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(54) Title:  METHOD FOR OBTAINING RECEPTOR AGONIST ANTIBODIES

(57) Abstract  
A method is provided for generating antibodies which demonstrate the agonist properties of the naturally occurring ligand of a receptor molecule by using as an immunogen a recombinant immunogen which corresponds to a multimeric form of a receptor. In this immunogen the extracellular domains are in a similar disposition to that expected for receptors having two or more subunits on the surface of the cell.
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METHOD FOR OBTAINING RECEPTOR AGONIST ANTIBODIES

Cross Reference to Related Applications

This application is a continuation-in-part of co-pending U.S. application serial number 08/474,673, filed June 7, 1995, the contents of which is incorporated hereby by reference.

Field of the Invention

The present invention relates generally to the generation of monoclonal antibodies by use of a selected antigen; and more particularly to the generation of monoclonal antibodies which are receptor agonists.

Background of the Invention

A vast majority of receptors of the single transmembrane class respond to ligand binding by some form of aggregation. This aggregation can be between identical receptor subunits (as in homodimerization, homotrimerization, etc.) or between different receptor subunits (as is heterodimerization, heterotrimerization, etc). This aggregation appears to be part of the signal for the target cell to respond biologically, in that mutants of the ligand which are unable to interact with the second subunit are still able to bind, but no longer cause dimerization and biological activation of the receptor [P. R. Young, Curr. Opin. Biotech., 2:408-421 (1992)].

For example, there is evidence in the literature that suggests dimerization of the erythropoietin receptor (EpoR) upon ligand binding [S. S. Watowich et al., Molec. Cell Biol., 14:3535-3549 (1992) and S. S. Watowich et al., Proc. Natl. Acad. Sci., USA, 89:2140-2144 (1992)]. Reports about IL-6 have indicated that its second subunit gp130 may dimerize upon IL-6 binding [M. Murkami et al., Science, 260:1808-1810 (1993)]. For some receptors in which homodimerization is induced by ligand binding, monoclonal antibodies (mAbs) were discovered which had agonist properties. These include mAbs to EGF, TNF and growth hormone receptors [A. B. Schrieber et al., J. Biol. Chem., 258:846-853 (1983); L. H. K.
Defize et al., EMBO J., 5:1187-1192 (1986); H. Engelmann et al., J. Biol. Chem., 265:14497-14504 (1990); and G. Fuh et al., Science, 256:1677-1680 (1992)]. In each case, these mAbs, by virtue of their two antigen recognition sites, were able to bring together two receptors and thus activate them. Fab fragments made from these mAbs were inactive. In some cases, the apparent affinity of the antibody for receptor was comparable to that of the ligand, e.g., growth hormone [Fuh et al., cited above].

It has also been discovered that antibodies to IL-3 receptor have agonist properties [Sugiuwara et al., J. Immunol., 140:526-530 (1988)]. Previous literature has described the production of anti-erythropoietin receptor antibodies [A. D’Andrea et al., Blood, 82:46-52 (1993); A. D’Andrea et al., Blood, 84:1982-1991 (1994) and M-G Yet et al., Blood, 82: 1713-1719 (1993). See also, PCT Application WO96/03438 published 8 February 1996. While the Yet et al., reference suggests the occurrence of possible EPO-like activity in one mAb, the mAb is not characterized. Neither Yet et al., nor the other literature provides any reproducible manner of generating agonist mAbs.

There remains a need in the art for the development of additional mAbs which have an affinity for receptors comparable to that of the ligand, and which can act as agonists of the receptor.

Summary of the Invention

In one aspect, the present invention provides a method for reliably generating an antibody which is an agonist of a receptor. This method employs as the immunizing antigen, a recombinant immunogen which consists of a first extracellular domain of a receptor molecule spaced apart from a second extracellular domain from that receptor by a bridging moiety. The bridging moiety places the first domain and the second domain into a functional proximity which mimics the functional domain orientation and proximity of the naturally occurring multimeric receptor. The bridging moiety can be an amino acid spacer peptide, an organic molecule, an Fc portion of a human immunoglobulin or an amphipathic helix, for example.
In another aspect, the invention includes antibodies produced by the above-described method. The antibodies so generated are characterized by the ability to bind to the naturally occurring receptor and by such binding initiate the biological activity of the receptor. The antibodies of the invention may be chimeric antibodies, humanized antibodies, monoclonal antibodies or polyclonal antibodies.

In still another aspect, the invention provides a recombinant polynucleotide sequence comprising a nucleotide sequence encoding the extracellular domain of a receptor molecule fused in frame to a nucleotide sequence encoding a specific proteolytic cleavage site, said cleavage sequence associated with a bridging moiety.

Yet a further aspect of the invention is the recombinant multimeric immunogen itself.

Additional aspects of this invention include a vector comprising a polynucleotide sequence described above under the control of suitable regulatory sequences capable of directing replication and expression of the polynucleotide sequence in a host cell, and a transformed host cell.

Still other aspects of the invention include therapeutic reagents comprising the antibodies produced by the method of this invention, as well as a method of treating a disease condition by administering a pharmaceutical composition of the invention.

Other aspects and advantages of the present invention are described further in the following detailed description of the preferred embodiments thereof.

Brief Description of the Drawings

Fig. 1 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation of UT-7 Epo cells, which plots % positive control vs. concentration (ng/ml).

Fig. 2 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation of DA3Epowt cells, which plots % positive control vs. concentration (ng/ml).
Fig. 3 is a graph illustrating the effects of the dimeric fusion protein EpoRFc and the monomeric protein mEpoR on erythropoietin stimulation in an CFU-E assay, which plots % positive control vs. concentration (ng/ml).

Fig. 4 is a bar graph demonstrating the binding of antibodies developed in response to immunization with the EpoRFc fusion protein to 32D/Epo wt and parental 32D Fig. (4a) and UT7-EPO, cells, plotting %Isotype control log fluorescence vs. control (CTL) and supernatant designations.

Fig. 5 is a bar graph demonstrating the results of an UT7-Epo proliferation assay with three mAbs to EpoRFc, plotting %Epo maximum vs. dilutions of supernatants. The supernatant designations are listed above the bars.

Fig. 6 is a bar graph demonstrating the ability of 4 mAbs to EPORFc to stimulate the proliferation and differentiation of human bone marrow progenitor cells to form red blood cell colonies (CFU-E), plotting Numbers of CFU-E vs. positive and negative EPO controls and supernatant dilutions. The supernatant designations are listed above the bars.

Fig. 7 is the DNA sequence [SEQ ID NO: 1] for plasmid mtalsEpoRFc containing the sequence encoding the EpoRFc fusion protein under control of a Drosophila S2 mtn promoter. Nucleotides 1 to 897 contain the promoter sequence. The EpoRFc fusion protein [SEQ ID NO: 2] is encoded by the following:

nucleotides 898 to 1647 encode the EpoR extracellular domain protein; nucleotides 1648 to 1659 encode the Factor Xa cleavage sequence; nucleotides 1660 to 2361 encoding the human IgG1 Fc sequence. The remainder of the sequence is derived from the plasmid parent. See Example 1.

Fig. 8 is the DNA sequence [SEQ ID NO: 3] of CosFcLink vector from which a KpnI/XbaI insert containing the IgG1 Fc region was obtained.

Detailed Description of the Invention

The present invention provides a reproducible and reliable composition and method for generating antibodies which demonstrate the agonist properties of the naturally occurring ligand of a receptor molecule. While, in principle, any purified preparation could be used to generate antibodies to a receptor, and from these some
may be agonists, this invention provides a specific immunogen which corresponds to a multimeric form of a receptor in which the extracellular domains are in a similar disposition to that expected for receptors having two or more subunits on the surface of the cell.

I. The Recombinant Immunogen

The recombinant immunogen of the present invention consists of an extracellular domain of a selected receptor molecule which is involved in ligand binding via the interaction of more than one extracellular domain. The extracellular domain is spaced apart from a second extracellular receptor domain by a bridging moiety.

By "extracellular domain" is meant that portion of a receptor protein which is localized on the extracellular surface of a cell and which contributes to interaction and binding with its native ligand.

The receptor subunit which contributes the extracellular domain(s) of the immunogen may be any receptor subunit which accomplishes ligand binding via a homodimer of the extracellular domain, a heterodimer of two different subunits of the receptor's extracellular domain, or a multimer of subunits. Receptors which homodimerize upon ligand binding and thus may contribute extracellular domains to the recombinant immunogen include those for, inter alia, erythropoietin (EPO) and thrombopoietin (CMPL), G-CSF, M-CSF, TGF-a, EGF, neu, growth hormone, prolactin, placental lactogen, c-kit (stem cell factor receptor), p50 and p75 receptor subunits of TNFa, and TNFβ. Other receptors which are anticipated to dimerize based on homology to TNF and which can be included in this list are Fas, CD40, CD27, CD30, 4-1BB and OX40.

Similarly, receptors which are already in a homodimeric form on the cell surface, prior to ligand binding may also contribute the extracellular domain(s) to the immunogen. These latter receptors include, inter alia, insulin, IGF1 and IGF2, and PDGF. PDGF includes dimeric ligand made of two chains A and B and two receptor subunits a and b. Ligands and receptors can associate as homo- or hetero-
Relaxin is also anticipated to be a dimeric receptor prior to ligand binding based on its homology to insulin and IGF.

Receptors which are formed by heterodimers of two different subunits may also be employed as contributors of extracellular domain(s) of one or both of the subunits to the recombinant. Such receptors include, \textit{inter alia}, GM-CSF, IL-3, IL-5, IL-6, Oncostatin M, CNTF, LIF, NGF, FGF, IL-4, IL-13, IFNa, IFN\(\beta\), IFNg, TGF\(\beta\)1, TGF\(\beta\)2 and IL-12. Receptors, such as IL-3, which involve more than one subunit may respond to this method, if dimerization of one of the subunits is required for signal transduction.

Receptors which form other aggregations, such as trimers of IL-2 receptor subunits, may also be used as sources of extracellular domain(s) for the recombinant immunogen of this invention.

The extracellular domains of the selected receptor useful in the recombinant immunogen may be isolated and/or otherwise obtained by resort to the published and publicly available receptor sequences. Methods conventional in the art may be employed to isolate or synthesize the appropriate nucleotide sequences encoding these domains for further manipulation in the generation of the immunogen of this invention.

By the term "bridging moiety" is meant a peptide or non-peptide sequence that stably associates with itself and places the first extracellular domain and the second extracellular domain into functional proximity (i.e., a relationship between the two or more domains which mimics the three dimensional functional proximity of the domains in the naturally occurring multimeric receptor, during or prior to ligand binding. Preferably, the bridging moiety is of a sufficient size to bring together the membrane proximal regions (i.e., the regions closest to the transmembrane region) of the extracellular domain. The precise dimensions of the bridging region can be estimated from the relevant crystal structures, such as those for growth hormone and its receptor, or TNF and its receptor [see, e.g., DeVos \textit{et al.}, \textit{Science}, 255:306 (1992)].

The suitability of a given "bridging moiety" can sometimes be evaluated empirically. For example, a dimeric form of a receptor which dimerizes upon
binding of ligand is expected to have a higher affinity for ligand than a monomeric form of the extracellular domain of the receptor, if the bridging group is appropriate. This would be evidenced either by direct binding studies of ligand to monomeric and dimeric receptor, or by the relative ability of the two forms of receptor to neutralize the biological activity of the ligand. For example, Fig. 1 shows the ability of dimeric EpoRFC to neutralize Epo activity about 100 times more effectively than monomeric EpoR.

An example of a bridging moiety is an amino acid spacer sequence (e.g., between 1 to 10 amino acids in length and optionally encoding a cleavage site) fused to a dimerization or oligomerization domain which permits the formation of dimers or oligomers, respectively. In the following example, for instance, a spacer encoding the Factor Xa cleavage site is part of the bridging moiety; and the dimerization domain is the hinge CH2CH3 region of a human IgG1. This IgG component consists of the CH2 and CH3 domains and the hinge region of IgG1 including cysteine residues contributing to inter-heavy chain disulfide bonding, for example residues 11 and 14 of the IgG1 hinge region [see, e.g., B. Frangione, *Nature*, 216: 939-941, (1967)]. Preferably the IgG1 component consists of amino acids corresponding to residues 1-4 and 6-15 of the hinge, 1-110 of CH2 and 1-107 of CH3 of IgG1 described by J. Ellison *et al.*, *Nucleic Acids Res.*, 10: 4071-4079 (1982). Residue 5 of the hinge is changed from cysteine in the published IgG1 sequence to alanine by alteration of TGT to GCC in the nucleotide sequence.

The bridging moiety can also be an Fc portion of any human immunoglobulin with an intact hinge CH2CH3 region, including those derived from IgG, IgE, IgM, IgA and IgG4. Still another suitable bridging moiety is an amino acid spacer fused to the C terminal domain of the TNF-like receptor of the shope fibroma poxvirus [C. A. Smith *et al.*, *BBRC*, 176: 335-342 (1991)]. The bridging moiety may also be an a dimerization domain like an amphipathic helix, such as a leucine zipper [see, e.g., P. Pack *et al.*, *Biochem.*, 21(6): 1579-1584 (Feb. 18, 1992)]. The bridging moiety may also be alkaline phosphatase.

Still other desirable embodiments of the bridging moiety are organic molecules which can functionally associate the receptor extracellular domains as
desired. Such organic molecules may be selected from among such molecules
known to associate peptide sequences to each other for other biological uses, e.g.,
bifunctional cross-linkers, such as carbodiimide, glutaraldehyde and DSS, BS3, and
others which may be obtained from several commercial sources. However, these
associations may require combination with specific target sequences for cross-
linking, e.g., an exposed Cys or His for nickel chelate, to achieve the appropriate
three dimensional disposition of receptor subunits. Choice of an appropriate cross-
linker can be determined by comparison to known crystal structures of homologous
receptors.

The suitability of a particular peptide or non-peptide entity as a bridging
moiety may be functionally assessed in a receptor ligand binding assay. The
suitability of the bridging moiety may be determined if the recombinant immunogen
binds the receptor's intended ligand with greater affinity than does the monomeric
sequence of the receptor extracellular domain. Ligand binding assays for the
selected receptors are known to those of skill in the art and may be readily selected
without undue experimentation. See, for example, the EpoR ligand assays described

Another bridging moiety or linker may be prepared by mutagenesis of a
receptor in the membrane proximal domain to create unpaired Cys, which can
disulfide bond to create a dimeric receptor. Such mutations can be evaluated for
appropriateness by observing whether the full length receptor containing such a
mutation is able to constitutively activate the ligand's activity upon transfection into
suitable target cells (e.g., EpoR mutations) [see, e.g., Watowich et al., Proc. Natl.
Acad. Sci., USA., 89:2140-2144 (1992)].

Thus, for example, the bridging moiety of the recombinant immunogen can
associate two identical extracellular domains into an immunogen that mimics a
homodimeric receptor. Alternately, for example, the bridging moiety can associate
two different extracellular domains of subunits of one receptor into an immunogen
that mimics a heterodimeric receptor. For example, for the heterodimereic
association of different subunits from a heterodimeric receptor, a different bridging
moiety could be used for each receptor subunit. The bridging moiety for a heterodimeric receptor is preferably a domain that cannot associate with itself, but which preferentially associates with a second domain. Thus, the first bridging moiety can be a CH1 region of a light chain of a selected immunoglobulin. Its complementary bridging moiety is the CH1 region of the heavy chain of the same immunoglobulin or the entire Fc region including the CH1, hinge, CH2, and CH3 regions of the heavy chain. It is also anticipated that various bridging entities may be employed in preparing other multimeric immunogens, e.g., trimers, by associating three identical domains or three extracellular domains from one, two or three subunits of a single receptor. It is presently preferred to use an Fc portion of an immunoglobulin as a bridging moiety to associate the extracellular receptor domains, as disclosed in Example 1 below.

II. Construction and Preparation of the Recombinant Immunogen

The present invention also provides the nucleic acid sequences encoding the recombinant immunogens described above. The nucleotide sequences encoding the extracellular domains of the receptors useful in the immunogens may be obtained from known receptor sequences by conventional means [see, J. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2d ed., Cold Spring Harbor Laboratory (1989)]. For example, the nucleotide sequences which form the immunogen of the invention may be isolated by conventional uses of polymerase chain reaction or conventional genetic engineering cloning techniques. Alternatively, these sequences may be constructed using chemical synthesis techniques.

Optionally, a nucleotide sequence which encodes a peptide sequence which provides an enzymatic cleavage site (of which many are well known in the art) is fused in frame to the extracellular domain nucleotide sequence prior to its association with the dimerization or oligomerization domain of the bridging moiety. This facilitates cleavage of the extracellular domain from the bridging moiety following expression.

According to the invention, the nucleic acid sequences encoding the extracellular domains may be modified as desired. It is within the skill of the art to
obtain other polynucleotide sequences encoding these receptor domains useful in the invention. Such modifications at the nucleic acid level include, for example, modifications to the nucleotide sequences which are silent or which change the amino acids, e.g. to improve expression or secretion. Such modifications include amino terminal, carboxy terminal or internal deletions of the nucleotide sequences, as desired.

Where the bridging moiety is an amino acid sequence, the nucleotide sequence encoding it is also obtained conventionally and fused to the extracellular domain sequence directly or via a cleavage site or via additional sequence intended as a spacer. For example, where the bridging moiety is an Fc portion of a human immunoglobulin with intact hinge CH2CH3 region, the nucleotide sequence encoding the Fc region is obtained from known antibody sequences, prepared by conventional techniques and fused in frame to the receptor sequences or to the sequence providing the enzymatic cleavage site.

To produce recombinant immunogens of this invention, a DNA sequence of the invention encoding the extracellular receptor domain, is fused in frame to an optional cleavage site and further fused to a nucleotide sequence encoding a peptide bridging moiety. Preparation of the nucleic acid sequences may be carried out chemically, enzymatically, or by a combination of the two methods, in vitro or in vivo as appropriate. Thus, the DNA sequences may be prepared by the enzymatic ligation of appropriate DNA fragments, by conventional methods such as those described by D. M. Roberts et al., Biochem., 24:5090-5098 (1985). The DNA fragments may be obtained by digestion of DNA containing the required sequences of nucleotides with appropriate restriction enzymes, by chemical synthesis, by enzymatic polymerization on DNA or RNA templates, or by a combination of these methods. These methods are generally provided by the commercial supplier of the reagents.

For example, digestion with restriction enzymes may be performed in an appropriate buffer at a temperature of 20-70°C, generally in a volume of 50ul or less with 0.1-10ug DNA. Enzymatic polymerization of DNA may be carried out in vitro using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an
appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10-37°C, generally in a volume of 50μl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer at a temperature of 4°C to ambient, generally in a volume of 50μl or less. The chemical synthesis of the DNA sequence or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in "Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual" (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait et al., Nucleic Acids Res., 10: 6243 (1982) and others. Preferably an automated DNA synthesizer is employed. The DNA sequence is preferably prepared by ligating two or more DNA molecules which together comprise a DNA sequence encoding the compound. The DNA molecules may be obtained by the digestion with suitable restriction enzymes of vectors carrying the required coding sequences or by use of polymerase chain reaction technology. The precise structure of the DNA molecules and the way in which they are obtained depends upon the structure of the desired product. The design of a suitable strategy for the construction of the DNA molecule coding for the recombinant immunogen is a routine matter for one skilled in the art.

Once the nucleotide sequence encoding the recombinant immunogen is designed, it is inserted into a suitable expression system. Systems for cloning and expression of a selected protein in a desired microorganism or cell, including, e.g. E. coli, Bacillus, Streptomyces, mammalian, insect, and yeast cells, are known and available from private and public laboratories and depositories and from commercial vendors.

Desirably, a recombinant molecule or vector is constructed in which the polynucleotide sequence encoding the immunogen is operably linked to a heterologous expression control sequence permitting expression of the human protein. Numerous types of appropriate expression vectors are known in the art for eukaryotic (including human) protein expression, by standard molecular biology techniques. Such vectors may be selected from among conventional vector types.
including mammalian, insects, e.g., baculovirus expression, Drosophila S2 cell, or yeast, fungal, bacterial or viral expression systems. Other appropriate expression vectors, of which numerous types are known in the art, can also be used for this purpose. Methods for obtaining such expression vectors are well-known. See, for example, Sambrook et al., cited above; Miller et al., Genetic Engineering, 8:277-298 (Plenum Press 1986) and Johansen et al., Genes and Develop., 3:882-889 (1989).

Suitable host cells or cell lines for transfection by this method include mammalian cells, such as Human 293 cells, Chinese hamster ovary cells (CHO), the monkey COS-1 cell line or murine 3T3 cells derived from Swiss, Balb-c or NIH mice may be used. Another suitable mammalian cell line is the CV-1 cell line. Still other suitable mammalian host cells, as well as methods for transfection, culture, amplification, screening, and product production and purification are known in the art. [See, e.g., Gething and Sambrook, Nature, 293:620-625 (1981), or alternatively, Kaufman et al., Mol. Cell. Biol., 5(7):1750-1759 (1985) or Howley et al., U. S. Patent 4,419,446].

Similarly bacterial cells are useful as host cells for the present invention. For example, the various strains of E. coli (e.g., HB101, MC1061, and strains used in the following examples) are well-known as host cells in the field of biotechnology. Various strains of B. subtilis, Pseudomonas, Streptomyces, other bacilli and the like may also be employed in this method.

Many strains of yeast cells known to those skilled in the art are also available as host cells for expression of the polypeptides of the present invention. Other fungal cells may also be employed as expression systems. Alternatively, insect cells such as Spodoptera frugiperda (Sf9) or Drosophila S2 may be used.

Thus, the present invention provides a method for producing a recombinant novel multimeric receptor immunogen which involves transfecting a host cell with at least one expression vector containing a recombinant polynucleotide as above-described under the control of a transcriptional regulatory sequence, e.g. by conventional means such as electroporation. The transfected host cell is then cultured under suitable conditions that allow expression of the product of the recombinant polynucleotide. During expression, the recombinant multimeric
immunogen is formed in the cell by the association of the bridging moiety with itself. The expressed multimeric protein is then recovered, isolated, and optionally purified from the culture medium (or from the cell, if expressed intracellularly) by appropriate means known to one of skill in the art.

To generate a heterodimeric receptor immunogen, a vector is designed as above to carry a first receptor subunit which is fused to, e.g., the CH1 region of the light chain. A vector is also designed to carry the second, different subunit of the receptor fused to e.g., the heavy chain sequence (e.g., CH1 region or the entire Fc region of the same Ig that contributes the light chain CH1, as described above).

Alternatively both sequences could be present on a single vector. The vectors are prepared as described above. However, for a heterodimeric receptor immunogen, the host cell must be co-transfected with both receptor-bridging moiety polynucleotide sequences. Expression of both sequences in the host cell causes the light chain CH1 and heavy chain CH1, or light chain CH1 and heavy chain Fc sequences to associate only complementarily (i.e., with each other not with their identical counterparts in the cell). Heterodimeric receptor immunogens are thereby formed in the manner of a "Fab"-like fragment or an antibody-like fragment, respectively.

Once expressed, the recombinant immunogen (e.g., homodimeric or heterodimeric) may be isolated following cell lysis in soluble form, or may be extracted using known techniques, e.g., in guanidine chloride. If the protein is secreted, it can be isolated from culture supernatant and purified. With an Fc bridging moiety, Protein A or Protein G Sepharose may be used to purify the immunogen. Where the immunogen is a receptor subunit sequence fused to a peptide epitope, a specific monoclonal antibody mAb to the peptide epitope can be used to purify the immunogen.

Another method of producing the novel multimeric receptor immunogen involves directly injecting the monomeric recombinant DNA (as, e.g., "naked DNA") into mice or rabbits intramuscularly. The multimeric receptor thus assembles in vivo, where it acts as an immunogen. See, e.g., Cohen, Science,
Association of a nucleotide sequence encoding the selected receptor extracellular domain and its optional cleavage site with a non-peptide bridging moiety may be by conventional covalent or ionic bonds, using conventional chemical linking agents. If the association is non-covalent, then cross-linking must occur either after purification or in freshly isolated cells prior to purification. Alternatively, the opportunity to cross-link could be enhanced by adding ligand to the receptor sequences to bring them into proximity to each other, add cross-linking agent and bridging moiety, and dissociate ligand by a conventional technique, e.g., low pH. The dimeric receptor could then be purified.

III. The Method of the Invention

The recombinant immunogens of this invention are thus useful as antigens for the development of anti-receptor antisera and antibodies to the multimeric receptor domain immunogen. Specific antisera and polyclonal antibodies may be generated by employing the recombinant multimeric immunogen as an immunogen using known techniques. See, Sambrook, cited above, Chapter 18, generally, incorporated by reference. Additionally, polyclonal antibodies and antisera may be generated to the immunogen formed in vivo following administration of the naked DNA.

The polyclonal antibodies developed in the immunized animal may be isolated from the animal's plasma, peripheral blood or other tissue in a conventional manner. Antibodies thus isolated may be employed in the methods described below for generation of mAbs, humanized and chimeric antibodies of the invention.

For example, monoclonal antibodies of the invention may be produced by conventional methods, including the Kohler and Milstein hybridoma technique, in which spleen cells from an immunized animal are fused with immortalized cells to create hybridoma cell lines which secrete a single mAb. Each hybridoma is then screen with a simple binding assay to detect agonist properties.
Other types of antibodies may be designed based on the agonist mAbs so identified. For example, recombinant techniques, such as described by Huse et al., Science, 246:1275-1281 (1988), or any other modifications thereof known to the art may be employed to generate antibodies. Thus, also encompassed within this invention are methods for generating humanized and chimeric agonist antibodies by employing the CDRs from the agonist antibodies produced as described above. Methods of identifying suitable human framework regions and modifying a mAb of the invention to contain same to produce a humanized or chimeric antibody of the invention, are well known to those of skill in the art. See, e.g., E. Mark and Padlin, "Humanization of Monoclonal Antibodies", Chapter 4, The Handbook of Experimental Pharmacology, Vol. 113, The Pharmacology of Monoclonal Antibodies, Springer-Verlag (June, 1994). Other types of recombinantly-designed antibodies are also encompassed by this invention.

As used in this specification and the claims, the following terms are defined as follows:

"Altered antibody" refers to a protein encoded by an altered immunoglobulin coding region, which may be obtained by expression in a selected host cell. Such altered antibodies are engineered antibodies (e.g., chimeric, humanized, reshaped human or reconstituted human antibodies) or antibody fragments lacking all or part of an immunoglobulin constant region, e.g., Fv, Fab, or F(ab')2 and the like.

"Altered immunoglobulin coding region" refers to a nucleic acid sequence encoding the altered antibody of the invention. When the altered antibody is a "reshaped human antibody", the sequences that encode the complementarity determining regions (CDRs) from a donor human immunoglobulin are individually inserted into a first immunoglobulin partner comprising human variable framework or as components of a variable region gene sequence attached to human constant sequences. If desired, the first immunoglobulin partner is operatively linked to a second fusion partner.

"First immunoglobulin partner" refers to a nucleic acid sequence encoding a human framework or human immunoglobulin variable region in which the native (or naturally-occurring) CDR-encoding regions are replaced by the CDR-encoding
regions of a donor human antibody. The human variable region can be an
immunoglobulin heavy chain, a light chain (or both chains), an analog or functional
fragments thereof. Such CDR regions, located within the variable region of
antibodies (immunoglobulins) can be determined by known methods in the art. For
example Kabat et al. (Sequences of Proteins of Immunological Interest, 4th Ed., U.S.
Department of Health and Human Services, National Institutes of Health (1987))
disclose rules for locating CDRs. In addition, computer programs are known which
are useful for identifying CDR regions/structures.

"Second fusion partner" refers to another nucleotide sequence encoding a
protein or peptide to which the first immunoglobulin partner is fused in frame or by
means of an optional conventional linker sequence (i.e., operatively linked).
Preferably the fusion partner is an immunoglobulin gene and when so, it is referred
to as a "second immunoglobulin partner". The second immunoglobulin partner may
include a nucleic acid sequence encoding the entire constant region for the same
(i.e., homologous - the first and second altered antibodies are derived from the same
source) or an additional (i.e., heterologous) antibody of interest. It may be an
immunoglobulin heavy chain or light chain (or both chains as part of a single
polypeptide). The second immunoglobulin partner is not limited to a particular
immunoglobulin class or isotype. In addition, the second immunoglobulin partner
may comprise part of an immunoglobulin constant region, such as found in a Fab, or
F(ab)2 (i.e., a discrete part of an appropriate human constant region or framework
region). A second fusion partner may also comprise a sequence encoding an integral
membrane protein exposed on the outer surface of a host cell, e.g., as part of a phage
display library, or a sequence encoding a protein for analytical or diagnostic
detection, e.g., horseradish peroxidase, β-galactosidase, etc.

The terms Fv, Fc, Fd, Fab, or F(ab')2 are used with their standard meanings
(see, e.g., Harlow et al., Antibodies A Laboratory Manual, Cold Spring Harbor
Laboratory, (1988)).

As used herein, an "engineered antibody" describes a type of altered
antibody, i.e., a full-length synthetic antibody (e.g., a chimeric, reconstituted human,
or reshaped human antibody as opposed to an antibody fragment) in which a portion
of the light and/or heavy chain variable domains of a selected acceptor antibody are
replaced by analogous parts from one or more donor antibodies which have
specificity for the selected epitope. For example, such molecules may include
antibodies characterized by an engineered heavy chain associated with an
unmodified light chain (or chimeric light chain), or vice versa. Engineered
antibodies may also be characterized by alteration of the nucleic acid sequences
encoding the acceptor antibody light and/or heavy variable domain framework
regions in order to retain donor antibody binding specificity. These antibodies can
comprise replacement of one or more CDRs (preferably all) from the acceptor
antibody with CDRs from a donor antibody described herein.

A "chimeric antibody" refers to a type of engineered antibody which contains
naturally-occurring variable region (light chain and heavy chains) derived from a
donor antibody in association with light and heavy chain constant regions derived
from an acceptor antibody from a heterologous species.

A "humanized antibody" refers to a type of engineered antibody having its
CDRs derived from a non-human donor immunoglobulin, the remaining
immunoglobulin-derived parts of the molecule being derived from one (or more)
human immunoglobulin(s). In addition, framework support residues may be altered
to preserve binding affinity (see, e.g., Queen et al., Proc. Nat'l. Acad. Sci. USA,

The term "reconstituted human antibody" refers to an antibody in which a
Fab is converted into a full length Mab by cloning the heavy chain of the Fab into a
human Ig constant region comprising the hinge region and CH-2 and CH-3 domains.
Preferably the constant region is one of the IgG isotypes IgG1- IgG4 or variants
thereof such as PE mutations. A reconstituted human antibody also includes
variants of the processes, mature NH₂ terminal regions of the light or heavy chain are
altered to conform with the predicted germ line parent sequence.

The term "donor antibody" refers to an antibody (monoclonal, or
recombinant) which contributes the nucleic acid sequences of its variable regions,
CDRs, or other functional fragments or analogs thereof to a first immunoglobulin
partner, so as to provide the altered immunoglobulin coding region and resulting
expressed altered antibody with the antigenic specificity and neutralizing activity characteristic of the donor antibody. One donor antibody suitable for use in this invention is 1C8.

The term "acceptor antibody" refers to an antibody (monoclonal, or recombinant) from a source genetically unrelated to the donor antibody, which contributes all (or any portion, but preferably all) of the nucleic acid sequences encoding its heavy and/or light chain framework regions and/or its heavy and/or light chain constant regions to the first immunoglobulin partner. Preferably a human antibody is the acceptor antibody.

"CDRs" are defined as the complementarity determining region amino acid sequences of an antibody which are the hypervariable regions of immunoglobulin heavy and light chains. See, e.g., Kabat et al., Sequences of Proteins of Immunological Interest, 4th Ed., U.S. Department of Health and Human Services, National Institutes of Health (1987). There are three heavy chain and three light chain CDRs (or CDR regions) in the variable portion of an immunoglobulin. Thus, "CDRs" as used herein refers to all three heavy chain CDRs, or all three light chain CDRs (or both all heavy and all light chain CDRs, if appropriate). CDRs provide the majority of contact residues for the binding of the antibody to the antigen or epitope. CDRs of interest in this invention are derived from donor antibody variable heavy and light chain sequences, and include analogs of the naturally occurring CDRs, which analogs also share or retain the same antigen binding specificity and/or neutralizing ability as the donor antibody from which they were derived.

By "sharing the antigen binding specificity or neutralizing ability" is meant, for example, that although antibody such as 1C8 may be characterized by a certain level of antigen affinity, a CDR encoded by a nucleic acid sequence of 1C8 in an appropriate structural environment may have a lower, or higher affinity. It is expected that CDRs of 1C8 in such environments will nevertheless recognize the same epitope(s) as in 1C8. A "functional fragment" is a partial heavy or light chain variable sequence (e.g., minor deletions at the amino or carboxy terminus of the immunoglobulin variable region) which retains the same antigen binding specificity and/or neutralizing ability as the antibody from which the fragment was derived.
An "analog" is an amino acid sequence modified by at least one amino acid, wherein said modification can be chemical or a substitution or a rearrangement of a few amino acids (i.e., no more than 10), which modification permits the amino acid sequence to retain the biological characteristics, e.g., antigen specificity and high affinity, of the unmodified sequence. For example, (silent) mutations can be constructed, via substitutions, when certain endonuclease restriction sites are created within or surrounding CDR-encoding regions.

Analogs may also arise as allelic variations. An "allelic variation or modification" is an alteration in the nucleic acid sequence encoding the amino acid or peptide sequences of the invention. Such variations or modifications may be due to degeneracy in the genetic code or may be deliberately engineered to provide desired characteristics. These variations or modifications may or may not result in alterations in any encoded amino acid sequence.

The term "effector agents" refers to non-protein carrier molecules to which the altered antibodies, and/or natural or synthetic light or heavy chains of the donor antibody or other fragments of the donor antibody may be associated by conventional means. Such non-protein carriers can include conventional carriers used in the diagnostic field, e.g., polystyrene or other plastic beads, polysaccharides, e.g., as used in the BIAcore (Pharmacia) system, or other non-protein substances useful in the medical field and safe for administration to humans and animals. Other effector agents may include a macrocycle, for chelating a heavy metal atom, or radioisotopes. Such effector agents may also be useful to increase the half-life of the altered antibodies, e.g., polyethylene glycol.

Also provided by the invention are human antibodies derived from human hybridomas, antibodies obtained by rescue from transgenic mice expressing human Ig domains, and antibodies made in primates. Any other modifications which are known to be useful to prepare mAbs as pharmaceutical agents may also be made to the antibodies of this invention.

Without wishing to be bound by theory, it is anticipated that the recombinant immunogens of this invention have configurations mimicking the form of the multimeric receptor on the cell surface. Thus, these immunogens generate a higher
frequency of antibodies which crosslink the multiple receptor subunits in a manner similar to that of the naturally occurring ligand and thereby are more likely than randomly obtained antibodies to be agonists of the receptor.

IV. Utilities

Polyclonal antisera, monoclonal antibodies and other antibodies of this invention, which bind to the novel immunogen as the antigen and can function as agonists are useful in substantially the same manner as is the naturally occurring ligand of the receptor. For example, an agonist antibody developed to the exemplary erythropoietin receptor dimeric fusion protein described in Example 2 may be used in therapeutic, diagnostic and research methods in which the ligand, erythropoietin, is useful. These antibodies may be used as research tools and as components for separation of the receptor proteins from other contaminants of living tissue, for example, are also contemplated for these antibodies.

Agonist antibodies to the receptor would have the same therapeutic utility as the natural ligand, but would have the advantage of longer half-life and hence prolonged activity in vivo. These agonists can thus be employed to activate the biological activity which results from receptor/ligand binding. Thus, these agonist antibodies are useful in the treatment of diseases in which the interaction of the receptor and its ligand is part of a biochemical cascade of events leading to a desired response. The advantages of such agonist antibodies include the ability to administer lower dosages of antibody than ligand, easier and less frequent administration of a pharmaceutical based on the agonist antibody, as well as easier purification. Agonist antibodies may demonstrate a different profile of activity in vivo than the non-agonist antibodies due to a different distribution.

Compositions and methods useful for the treatment of conditions associated with abnormal receptor or ligand levels are provided. The present invention provides pharmaceutical compositions useful in the treatment of: anemia associated with chronic renal failure; anemia associated with AIDS; pre-dialysis patients; patients in need of pre- and/or post surgery hematocrit boosting; cancer patients undergoing hematocrit decreasing radiation or chemotherapy; rheumatoid arthritis
and sickle cell anemia. These compositions contain a therapeutically effective amount of an agonist antibody of this invention and an acceptable pharmaceutical carrier. As used herein, the term "pharmaceutical" includes veterinary applications of the invention. The term "therapeutically effective amount" refers to that amount of a receptor agonist antibody, which is useful for alleviating a selected condition. Also provided are compositions and methods for inhibiting receptor activity in order to ameliorate an undesired response.

The receptor agonist antibodies of the invention can be formulated into pharmaceutical compositions and administered in the same manner as described for mature proteins [see, e.g., International Patent Application, Publication No. WO 90/02762 (Mar. 22 1990)]. These therapeutic compositions of the invention may be administered to mimic the effect of the normal receptor ligand. These compositions may contain a pharmaceutically acceptable carrier. Suitable carriers are well known to those of skill in the art and include, for example, saline. Alternatively, such compositions may include conventional delivery systems into which protein of the invention is incorporated. Optionally, these compositions may contain other active ingredients, e.g., chemotherapeutics.

Thus, the invention also provides improved methods of treating a variety of disorders in which receptor/ligand interactions are implicated, e.g., inflammation, autoimmune disorders, etc. For example, an agonist antibody developed to a dimeric EpoR of this invention can be employed to treat acute renal failure, anemia, AIDS, and any disorder which the ligand erythropoietin can be used for treatment, e.g., for cancer chemotherapy. Similarly, an agonist antibody developed to a dimeric TpoR receptor may be employed to treat conditions of low platelet count, i.e., patients undergoing chemotherapy for cancer, etc. An agonist antibody developed to the G-CSF dimeric receptor of this invention is useful to stimulate the polymorphonuclear cells, thereby for the treatment of conditions characterized by neutropenia, e.g., cancer chemotherapy, etc.

The invention encompasses methods of administering therapeutically effective amounts of a antibody or pharmaceutical composition of the invention to a patient. The dose, timing and mode of administration of these therapeutic or gene
therapy compositions may be determined by one of skill in the art, and may be less
than or equal to the amounts of the ligand known to be administered for similar
conditions. Such factors as the disease being treated, the age, physical condition,
and the level of the receptor detected by the diagnostic methods described above,
may be taken into account in determining the dose, timing and mode of
administration of the therapeutic compositions of the invention. Generally, where
treatment of an existing disorder is indicated, a therapeutic composition of the
invention is preferably administered in a site-directed manner and is repeated as
needed. Such therapy may be administered in conjunction with conventional
therapies for such conditions.

Generally, an agonist antibody of the invention is administered in an amount
between about 0.01 ng/kg body weight to about 1 g/kg and preferably about 0.01
ng/kg to 100 mg/kg per dose. Preferably, these pharmaceutical compositions are
administered to human or other mammalian subjects by injection. However,
administration may be by any appropriate internal route, and may be repeated as
needed, e.g., as frequently as one to three times daily for between 1 day to about
three weeks to once per week or once biweekly. Preferably, the agonist antibody is
administered less frequently than is the ligand, when it is used therapeutically.

Optionally, the pharmaceutical compositions of the invention may contain
other active ingredients or be administered in conjunction with other therapeutics.
Suitable optional ingredients or other therapeutics include those conventional for
treating conditions of this nature, e.g. other anti-inflammatories, diuretics, and
immune suppressants, among others.

According to the methods of this invention, and as described in detail in the
following examples, antibodies were generated to the erythropoietin receptor
(EpoR), which mimic the agonist properties of erythropoietin. The following
examples illustrate the construction and expression of exemplary multimeric
receptor proteins of the invention. These examples are illustrative only and do not
limit the scope of the invention.
Example 1 - Multimeric EpoR Antigen

A dimeric antigen of this invention was designed by fusing the extracellular domain of the erythropoietin receptor via an amino acid linker to the Fc portion of a human immunoglobulin, and expressing the fusion protein in a suitable host cell.

Using the following primers sets based on the published nucleotide sequence of EpoR (Jones et al., Blood, 76: 31-35 (1990)), the extracellular domain of the EpoR was amplified via PCR from a human fetal liver cDNA library (Clontech).

One primer was selected from: 5' GT ATC ATG GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG 3' [SEQ ID NO: 4] and 5' ATG GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG 3' [SEQ ID NO: 5]. A second primer was selected from: 3' GGA GAC GGG GGG TCG ATA CAC CGA ACG AGA ATC CTG TG 5' [SEQ ID NO: 6] and 3' GGA GAC GGG GGG TCG ATA CAC CGA ACG AGA ATC 5' [SEQ ID NO: 7]; 3' CAC AGC GAC GAC TGC GGA TCG CTG GAC CTG GGG atc act ga 5' [SEQ ID NO: 8] and 3' CAC AGC GAC GAC TGC GGA TCG CTG GAC GAC TCG GGG gtc 5' [SEQ ID NO: 9].

The resulting 250 bp DNA fragment so isolated was cloned into the vector PCR2000 (Invitrogen) and sequenced. The EpoR fragment was found to encode amino acids 1-250 [SEQ ID NO: 2] of the extracellular domain of the human EpoR [Jones et al., cited above; SWISSPROT Accession number P19235].

An SpeI/XbaI fragment was isolated from the PCR2000-derived vector by digestion containing this EpoR insert, nucleotide 898 to 1647 of SEQ ID NO: 1. The insert was then cloned into the Drosophila S2 vector mtl [see, European Patent No. 290,261 B, published November 9, 1988] at the equivalent linker sites, SpeI and XbaI. The resulting plasmid vector, pS2EpoR, contains the EpoR extracellular domain gene insert under the control of the Drosophila copper metallothionein (mtn) promoter [Johansen et al., Genes and Development, 3:882-889 (1989); Angelichio et al., Nucl. Acid Res., 19:5037-5043 (1991)].

The plasmid pS2EpoR was digested with BssH2 and XbaI and a C terminal fragment of the EpoR sequence was removed by this digestion. A plasmid
containing the whole EpoR gene fused to a bridging moiety was then prepared by cloning the following three fragments:

1. the large BssH2/XbaI fragment from the above digestion;
2. a synthetic BssH2/KpnI linker, which spanned nucleotide 1561 to nucleotide 1659 of SEQ ID NO: 1, which encoded the C terminus of the EpoR extracellular domain, aa 222 to 250 of SEQ ID NO: 2, linked in frame to the four amino acid recognition sequence for protease Factor Xa cleavage (IleGluGlyArg) [amino acids 251 to 254 of SEQ ID NO: 2], and
3. a KpnI/XbaI fragment containing the human IgG1 Fc region, spanning nucleotide 1660 to the XbaI site which appears at nucleotides 2371-2376 of SEQ ID NO: 1 [see, also Johansen et al., J. Biol. Chem., 270:9459-9471 (1995)].

This KpnI/XbaI fragment was constructed as follows:

Human IgG1 cDNA encoding CH1, the hinge, CH2 and CH3 described by J. Ellison et al., Nucleic Acids Res., 10: 4071-4079 (1982) was cloned from the human IgG plasma cell leukemia ARH-77 (American Type Tissue Collection), using RT-PCR. This cDNA was fully sequenced to confirm identity with the published sequence [see, International patent publication WO 92/00985]. This sequence was inserted into a pUC18 vector (pUC18-Fc). This vector was digested with KpnI and SacII, deleting the CH1, hinge and part of CH2. The deleted region was replaced with a PCR amplified fragment containing the hinge-CH2 region as follows.

Using the following PCR primers: 5’ TCG AGC TCG GTA CCG AGC CCA AAT CGG CCG ACA AAA CTC ACA C 3’ [SEQ ID NO: 10] and 5’ GTA CTG CTC CTC CCG CGG CTT TGT CTT G 3’ [SEQ ID NO: 11], a DNA fragment containing the hinge-CH2 region was amplified from pUC18-Fc, digested with KpnI and SacII, gel purified and cloned back into the KpnI/SacII digested pUC18-Fc vector. The Cys, which occurs at position 230 [Kabat numbering; Kabat et al., "Sequences of Proteins of Immunological Interest, 5th Edition, US Department of Health and Human Services, NIH Publication No. 91-3242 (1991); this is also residue 5 of the hinge of the IgG1 heavy chain; residue 261 of SEQ ID NO: 2] was altered to an Ala through a TGT to GCC substitution in the nucleotide sequence to
avoid having the unpaired Cys present which is usually involved in light chain-heavy chain crosslinking.

An altered DNA sequence in one of the PCR primers introduced a unique KpnI site at the 5' end of the hinge. The resulting plasmid was called pUC18Fcmod, and the junctions and PCR amplified region were sequenced for confirmation.

The entire hinge-CH2-CH3 insert in pUC18-Fcmod was removed in a single DNA fragment with KpnI and XbaI, gel purified, and ligated into SFcR1Cos4 cut with KpnI and XbaI to create COSFc. SFcR1Cos4 is a derivative of pST4DHFR [K. Deen et al, Nature, 331: 82 (1988)] and contains the soluble Fc receptor type I (sFcR1) inserted between the cytomegalovirus (CMV) promoter and bovine growth hormone (BGH) polyadenylation regions, and also contains the dihydrofolate reductase (DHFR) cDNA inserted between the b-globin promoter and SV40 polyadenylation regions, an SV40 origin of replication, and an ampicillin resistance gene for growth in bacteria.

Cutting the vector with KpnI and XbaI removes the sFcR1 coding region, so that the COSFc vector contains the hinge-CH2-CH3 region inserted between the CMV promoter and BGH polyA regions. The COSFcLink vector was made from COSFc by inserting an oligonucleotide linker at the unique EcoRI site of the vector, which recreates this EcoRI site, and also introduces BstEII, PstI and EcoRV cloning sites. The oligonucleotides used were:

5' AATTCCGTTACCTGCAGATATCAAGCT 3' [SEQ ID NO: 12] and
3' GCCAATGGACGTCTATAGTTCGATTAA 5' [SEQ ID NO: 13]. The junction was sequenced to confirm orientation in the vector. The size of the final vector is 6.37 kb and is reported as SEQ ID NO: 3. The KpnI/XbaI fragment used in the dimeric immunogen described herein was obtained from COSFcLink.

The resulting plasmid DNA construct containing the three fragments described above was called pmtalsEpoRFc [SEQ ID NOS: 1 and 2]. The plasmid sequence contains the heterologous fusion sequence comprising an intact EpoR extracellular domain encoding amino acids 1-250 of SEQ ID NO: 2 linked via a four amino acid linker (aa 251-254 of SEQ ID NO: 2) to a human IgG1 Fc region (aa 255
to 488 of SEQ ID NO: 2). In the plasmid, the fusion sequence was under the control of the mtn promoter, described above.

Plasmid pmtalsEpoFc was cotransfected into Drosophila S2 cells with a vector encoding hygromycin resistance [see, EP No. 290,261B, cited above]. Stable co-transfectants were selected in hygromycin, and expression of the EpoR induced by CuSO₄ according to published protocols [Johansen et al., cited above; Angelichio et al., cited above].

The co-transfected cells secreted the EpoRFc protein as a dimeric molecule due to the natural affinity of the Fc sequence for itself. Under reducing conditions in SDS-PAGE, the EpoRFc protein ran as a monomer. The dimeric protein was purified from Drosophila medium by passage over a Protein A Sepharose column.

To obtain the monomeric EpoR extracellular protein apart from its Fc fusion, the EpoRFc fusion protein is treated as follows: EpoR-Fc was dialyzed into 20 mM Tris, 100 mM NaCl, 2 mM CaCl₂, pH 8. Factor Xa (New England Biolabs) was added at a ratio of 1 mg Factor Xa per 25 mg EpoRFc and incubated at 6°C for 18-20 hours.

The digest was then added to Protein A Sepharose 4 Fast Flow [Pharmacia], washed with 100 mM Tris, pH 8, giving a ratio of 0.5 ml packed resin per mg protein. Following a 90 minute incubation with mixing, at 6°C, the resin was separated from the supernatant by centrifugation. SDS/PAGE and Western blots showed that EpoR, free of Fc, was present in the supernatant. The N-terminal sequence for EpoR was correct. The final product was sterile filtered.

**Example 2 - Erythropoietin Binding Assay**

The activity of the dimeric fusion protein EpoRFc and the monomeric single-stranded mEpoR protein cleaved from EpoRFc were tested in various biological assays by their ability to neutralize the activity of erythropoietin (Epo).

A. *Inhibition of Epo-induced proliferation of UT7-Epo Cells*

An assay for the measurement of Epo activity on the proliferation of UT7Epo cells, which are dependent on Epo for growth [Komatsu et al., *Blood*, 82:456-464 (1993)] was performed as follows. The Epo used in the experiment is Epogen (2000
U/ml) [Amgen, Thousand Oaks, Ca], diluted in phosphate buffered saline (PBS) and human serum albumin (HSA) for storage at 4°C at 200 U/ml. Dilutions of cleaved EpoR or EpoRFc protein samples were made, so that final concentrations ranged from 0.001 to 100 ng/ml.

Samples of either EpoR or EpoRFc were added to wells at 10 ul/well in quadruplicate. Epo (0.2 U/ml) was added to each well. UT7Epo cells (1 x 10^5 cells/ml) were plated at 100 ul/well. After the plates were incubated at 37°C for three days, 10 ul/well of ^3H-thymidine (diluted to 100 mCi/ml in IMDM+10% fetal calf serum (FCS)) were added to a final concentration of 10 uCi/ml. Plates were incubated at 37°C for four hours with ^3H-thymidine. The 96-well plates were harvested onto glass fiber filters using the Tomtec plate harvester with 10% cold TCA and cold 95% ethanol. Solid scintillant was melted onto the filters and the samples counted. The mean and standard error of quadruplicate samples was determined.

The data were reported as the percent of positive (0.2 U/ml) Epo control and are illustrated in Fig. 1.

**B. Inhibition of Epo-induced proliferation of 32D/Epo wt Cells**

Another assay was performed for the measurement of Epo activity on the proliferation of 32D/Epo wt cells (Miura, O. *et al.*, Mol. Cell. Biol., 13:1788-1795 (1993)). 32D/Epo wt is an IL-3 dependent cell line transfected with the human Epo receptor.

This assay is performed as described in Part A above, with the modification that the each well contains 1 U/ml Epo. The results of this assay are illustrated in Fig. 2.

**C. Inhibition of Epo-induced CFU-E colony formation**

A third assay for measuring the ability of Epo to stimulate the differentiation of murine bone marrow cells to hemoglobin producing mature erythrocytes (CFU-E assay) was performed as described below.

Murine bone marrow cells were flushed from the femur of female B2D6F1 mice. The marrow cells (1 x 10^5 cells/ml final concentration) were mixed with IMDM, 25% FCS (final) and methylcellulose (0.8 % final). 0.4 ml cells was plated
per well of 24-well TC plate. EpoRFc (40 ul/well) samples were added and Epo (1 U/ml) was added to each well. Plates were incubated at 37°C, 5% CO2, 6% O2 for two days.

CFU-E colonies containing 8 or more red cells were counted. The mean and standard error of triplicate samples were determined and data reported as the percent of the positive (1 U/ml) Epo control. The results of this assay are illustrated in Fig. 3.

In all three assays, both the monomeric extracellular domain protein EpoR and the dimeric fusion protein EpoRFc were able to bind, and thereby neutralize, the biological activity of the ligand Epo. However, the dimeric fusion protein EpoRFc was consistently 10 to 100 fold more effective at neutralizing Epo activity than non-fused, monomeric extracellular domain protein EpoR, suggesting that the dimeric antigen had a higher affinity to the natural ligand, Epo. The assay results also confirm that the dimeric fusion protein mimics the cell surface form of the natural EpoR in being able to bind to Epo with greater affinity than the mEpoR domain protein.

Example 3 - Agonist Antibodies of the Invention

A. Generation of Hybridomas

Mice were immunised s.c. with recombinant EpoRFc (34ug) in Freund’s complete adjuvant and then boosted i.p. 4 weeks later (34ug) with Freund’s incomplete adjuvant. One and three days before fusion mice received 20ug in PBS i.p.. The spleens were harvested and fused with myeloma cells according to the method described in Zola. (Monoclonal Antibodies: A Manual of Techniques, Zola H. ed., Boca Raton, Fl: CRC Press, 1987)

Positive hybridomas were selected through a primary screen described below. Positives were rescreened using a competitive immunoassay and then BIAcore was used to select hybridomas that expressed high affinity monoclonal antibodies which were then cloned twice by the limiting dilution method.
A1. **Primary Screening assay of anti EpoRFc Hybridomas**

96 well microtitre plates were coated with 100ul/well of EpoRFc at 0.5ug/ml in coating buffer (50mM Na₂HPO₄, 150mM NaCl, 0.02% v/v Kathon, pH 7.4) and incubated overnight at 4°C. The wells were aspirated and 250ul/well blocking buffer (1% w/v BSA, 50mM Tris, 150mM NaCl, 0.1% v/v Kathon, pH 7.4) added for 1 hour at 37°C. The wells were washed X4 with wash buffer (10mM Tris, 150mM NaCl, 0.05% Tween 20, pH 7.4) and 50ul/well of 40ug/ml human IgG diluted in assay buffer (0.5% w/v BSA, 0.05% w/v bovine γ globulin, 50mM Tris, 150mM NaCl, 7.86mg/L DTPA, 0.1g/L Tween-40, 0.02% v/v Kathon, pH 7.4) added, followed by 50ul hybridoma supernatant. The plates were incubated for 1 hour at 37°C on a plate shaker, the wells washed X4 and then 100ul/well Europium conjugated anti-mouse IgG added (0.5ug/ml in assay buffer). After incubation for 1 hour at 37°C on the plate shaker the wells were again washed X4 and 100ul/well of enhancement solution added into each well and incubated for 2 min at 22°C on the plate shaker and the counts read on a Delfia plate reader.

A2. **Isolation of Hybridomas Producing High Affinity Antibodies that Recognise Solution Phase EpoRFc using a competitive immunoassay.**

Microtitre plates were coated with EpoRFc and blocked as above. The wells were washed X4 and then 50ul of either EpoRec at 6ug/ml diluted in assay buffer or 50ul human IgG at 40ug/ml diluted in assay buffer or 50ul assay buffer alone were added followed by 50ul hybridoma supernatant. After incubation for 1 hour at 37°C on the plate shaker the wells were washed X4 followed by addition of 100ul/well Europium conjugated anti-mouse IgG at 0.5ug/ml (diluted in assay buffer). After incubation for 1 hour at 37°C on the plate shaker the wells were again washed X4 and 100ul/well of enhancement solution added into each well and incubated for 2 min at 22°C on the plate shaker and the counts read on a Delfia plate reader.

Positive antibodies would be displaced by solution phase EpoRFc and these would thus show a reduction in counts when compared to wells with only assay buffer, antibodies giving a reduction in counts with human IgG would be non specific. High affinity antibodies would show >80% reduction in counts.
A hybridoma designated herein as 1C8 (or alternatively as 5-1C8) has been deposited at the European Collection of Animal Cell Cultures, Porton Down, Salisbury, Wiltshire, SP4 OJG United Kingdom on 5 June 1996 and assigned provisional Accession number 96060519. The deposit referred to herein will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for purposes of Patent Procedure. These deposits are provided merely as convenience to those of skill in the art and are not an admission that a deposit is required under 35 U.S.C. §112. The sequence of the polynucleotides contained in the deposited material, as well as the amino acid sequence of the polypeptides encoded thereby, are incorporated herein by reference and are controlling in the event of any conflict with any description of sequences herein. A license may be required to make, use or sell the deposited material, and no such license is hereby granted.

A3. Binding Analysis in the BIAcore for the Selection of Antibodies that Bound EpoR with High Affinity

Rabbit anti-mouse Fc (RAMFc) was immobilised to the sensor chip surface (Pharmacia BIosensor protocol) and used as a capture antibody. The run buffer used was 10mM HEPES, 150mM NaCl, 3.4mM EDTA, 0.005% (v/v) Surfactent P 20 at a flow rate of 5ul/min. 20ul hybridoma supernatant was injected over the sensor chip surface followed by 20ul EpoR or EpoRFc (3ug/ml diluted in run buffer). The surface was regenerated by injection of 15ul HCL (100mM) and then 10ul SDS (0.025%).

High affinity monoclonal antibodies were selected on the basis of a fast on rate and slow off rate with respect to EpoR binding.

B. Assays to Test Agonist Characteristic of Antibodies

The hybridoma supernatants or purified antibodies were then tested for their ability to bind to the naturally occurring EpoR on UT7 or 32D/Epo wt cells in flow cytometry as follows. $5 \times 10^5$ cells per sample of 32D/Epo wt or UT7-Epo cells were resuspended in 50 ul PBS/10%BSA. 5.0 ul of each purified anti-EpoRFc was
diluted to 30μg/ml and incubated on ice for 45 minutes. Cells were washed and
resuspended in 50 ul PBS/10% bovine serum albumin (BSA) and 7 ul FITC-labelled
goat anti-mouse IgG (Fab')2 [Tago] added for 45 minutes on ice. Cells were again
washed in PBS/10%BSA and resuspended in 0.4 ml PBS/10% BSA and then 0.2 ml
3.2% paraformaldehyde added followed by vortexing. Cells were stored at 4°C until
analysis on Becton-Dickinson's FACSscan fluorescence activated cell sorter.

The data, illustrated in Figure 4, was reported as the % of control antibody
fluorescence. As can be seen, four antibodies stained UT7Epo cells while three did
not. Interestingly, only one of the four positive antibodies on UT7Epo cells was able
to recognize the transfected human Epo receptor in 32D/Epo wt cells, suggesting
some differences in the disposition of the Epo receptor in these cell lines. This also
suggests differences in the epitopes recognized by these antibodies.

C. Assays to Test agonist activity of antibodies.

(1) Hybridoma supernatants or purified antibodies were tested for their
ability to mimic the activity of Epo by stimulating the proliferation of UT7-Epo cells
in an assay performed as described in Example 2A above, with the modification that
Epo is absent from wells containing the hybridoma supernatants. The only sample
was dilutions of the monoclonal antibodies against EpoRFc dimeric protein. Results
of this assay are shown in Figure 5 for some purified EpoRF reactive monoclonal
antibodies. Four of the antibodies gave significant proliferative activity, in one case
approaching that of Epo itself (1C8). The variability of the extent of peak activity
relative to Epo suggests that the way in which the antibodies bind may be an
important determinant of activity.

(2) Hybridoma supernatants or purified antibodies were tested for their
ability to mimic the activity of Epo by stimulating the proliferation and
differentiation of human bone marrow progenitor cells to form red blood cell
colonies (CFU-E) similar to the assay described above in Figure 6. The present
assay differed in that the progenitor cells were of human origin.

In this procedure, light density cells from human bone marrow centrifuged
over Histopaque 1077 were washed and resuspended at 2.5 x 10^6 cells/ml in X-vivo

31
medium (BioWhittaker). The purified monoclonal antibodies were diluted in X-vivo medium, and the Epo positive control was 4 U/ml. For the assay, 0.3 ml cells, 0.3 ml Mab sample (or Epo control) and 0.7 ml X-vivo medium were incubated in a polypropylene tube for 30 min at RT, then 0.9 ml FCS, 0.3 ml 10% BSA and 0.8 ml 3.2% methylcellulose were added. 0.4 ml were plated per well of a 24-well TC dish (Nunc). This procedure departs from the standard assay in the pre-incubation of cells, X-Vivo and Mab alone for 30 min (without serum, BSA or methylcellulose) prior to plating in methylcellulose.

The results are shown in Figure 6. Once again, all four antibodies were positive in the UT7Epo proliferation assay were also able to stimulate the generation of mature red blood cells. Again the most potent was antibody 1C8 which gave almost 50% of the maximal activity of human Epo on the same cells. The order of maximal activity was similar to that observed in the proliferative assay.

D. Epitope mapping of agonist antibodies.

It was likely that one contributor to the different activities of the agonist antibodies was the epitope recognized on the erythropoietin receptor. To determine if the antibodies recognized overlapping epitopes on the Epo receptor, the ability of antibodies to compete with each other in binding to EpoR or EpoRFc was measured. RAMFc was immobilised to the sensor chip surface and using a flow rate of 5ul/min the following sequential injections were used. 5ul of first monoclonal antibody (25 or 30ug/ml), 10ul EpoR (5ug/ml) or EpoRFc (6ug/ml), 2 X 5ul of nonspecific monoclonal antibodies, (100ug/ml each of IgG 2bk, IgG 3k, IgG 1k and IgG 2ak), 5ul of second monoclonal antibody. The surface was regenerated with 15ul 0.1M phosphoric acid and 8ul 0.025% SDS at 10ul/min. The data are shown in Table I.

Table I  Competition of different monoclonal antibodies for epitopes on Epo receptor as measured by BIAcore. Results are expressed as Response Units. The antibody attached to the chip via protein A is shown in the left hand column and
each row shows the binding of each monoclonal after prebinding of EpoR or EpoRFc.

<table>
<thead>
<tr>
<th>Epo-R</th>
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<tr>
<td></td>
<td>5-1C8</td>
</tr>
<tr>
<td>5-1C8</td>
<td>-25</td>
</tr>
<tr>
<td>3-2B6</td>
<td>-3</td>
</tr>
<tr>
<td>5-2G6</td>
<td>-13</td>
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<table>
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<tr>
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<td>-7</td>
</tr>
<tr>
<td>5-2G6</td>
<td>-11.6</td>
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</table>

The data indicate that of the three agonist antibodies, 2B6 and 2G6 bind to two non-overlapping epitopes since they do not compete for binding to the Epo receptor. In contrast, 1C8 competes with both 2B6 and 2G6, suggesting that it binds to a third distinct epitope which overlaps the other two. Thus there is no single epitope which can lead to agonist activity, but the precise epitope can very likely affect the extent of bioactivity observed. Use of the EpoRFc as antigen seems to generate a wide variety of agonist epitopes.

E. Competition of antibody binding with Epo.

Another measure of differences between the agonist antibodies is provided by studies which measure the ability of monoclonal antibody to compete with Epo binding to the receptor. These experiments were conducted in two ways. First, a goat anti-human IgG was attached to the BIAcore chip, followed by sequential binding of EpoRFc, Epo and finally monoclonal antibody. In this experiment, pre-binding of Epo blocked the binding of 1C8 by more than 75%, but had only a limited effect on the binding of 1D9, 2G6 and 2B6. Second, if the EpoRFc binding to the chip was followed first by monoclonal antibody and then by Epo, all four monoclonal antibodies could block subsequent binding of Epo. More specifically, a goat anti-human IgG, Fc specific antibody was immobilised on the sensor chip surface.
Injection of 25ul EpoRFc (2ug/ml) at 5ul/min was followed by injection of 25ul Epo (5ug/ml) then 25ul Mab (10ug/ml) at 5ul/min. RU recorded. The surface was regenerated with injections of 15ul 0.1M phosphoric acid (5ul/min) and 8ul 0.025% SDS at 10ul/min. and as mentioned above the experiment was repeated reversing the order of addition for Epo and mAb, i.e. inject Mab first, then displaced with Epo.

These data, shown in Table II suggest that all four antibodies may block access of Epo to its binding site, but only the 1C8 antibody overlaps substantially with the Epo binding site on the Epo receptor.

Table II Competition of monoclonal antibody binding with Epo binding to Epo receptor as measured by BIAcore. The order of addition is left to right as indicated. The identity of each monoclonal antibody is listed in the left column.

<table>
<thead>
<tr>
<th>EpoRFc + Epo + Mab</th>
<th>Mab</th>
<th>Epo R.U.</th>
<th>Epo R.U.</th>
<th>Mab R.U.</th>
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</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>557</td>
<td>118</td>
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<tr>
<td>1D9</td>
<td>475</td>
<td>106</td>
<td>173</td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>471</td>
<td>106</td>
<td>264</td>
<td></td>
</tr>
<tr>
<td>2G6</td>
<td>468</td>
<td>104</td>
<td>300</td>
<td></td>
</tr>
<tr>
<td>1C8</td>
<td>465</td>
<td>104</td>
<td>77</td>
<td></td>
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<table>
<thead>
<tr>
<th>EpoR + Mab + Epo</th>
<th>Mab</th>
<th>Epo R.U.</th>
<th>Mab R.U.</th>
<th>Epo R.U.</th>
</tr>
</thead>
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<td>105</td>
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<tr>
<td>1D9</td>
<td>452</td>
<td>173</td>
<td>101</td>
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<td>2B6</td>
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<td>455</td>
<td>9.2</td>
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</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>1C8</td>
<td>447</td>
<td>436</td>
<td>-196</td>
<td></td>
</tr>
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</table>

F. Affinity of the monoclonal antibodies to the Epo receptor

The binding kinetics and affinities of the agonist antibodies was determined by measurements in the BIAcore with both the soluble EpoR and with the dimeric EpoRFc. Specifically, RAMFc was immobilised to the sensor chip surface and a flow rate of 5ul/min was used with run buffer. The Mab was first bound to the RAMFc (5ul injection) followed by a 20ul injection of of EpoR (0-4ug/ml) or EpoRFc (0-6ug/ml) then buffer flow for 120 sec and regeneration with 15ul 0.1M phosphoric acid and 8ul 0.025% SDS at 10ul/min.
The four antibodies showed quite different kinetics and binding constants as listed in Table III.

Table III  Affinities and kinetics of binding of monoclonal antibodies to Epo receptor as measured by BIAcore.

<table>
<thead>
<tr>
<th>Mab</th>
<th>Kass. M⁻¹ s⁻¹</th>
<th>Kdiss. (s)</th>
<th>K₀ (M)</th>
<th>(2nd Dissoc. K₀)</th>
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</thead>
<tbody>
<tr>
<td>1C8</td>
<td>2.54 x 10⁵</td>
<td>5.9 x 10³</td>
<td>2.3 x 10⁻⁴</td>
<td>4.3 x 10⁻⁴</td>
</tr>
<tr>
<td>2G6</td>
<td>1.64 x 10⁵</td>
<td>4.3 x 10⁻⁴</td>
<td>2.6 x 10⁻⁹</td>
<td></td>
</tr>
<tr>
<td>2B6</td>
<td>2.4 x 10⁵</td>
<td>1.9 x 10⁻⁴</td>
<td>7.8 x 10⁻¹⁰</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mab</th>
<th>Kass. M⁻¹ s⁻¹</th>
<th>Kdiss. (s)</th>
<th>K₀ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1C8</td>
<td>4 x 10⁵</td>
<td>N/D</td>
<td>&lt; 5 x 10⁻¹⁰</td>
</tr>
<tr>
<td>2G6</td>
<td>1.77 x 10⁵</td>
<td>N/D</td>
<td>&lt; 5 x 10⁻¹⁰</td>
</tr>
<tr>
<td>2B6</td>
<td>1.96 x 10⁵</td>
<td>N/D</td>
<td>&lt; 5 x 10⁻¹⁰</td>
</tr>
</tbody>
</table>

N/D No Dissociation

On the monomeric EpoR, the affinities ranged between 0.75nM and 23nM.

Interestingly, the most biologically potent antibody, 1C8, had the lowest dissociation constant of 23nM, and also had a second binding mode with a dissociation constant of 4.3nM. It was the only antibody to show this phenomenon. In contrast, with the dimeric receptor EpoRFc, all four antibodies had non-measurable dissociations, suggesting dissociation constants of less than 500pM. These data suggest that kinetics may also play a role in the relative agonist activity of different monoclonal antibodies.
All documents cited above are incorporated herein by reference. Numerous modifications and variations of the present invention are included in the above-identified specification and are expected to be obvious to one of skill in the art. Such modifications and alterations to the compositions and processes of the present invention are believed to be encompassed in the scope of the claims appended hereto.
(i) APPLICANT: Young, Peter R.
    Erickson-Miller, Connie

(ii) TITLE OF INVENTION: Method for Obtaining Receptor Agonist Antibodies

(iii) NUMBER OF SEQUENCES: 13

(iv) CORRESPONDENCE ADDRESS:
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    (E) COUNTRY: USA
    (F) ZIP: 19406-2799

(v) COMPUTER READABLE FORM:
    (A) MEDIUM TYPE: Floppy disk
    (B) COMPUTER: IBM PC compatible
    (C) OPERATING SYSTEM: PC-DOS/MS-DOS
    (D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
    (A) APPLICATION NUMBER: US
    (B) FILING DATE:
    (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
    (A) NAME: Jervis, Herbert H.
    (B) REGISTRATION NUMBER: 31,171
    (C) REFERENCE/DOCKET NUMBER: SBC P50349-1

(ix) TELECOMMUNICATION INFORMATION:
    (A) TELEPHONE: 610-270-5015
    (B) TELEFAX: 610-270-5090
(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 4990 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: double
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
   (A) NAME/KEY: CDS
   (B) LOCATION: 898..2361

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAGTAAAGAGT GCCTGGCGAT GCCCTCAAGT GCCCAACCAAG AGTTTTGATG CCCATAAC  600
TCCCCAAGAT GGAAGGGCAG ACAAATCTTT CGCGGGCGAG ACAAAAGCTT CTGACACAGT  660
CTCCACTGGA ATTTGAGGCC GGCGGGCGTT TGCAAAAGAG GTGAACTCGA CGAAGACCC  720
GTTGTAAAG CGCGGTTTCC AAAATGTA AAACCGGAG CATCTGGCCA ATGTGCACTCA  780
GTTGTGGTCA GCAGCAAAAAT CAAGTGGAATC ATCTCAGTGC AACTAAAGGS GGGATCGGAT  840
ATCCAAGGTTC ACGCGGACT AGTCTAGTAA CGGCGGCCAG TGTGCTGAAA TCGGCT
897

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Val Gln Arg Val Glu Ile Leu Glu Gly Arg Thr Glu Cys Val Leu Ser
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Ala Glu Pro Ser Phe Gly Gly Phe Trp Ser Ala Trp Ser Glu Pro Val
225 230 235 240

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Ser Leu Leu Thr Pro Ser Asp Leu Asp Pro Ile Glu Gly Arg Gly Thr
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TATCATGCAA CTGCTTTAGGAC AGGTGGCGGC ACGGCTTCTGG GTCACTTTTGG GCGAGGACCG 2811
CTTTCGCTGG AGCGGACAGGA TGATCGGCCC GTGTCCCTGG GTATTCGGAA CTCTGCAAGC 2871
CCTCGCTCAG GCTTTCGTCA CTGTCCCCGC CACAAACGTT TCCGGCAGGA AGCAGGCCAT 2931
TATCGCGCAG ATGCGGCGCC AGCGGCTGGG CTACGTCTTG CTGGGCGTGG CGACGGCGAG 2991
CTGGATGGCC TTCCCCATTA TGATTTCTTC GCTTTGGGGC GCATCGGGGA TGCCGGCGTT 3051
GCAGGCATG CTGTCCAGGC AGGTATGATGA CGACCATCAG GCAGACGTCC AAGGATCGCT 3111
CCGCGGTCTTT ACCGCCAGG AAAAAAGCG GAAAAGCGGTAA AAGGCAGCGT TGCTTGGCGTT 3171
TTTCCATAGG CTCGCCCTTT CGGACGAGCGA TCAAAAAAAAT CGACGCTTTA ATGCAGATCTG 3231
GCCAAGCCCG ACAGGACTAT AAAGATACCA GCGTTTTCCC CCTGAAAGCT CCCCCGACG 3291
CTCTCTTGTG CTGCCCTGTC CGCTTACCGG ATACCTGTCC GCTTTCTCCC CTGGGGAAAG 3351
CGTGGGCTTT TCTCAATGCT CGGCTTGA GTATCTCAGT TGCGTGGAGG TGTTTCGTCTC 3411
CAAGCTGGGC TGTTGACGAG AAAAAAGGCG TCAAGCCCCGC CGTGGCGCCT TATCCGGTA 3471
CTATCTCTTT GAGTCAACCG CGTAAAGACA CGACTTAGCC CACCTGGCAG CAGCCACTGG 3531
TAACAGGATT AGCGAGGCGA GTATGATTG CGGTGCTACA GAGTCTTTGG AGTGGTGCCC 3591
TAACCTCAGGC TACACTAGAA GGACAGATTG TTTGATCTCG GTCTCTCGTGAG AGCCAGTTAC 3651
CTCGGAAAA AGGATTGTTGA GTCTCTGATC CGGCAAAACAA ACCCGCGCTG GTAGCGGTGG 3711
TTTTTATTGG TTGCAAGCAGC AGGATCGGCGG CAGAAAAAAA GGAATCTCAAG AAGATCCTTTT 3771
GATCTTTTCTT ACGGGGTCTTG ACGCTCATGG GAACGAAAAAC TACGGTTAAG GAATTTTTGT 3831

42
CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTTAAAAT GAAGTTTTAA
ATCAATCTAA AGTATATATG AGTAAACCTG GTCTGACAGT TACCAATGCT TAATCACTGA
GGCACCTATC TCACCGATCT GTCTATTTTCG TTCTATCATA GTTGCTTGAC TCCCCGTGCT
GTAGATAACT ACAGATACGG AGGCTTTACC ATCTGGCCCC AGTGCCTGAA TGATACCGCG
AGACCACACG TCACCGGCTC CAGATATTTC AGCAATAAAC CGACCAGCCG GAAGGGCGGA
GCCACAAGGT GGTCTTGCAA CTTTATCAGG CTCATCCACG TCTATTAAATT GTTGCCCGGA
AGCTAGAGTA AGTAGGTCGG ACATTTAGAT TTTGCCCAAC GTGTTGCGCA TTGCGCTGAG
CATGTTGCTG TCACGGTCGG CGTTTGGTAT GGCCTCATTC AGCTCCGCGT CCCAACGATC
AAGGCGAGTT ACATGATCCC CCATGTGTG CAAAAGAGCG GTAGCTCTCT TCAGTCTCCC
GATCGTGGTG AGAAGTAAGT TGCCCGCAGT GTTATCACCT ATGGTTATGG CAGCACTGCA
TAATTCCTTC ACTGTCAAGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG AGTACTCAAC
CAAGTCATTC TGAAGACTAG GTATGCCGGCG ACCGAGTTGC TCTTGCCCGG GTCAACACG
GGATATACCC GCGCCACATA GCAGAACTTT AAAAGTGCTC ATCAATTGAA AACGTTCTTC
GGGGCGAAAA CTCTCAAGGA TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG
TGACCCCAAC TGATCTGACG CATCCTTATC TTTCACCACG GTTTCTGGGT GAGCAAAAAC
AGGAAGCCAA AATGCCGAAC AAAAGGGAAAT AAGGGCGACA CGGAAATGTG GAATACCTAC
ACTCTCCCTT TTCTAAATTT ATTGAAGCAT TTATCAGGGT TATTTGCTCA TGAGCGGATA
CATATTGCAA GTTAGGAGAA AAAATAAAAA AATAGGGGTT CCGGCAGCAT TCCCCGAAA
AGTGCCACCT GACGTCTAAG AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGGC
TATCACGAGG CCCCTTTGT
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 488 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Asp His Leu Gly Ala Ser Leu Trp Pro Gln Val Gly Ser Leu Cys
1     5     10     15

Leu Leu Leu Ala Gly Ala Ala Trp Ala Pro Pro Asn Leu Pro Asp
20    25    30

Pro Lys Phe Glu Ser Lys Ala Ala Leu Leu Ala Ala Arg Gly Pro Glu
35    40    45

Glu Leu Leu Cys Phe Thr Glu Arg Leu Glu Asp Leu Val Cys Phe Trp
50    55    60

Glu Glu Ala Ala Ser Ala Gly Val Gly Pro Gly Asn Tyr Ser Phe Ser
65    70    75    80

Tyr Gln Leu Glu Asp Glu Pro Trp Lys Leu Cys Arg Leu His Gln Ala
85    90    95

Pro Thr Ala Arg Gly Ala Val Arg Phe Trp Cys Ser Leu Pro Thr Ala
100   105   110

Asp Thr Ser Ser Phe Val Pro Leu Glu Leu Arg Val Thr Ala Ala Ser
115   120   125

Gly Ala Pro Arg Tyr His Arg Val Ile His Ile Asn Glu Val Val Leu
130   135   140

Leu Asp Ala Pro Val Gly Leu Val Ala Arg Leu Ala Asp Glu Ser Gly
145   150   155   160

His Val Val Leu Arg Trp Leu Pro Pro Pro Glu Thr Pro Met Thr Ser
165   170   175
Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser
405 410
Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr
420 425 430
Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr
435 440 445
Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe
450 455 460
Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys
465 470 475 480
Ser Leu Ser Leu Ser Pro Gly Lys
485

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6367 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACGTGACG GATCGGGGAGA TCGGGGATCG ATCCGTCGAC GTACGACTAG TTATTAATAG 60
TAATCAATTAG CGGGGTCATT AGTTCATAGC CCATATAGG AGTTCGCGGT TACATAACTT 120
ACGGTTAAATG GGGCCGCTGG CTGACCCGCC ACGACCCCC CCCATGGAGC GTCAATAATG 180
ACGTATGGTC CCATAGTAGC GCCAATAGGG ACGTTCATT GAGTCGCAATG GTGCGACTAT 240
TTACGTTAAAT GGGCAGTGATG CGAGTGATC ATATGCAAG TACCAGCCCT 300
ATTGACGTCA ATGACGGTAA ATGGGCCCAG TGGCGATTAG CCGTACAT GACCTTATGG 360
GACTTTCCTA CTTGGGAGTA CATCTACGTA TTATGTCATG CTATTTACCAT GTGTGGATTGG 420
TTTGGCCAGT ACATCAATGG GGCTTGGATAG CAGGTGGACT CACGGGGATT TCAAATGTCCT 480
CACCCCATTT AGCTGTAATGG GATTTTGATTT TGCCACCAA AATCAACGGGA CTTTCCAAAA 540
TGTCGTAACA ACTCCGCCA AATGGACGCA AATGGCGGTA GGCGGTGCAG GTGGAGGATT 600
TATATAAGCA GACGCTGGGT CAGTGAACGT CAGATCGCCT GGAGACGCCA TCGAAATCCG 660
TTACCTGCAG ATATAAAGT AATCTGCGTAC CGAGCCCAA TCGGGCGACA AAACCTCACAC 720
ATGCCCACCAC TGCCCGACAC CTAAGCTCCT GGGGGGCCGG TCAAGTCTCT TCTTCCCCCC 780
AAACACCAAAG GACACCTCTCA TGATCTCCCG GACCCCTGAC GTCAATGTGAG GTGTGGTTGA 840
CGTGAGCCCA GAAGACCCCTT AGTCAGCTGT CAACTGGTAC GTGGACGGCG CGGAGATTGCA 900
TAATGCAAGA ACAAGCCGCG GGAGGAGCGA GTCAACACGC ACGTACCGGG TGTCAGCGCT 960
CCTCACCCTC CTGCACCAGGG ACTGCTGCTTA TGGCAAGGAG TACAATGTCA AGGTCTCCCA 1020
CAAAGCCCTC CGGCCCCATA TGAGGAACAC CATCTCCAAA GCCAAAGGCC AGCCCCGAGA 1080
ACCACAGGTG TACACCTCTG CCCCATCCCG GGTAGAGCTG ACCAAGAAACC AGGTACACCT 1140
GACCTGCTGT GTCAAGGCCT TCTATCCCGC CGACACCGCC GTGGAGTGAG AGAGCAAATTG 1200
GCAGCGGGAG AACAACCTACA AGACCCAGCC TCCCGTGCTG GACTCCGACG GCTCCTCTCTT 1260
CCTCTAAGCG AAGCTCAGGG TGGGAAAGCG CAGGGTGCCG CAGGGGAACG TCTTCTCATG 1320
CTCCTGCACT CATGAGGCTC TGCAACACCA CTACACGGCA AAGACCTCTT CCTGGTCCTCC 1380
GGGTAAATGA GTGTAGCTGA GACGCGCTGT ACTAGCTCAG ACTGTCCTCT CTAGTGGCCA 1440
GCCATCTGTT GTTTGGCCCT CCCCGTCGTT TTCTTGGACC CTGGGAAGGGT CCACCTCCAC 1500
TGCTCTCTTG TAATAAAATG AGGAAATGTC ACGCATGATT GTGAGTAAGT GTCAATCTAT 1560
TCTGCGGGGT GGTTGGGCGG AGGACAGCAA GGGGGAGGAT TGGAAGACAA ATAGCAGGCA 1620
TGCTGGGGAT GCGGTGGGCT CTATGGAAAC AGCTGGGGCT CGAGGGGGGA TCTCCCGAATC 1680
CCAGCTTTGG CTTCCTCAATT TCTTATTTTGC ATAATGAGAA AAAAGAGAAA ATTAATTTTA 1740
ACACCAATTG AGTAGTGGAT TGAGCAAATG CGTGGCCAAA AAGGATGCTT TAGAGACAGT 1800
GTCTCTGCA CAGATAAGGA CCAACATATT TCAGAGGGGAG TACCCAGAGC TGAGACTCCT 1860
AAGCCAGTGAC GTGGCAGACG ATTCCTAGGGA GAAATATGCT TGTCATCCAC GAGGCCGATG 1920
TCGCTAGAGC CACACCCTGG TAAGGCCCAAC TCTGCTCAAC CAGGATAGAG AAGGCCAGAG 1980
CCAGGGCAAGA GCATATAAGG TGAGTGGGCA TCAGTTGCTC TCTCATTGTC TCTCTGACAT 2040
AGTTGATTTG GGAGCTGAGA TAGCTTGAGGC AGCTCAGGGC TGCGATTTTG CGCCCAAACCT 2100
GACGGCAATCT CTAAGCTTGA AAAGCTTGGTT AGTTTATCCC ACGCTGCAATC ATGGTTCGAC 2160
CATGGACTGC CATCCTGCAGC GTGCTCCCAAA ATATGGGGAT TGCCAGAGAC GAGAACCTAC 2220
CCTGCGCTCC GCTCAGGAAC GAGTTCAAGT ACTTCTAAAG AATGACCACA ACCCTCTTCA 2280
TGGAAGGTAA ACAGAATCTG GTGATTATGG GTAGGAAAAA CGTTCTTCAC ATTTCTGAGA 2340
AGAATCGACC TTTAAAGGCG AGAATATAAT TAGTTTCAGC TAGAGACCTC AAAGACCGAC 2400
CACGAGGAGC TCATTTCTCT GCCAAAGTTT TGATGATGTC CTTAAGACCTT ATGGACAAC 2460
CGAATAAGTG AAGTAAAGTG GACATGGTTT GAGTAGCTCGG AGGCGAGTTCT GTTTACCAAG 2520
AAGCCATGAA TCAACCGAGC CACCTTAGAC TCTTGTGAGC AAGGATCGTG CAGGAAATTG 2580
AAAGTAGACG GTTTTTCCCA GAATAATGATT TGGGGAAATA TAAACTCTCC CCAGAATACC 2640
CAGCCGCTCT CTCTGAGGTC CAGGAGGAAA AAGGATCAAAT GATAAGTGTG GAACTCTACG 2700
AGAAGAAAGA CTAACAGGAA GATGCTTCTCA AGTTCTCTGC TCCCCCTCTTA AAGCTATGCA 2760
TTTTTATAG ACCAGCTTGG TGAGAATGTG TTTATGCGAG CTTTAAATGG TTACAATAAA 2820
AGCAATAGCA TCACAATTTC CACAAATAAA GCAATTAAAA CACCCCAATC TAGTTTGCTG 2880
TTGCTCAAAC TCATCAATGG ATCTTATCAT GTCTGGATCA ACGATAGCTT ATCTGTTGGC 2940
GATGCGAAGC ACCTGAGGTG TCAGTGTTTTC CTTGCTACTGA TTTAGAAGGCC ATTTGCCCCC 3000
WO 96/40231

PCT/US96/09613

TGAGTGGGGAT TTGGGAGCCG TAAACTTCTTC TTTCAAGGA AGCAATGCGAG AAAGAAAAGC 3060

ATACAAAGTAA TAAGCTGGCA CTGTAATATG GAAGAGTATG AGGTGTGATG AATTAGATT 3120

ACATACTTCT GAATTGAAAC TAAAACCTTT TAAATATTGA AATATATAAC ACATTCTATA 3180

TGAAAGTGATT TTAACATAGTG AACACAGATA CTAAGAAAGG AAAGCTAATG ATAGGTGTCG 3240

CTAAAAGATTC ATTTATTTAT TCTACAATAG ATGAGCTGCA CATCAAATTT CCGCTCAAT 3300

TCTTCAACAGA ATTGAAGAGA GCAATCCTCA AACTCATCTG GAATAACAAA AAACCCAGGA 3360

TAGCAAAAC CCTTCTCAAG GATAAAGAGA CCTCTGCTGG AATCACCAGAT CTCGACCTAA 3420

AGCTCTACTA CAGAGCAATTT TGATATAAAA CTGCAATGGA TGATATAGA AAGGACAAG 3480

TAGACCAATTG GAAATGAACC CACACAGCTA TGTCACCTTG ATCTTCAAAC AGAGAGCTAA 3540

AACCATCCAC TGGAAAAAG AGACATTTTT CAACAAATGG TGCTGGCACA ATGGTGCGTT 3600

ATCATGGAGA AGAATGTTGA TTGGACTATTCCAATTCCTCTTGACTAATTGAGGTCAATC 3660

GTGGACTCAA GAACTCCCAA TAAAACAGA GACACTGAAA CTATAGAGG AGAAAGTGGG 3720

GAAAGCCCTC GAAGATATGG GCACAGGGGA AAATTCCTTG AATAGAAGG CATGCGCTTG 3780

TGCTGTAAAGA TGAGAATTTG ACAATGGAGA CCTCACAGAAA CTCCAAAGCT ATCGGATCAA 3840

TCTCTCCAAA AAAGCCCTCCT CACTACTCTT GGAATAGCTC AGAGGCGCGG GCGGCCTCGG 3900

CCTCTGCATA AATAAAAAAG ATAGTCAGC CATGATCGGG GCGGAGATGG GCGGAACCTG 3960

GGCGAGAGTAA GGGCGGGGAT GGGCGAGTT AGGGCCGAGA CTATGTTTG TCAGCTAATTG 4020

AGATGCAATGC TTGGCATCAT TGGCTCGTGG GGGAGGCTTG GGCAGTTTAC ACACCTGTT 4080

GCTGACTATAT TGAGAATCATG TCTTTGACATA TTTCTGCTTG CTGGGAGGCC TGGGACTTT 4140

CCACACCTTA ACTGACACAC ATTTCCACAGA ATTAATTCGG TATCCCGTAG ACCTCGAGAG 4200

CTTGGCGTTA AACAGGATGCAG TACTGTATTC TGTGTGAATG TTATTCTCGGC TCACAATTCC 4260

ACACAAACATA CGAGCCGGAA GCATAAAGTG TAAAGCCTGG GTGCCTAAT GAGTGAGCTA 4320
ACTCACATTAA ATTTGGCGCT CGTCTCTGCC CGCTGTTCGAC TCCTGAAAAC TGGTTGACCA 4380
GCTGCAATTTA TGAATCGGCG AACCGCGGAG GAGAGCCCGT TTGCTATTTG GGGCGCTCTTC 4440
CGCTTCCTCG CTCTACTGACT CGTGTGCGTCT CTCGTGGTCTGG CTCGGCGGAG CGGTATCAGC 4500
TCATCTAAAG GCGTTAATAC GGGTTACCAG AGAATACGGG GATAACCCAG GAAGAAACAT 4560
GTGAGCAAAA GGCCAGCAAAG AGCGCGAGAA CGGTAAAAAG GCCGCGTTGC TGGCGTTTTT 4620
CCATAGGACT TCAGCCCCCTG AGGACAGATCA CAAAAATCGA CGCTCAAGTC AGAGTGGCCG 4680
AAACCCAGACA GAGCTATAAA GATACCAGGC GTTTCCCTTG GGAAGCTTCCG TGCTGCGTCTC 4740
TCCCTGTCCG ACCCTCGGCAC TTACCAGTAT CCTGTCCGCC TCTCTCCCTT GGGGAGCGT 4800
GGCGCTTTCT CAATGCTCACG TCGTGTAGGTA TCTCAGTGTCG GTGTAAGGTCG TCCGGCTCAA 4860
GCTGGGCTGT GTGCAAGAAACCCCCGTTCAA GCCGCGACGC TGGCCTTTAT CGGTAATCACTA 4920
TGCTCTGAG TCCAAAGCCG TAAGACACAG CTTATCGCAA CTGGCCGACG CCACCTGTTAA 4980
CAGGATTAGC AGAGCGAGGT ATGTAAGGCGG TGCTCAAGAG TTCTGAAAGT GGGGCTCTAA 5040
CTACGGCTAC ACTAGAAGGGA CAGTATTTTG TATCTCAGCCT CTCGTGAAGC CATGGTCCCTT 5100
CGAAAAAGA GGGGTAAGCT TTTGATCCGC AAACAAAGCG ACCGCTGGTA GCGGGTTTTT 5160
TTCTGTTTGC AAGCAGGAGA TTACCAGGCA AAAAAGGGA TCTCAAGGAAG ATCTCTTGAT 5220
CTTTCTCATG GGGTCTGAGC CTCAATGCGA CGAAACTCAA CTGGAAAGGA TTTGGTCCAT 5280
GAGGATTACA AACACGCTCT TCACCTCTAA CCTTTTAAAT TAAAAATGAA GGTGAAAAATC 5340
AAACTAAAGT ATATAAGCAT AAACCTAGGGT TCAGAGTTAC CAATGCTTAAA TCAATGGGC 5400
ACCTATCTCA GCGATCTGTC ATTTGCTTGG ATCCCATAGTT GCCTGACCTCC CGTGGTGTGA 5460
GATAACTACG ATACGGGAGG GCTTACCACG TGCCCCCAGTG CTCGCAATGA TACCGCGGAGA 5520
CCCACGCTCA CGCGCTCCAG ATTTATCAGC AAAAAACCG CCCAGCGCAA GGGCGGAGCG 5580
CAGAAGTGCG CCTGCAACTT TATCGGCCCTC CATCCAGTCT ATTTATTATG GCCGGGAAAGC 5640
(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 38 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: unknown
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTATCATGGA CCACCTCGGG GCGTCCCTCT GGC CCCAG

38
(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:
ATGGACCACC TCGGGCGTC CCTCTGGCCC CAG

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:
GTGTCCTAAG AGCAAGCCAC ATAGCTGGGG GCCAGAGG

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTAAGAGCAA GCCACATAGC TGGGGGCAG AGG

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 41 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: unknown
  (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGTCACTAGG GGTCCAGGTC GCTAGGGTC AGCAGCGACA C

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 36 base pairs
  (B) TYPE: nucleic acid
  (C) STRANDEDNESS: unknown
  (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CTGGGGGTCC AGTCGCTAG GGTACACAG CGACAC
(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 43 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: unknown
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TCGAGCTCGG TACCGAGCCC AAATCGGCGG ACAAACACTCA CAC

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 28 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: unknown
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

GTACTGCTCC TCCGCGGCT TGCTCTTG

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
   (A) LENGTH: 27 base pairs
   (B) TYPE: nucleic acid
   (C) STRANDEDNESS: unknown
   (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

AATTCGGTTA CCTGCAGATA TCAAGCT

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: unknown
(D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

AATTAGCTTG ATATCTGAG GTAACCG
WHAT IS CLAIMED IS:

1. A method for generating an antibody capable of acting as an agonist of a receptor comprising: introducing into an immunocompetent animal or isolated immunocompetent cells thereof an antigenically effective amount of a recombinant immunogen comprising a first extracellular receptor domain spaced apart from a second extracellular receptor domain by a bridging moiety which places said first domain and said second domain in functional proximity which mimics the functional domain proximity of a native multimeric receptor.

2. The method according to claim 1 wherein said native multimeric receptor is a homodimer and said first domain and said second domain are the same.

3. The method according to claim 1 wherein said native multimeric receptor is a heterodimer and said first domain and said second domain are different subunits of the same receptor.

4. The method according to claim 1 wherein said bridging moiety is peptidic.

5. The method according to claim 4 wherein said bridging moiety is an amino acid amphipathic helix.

6. The method according to claim 5 wherein said helix is a leucine zipper.

7. The method according to claim 4 wherein said bridging moiety is an Fc portion of a human immunoglobulin with an intact hinge region.

8. The method according to claim 1 wherein said bridging moiety is an organic non-peptidic molecule.
9. The method according to claim 8 wherein said organic molecule is a bifunctional cross linker.

10. The method according to claim 9 wherein said cross-linker is selected from the group consisting of carbodiimide, glutaraldehyde, DSS, and BS3.

11. In a method for generating a monoclonal antibody comprising introducing an immunogen into an animal or to isolated cells thereof, isolating antibody producing cells therefrom, fusing said antibody producing cells with immortalized cells and isolating a hybridoma cell line which secretes a monoclonal antibody to said immunogen, the improvement comprising employing as the immunogen the immunogen of claim 1.


14. An altered antibody comprising a portion of the antibody selected from the group consisting of the antibody of Claim 12 and Claim 13.

15. A recombinant immunogen comprising a first extracellular receptor domain spaced apart from a second extracellular receptor domain by a bridging moiety which places said first domain and said second domain in functional proximity which mimics the functional domain proximity of a native multimeric receptor.

16. The immunogen according to claim 15 wherein said multimeric receptor is a homodimer or heterodimer.
17. The immunogen according to claim 15 wherein said bridging moiety is peptidic domain.

18. The immunogen according to claim 15 wherein said bridging moiety is an organic non-peptidic molecule.

19. A recombinant polynucleotide comprising a nucleotide region encoding a first extracellular domain of a receptor molecule fused in the correct reading frame to a nucleotide region encoding a peptidic bridging moiety which is fused in the correct reading frame to a nucleotide region encoding a second extracellular domain of a receptor molecule.

20. The recombinant polynucleotide according to claim 19 comprising a nucleotide region encoding a first extracellular domain of a receptor molecule fused in the correct reading frame to a nucleotide region encoding a peptidic bridging moiety.

21. The polynucleotide according to claim 19 wherein a further nucleotide region encoding an enzymatically cleavable peptide sequence is optionally inserted in the correct reading frame between: (a) the region encoding said first extracellular domain and the region encoding said bridging moiety; or (b) the region encoding the bridging moiety and the region encoding said second extracellular domain; or both (a) and (b).

22. The polynucleotide according to claim 20 wherein a further nucleotide region encoding an enzymatically cleavable peptide sequence is optionally inserted in the correct reading frame between the region encoding said first extracellular domain and the region encoding said bridging moiety.
23. A vector comprising a polynucleotide sequence of any of the claims 19-22 under the control of suitable regulatory sequences capable of directing replication and expression of said polynucleotide sequence in a host cell.

24. A host cell transformed with the vector according to claim 23.

25. A recombinant immunogen comprising a first extracellular receptor domain of the erythropoietin receptor spaced apart from a second erythropoietin receptor extracellular domain by an Fc region with intact hinge region of a human antibody, which places said first domain and said second domain in functional proximity which mimics the functional domain proximity of the native dimeric erythropoietin receptor.

26. The immunogen according to claim 25, which is EpoRFc.

27. An EPO agonist antibody characterized as possessing at least 20% of EPO activity when measured in a CFU(E) assay or at least 40% of EPO activity when measured in a proliferation assay or having an affinity for EpoR as measured by Kd equal to or less than about 23nM, or having an affinity for EpoRFc as measured by Kd of equal to or less than 500pM.

28. The antibody according to claim 27 wherein said antibody possesses at least 40% of EPO activity when measured in a CFU(E) assay or at least 60% of EPO activity when measured in a proliferation assay.

29. The antibody according to claim 27 wherein the antibody is selected from the group consisting of 1C8 and 2G6.

30. A method of treating anemia in a patient comprising administering a therapeutically effective amount of the antibody of claim 27.
31. A method for modulating the endogenous activity of an EPO receptor in a mammal comprising administering a modulating effective amount of the antibody of claim 27.
Fig. 3

Concentration (ng/ml)

% Positive Cell

Epo R-Fc  Epo R
Epo R-Fc McAb Binding to 32D/epo wt and Parental Cells

A

Epo R-Fc McAb Binding to UT7-Epo Cells

B

Fig. 4
Fig. 5
Fig. 6
plasmid mtalsEpoRFc  [SEQ ID NO: 1 and 2]
TCGCGCGTTT CGGTGATGAC GGTGAAAACC TCTGACACAT GCAGCTCCCG 50
GAGACGTTCA CAGTTTTGCT GTAAGCCGAT GCCGCGAGCA GACAAGCCCG 100
TCAGGGCGCG TGAGGCGGTG TGCCGGGCTGG TGCCGGCTGG CCTAACATATG 150
CGGCATCGA GCGATTGTGA GTGAGCTGCG ACCATATGCG GTGTAATAA 200
CCGCACAGAT GCGTAAGGAG AAAATACCGC ATCGAGGCGCC ATTCGCCATT 250
CAGGCTGCGC AACTGTTGAG AAGGCGATGC GTGCGGCCC TCTTGCTAT 300
TACGCGACCT GCGAAAAGGG GAGATGTCTG CAAGGCGATT AAGTTGAGTA 350
ACGCCAGGTT TTTCCCAAGTC AGCAGGTTGTA AAAACGACGG CCAGTGCCAG 400
TGAATTCCGG CTGAGCACAGG ATGGCGTGCC CGATGCGACT AGCTCCTTGC 450
TGCGAGCCGT CCTATCTTCT GTTCCGATA AGAGACCGAG AACTCAGGCC 500
CCCCACCCGG CACCGCCACC CCCACATA TGTGCTAGCC AAGTAAAGCT 550
GCCTGCACGT GCCCATGTG CCCAACCAG ACTTTTGTAC ATCCATAACAG 600
TCCCAAGGT GGAGAACCGA ACCATCTCTT CGCGCCGAG ACAAAAGCCT 650
CTGCACACGT CTCCACCTGCA ATTTGAGGCC GCGCAGGCTG TGGAAAGAG 700
GTGAATCGAA CGAAAAGACC GTGCTAAAG CCGCGTTCAG AAAATGTATA 750
AAACCGAGAG CATCTTGCCA ATGGCATCA GTGCGTGTCA GCAGCAATA 800
CAAGTGAATC ATCCTCAGTG AACTAAGGG GGATCCGAT ATCCAGGTT 850
ACCGCGAGCT AGTCTAGGA A CGCGCCGAG TGTGCTGGAA TCTGGCT ATG

GAC CAC CTC GGG GCG TCC CTC TGG CCC CAG GTC GGC TCC CTT 942
Asp His Leu Gly Ala Ser Leu Trp Pro Gln Val Gly Ser Leu
5 10 15
TGT CTC CTG CTC GCT GGG GCC GCC TGG GCG CCC CCC CCT AAC 984
Cys Leu Leu Leu Ala Gly Ala Ala Ala Trp Ala Pro Pro Pro Asn
20 25
CTC CCG GAC CCC AAG TTC GAG AGC AAA GCG GCC TCG CTG GCG 1026
Leu Pro Asp Pro Lys Phe Glu Ser Lys Ala Ala Leu Leu Leu Leu
30 35 40

Fig. 7A
Fig. 7B
GAG CCG AGC TTC GGC GCC TTC TGG AGC GCC TGG TCG GAG CCT 1614
Glu Pro Ser Phe Gly Gly Phe Trp Ser Ala Trp Ser Glu Pro 230 235

GTG TCG CTG CTG AGC CCT AGC GAC CTG GAC CCC ATT GAG GGC 1656
Val Ser Leu Leu Thr Pro Ser Asp Leu Asp Pro Ile Glu Gly 240 245 250

CGT GGT ACC GAG CCC AAA TCG GCC GAC AAA ACT CAC ACA TGC 1698
Arg Gly Thr Glu Pro Lys Ser Ala Asp Lys Thr His Thr Cys 255 260 265

CCA CCG TGC CCA GCA CCT GAA CTC CTG GGG GGA CCG TCA GTC 1740
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val 270 275 280

TTC CTC TTC CCC CCA AAA CCC AAG GAC ACC CTC ATG ATC TCC 1782
Phe Leu Phe Pro Pro Lys Pro Asp Thr Leu Met Ile Ser 285 290 295

CGG ACC CCT GAG GTC ACA TGC GTG GTG GAC GTG AGC CAC 1824
Arg Thr Pro Glu Val Thr Cys Val Val Asp Val Ser His 300 305

GAA GAC CCT GAG GTC AAG TTC AAC TGG TAC GTG GAC GGC GTG 1866
Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly Val 310 315 320

GAG GTG CAT AAT GCC AAG ACA AAG CCG GAG GAG CAG TAC 1908
Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr 325 330 335

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Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His 340 345 350

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Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser 355 360 365

AAC AAA GCC CTC CCA GCC CCC ATC GAG AAA ACC ATC TCC AAA 2034
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys 370 375

GCC AAA GGG CAG CCC CGA GAA CCA CAG GTG TAC ACC CTG CCC 2076
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro 380 385 390

CCA TCC CGG GAT GAG CTG ACC AAG AAC CAG GTC AGC CTG ACC 2118
Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr 395 400 405

Fig. 7C
Fig. 7D
AGGTAGATGA CGACCATCAG GGACAGCTTC AAGGATCGCTT CGCGGCTCTT 3121
ACCGCCAGC AAAAGGCCAG GAACCGTAAA AAGGCCGCTT TGCTGGCGTT 3171
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CCTGGAAGCT CCTCTGGTGC CGTCTCGTGT CCCGACCGTG CGCTTACCGG 3321
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Fig. 7E

SUBSTITUTE SHEET (RULE 26)
GATCGTTGTC AGAAGTAAGT TGCCGCAGT GTTATCACTC ATGGTTATGG 4421
CAGGACTGCA TAAATCTCTT ACTGTCATGC CATCGTGAAG ATGCTTTCCT 4471
GTGACCTGGT AGTACTCAAC CAAGTCATTG TGAGAAATAGT GTATGCGGCG 4521
ACCGAGTTGC TCTTGCCCAG CGTCAAACAG GGATAATACC GCGGCACATA 4571
GCAGAAGCTT AAAAGTGCTC ATCATGGAAA AACGTTCTTC GGGCGAAAA 4621
CTCTCAAGGA TCTTACCGGT GTGAGATCC AGTTGATGTT AACCCACCTCG 4671
TGCACCCAAC TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT 4721
GAGCAAAAAAC AGGAAGGCAA AATGCGCGAA AAAAGGGAAT AAGGGGACA 4771
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Fig. 7F

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Fig. 8A

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Fig. 8B
Fig. 8C

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**Fig. 8D**

**SUBSTITUTE SHEET (RULE 26)**
Fig. 8E

SUBSTITUTE SHEET (RULE 26)
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/133.1, 192.1, 194.1; 530/387.1; 435/240.27, 172.3, 70.21, 71.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG, BIOSIS, SCISEARCH, EMBASE, MEDLINE, WPI, search terms: immunogen, antigen, extracellular, surface, receptor, antibody?, immunoglobulin?, erythropoietin, dimer, authors.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tr>
<td>Y</td>
<td>J. Immunol., Volume 142, Number 3, issued 01 February 1989, SCHREURS et al., &quot;A monoclonal antibody with IL-3-like activity blocks IL-3 binding and stimulates tyrosine phosphorylation&quot;, pages 819-825, see entire document.</td>
<td>1-31</td>
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<td>Y</td>
<td>Blood, Volume 82, Number 6, issued 15 September 1993, YET et al., &quot;The extracytoplasmic domain of the erythropoietin receptor forms a monomeric complex with erythropoietin&quot;, pages 1713-1719, see entire document.</td>
<td>1-31</td>
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Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier document published on or after the international filing date
  * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  * "O" document referring to an oral disclosure, use, exhibition or other means
  * "P" document published prior to the international filing date but later than the priority date claimed

T Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X Document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y Document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

Date of the actual completion of the international search: 27 AUGUST 1996

Date of mailing of the international search report: 13 SEP 1996

Name and mailing address of the ISA/US Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
HEATHER BAKALYAR

Telephone No. (703) 305-2596

Form PCT/ISA/210 (second sheet)(July 1992)*
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<td>Science, Volume 256, issued 19 June 1992, FUH et al., &quot;Rational design of potent antagonists to the human growth hormone receptor&quot;, pages 1677-1680, see entire document.</td>
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<td>J. Immunol., Volume 140, Number 2, issued January 1992, SUGAWARA et al., &quot;Monoclonal autoantibodies with interleukin 3-like activity derived from a MRL/lpr mouse&quot;, pages 526-530, see entire document.</td>
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INTERNATIONAL SEARCH REPORT

Box I  Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. □ Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. □ Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. □ Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II  Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. X As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. □ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. □ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. □ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest □ The additional search fees were accompanied by the applicant’s protest.

□ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)
A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):
A61K 39/00, 39/385, 39/395, 39/40, 39/42; C07K 16/00; C12N 5/00, 15/00; C12P 21/04

A. CLASSIFICATION OF SUBJECT MATTER:
US CL:
424/133.1, 192.1, 194.1; 530/387.1; 435/240.27, 172.3, 70.21, 71.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains claims directed to more than one species of the generic invention. These species are deemed to lack Unity of Invention because they are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for more than one species to be examined, the appropriate additional examination fees must be paid. The species are as follows:

Species 1. Immunogens having a bridging moiety which is peptidic; and
Species 2. Immunogens having a bridging moiety which is an organic non-peptidic molecule.

The claims are deemed to correspond to the species listed above in the following manner:
Species 1, claims 4-7, 17 and 19-31, and
Species 2, claims 8-10 and 18

The following claims are generic: 1-3 and 11-16.

The species listed above do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, the species lack the same or corresponding special technical features for the following reasons: immunogens having bridges such as peptides are structurally different from those immunogens having organic non-peptidic linkers. Further, the immunogens are made differently, using materially different process steps and reagents.