fragment B is created using the forward primer 2G12HtoXF (5' ACT ATG AAC TGG GTA CGT CGT GTA CCA 3') (SEQ. ID. NO: 25) which anneals to the region encoding the last three amino acids of the HC CDR1 and the first six amino acids region of framework 2, and the reverse primer Ndp-bsiWI (SEQ. ID NO: 21). After the fragments are generated they are gel isolated and combined for overlap extension PCR. The new CDR coding primers contain a complementary 21 base overlap region. The vector specific primers Lead VH and Ndp-bsiWI are used for the overlap PCR protocol (as detailed previously). After generation the fragment is gel isolated and digested with Xho I and Apa I. The insert is again gel purified and ligated into the similarly digested pRL5 –Bsi WI vector which already contains the 2G12 light chain shown in Fig. 4. The DNA is precipitated and transformed into TOP 10 F' bacteria for the growth for plasmid isolation as a library pool for continuation into step 2. In step 2, the GLP-1 mimetic peptide is placed into the HC CDR 3 using overlap extension PCR similar to above, with the exception that fragment A is generated using the reverse primer 2G12H3GLP-1R (5' TTC CAA ATA AGA ACT TAC ATC ACT GGT AAA GGT CCC TTC AGC ATG MNN MNN ACG TGC GCA GTA GTA AAT AGC 3') (SEQ. ID. NO: 33), which binds to the end of framework 3 of the 2G12 heavy chain, and GLP-1 int-R (5' CAA TGA ATT CCT TGG CAG CTT GGC CTT CCA AAT AAG AAC TTA CAT CAC TG 3') (SEQ. ID. NO: 34), which allows the extension of the amplified product. 2G12H3GLP-1R and GLP-1 int-R primers are mixed 1:10 in the PCR reaction. Fragment B is created using the forward primer

2G12H3GLP-1F (5' GGC CAA GCT GCC AAG GAA TTC ATT GCT TGG CTG GTG AAA GGC CGA NNK NNK TGG GGT CCA GGT ACC GTA GTA 3') (SEQ. ID. NO: 35), which binds to the beginning of framework 4, and GLP-1 int-F (5' CAG TGA TGT AAG TTC TTA TTT GGA AGG CCA AGC TGC CAA GGA ATT CAT TG 3') (SEQ. ID. NO: 36), which also allows the extension of the amplified product and is complement reverse to the GLP-1 int-R primer. 2G12H3GLP-1F and GLP-1 int-F primers are mixed 1:10 in the PCR reaction. The fragments are gel isolated and combined for overlap extension PCR as detailed above. The fragment is again gel isolated and then digested with BsmBI and Apa I. This fragment is gel isolated and ligated into the pooled library vector plasmid prep in which the H32 was randomized (as described above), that has been similarly digested with BsmBI and Apa I. The DNA is precipitated and transformed into bacteria for expression of the 2G12 CDR3 library.

Example 6

An antibody Fab fragment containing a single peptide within a HC CDR region conferring binding to a single cell surface receptor, is converted to a potential therapeutic reagent capable of binding, dimerizing and activating two receptors by conversion of the normal Fab scaffold to a domain exchanged scaffold structure. This domain exchanged structure contains two light chains, and two heavy chains each of which contains a binding peptide. Thus, the domain exchanged molecule contains two binding peptides. Conversion of the scaffold structure is accomplished by changing Ala113 to Pro (Kabat numbering system).

As an example of applying this strategy, the cMpl-R binding Fab clone X4b is modified to achieve a domain exchanged scaffold. Fab clone X4b (see Figs. 8 and 9)

contains a single cMpl-R binding peptide in the heavy chain CDR3 region of a human Fab scaffold, and therefore is capable of binding the cMpl-R. However, the monomeric Fab does not stimulate the activation of the cMpl-R. This Fab is converted to a molecule with cMpl-R activating potential by conversion of the normal human Fab scaffold to a domain exchanged scaffold. The first step in the conversion process is to convert the alanine in X4b at amino acid position 113 (Kabat numbering system) to a proline. This is accomplished through use of overlap PCR. Fragment A is created using the forward primer Lead VH (5'-GCT GCC CAA CCA GCC ATG GCC-3') (SEQ. ID. NO: 18) and the reverse primer DomainXR (5'-GGA GAC GGT GAC CGT GGT CCC TTG GCC-3') (SEQ. ID. NO: 41). Fragment B is created using the forward primer DomainXF (5'-CAA GGG ACC ACG GTC ACC GTC TCC CCG GCC TCC ACC AAG GGC CCA TCG GTC-3') (SEQ. ID. NO: 42) and the reverse primer N-dp (5'-AGC GTA GTC CGG AAC GTC GTA CGG-3') (SEQ. ID. NO: 43). After the fragments are generated by PCR they are gel purified and combined for overlap extension PCR. A 24 bp overlap is provided in the DomainXF and R primers. The Lead VH and N-dp primers are used in the overlap extension PCR to generate the new heavy chain. Following construction of the new heavy chain, which incorporates the proline at position 113, the fragment is gel isolated and then digested with Xho I and Age I. This fragment is again gel isolated and ligated into the pRL5-X4b vector which is similarly digested and gel isolated, thereby replacing the original heavy chain with the modified one.

Additionally, further changes are introduced to help stabilize the domain exchanged structure. Changes along the interface between the light chain and the

exchanged heavy chain, which stabilize their interaction, are made. Specifically, the Lys, Ala and Thr at positions 19, 57 and 77, respectively, can be randomized to stabilize the domain exchanged structure. These changes are made using ovelap extension PCR in a manner similar to that described above.

To confirm that the above changes convert the scaffold to a domain exchanged conformation, bacterial supernatants containing the antibody product from the converted clones are run on isoelectric focusing gels and compared to the non-converted clones. Conversion to a domain exchanged structure doubles the molecular weight of the antibody and alters its mobility on an isoelectric focusing gel.

Additionally, size exclusion chromatography is used to detect the increased molecular weight. A medium such as Bio-Gel 100 commercially available from Bio-Rad is used to separate a domain exchanged Fab structure with a molecular weight of approximately 100Kd from a standard Fab structure with a molecular weight of 50 Kd. Commercially available molecular weight standards are used during the chromatography to confirm the molecular weights of the Fab products. Mutations which induce a domain exchanged structure result in the production of the higher molecular weight (100Kd) antibody form. Although some normal monomeric Fab (MW 50Kd) or individual light and heavy chains (MW 25Kd) may still be observed, an increased molecular weight form (100Kd) is detectable. Further mutations within the interface between the light chain and exchanged heavy chain which stabilize the domain exchanged structure, result in increased production of the100 Kd molecular weight domain exchanged form as a percentage of the total antibody products.

Further screening is conducted looking at potential activity. Since the monomeric Fab with a single peptide will not stimulate the cMpl-R, only mutations which convert the Fab or a portion thereof to the domain exchanged conformation have activity. Thus clones are screened for their potential for activating the cMpl-R using the high throughput luciferase activity screens, such as is described in WO 02/46238A2 (see Example 1), the disclosure of which is incorporated herein by this reference.

It will be understood that various modifications may be made to the embodiments disclosed herein. For instance, substitutions of single or multiple amino acids in the antibody sequence can frequently be made without destroying the functionality of the antibody or fragment. Thus, it should be understood that polypeptides or antibodies having a degree of identity greater than 70% to the specific antibodies described herein are within the scope of this disclosure. In particularly useful embodiments, antibodies having an identity greater than about 80% to the specific antibodies described herein are contemplated. In other useful embodiments, antibodies having an identity greater than about 90% to the specific antibodies described herein are contemplated. Therefore, the above description should not be construed as limiting, but merely as exemplifications of preferred embodiments. Those skilled in the art will envision other modifications within the scope and spirit of this disclosure.

WHAT IS CLAIMED IS:

1. An altered domain-exchanged antibody or a fragment thereof comprising a complementarity determining region (CDR) wherein either i) at least a portion of said CDR has been replaced by a biologically active peptide or ii) a biologically active peptide has been inserted into said CDR.

2. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein the biologically active peptide is selected from the group consisting of TPO, TPO mimetics, EPO, EPO mimetics, hBNP, hBNP mimetics, GLP-1, GLP-1 mimetics, GLP-2, GLP-2 mimetics, exendin, exendin mimetics, glucagon, glucagon mimetics, ANP, ANP mimetics, functional fragments of adiponectin and functional fragments of PACAP.

3. An altered domain-exchanged antibody or a fragment thereof according to claim 1 further comprising at least one flanking sequence including at least one amino acid covalently linked to at least one end of the peptide.

4. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein the biologically active peptide comprises an amino acid sequence selected from the group consisting of SEQ. ID. NOS: 1 through 10 and 30.

5. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein the CDR is selected from the group consisting of heavy chain CDR2 and heavy chain CDR3.

6. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein at least one residue in the heavy chain of the domain-exchanged antibody is mutated.

7. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein at least one residue in the heavy chain CDR1 of the domain-exchanged antibody is mutated.

8. An altered domain-exchanged antibody or a fragment thereof according to claim 7 wherein a histidine residue in the heavy chain CDR1 of the domain-exchanged antibody is mutated.

9. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein at least one residue in the light chain of the domain-exchanged antibody is mutated.

10. Nucleic acid encoding an altered domain-exchanged antibody or a fragment thereof according to claim 1.

11. An expression vector comprising nucleic acid according to claim 10.

12. A host cell transformed with an expression vector according to claim 11.

13. A method of producing an altered domain-exchanged antibody or a fragment thereof comprising culturing a host cell according to claim 12 under conditions suitable for expression of the altered domain-exchanged antibody or a fragment thereof.

14. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein at least a portion of said CDR has been replaced by a biologically active peptide.

15. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein a biologically active peptide has been inserted into said CDR.

16. A composition comprising an altered domain-exchanged antibody or a fragment thereof according to claim 1 and a pharmaceutically acceptable carrier.

17. A method of engineering an antibody or fragment thereof to exhibit an activity of a biologically active peptide comprising:

a) providing nucleic acid encoding a domain-exchanged antibody; and

b) either i) replacing at least a portion of at least one CDR encoding region with nucleic acid encoding a biologically active peptide or ii) inserting nucleic acid encoding a biologically active peptide into at least one CDR encoding region.

18. A method of stimulating proliferation, differentation, or growth of promegakaryocytes or megakaryocytes, comprising contacting promegakaryocytes or megakaryocytes with an effective amount of a domain-exchanged antibody having one or more CDR regions replaced with a TPO mimetic peptide.

19. A method of increasing the production of red blood cells comprising contacting hemopoietic stem cells or progenitors thereof with an effective amount of domain-exchanged antibody having one or more CDR regions replaced with an EPO mimetic peptide.

20. A library comprising varied domain-exchanged antibodies wherein either i) amino acid residues corresponding to at least a portion of at least one CDR are replaced by a biologically active peptide or ii) a biologically active peptide has been inserted into at least one CDR, the biologically active peptide having at least one flanking sequence which has been randomized to generate antibodies having variable amino acid sequences.

21. The library of claim 20 wherein said domain-exchanged antibodies are 2G12.

22. A nucleic acid library encoding the library of claim 20.

23. A library comprising varied domain-exchanged antibodies or fragments thereof comprising a heavy chain CDR1, wherein either i) amino acid residues of at least a portion of one or more CDRs are replaced by a biologically active peptide or ii) a biologically active peptide is inserted into one or more CDRs, and further wherein a heavy chain CDR1 of the antibodies or fragments thereof comprises one or more randomized amino acid residues.

24. The library of claim 23 wherein a histidine of a heavy chain CDR1 is randomized.

25. The library of claim 23 wherein said domain exchanged antibodies are 2G12.

26. A nucleic acid library encoding the library of claim 23.

27. A method comprising:

providing an antibody having a biologically active peptide inserted into at least one CDR or in place of at least a portion of the amino acids of at least one CDR; and

modifying the antibody to provide a domain-exchanged antibody.

28. The method of claim 27 wherein the step of modifying the antibody comprises weakening the VH/VL interface contacts.

29. The method of claim 27 wherein the step of modifying the antibody comprises substituting amino acid residues in the region of the antibody linking the V_H and C_H1 domains.

30. The method of claim 29 wherein the step of substituting amino acids in the region of the antibody linking the V_H and C_H1 domains comprises substituting a

proline for another amino acid residue in the region of the antibody linking the V_H and C_H1 domains.

31. A method as in claim 29 wherein the step of substituting amino acid residues in the region of the antibody linking the V_H and C_H1 domains comprises substituting a proline for another amino acid occurring at position 113 according to the Kabat numbering system.

32. A method as in claim 27 wherein the step of modifying the antibody comprises creating a favorable V_H/V_H' interface.

33. A method of performing a diagnostic assay, wherein said method comprises a step of detecting, wherein said detecting is by means of an antibody or fragment thereof of claim 1.

34. The method of claim 33 wherein said antibody or fragment thereof comprises a detectable moiety.

35. A kit for performing a diagnostic assay, said kit comprising the antibody or fragment thereof of claim 1.

36. A method of purifying a protein by contacting said protein with an antibody or fragment thereof of claim 1 under conditions wherein said protein binds to said antibody or fragment thereof.

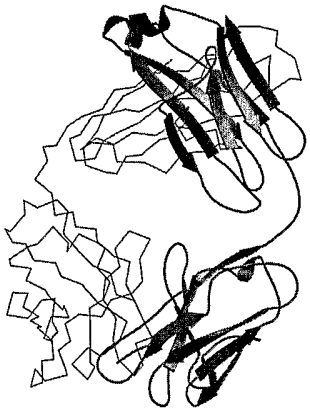
37. An altered domain-exchanged antibody or a fragment thereof according to claim 1 further comprising a detectable moiety.

38. An altered domain-exchanged antibody or a fragment thereof according to claim 1 wherein said domain exchanged antibody is 2G12.

39. A method of treating a subject in need thereof, said method comprising administering to said subject an effective amount of an antibody or fragment thereof of claim 1.

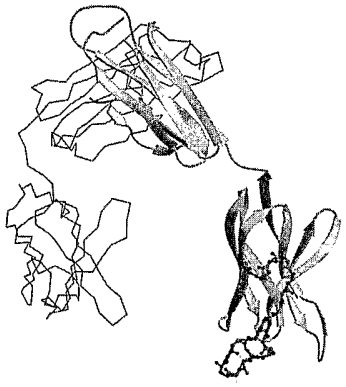
40. The method of claim 39 wherein said antibody or fragment thereof comprises a biologically active peptide selected from the group consisting of TPO, TPO mimetics, EPO, EPO mimetics, hBNP, hBNP mimetics, GLP-1, GLP-1 mimetics, GLP-2, GLP-2 mimetics, exendin, exendin mimetics, glucagon, glucagon mimetics, ANP, ANP mimetics, functional fragments of adiponectin having the biological activity of adiponectin, and functional fragments of PACAP-38 having the biological activity of PACAP-38.

FIGURE 1



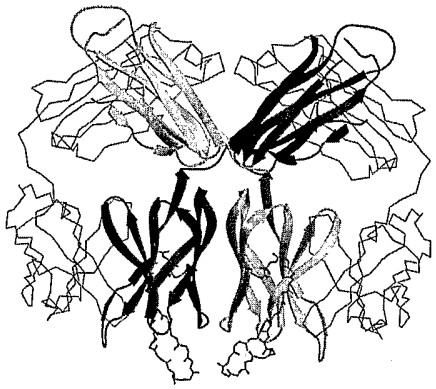
A typical structure of an antibody Fab domain.

FIGURE 2A



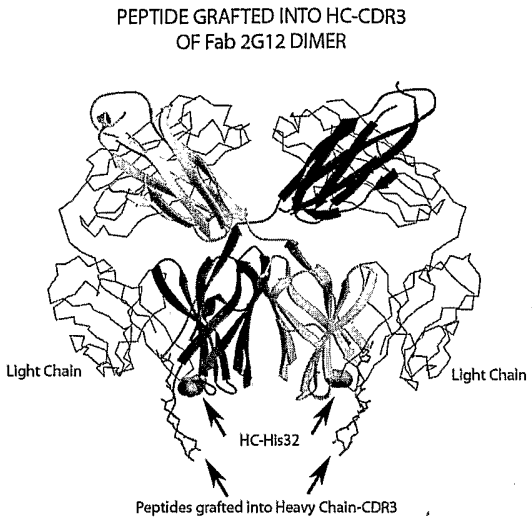
A model of 2G12 with TPO peptide grafted into HC-CDR2. Point mutations in a typical monomer antibody scaffold cause one domain of the heavy chain to move away from the antibody light chain.

FIGURE 2B



The 2G12 monomer spontaneously forms a dimer resulting in a cross heavy chain dimer motif. TPO peptides grafted into the HC-CDR2 regions would result in peptides in close proximity to one another in an anti-parallel fashion.

FIGURE 3



A model of a 2G12 dimer with TPO peptides grafted into the HC-CDR3 regions. Modeling of the structure shows that the histidine residue at position 32 comes into close contact with the peptide. Randomly mutating this position in conjunction with randomly mutating the flanking positions of the peptide may lead to an optimized presentation of the peptides

Figure 4

2G12 Light Chain codon optimized variant: 2G12-LCwt

E L D V V M T Q S P S T L S A S V G D
gag ctc GAT GTT GTT ATG ACC CAA TCC CCT TCT ACC CTG TCC GCG TCC GTA GGC GAT

T I T I T C R A S Q S I E T W L A W Y
ACT ATT ACT ATT ACT TGT CGT GCT TCT CAA TCC ATC GAA ACC TGG CTG GCA TGG TAC

Q Q K P G K A P K L L I Y K A S T L K
CAA CAA AAA CCT GGC AAA GCT CCT AAA CTG CTG ATC TAC AAA GCT TCC ACT CTG AAA

T G V P S R F S G S G S G T E F T L T
ACT GGC GTT CCT TCT CGT TTC TCC GGC TCC GGC TCT GGC ACC GAA TTT ACC CTG ACT

I S G L Q F D D F A T Y H C Q H Y A G
ATT TCC GGC CTG CAA TTC GAC GAC TTT GCG ACC TAT CAC TGT CAA CAT TAC GCT GGC

Y S A T F G Q G T R V E I K R T (SEQ ID NO 10)
TAC TCT GCA ACT TTT GGC CAA GGC ACC CGT GTT GAA ATC AAA cgt acg (SEQ ID NO 11)

Figure 5

2G12 Heavy Chain codon optimized variant: 2G12-HCwt

```
    L  E  V  Q  L  V  E  S  G  G  G  L  V  K  A  G  G  S  L  I  L
5′ ctc gag GTG CAA CTG GTT GAA TCT GGT GGT CTG GTT AAA GCT GGT GGT TCT CTG ATT CTG

 S  C  G  V  S  N  F  R  I  S  A  H  T  M  N  W  V  R  R  V  P  G
TCT TGT GGT GTT TCT AAT TTC CGT ATT TCT GCA CAT ACT ATG AAC TGG GTA CGT CGT GTA CCA GGG

 G  G  L  E  W  V  A  S  I  S  T  S  S  T  Y  R  D  Y  A  D  A  V
GGT GGT CTG GAA TGG GTA GCA TCG ATC TCT ACT TCC TCT ACT TAC CGT GAT TAT GCC GAT GCC GTT

 K  G  R  F  T  V  S  R  D  D  L  E  D  F  V  Y  L  Q  M  H  K  M
AAA GGT CGT TTT ACC GTC TCC CGT GAT GAT CTG GAA GAT TTT GTT TAC CTG CAA ATG CAC AAA ATG

 R  V  E  D  T  A  I  Y  Y  C  A  R  K  G  S  D  R  L  S  D  N  D
CGT GTC GAA GAT ACT GCT ATT TAC TAC TGC GCA CGT AAA GGC TCT GAC CGT CTG TCT GAT AAT GAT

 P  F  D  A  W  G  P  G  T  V  V  T  V  S  P  A  S  T  K  G (SEQ ID NO 12)
CCG TTT GAT GCA TGG GGT CCA GGT ACC GTA GTA ACC GTT AGT CCA GCA AGT ACG aag ggc cc 3′
                                                              (SEQ ID NO 13)
```

Figure 6

Peptide Grafted 2g12 Heavy Chain codon optimized variant: 2gHCvarH2

```
    L   E   V   Q   L   V   E   S   G   G   G   L   V   K   A   G   G   S   L   I   L
5' ctc gag GTG CAA CTG GTT GAA TCT GGT GGT GGT CTG GTT AAA GCT GGT GGT TCT CTG ATT CTG

 S   C   G   V   S   N   F   R   I   S   A   H   T   M   N   W   V   R   R   V   P   G
TCT TGT GGT GTT TCT AAT TTC CGT ATT TCT GCA CAT ACT ATG AAC TGG GTA CGT CGT GTA CCA GGG

 G   G   L   E   W   V   A   S   I   X   X   I   E   G   P   T   L   R   Q   W   L   A
GGT GGT CTG GAA TGG GTA GCA TCG ATC NNK NNK ATT GAA GGT CCG ACT CTG CGT CAA TGG CTG GCT

 A   R   A   X   X   R   F   T   V   S   R   D   D   L   E   D   F   V   Y   L   Q   M
GCG CGT GCT NNK NNK CGT TTT ACC GTC TCC CGT GAT GAT CTG GAA GAT TTT GTT TAC CTG CAA ATG

 H   K   M   R   V   E   D   T   A   I   Y   Y   C   A   R   K   G   S   D   R   L   S
CAC AAA ATG CGT GTC GAA GAT ACT GCT ATT TAC TAC TGC GCA CGT AAA GGC TCT GAC CGT CTG TCT

 D   N   D   P   F   D   A   W   G   P   G   T   V   V   T   V   S   P   A   S   T   K
GAT AAT GAT CCG TTT GAT GCA TGG GGT CCA GGT ACC GTA GTA ACC GTT AGT CCA GCA AGT ACG AAG

 G
GGC CC
```

****Underlining shows positions modified relative to 2gHCwt2**

Figure 7

Peptide Grafted 2g12 Heavy Chain codon optimized variant: 2gHCvarH3

```
L   E   V   Q   L   V   E   S   G   G   G   L   V   K   A   G   G   S   L   I   L
5′ ctc gag GTG CAA CTG GTT GAA TCT GGT GGT GGT CTG GTT AAA GCT GGT GGT TCT CTG ATT CTG

 S   C   G   V   S   N   F   R   I   S   A   X   T   M   N   W   V   R   R   V   P   G
TCT TGT GGT GTT TCT AAT TTC CGT ATT TCT GCA NNK ACT ATG AAC TGG GTA CGT CGT GTA CCA GGG

 G   G   L   E   W   V   A   S   I   S   T   S   S   T   Y   R   D   Y   A   D   A   V
GGT GGT CTG GAA TGG GTA GCA TCG ATC TCT ACT TCC TCT ACT TAC CGT GAT TAT GCC GAT GCC GTT

 K   G   R   F   T   V   S   R   D   D   L   E   D   F   V   Y   L   Q   M   H   K   M
AAA GGT CGT TTT ACC GTC TCC CGT GAT GAT CTG GAA GAT TTT GTT TAC CTG CAA ATG CAC AAA ATG

 R   V   E   D   T   A   I   Y   Y   C   A   R   X   X   I   E   G   P   T   L   R   Q
CGT GTC GAA GAT ACT GCT ATT TAC TAC TGC GCA CGT NNK NNK ATT GAA GGT CCG ACT CTG CGT CAA

 W   L   A   A   R   A   X   X   W   G   P   G   T   V   V   T   V   S   P   A   S   T
TGG CTG GCT GCG CGT GCT NNK NNK TGG GGT CCA GGT ACC GTA GTA ACC GTT AGT CCA GCA AGT ACG


 K   G     (SEQ ID NO: 23)
aag ggc cc 3′  (SEQ ID NO: 22)
```

****Underlining shows positions modified relative to 2gHCwt2**

Figure 8
X4b Heavy Chain (VH) Sequence

```
                                                                         19
    E   V   Q   L   L   E   Q   S   G   A   E   V   K   K   P   G   S   S   V   K   V
5’ GAG GTG CAG CTG CTC GAG CAG TCT GGG GCT GAG GTG AAG AAG CCT GGG TCC TCG GTG AAG GTC

 S   C   R   A   S   G   G   T   F   N   N   Y   A   I   S   W   V   R   Q   A   P   G
TCC TGC AGG GCT TCT GGA GGC ACC TTC AAC AAT TAT GCC ATC AGC TGG GTG CGA CAG GCC CCT GGA

                                                              57
 Q   G   L   E   W   M   G   G   I   F   P   F   R   N   T   A   K   Y   A   Q   H   F
CAA GGG CTT GAG TGG ATG GGA GGG ATC TTC CCT TTC CGT AAT ACA GCA AAG TAC GCA CAA CAC TTC

                                                      77
 Q   G   R   V   T   I   T   A   D   E   S   T   G   T   A   Y   M   E   L   S   S   L
CAG GGC AGA GTC ACC ATT ACC GCG GAC GAA TCC ACG GGC ACA GCC TAC ATG GAG CTG AGC AGC CTG

 R   S   E   D   T   A   I   Y   Y   C   A   R   L   P   I   E   G   P   T   L   R   Q
AGA TCT GAG GAC ACG GCC ATA TAT TAT TGT GCG AGA TTG CCA ATT GAA GGG CCG ACG CTG CGG CAA

                                                                          113
 W   L   A   A   R   A   P   V   W   G   Q   G   T   T   V   T   V   S   A   A   S   T
TGG CTG GCG GCG CGC GCG CCT GTT TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC GCA GCC TCC ACC

 K   G   P      (SEQ ID NO: 38)
AAG GGC CC 3’   (SEQ ID NO: 37)
```

***Boxes show amino acids to be modified. Kabat based amino acid numbering included.**
****Underlining shows position of grafted c-MplR binding peptide**

Figure 9
X4b Light Chain (VL) Sequence

```
     E  L  T  Q  S  P  G  T  L  S  L  S  P  G  E  R  A  T  L  S  C
5'   GAG CTC ACG CAG TCT CCA GGC ACC CTG TCT TTG TCT CCA GGG GAA AGA GCC ACC CTC TCC TGC

  R  A  S  H  S  V  S  R  A  Y  L  A  W  Y  Q  Q  K  P  G  Q  A  P
AGG GCC AGT CAC AGT GTT AGC AGG GCC TAC TTA GCC TGG TAC CAG CAG AAA CCT GGC CAG GCT CCC

  R  L  L  I  Y  G  T  S  S  R  A  T  G  I  P  D  R  F  S  G  S  G
AGG CTC CTC ATC TAT GGT ACA TCC AGC AGG GCC ACT GGC ATC CCA GAC AGG TTC AGT GGC AGT GGG

  S  G  T  D  F  T  L  T  I  S  R  L  E  P  E  D  F  A  V  Y  Y  C
TCT GGG ACA GAC TTC ACT CTC ACC ATC AGC AGA CTG GAG CCT GAA GAT TTT GCA GTG TAC TAC TGT

  Q  Q  Y  G  G  S  P  W  F  G  Q  G  T  K  V  E  L  K  R  T (SEQ ID NO: 40)
CAG CAG TAT GGT GGC TCA CCG TGG TTC GGC CAA GGG ACC AAG GTG GAA CTC AAA CGA ACT(SEQ ID NO: 39)
```