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Description

FIELD OF THE INVENTION

[0001] The invention relates to recombinantly constructed proteins useful for treating pathological neovascularization, *e.g.*, asthma, arthritis, cancer, and macular degeneration.

BACKGROUND OF THE INVENTION

[0002] Pathological neovascularization is a key component of diseases like wet age-related macular degeneration (AMD), proliferative diabetic retinopathy, rheumatoid arthritis, osteoarthritis, and asthma. It also plays an important role in growth and spread of tumors. Neovascularization is regulated by an exquisite balance of pro- and anti-angiogenic factors.

[0003] Vascular endothelial growth factor (VEGF) is known to be necessary for neovascularization. Inhibition of VEGF activity has been shown to inhibit neovascularization in animal models of AMD, arthritis and in various tumor models. Methods used to inhibit VEGF activity include antibodies, receptor fusion proteins, peptides and small molecules.

[0004] VEGF-R1 (Flt-1) and VEGF-R2 (KDR) proteins have been shown to bind VEGF with high affinity. Both Flt-1 and KDR have seven Ig-like domains in their extracellular region. Domain 2 has been shown to be essential for VEGF binding. Fusions of each of the full-length, soluble receptor (domains 1-7) and domains 1-3 to IgG Fc bind VEGF efficiently IgG Fc fusions to Ig-like domain 2 alone was, however, incapable of binding VEGF, as was a combination of Ig-like domain 1 and 2. Davis-Smyth, 1996. Therefore, Ig-like domains 1 and 3 seem to be required along with domain 2 for efficient VEGF binding.

[0005] Holash et al (2002) Proc. Natl. Acad. Sci. vol. 99(17): 11393-11398 discloses further fusion polypeptides comprising portions of the VEGF-R1 receptor fused to the Fc portion of the human IgG1 heavy chain. Vangelista et al (2002) Protein Engineering vol. 15(1): 51-57 discloses a fusion polypeptide in which the two extracellular domains of the human FcεRI α-chain are fused to the dimerizing C-terminal domain of the human IgG1 heavy chain.

BRIEF SUMMARY OF THE INVENTION

[0006] According to one aspect of the present disclosure, a fusion protein is provided. The fusion protein has the formula X-Y-Z. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a 5 - 25 amino acid residue polypeptide. Z is a CH3 region of an IgG heavy chain molecule.

[0007] Another aspect of the present disclosure is a polypeptide of the formula X-Y-Z. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a linker moiety which provides the spatial separation of 5-25 amino acid residue. Z is a CH3 region of an IgG heavy chain molecule.

[0008] Yet another aspect of the present disclosure is a fusion protein of the formula X-Y-Z. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a 5 - 25 amino acid residue polypeptide. Z is an Fc portion of an antibody molecule.

[0009] A fusion protein of the formula X-Y-Z is also provided by the present disclosure. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a linker moiety which provides the spatial separation of 5-25 amino acid residues. Z is an Fc portion of an antibody molecule.

[0010] Still another aspect of the present disclosure is a method of multimerizing a polypeptide X. A polypeptide X is linked to a polypeptide Z via a polypeptide Y to form polypeptide XYZ. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a 5 - 25 amino acid residue polypeptide. Z is a CH3 region of an IgG heavy chain molecule. Polypeptide XYZ which is formed multimerizes.

[0011] Yet another aspect of the present disclosure provides a method of multimerizing a polypeptide X. Polypeptide X is linked to a polypeptide Z via a moiety Y to form polymer XYZ. X comprises a polypeptide selected from the group consisting of an extracellular receptor, an antibody variable region, a cytokine, a chemokine, and a growth factor. Y consists essentially of a linker moiety which provides the spatial separation of 5-25 amino acid residues. Z is a CH3 region of an IgG heavy chain molecule. Polypeptide XYZ which is so formed multimerizes.

[0012] In one aspect of the present disclosure a nucleic acid molecule is provided. The nucleic acid molecule encodes a fusion protein which comprises an Ig-like domain 2 of VEGF-R1 (Flt-1); a linker, and a multimerization domain. The fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

[0013] In another aspect of the present disclosure a fusion protein is provided. The fusion protein comprises an Ig-like domain 2 of VEGF-R1 (Flt-1), a linker, and a multimerization domain. The fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

[0014] In another aspect of the present disclosure an *in vitro* method is provided. A nucleic acid molecule is delivered to an isolated mammalian cell. The nucleic acid molecule encodes a fusion protein which comprises an Ig-like domain 2 of VEGF-R1 (Flt-1); a linker; and a multimerization domain. The fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25. Expression of the fusion protein is controlled by a promoter. A cell is formed which expresses a fusion protein.

[0015] Still another aspect of the present disclosure is a method for delivering a fusion protein to a mammal. A mammalian cell which expresses the fusion protein is delivered to a mammal. The cell expresses and secretes the fusion protein thereby supplying the fusion protein to the mammal. The fusion protein comprises an Ig-like domain 2 of VEGF-R1 (Flt-1), a linker, and a multimerization domain. The fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

[0016] Another aspect of the present disclosure is a method for supplying a fusion protein to a mammal. A fusion protein which comprises an Ig-like domain 2 of VEGF-R1 (Flt-1), a linker, and a multimerization domain is delivered to a mammal. The fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25. Alternatively, a nucleic acid construct which encodes said fusion protein can be delivered to the mammal, whereby the fusion protein is expressed by the mammal.

[0017] These and other aspects of the present disclosure, which will be described in more detail below, provide the art with methods and agents for treating diseases related to vascular proliferation and inflammation. The agents may provide increased stability and bioavailability relative to natural forms of the proteins.

[0018] Now a part of the disclosure that is contained herein provides the aspects and embodiments of the present invention. More particularly, a first aspect of the present invention provides a fusion protein of the formula X-Y-Z, wherein: X comprises the Ig-like domain 2 of VEGF-R1 but lacks the Ig-like domains 1 and 3 of VEGF-R1, with said Ig-like domain 2 of VEGF-R1 being covalently linked to moiety Z via moiety Y; Y consists of a 5 - 25 amino acid residue polypeptide; and Z is a CH3 region of an IgG heavy chain molecule or an Fc portion of an antibody molecule.

[0019] In a related aspect, the present invention provides a composition comprising this fusion protein of the invention, which composition further comprises one or more pharmaceutically acceptable excipients or carriers.

[0020] In a further aspect, the present invention provides a method of multimerizing a polypeptide X, the method comprising: linking a polypeptide X to a polypeptide Z via a polypeptide Y to form a polypeptide XYZ, wherein: X comprises the Ig-like domain 2 of VEGF-R1 but lacks the Ig-like domains 1 and 3 of VEGF-R1, with said Ig-like domain 2 of VEGF-R1 being covalently linked to polypeptide Z via polypeptide Y; Y consists of a 5 - 25 amino acid residue polypeptide; and Z is a CH3 region of an IgG heavy chain molecule or an Fc portion of an antibody molecule; whereby the polypeptide XYZ multimerizes.

[0021] In yet further aspects, the present invention provides a nucleic acid molecule which encodes the fusion protein of the present invention, a vector which comprises said nucleic acid molecule, a mammalian cell which comprises the nucleic acid molecule, and an *in vitro* method comprising delivering the nucleic acid molecule to an isolated mammalian cell so as to form a cell which expresses said fusion protein.

[0022] In still further aspects, the present invention provides the fusion protein, nucleic acid, vector or mammalian cell of the present invention, for use in treating a mammal. In one embodiment, the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy. In another embodiment, the mammal has cancer. In another embodiment, the mammal has rheumatoid arthritis. In another embodiment, the mammal has asthma. In another embodiment, the mammal has osteoarthritis.

[0023] Additional aspects and embodiments of the present invention are set out in the appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

[0024] Fig. 1. Flexible region of 9-Gly linker in D2-9Gly-Fc construct. The predicted relative flexibility by Karpus and Schultz (1985) method shows the polyglycine 9-mer (9-Gly) linker (aa 94 to 103) in D2-9Gly-Fc protein as a region with greater flexibility than the average (>1) as compared to D2-Fc construct that does not contain 9-Gly linker. Both fusion proteins contain identical amino acid sequences enclosed in boxes: sp - signal peptide (aa -24 to -1), Flt-1 domain 2 (aa 1 to 93) and IgGl-Fc residues (244 aa). The arrow represents the signal peptidase cleavage site as predicted using the SignalP V2.0 program (Nielsen et al., 1997).

[0025] Fig. 2. Biological activity of D2-9Gly-Fc vs. D2-Fc. 293 cells were grown in the starvation media (M199 + 5% FCS) and transfected with plasmids containing D2-9Gly-Fc and D2-Fc expression cassettes under control of CMV promoter. Conditioned media (CM) was collected 72 h later. HUVECs were seeded into 96 well plate (2E3 cells/well) in starvation media + VEGF (10 ng/mL) and 50 ul CM plus VEGF (10 ng/mL) was added 24 h later. The controls (+/- VEGF) were incubated with CM from the control pEGFP (Clontech; pEGFP carries a red-shifted variant of wild-type green

fluorescent protein (GFP) which has been optimized for brighter fluorescence and higher expression in mammalian cells) plasmid transfection The positive control was treated with 50 ng of Flt-1-IgG recombinant protein (R&D Systems). The HUVECs were assayed for proliferation 3 days post treatment using CellTiter 96® AQ_{ueous} reagent (Promega). The data represent the means of the average values of OD_{490} of two experiments each assayed in triplicates.

[0026] Fig. 3. Western blot analysis of D2-9Gly-Fc and D2-Fc. The size of both D2-9Gly-Fc and D2-Fc proteins appears to be twice as large while migrating in non-reducing gel as compared to migration in reducing gel. The proteins were loaded from the conditioned media following 293 cell transfection of plasmids expressing D2-9Gly-Fc and D2-Fc were separated by SDS-electrophoresis and transferred to PVDF membrane. The blot was probed with goat anti-human anti-IgG1 Fc and rabbit anti-goat IgG-HRP antibodies.

[0027] Fig. 4. sFlt-1 hybrid proteins containing 9Gly linker and VEGF Ex3. Structure comparison of D2-9Gly-Ex3/CH3 to previously constructed proteins. All three proteins contain identical amino acid sequence of Flt-1 domain 2, consisting of 24 aa of Flt-1 signal peptide and 93 aa of Flt-1 domain 2. D2-9Gly-Ex3/CH3 contains 9 aa of 9Gly linker, 14 aa of VEGF Ex3 and 120 aa of the CH3 region of human IgG1 heavy chain Fc.

[0028] Fig. 5. Biological activity of D2-9Gly-Ex3/CH3 vs. D2-9Gly-Fc. Protein D2-9Gly-Ex3/CH3, where domain 2 is connected to the CH3 region through 9Gly linker and VEGF Ex 3, is also efficiently inhibiting VEGF-dependent HUVECs proliferation as compared to control proteins D2-9Gly-Fc and D2-Fc. 50 ng of the recombinant Flt-1-IgG (R&D Systems) was used as a control.

[0029] Fig. 6. HUVECs proliferation assay comparing D2-$(Gly_4Ser)_3$-Fc protein activity with D2-9Gly-Fc and D2-9Gly-Ex3/CH3.

[0030] Fig. 7. Western blot Proteins (non-reduced and reduced) from conditioned medium of transfected 293 cells (15 ul of CM) with plasmids expressing (1): D2-9Gly-Fc; (2): D2-$(G_4S)_3$-Fc and (3) - EGFP proteins were separated by SDS-electrophoresis and transferred to PVDF membrane. The blot was probed with goat anti-human IgG1 Fc and rabbit anti-goat IgG-HRP antibodies.

[0031] Fig. 8. Combinations of proteins with/without 9Gly linker or VEGF Ex3. Structure comparison of three novel proteins with or without 9Gly linker and/or VEGF Ex3, D2-9Gly-CH3, D2-CH3 and D2-Ex3/CH3.

[0032] Fig. 9. HUVECs proliferation assay with the Flt-1(D2) constructs with 9Gly, Ex3 and CH3 combinations. Conditioned media from 293 cells (5 ul) containing proteins D2-Ex3/CH3, D2-9Gly-CH3 and D2-CH3 were compared to D2-9Gly-Fc and D2-9Gly-Ex3/CH3.

[0033] Fig. 10 Western blot 293 cells were transfected with plasmids expressing: (1) D2-9Gly-Fc; (2) D2-9Gly-CH3 (52/26 kDa); and (3) D2-CH3 (50/25 kDa). Proteins from 293 cells conditioned medium (15 ul of CM non-reduced and/or reduced) were separated by SDS-electrophoresis and transferred to PVDF membrane. The blot was probed with anti-human VEGF-R1 HRP conjugate (R&D Systems).

[0034] Fig. 11. VEGF "in vitro" binding assay. Conditioned media from 293 cells containing known concentrations of both D2-9Gly-Fc and Flt-1 D(1-3) control soluble receptors (ranging in concentrations from 0.29 -150 pM) were serially diluted and mixed with 10 pM VEGF. The amount of unbound VEGF was then measured by ELISA. D2-9Gly-Fc binds VEGF with higher affinity than all other constructs. "n" represents the number of independent experiments (transfection and binding assay).

[0035] Fig. 12. The binding kinetics of the soluble Flt-1 constructs were measured by surface plasmon resonance with a BIAcore instrument. sFlt-1 constructs were immobilized onto a sensor chip, and VEGF165 was injected at concentrations ranging from 0.2 to 15 nM. The sensorgrams were evaluated using the BIA Evaluation program, the rate constants Ka and Kd were determined and the dissociation constant (KD) calculated from the ratio of Kd/Ka = KD. The lower KD value means better affinity.

[0036] Fig. 13A-13C. Fig 13A shows expression levels of Flt-1 constructs having various linkers. Fig. 13B shows dimerization or multimerization of Flt-1 constructs having various linkers and a CH3 moiety of Fc of IgG1. The difference between the non-reduced and the reduced conditions indicates that the proteins had multimerized. Fig. 13C shows the inhibitory bioactivity of indicated Flt-1 constructs present in condition medium in a HUVEC proliferation assay in the presence of VEGF. Each of the constructs demonstrated inhibitory activity approaching proliferation levels of the HUVEC in the absence of VEGF.

[0037] Fig. 14. Using a murine oxygen-induced retinopathy (OIR) model of retinal neovascularization (NV), one of the Flt-1 constructs was administered to the mouse eyes and neovascularization was determined. The mice were exposed to hyperoxic conditions. The number of neovascular events was determined in the treated eyes compared to the events in the untreated eyes of the same animals. The animal was considered a "responder" if there was a greater than 50% reduction in neovascular events.

DETAILED DESCRIPTION OF THE INVENTION

[0038] It is a discovery of the present inventors that a Flt-1 Ig-like domain 2 without domains 1 and 3 is capable of efficiently binding VEGF and inhibiting VEGF-dependent endothelial cell proliferation. Domain 2 can be covalently linked

to a multimerization domain via a linker. Linkers are typically polypeptide chains. The length of the chain may be 6, 7, 9, 11, 13, 15 or more amino acid residues, but typically is between 5 and 25 residues. Depending upon the length and side chain composition, a linker may have but need not have greater than average flexibility. Flexibility can be calculated using algorithms known in the art. Multimerization domains are those portions of multimeric proteins which promote the association of subunits to form, for example, dimers, trimers, tetramers, etc. Suitable recombinant proteins for efficiently binding VEGF and/or inhibiting VEGF-dependent endothelial cell proliferation are selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

[0039] Moreover, the inventors have found that the multimerization domains and linkers can be used with a variety of other proteins or portions of proteins to induce multimerization. Such proteins may be those which bind to ligand or receptor only when multimerized, or may be those whose binding affinity is enhanced when multimerized. Suitable proteins for multimerization include extracellular receptors (which include portions thereof), antibody variable regions, cytokines, chemokines, and growth factors. Suitable proteins include tyrosine kinase receptors and serine thereonine kinase receptors. Specific examples of extracellular receptors include EGF-receptor, G protein coupled receptors, FGF receptor, Fc receptors, T cell receptors, etc. Examples of antibody variable regions include Fab, $F(ab')_2$, and ScFv. Examples of cytokines include GM-CSF, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-18, IL-21, IL-23, IFN-α, IFN-β, IFN-γ, MIP-1α, MIP-1β, TGF-β, TNFα, and TNF-β. Examples of chemokines include BCA-1 / BLC, BRAK, Chemokine CC-2, CTACK, CXCL-16, ELC, ENA, ENA-70, ENA-74, ENA-78, Eotaxin, Exodus-2, Fractalkine, GCP-2, GRO, GRO alpha (MGSA), GRO-beta, GRO-gamma, HCC-1, HCC-4, 1-309, IP-10, I-TAC, LAG-1, LD78-beta, LEC / NCC-4, LL-37, Lymphotactin, MCP, MCAF (MCP-1), MCP-2, MCP-3, MCP-4, MDC, MDC, MDC-2, MDC-4, MEC / CCL28, MIG, MIP, MIP-1 alpha, MIP-1 beta, MIP-1 delta, MIP-3 / MPIF-1, MIP-3 alpha, MIP-3 bet, MIP-4 (PARC), MIP-5, NAP-2 , PARC, PF-4, RANTES, RANTES-2, SDF-1 alpha, SDF-1 beta, TARC, and TECK. Examples of growth factors include Human Amphiregulin, Human Angiogenesis Proteins, Human ACE, Human Angiogenin, Human Angiopoietin, Human Angiostatin, Human Betacellulin, Human BMP, Human BMP-13 / CDMP-2, Human BMP-14 /CDMP-1, Human BMP-2, Human BMP-3, Human BMP-4, Human BMP-5, Human BMP-6, Human BMP-7, Human BMP-8, Human BMP-9, Human Colony Stimulating Factors, Human flt3-Ligand, Human G-CSF, Human GM-CSF, Human M-CSF, Human Connective Tissue Growth Factor, Human Cripto-1, Human Cryptic, Human ECGF, Human EGF, Human EG-VEGF, Human Erythropoietin, Human Fetuin, Human FGF, Human FGF-1, Human FGF-10, Human FGF-16, Human FGF-17, Human FGF-18, Human FGF-19, Human FGF-2, Human FGF-20, Human FGF-3, Human FGF-4, Human FGF-5, Human FGF-6, Human FGF-7 / KGF, Human FGF-8, Human FGF-9, Human FGF-acidic, Human FGF-basic, Human GDF-11, Human GDF-15, Human Growth Hormone Releasing Factor, Human HB-EGF, Human Heregulin, Human HGF, Human IGF, Human IGF-I, Human IGF-II, Human Inhibin, Human KGF, Human LCGF, Human LIF, Human Miscellaneous Growth Factors, Human MSP, Human Myostatin, Human Myostatin Propeptide, Human Nerve Growth Factor, Human Oncostatin M, Human PD-ECGF, Human PDGF, Human PDGF (AA Homodimer), Human PDGF (AB Heterodimer), Human PDGF (BB Homodimer), Human PDGF (CC Homodimer), Human PIGF, Human PIGF, Human PIGF-1, Human PIGF-2, Human SCF, Human SMDF, Human Stem Cell Growth Factor, Human SCGF-alpha, Human SCGF-beta, Human Thrombopoietin, Human Transforming Growth Factor, Human TGF-alpha, Human TGF-beta, and Human VEGF.

[0040] Flt-1 receptor protein has an extracellular portion which comprises seven Ig-like domains. These are located at residue numbers 32...123, 151...214, 230...327, 335...421, 428...553, 556...654, and 661...747 of Genbank accession no. P17948, see also SEQ ID NO: 15. Residue numbers 1-26 comprise a signal sequence. Flt-1 protein is encoded by the DNA sequence shown at Genbank accession no. NM_002019 (SEQ ID NO: 14).

[0041] Multimerization domains that can be used in accordance with the present disclosure are known in the art. Sequences of the Fc portion of IgG1 or IgG2 gamma heavy chain can be used, for example, CH3 alone (aa 371-477) or both of CH2 and CH3 domains (aa 247-477). Fc portion of Ig molecules is that which is obtained by cleavage of whole antibody molecules with the enzyme papain. Other means can be used to obtain these portions. For the IgG1 gamma heavy chain protein sequence, see Genbank accession no Y144737 and SEQ ID NO: 10. Other Fc regions can be used for example from other IgG types and from IgA, IgM, IgD, or IgE antibodies. The multimerization region of VEGF can also be used. A DNA sequence encoding VEGF is shown at Genbank accession no. NM_003376 and SEQ ID NO: 11. An amino acid sequence of VEGF is shown at Genbank accession no. CAC19513 and SEQ ID NO: 12. The multimerization region of VEGF (SEQ ID NO: 13), encoded by VEGF exon 3 (VEGF Ex3), is at about amino acid residues 75-88 of VEGF protein (SEQ ID NO: 12). Multimerization domains will cause at least 5 %,10%,20%,30%,40%,50%,60%,75%, 80%,85%,90%, or 95% of the monomeric fusion proteins to migrate on a non-denaturing polyacrylamide gel at a rate appropriate for a multimer. Glycosylation can affect the migration of a protein in a gel. Although particular sequences are shown here, variants such as allelic variants can be used as well. Typically such variants will have at least 85 %, 90 %, 95 %, 97 %, 98 %, or 99 % identity with the disclosed sequence.

[0042] Multimerization can be assayed, for example, using reducing and non-reducing gels, as demonstrated herein. Multimerization can also be assayed by detection of increased binding affinity of a protein for its ligand/receptor. BiaCore™ surface plasmon resonance assays can be used in this regard. These assays detect changes in mass by measuring changes in reactive index in an aqueous layer close to a sensor chip surface. Any method known in the art can be used

to detect multimerization.

[0043] Linker moieties according to the present disclosure can be comprised of for example 5-100 amino acid residues, 5-75 amino acid residues, 5-50 amino acid residues, 5-25 amino acid residues, 5-20 amino acid residues, 5-15 amino acid residues, 5-10 amino acid residues, or 5-9 amino acid residues. Examples of useful linkers include: gly_9 (SEQ ID NO: 27), glu_9 (SEQ ID NO: 28), ser_9 (SEQ ID NO: 29), $gly_5cyspro_2cys$ (SEQ ID NO: 30), $(gly_4ser)_3$ (SEQ ID NO: 31), SerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 32), Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn (SEQ ID NO: 13), Gly Asp Leu Ile Tyr Arg Asn Gln Lys (SEQ ID NO: 26), and Gly_9ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 34). Other polypeptide linkers which can be used include a polyglycine of different lengths, including of 5, 7, or 30 residues. Additionally, other portions of Flt-1 can be used as a linker, for example domain 3 of Flt-1. See SEQ ID NO: 15. Linker moieties can also be made from other polymers, such as polyethylene glycol. Such linkers can have from 10 to 1000, 10-500, 10-250, 10-100, or 10-50 ethylene glycol monomer units. Suitable polymers should be of a size similar to the size occupied by the appropriate range of amino acid residues. A typical sized polymer would provide a spacing of from about 10-25 angstroms.

[0044] Notwithstanding the generality of the foregoing disclosure, however, the present invention relates to a fusion protein of the formula X-Y-Z, wherein: X comprises the Ig-like domain 2 of VEGF-R1 but lacks the Ig-like domains 1 and 3 of VEGF-R1, with said Ig-like domain 2 of VEGF-R1 being covalently linked to moiety Z via moiety Y; Y consists of a 5 - 25 amino acid residue polypeptide; and Z is a CH3 region of an IgG heavy chain molecule or an Fc portion of an antibody molecule.

[0045] Fusion proteins according to the present disclosure, including the fusion proteins of the present invention, can be made by any means known in the art. While such proteins can be made synthetically, or by linking portions which are made, recombinant production can also be used. A fused gene sequence can be produced using the standard tools of recombinant DNA. The fused gene sequence can be inserted into a vector, for example a viral or plasmid vector, for replicating the fused gene sequence. A promoter sequence which is functional in the ultimate recipient cell can be introduced upstream of the fused gene sequence. Promoters used can be constitutive, inducible or repressible. Examples of each type are well-known in the art. The vector can be introduced into a host cell or mammal by any means known in the art. Suitable vectors which can be used include adenovirus, adeno-associated virus, retrovirus, lentivirus, and plasmids. If the vector is in a viral vector and the vector has been packaged, then the virions can be used to infect cells. If naked DNA is used, then transfection or transformation procedures as are appropriate for the particular host cells can be used. Formulations of naked DNA utilizing polymers, liposomes, or nanospheres can be used for fusion gene delivery. Cells which can be transformed or transfected with recombinant constructs according to the invention may be any which are convenient to the artisan. Exemplary cell types which may be used include bacteria, yeast, insects, and mammalian cells. Among mammalian cells, cells of many tissue types may be chosen, as is convenient. Exemplary cells which may be used are fibroblasts, hepatocytes, endothelial cells, stem cells, hematopoietic cells, epithelial cells, myocytes, neuronal cells, and keratinocytes. These cells can be used to produce protein *in vitro*, or can be delivered to mammals including humans to produce the encoded proteins *in vivo*. This means of delivery is an alternative to delivering nucleic acid to a mammal, delivering viral vector to a mammal, and delivering fusion protein to a mammal.

[0046] Compositions of protein or nucleic acids can be in carriers, such as buffers, aqueous or lipophilic carriers, sterile or non-sterile, pyrogenic or non-pyrogenic vehicles. Non-pyrogenic vehicles are useful for injectible formulations. Formulations can be liquid or solid, for example, lyophilized. Formulations can also be administered as aerosols. Compositions may contain one or more fusion proteins or one or more nucleic acids, or both fusion proteins and nucleic acids. The fusion proteins and or nucleic acids in a composition may be homogeneous, in which case homomultimer proteins will form, or they may be heterogeneous in the composition, in which case heteromultimer proteins will form. In the case of heteromultimers, typically the X moiety will vary between fusion proteins, but the Z moiety will be the same between fusion proteins.

[0047] Fusion proteins can be provided to a cell or mammalian host by any means known in the art. Protein can be delivered to the cell or host. Nucleic acid can be administered to the cell or host. Transformed or transfected cells can be administered to the cell or host. In the latter case, cells of the same genetic background are desired to reduce transplantation rejection.

[0048] Suitable cells for delivery to mammalian host animals include any mammalian cell type from any organ, tumor, or cell line. For example, human, murine, goat, ovine, bovine, dog, cat, and porcine cells can be used. Suitable cell types for use include without limitation, fibroblasts, hepatocytes, endothelial cells, keratinocytes, hematopoietic cells, epithelial cells, myocytes, neuronal cells, and stem cells.

[0049] Means of delivery of fusion proteins or nucleic acids encoding fusion proteins include delivery of cells expressing the fusion proteins, delivery of the fusion proteins, and delivery of nucleic acids encoding the fusion proteins. Fusion proteins, cells, or nucleic acids can be delivered directly to the desired organ or tumor, for example by injection, catheterization, or endoscopy They can also be delivered intravenously, intrabronchially, intra-tumorally, intrathecally, intramuscularly, intraocularly, topically, subcutaneously, transdermally or *per os.* Patients who can be effectively treated include those with wet age-related macular degeneration, proliferative diabetic retinopathy, rheumatoid arthritis, oste-

oarthritis, uveitis, asthma, and cancer. The treatments will improve symptoms and/or markers of disease and/or disease severity.

[0050] Nucleic acids can be delivered to mammals, and in particular to humans, in any desired vector. These include viral or non-viral vectors, including adenovirus vectors, adeno-associated virus vectors, retrovirus vectors, lentivirus vectors, and plasmid vectors. Exemplary types of viruses include HSV (herpes simplex virus), adenovirus, AAV (adeno associated virus), HIV (human immunodeficiency virus), BIV (bovine immunodeficiency virus), and MLV (murine leukemia virus). Nucleic acids can be administered in any desired format that provides sufficiently efficient delivery levels, including in virus particles, in liposomes, in nanoparticles, and complexed to polymers.

[0051] Combinations of protein and nucleic acid treatments can be used. For example, a fusion protein according to the present disclosure, e.g. a fusion protein according to the present invention, can be administered to a patient. If a favorable response is observed, then a nucleic acid molecule encoding the fusion protein can be administered for a long term effect. Alternatively, the protein and nucleic acid can be administered simultaneously or approximately simultaneously. In another alternative, an antibody or fusion protein for a ligand can be administered followed by or concomitantly with an antibody or fusion partner for a receptor. Another option employs a combination of nucleic acids in which one encodes an antibody and another encodes a fusion protein. Some antibodies that can be employed in combination with the Flt-1 constructs of the present disclosure, e.g. in combination with the Flt-1 constructs of the present invention, (whether in the protein or nucleic acid form) are bevacizumab and ranibizumab, both directed to VEGF. These are particularly useful for treating cancer and macular degeneration, respectively.

[0052] The practice of the present disclosure, including the practice of the present invention, employs, unless otherwise indicated, conventional techniques of molecular biology (including recombinant techniques), microbiology, cell biology, biochemistry and immunology, which are within the skill of the art. Such techniques are explained fully in the literature, such as, Molecular Cloning: A Laboratory Manual, Second Edition (Sambrook et al., 1989); Current Protocols In Molecular Biology (F.M. Ausubel et al., eds., 1987); Oligonucleotide Synthesis (M.J. Gait, ed., 1984); Animal Cell Culture (R.I. Freshney, ed., 1987); Methods In Enzymology (Academic Press, Inc.); Handbook Of Experimental Immunology (D.M. Wei & C.C. Blackwell, eds.); Gene Transfer Vectors For Mammalian Cells (J.M. Miller & M.P. Calos, eds., 1987); PCR: the Polymerase Chain Reaction, (Mullis et al., eds., 1994); Current Protocols In Immunology (J.E. Coligan et aL, eds., 1991); Antibodies: A Laboratory Manual (E. Harlow and D. Lane eds. (1988)); and PCR 2: A Practical Approach (M.J. MacPherson, B.D. Hames and G.R. Taylor eds. (1995)).

[0053] A gene delivery vehicle is any molecule that can carry inserted polynucleotides into a host cell. Examples of gene delivery vehicles are liposomes, biocompatible polymers, including natural polymers and synthetic polymers; lipoproteins; polypeptides; polysaccharides; lipopolysaccharides; artificial viral envelopes; metal particles; and bacteria, viruses, such as baculovirus, adenovirus and retrovirus, bacteriophage, cosmid, plasmid, fungal vectors and other recombination vehicles typically used in the art which have been described for expression in a variety of eukaryotic and prokaryotic hosts, and may be used for gene therapy as well as for simple protein expression.

[0054] Gene delivery, gene transfer, and the like as used herein, are terms referring to the introduction of an exogenous polynucleotide (sometimes referred to as a "transgene") into a host cell, irrespective of the method used for the introduction. Such methods include a variety of well-known techniques such as vector-mediated gene transfer (by, *e.g.*, viral infection/transfection, or various other protein-based or lipid-based gene delivery complexes) as well as techniques facilitating the delivery of "naked" polynucleotides (such as electroporation, "gene gun" delivery and various other techniques used for the introduction of polynucleotides). The introduced polynucleotide may be stably or transiently maintained in the host cell. Stable maintenance typically requires that the introduced polynucleotide either contains an origin of replication compatible with the host cell or integrates into a replicon of the host cell such as an extrachromosomal replicon (*e.g.*, a plasmid) or a nuclear or mitochondrial chromosome. A number of vectors are known to be capable of mediating transfer of genes to mammalian cells, as is known in the art and described herein.

[0055] The exogenous polynucleotide is inserted into a vector such as adenovirus, partially-deleted adenovirus, fully-deleted adenovirus, adeno-associated virus (AAV), retrovirus, lentivirus, naked plasmid, plasmid/liposome complex, etc. for delivery to the host via intravenous, intramuscular, intraportal or other route of administration. Expression vectors which can be used in the methods and compositions of the present disclosure, e.g. in the compositions of the present invention, include, for example, viral vectors. One of the most frequently used methods of administration of gene therapy, both *in vivo* and *ex vivo*, is the use of viral vectors for delivery of the gene. Many species of virus are known, and many have been studied for gene therapy purposes. The most commonly used viral vectors include those derived from adenoviruses, adeno-associated viruses (AAV) and retroviruses, including lentiviruses, such as human immunodeficiency virus (HIV).

[0056] Adenovirus is a non-enveloped, nuclear DNA virus with a genome of about 36 kb, which has been well-characterized through studies in classical genetics and molecular biology (Hurwitz, M.S., Adenoviruses Virology, 3rd edition, Fields et al., eds., Raven Press, New York, 1996; Hitt, M.M. et al., Adenovirus Vectors, The Development of Human Gene Therapy, Friedman, T. ed., Cold Spring Harbor Laboratory Press, New York 1999). The viral genes are classified into early (designated E1-E4) and late (designated L1-L5) transcriptional units, referring to the generation of two temporal

classes of viral proteins. The demarcation of these events is viral DNA replication. The human adenoviruses are divided into numerous serotypes (approximately 47, numbered accordingly and classified into 6 groups: A, B, C, D, E and F), based upon properties including hemaglutination of red blood cells, oncogenicity, DNA and protein amino acid compositions and homologies, and antigenic relationships.

[0057] Recombinant adenoviral vectors have several advantages for use as gene delivery vehicles, including tropism for both dividing and non-dividing cells, minimal pathogenic potential, ability to replicate to high titer for preparation of vector stocks, and the potential to carry large inserts (Berkner, K.L., Curr. Top. Micro. Immunol. 158:39-66, 1992; Jolly, D., Cancer Gene Therapy 1:51-64 1994). Adenoviral vectors with deletions of various adenoviral gene sequences, such as pseudoadenoviral vectors (PAVs) and partially-deleted adenoviral (termed "DeAd"), have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for delivery of nucleic acids to recipient cells.

[0058] In particular, pseudoadenoviral vectors (PAVs), also known as 'gutless adenovirus' or mini-adenoviral vectors, are adenoviral vectors derived from the genome of an adenovirus that contain minimal *cis*-acting nucleotide sequences required for the replication and packaging of the vector genome and which can contain one or more transgenes (*See,* U.S. Patent No. 5,882,877 which covers pseudoadenoviral vectors (PAV) and methods for producing PAV). PAVs have been designed to take advantage of the desirable features of adenovirus which render it a suitable vehicle for gene delivery. While adenoviral vectors can generally carry inserts of up to 8kb in size by the deletion of regions which are dispensable for viral growth, maximal carrying capacity can be achieved with the use of adenoviral vectors containing deletions of most viral coding sequences, including PAVs. *See* U.S. Patent No. 5,882,877 of Gregory et al.; Kochanek et al., Proc. Natl. Acad. Sci. USA 93:5731-5736, 1996; Parks et al., Proc. Natl. Acad. Sci. USA 93:13565-13570, 1996; Lieber et al., J. Viral. 70:8944-8960, 1996; Fisher et al., Virology 217:11-22, 1996; U.S. Patent No. 5,670,488; PCT Publication No. WO96/33280, published October 24, 1996; PCT Publication No. WO96/40955, published December 19, 1996; PCT Publication No. WO97/25446, published July 19, 1997; PCT Publication No. WO95/29993, published November 9, 1995; PCT Publication No. WO97/00326, published January 3, 1997; Morral et al., Hum. Gene Ther. 10: 2709-2716,1998. Such PAVs, which can accommodate up to about 36 kb of foreign nucleic acid, are advantageous because the carrying capacity of the vector is optimized, while the potential for host immune responses to the vector or the generation of replication-competent viruses is reduced. PAV vectors contain the 5' inverted terminal repeat (ITR) and the 3' ITR nucleotide sequences that contain the origin of replication, and the cis-acting nucleotide sequence required for packaging of the PAV genome, and can accommodate one or more transgenes with appropriate regulatory elements, *e.g.* promoter, enhancers, etc.

[0059] Other, partially deleted adenoviral vectors provide a partially-deleted adenoviral (termed "DeAd") vector in which the majority of adenoviral early genes required for virus replication are deleted from the vector and placed within a producer cell chromosome under the control of a conditional promoter. The deletable adenoviral genes that are placed in the producer cell may include E1A/E1B, E2, E4 (only ORF6 and ORF6/7 need be placed into the cell), pIX and pIVa2. E3 may also be deleted from the vector, but since it is not required for vector production, it can be omitted from the producer cell. The adenoviral late genes, normally under the control of the major late promoter (MLP), are present in the vector, but the MLP may be replaced by a conditional promoter.

[0060] Conditional promoters suitable for use in DeAd vectors and producer cell lines include those with the following characteristics: low basal expression in the uninduced state, such that cytotoxic or cytostatic adenovirus genes are not expressed at levels harmful to the cell; and high level expression in the induced state, such that sufficient amounts of viral proteins are produced to support vector replication and assembly. Preferred conditional promoters suitable for use in DeAd vectors and producer cell lines include the dimerizer gene control system, based on the immunosuppressive agents FK506 and rapamycin, the ecdysone gene control system and the tetracycline gene control system. Also useful in the present disclosure, including the present invention may be the GeneSwitch™ technology (Valentis, Inc., Woodlands, TX) described in Abruzzese et al., Hum. Gene Ther. 1999 10:1499-507. The partially deleted adenoviral expression system is further described in WO99/57296.

[0061] Adeno-associated virus (AAV) is a single-stranded human DNA parvovirus whose genome has a size of 4.6 kb. The AAV genome contains two major genes: the rep gene, which codes for the rep proteins (Rep 78, Rep 68, Rep 52, and Rep 40) that are involved in AAV replication, rescue, transcription and integration; and the cap gene, which codes for the cap proteins that form the AAV viral particle. AAV derives its name from its dependence on an adenovirus or other helper virus (*e.g.*, herpesvirus) to supply essential gene products that allow AAV to undergo a productive infection, i.e., reproduce itself in the host cell. In the absence of helper virus, AAV integrates as a provirus into the host cell's chromosome, until it is rescued by superinfection of the host cell with a helper virus, usually adenovirus (Muzyczka, Curr. Top. Micor. Immunol. 158:97-127,1992).

[0062] Interest in AAV as a gene transfer vector results from several unique features of its biology. At both ends of the AAV genome is a nucleotide sequence known as an inverted terminal repeat (ITR), which contains the cis-acting nucleotide sequences required for virus replication, rescue, packaging and integration. The integration function of the ITR mediated by the rep protein in trans permits the AAV genome to integrate into a cellular chromosome after infection,

in the absence of helper virus. This unique property of the virus has relevance to the use of AAV in gene transfer, as it allows for the integration of a recombinant AAV containing a gene of interest into the cellular genome. Therefore, stable genetic transformation, ideal for many of the goals of gene transfer, may be achieved by use of rAAV vectors. Furthermore, the site of integration for AAV is well-established and has been localized to chromosome 19 of humans (Kotin et al., Proc. Natl. Acad. Sci. 87:2211-2215, 1990). This predictability of integration site reduces the danger of random insertional events into the cellular genome that may activate or inactivate host genes or interrupt coding sequences, consequences that can limit the use of vectors whose integration of AAV, removal of this gene in the design of rAAV vectors may result in the altered integration patterns that have been observed with rAAV vectors (Ponnazhagan et al., Hum Gene Ther. 8: 275-284,1997).

[0063] There are other advantages to the use of AAV for gene transfer. The host range of AAV is broad. Moreover, unlike retroviruses, AAV can infect both quiescent and dividing cells. In addition, AAV has not been associated with human disease, obviating many of the concerns that have been raised with retrovirus-derived gene transfer vectors.

[0064] Standard approaches to the generation of recombinant rAAV vectors have required the coordination of a series of intracellular events: transfection of the host cell with an rAAV vector genome containing a transgene of interest flanked by the AAV ITR sequences, transfection of the host cell by a plasmid encoding the genes for the AAV rep and cap proteins which are required in trans, and infection of the transfected cell with a helper virus to supply the non-AAV helper functions required in trans (Muzyczka, N., Curr. Top. Micor. Immunol. 158:97-129, 1992). The adenoviral (or other helper virus) proteins activate transcription of the AAV rep gene, and the rep proteins then activate transcription of the AAV cap genes. The cap proteins then utilize the ITR sequences to package the rAAV genome into an rAAV viral particle. Therefore, the efficiency of packaging is determined, in part, by the availability of adequate amounts of the structural proteins, as well as the accessibility of any cis-acting packaging sequences required in the rAAV vector genome.

[0065] Retrovirus vectors are a common tool for gene delivery (Miller, Nature (1992) 357:455-460). The ability of retrovirus vectors to deliver an unrearranged, single copy gene into a broad range of rodent, primate and human somatic cells makes retroviral vectors well suited for transferring genes to a cell.

[0066] Retroviruses are RNA viruses wherein the viral genome is RNA. When a host cell is infected with a retrovirus, the genomic RNA is reverse transcribed into a DNA intermediate which is integrated very efficiently into the chromosomal DNA of infected cells. This integrated DNA intermediate is referred to as a provirus. Transcription of the provirus and assembly into infectious virus occurs in the presence of an appropriate helper virus or in a cell line containing appropriate sequences enabling encapsidation without coincident production of a contaminating helper virus. A helper virus is not required for the production of the recombinant retrovirus if the sequences for encapsidation are provided by co-transfection with appropriate vectors.

[0067] The retroviral genome and the proviral DNA have three genes: the gag, the pol, and the env, which are flanked by two long terminal repeat (LTR) sequences. The gag gene encodes the internal structural (matrix, capsid, and nucleocapsid) proteins; the po1 gene encodes the RNA-directed DNA polymerase (reverse transcriptase) and the env gene encodes viral envelope glycoproteins. The 5' and 3' LTRs serve to promote transcription and polyadenylation of the virion RNAs. The LTR contains all other cis-acting sequences necessary for viral replication. Lentiviruses have additional genes including vit vpr, tat, rev, vpu, nef, and vpx (in HIV-1, HIV-2 and/or SIV). Adjacent to the 5' LTR are sequences necessary for reverse transcription of the genome (the tRNA primer binding site) and for efficient encapsidation of viral RNA into particles (the Psi site). If the sequences necessary for encapsidation (or packaging of retroviral RNA into infectious virions) are missing from the viral genome, the result is a cis defect which prevents encapsidation of genomic RNA. However, the resulting mutant is still capable of directing the synthesis of all varion proteins.

[0068] Lentiviruses are complex retroviruses which, in addition to the common retroviral genes gag, po1 and env, contain other genes with regulatory or structural function. The higher complexity enables the lentivirus to modulate the life cycle thereof, as in the course of latent infection. A typical lentivirus is the human immunodeficiency virus (HIV), the etiologic agent of AIDS. *In* vivo, HIV can infect terminally differentiated cells that rarely divide, such as lymphocytes and macrophages. In vitro, HIV can infect primary cultures of monocyte-derived macrophages (MDM) as well as HeLa-Cd4 or T lymphoid cells arrested in the cell cycle by treatment with aphidicolin or gamma irradiation. Infection of cells is dependent on the active nuclear import of HIV preintegration complexes through the nuclear pores of the target cells. That occurs by the interaction of multiple, partly redundant, molecular determinants in the complex with the nuclear import machinery of the target cell. Identified determinants include a functional nuclear localization signal (NLS) in the gag matrix (MA) protein, the karyophilic virion-associated protein, vpr, and a C-terminal phosphotyrosine residue in the gag MA protein. The use of retroviruses for gene therapy is described, for example, in United States Patent 6,013,516; and U.S. Patent 5,994,136.

[0069] Other methods for delivery of DNA to cells do not use viruses for delivery. For example, cationic amphiphilic compounds can be used to deliver the nucleic acid of the present disclosure, e.g. the nucleic acid of the present invention. Because compounds designed to facilitate intracellular delivery of biologically active molecules must interact with both non-polar and polar environments (in or on, for example, the plasma membrane, tissue fluids, compartments within the cell, and the biologically active molecular itself), such compounds are designed typically to contain both polar and non-

polar domains. Compounds having both such domains may be termed amphiphiles, and many lipids and synthetic lipids that have been disclosed for use in facilitating such intracellular delivery (whether for *in vitro* or *in vivo* application) meet this definition. One particularly important class of such amphiphiles is the cationic amphiphiles. In general, cationic amphiphiles have polar groups that are capable of being positively charged at or around physiological pH, and this property is understood in the art to be important in defining how the amphiphiles interact with the many types of biologically active (therapeutic) molecules including, for example, negatively charged polynucleotides such as DNA.

[0070] The use of compositions comprising cationic amphiphilic compounds for gene delivery is described, for example, in United States Patent 5,049,386; US 5,279,833; US 5,650,096; US 5,747,471; US 5,767,099; US 5,910,487; US 5,719,131; US 5,840,710; US 5,783,565; US 5,925,628; US 5,912,239; US 5,942,634; US 5,948,925; US 6,022,874; U.S. 5,994,317; U.S. 5,861,397; U.S. 5,952,916; U.S. 5,948,767; U.S. 5,939,401; and U.S. 5,935,936.

[0071] In addition, nucleic acid of the present disclosure, e.g. nucleic acid of the present invention, can be delivered using "naked DNA". Methods for delivering a non-infectious, non-integrating DNA sequence encoding a desired polypeptide or peptide operably linked to a promoter, free from association with transfection-facilitating proteins, viral particles, liposomal formulations, charged lipids and calcium phosphate precipitating agents are described in U.S. Patent 5,580,859; U.S. 5,963,622; U.S. 5,910,488.

[0072] Gene transfer systems that combine viral and nonviral components have also been reported. Cristiano et al., (1993) Proc. Natl. Acad Sci. USA 90:11548; Wu et al. (1994) J. Biol. Chem. 269:11542; Wagner et al. (1992) Proc. Natl. Acad Sci. USA 89:6099; Yoshimura et al. (1993) J. Biol. Chem. 268:2300; Curiel et al. (1991) Proc. Natl. Acad. Sci. USA 88:8850; Kupfer et al. (1994) Human Gene Ther. 5:1437; and Gottschalk et al. (1994) Gene Ther. 1:185. In most cases, adenovirus has been incorporated into the gene delivery systems to take advantage of its endosomolytic properties. The reported combinations of viral and nonviral components generally involve either covalent attachment of the adenovirus to a gene delivery complex or co-internalization of unbound adenovirus with cationic lipid: DNA complexes.

[0073] For delivery of DNA and protein to the eye, administration will typically be local. This has the advantage of limiting the amount of DNA that needs to be administered and limiting systemic side-effects. Many possible modes of delivery can be used, including, but not limited to: topical administration on the cornea by a gene gun; subconjunctival injection, intracameral injection, via eye drops to the cornea, injection into the anterior chamber via the temporal limbus, intrastromal injection, corneal application combined with electrical pulses, intracorneal injection, subretinal injection, intravitreal injection, and intraocular injection. Alternatively cells can be transfected or transduced *ex vivo* and delivered by intraocular implantation. See, Auricchio, Mol. Ther. 6: 490-494, 2002; Bennett, Nature Med. 2: 649-654, 1996; Borrás, Experimental Eye Research 76: 643-652, 2003; Chaum, Survey of Ophthalmology 47: 449-469, 2002; Campochiaro. Expert Opinions in Biological Therapy 2: 537-544 (2002); Lai, Gene Therapy 9. 804 813, 2002; Pleyer, Progress in Retinal and Eye Research, 22: 277-293, 2003.

[0074] The effects of various proposed therapeutic agents and administrations can be tested in suitable animal models for particular diseases. For example, retinopathy of prematurity can be tested in an oxygen-induced retinopathy model in the mouse as described in Smith, Investigative Ophthalmology & Visual Science, 35: 101-111, 1994. Laser-induced choroidal neovascularization in a mouse can be used as a model for human choroidal neovascularization (CNV), which occurs in diseases such as age-related macular degeneration. Tobe, American Journal of Pathology 153: 1641-1646, 1998. Other models of CNV have been developed in primates, rats, minipigs, and rabbits. Mouse models of age-related macular degeneration have been developed in genetically-deficient mice. Mice deficient in either monocyte chemoattractant protein-1 or C-C chemokine receptor-2 develop features of age-related macular degeneration. Ambati, Nature Med. 9: 1390-1397, 2003.

[0075] While the invention has been described with respect to specific examples including presently preferred modes of carrying out the invention, those skilled in the art will appreciate that there are numerous variations and permutations of the above described systems and techniques that fall within the scope of the appended claims.

EXAMPLES

Example 1

[0076] Two constructs were generated: the first, D2-9Gly-Fc, containing a polyglycine 9-mer (9Gly) linker and the second, D2-Fc, with the same sequence except the 9Gly linker (Fig. 1).

[0077] We analyzed the amino acid sequences of D2-9Gly-Fc and D2-Fc proteins using the Protein Analysis Toolbox of the sequence analysis program Mac Vector 6.5.1. (IBI, New Haven, CT). The polyglycine 9-mer linker in the D2-9Gly-Fc sequence was identified as a region with higher than average flexibility by the flexibility prediction method of Karpus and Schultz (1985) Naturwiss, 72: 212-213. No such region was detected in the D2-Fc sequence (Fig. 1).

Example 2

[0078] We tested an isolated Flt-1 Ig-like domain 2 connected to the IgG1 Fc region by a flexible polyglycine 9-mer linker (D2-9Gly-Fc). The D2-9Gly-Fc fusion protein is capable of efficiently binding VEGF and of inhibiting VEGF-dependent human umbilical vein endothelial cell (HUVEC) proliferation. See Fig. 2. In contrast, when Flt-1 Ig-like domain 2 is linked directly to the IgG1 heavy chain (Fc) to form D2-Fc, only minimal VEGF binding was observed. See Fig. 2. Both the dimerization via IgG1 Fc and the insertion of a flexible linker appear to facilitate VEGF binding to Flt 1 domain 2. The presence of dimeric forms in both D2-9Gly-Fc and D2-Fc were confirmed by the Western blot analysis. See Fig. 3.

Example 3

[0079] An intravitreal injection of AAV vector (1×10^8 to 1×10^9 particles in a volume of 0.0005 mL) is administered to newborn (P0) or 1 day old (P1) C57BL/6 mice. Retinal neovascularization (NV) is induced in C57BL/6 mice by exposing P7 pups and their nursing dam to hyperoxia for 5 days. The pups are returned to room air on P12 and are euthanized at P17 (time of peak NV). (Smith LEH, Weslowski E, McLellan A, Kostyk SK, D'Amato R, Sullivan and D'Amore PA. Oxygen-Induced Retinopathy in the Mouse. Invest Opth Vis Sci. 1994;35:101-111.) Entire paraffin embedded eyes are serially cross sectioned at 5 micron intervals. The degree of NV is determined by counting the number of endothelial cell nuclei internal to the inner limiting membrane in sections taken every 100 microns.

[0080] Cohorts of animals treated with the AAV vectors coding for the anti-angiogenic agents are compared to cohorts treated with vectors coding for irrelevant transgenes or with vectors that do not code for a transgene. The average number of endothelial cell nuclei in each treated eye is compared to each animal's untreated fellow eye.

Example 4

Generation of D2-9Gly-Ex3/CH3

[0081] Domain 2 of Flt-1 has been shown to be essential for $VEGF_{165}$ binding. However, it was demonstrated that Flit-1 domain 2 alone was incapable of binding VEGF A. (Davis-Smyth et al., 1996.) VEGF A, when present as a dimer, binds to Flt-1 through acidic residues (amino acids 63-67 of the mature protein) that allows a possible mechanism for ligand-induced dimerization of receptor (Keyt et al., 1996).

[0082] Therefore, a dimerization of domain 2 of Flt-1 was used as a strategy to restore the binding of domain 2 of Flt-1 to VEGF A. Fusion with a fragment of IgG heavy chain can be used for dimerization of proteins (Davis-Smyth et al., 1996). Here we demonstrate that amino acids 75-88 (*i.e.*, PSCVPLMRCGGCCN; SEQ ID NO: 13) of VEGF A (SEQ ID NO: 12) increase the biological activity of sFlt-1 hybrid proteins.

[0083] Initially, three hybrid proteins were engineered: D2-9Gly-Fc, D2-Fc and D2-9Gly-Ex3/CH3 (Fig. 4). All three hybrid proteins contain the same Flt-1 domain D2 as D2-9Gly-Fc. No VEGF binding was observed with D2-Fc, which does not contain the polyglycine 9-mer (9Gly) linker. The third protein, D2-9Gly-Ex3/CH3, contains the polyglycine 9-mer (9Gly) linker and the multimerization domain of VEGF (aa PSCVPLMRCGGCCN; SEQ ID NO: 13; VEGF Ex3), but it also contains the CH3 region of human IgG1 heavy chain Fc (aa 371-477 of the SEQ ID NO: 10).

[0084] The protein D2-Fc did not show efficient inhibitory activity in the HUVEC proliferation assay (Fig. 5) and by implication did not bind to $VEGF_{165}$ efficiently. However, the third hybrid protein, D2-9Gly-Ex3/CH3, which comprises domain 2 of Flt-1 fused to the CH3 region via both the 9Gly linker and the dimerization region of VEGF165 (Ex 3), did demonstrate inhibitory activity in a VEGF-dependent HUVECs proliferation assay (Fig. 5). This implies that this hybrid protein binds to $VEGF_{165}$ efficiently.

Example 5

Using linker $(Gly_4Ser)_3$ in Flt-1 D2 construct

[0085] The use of several polyglycine linkers has been previously described for improvement of protein features (Mouz et al., 1996; Qiu et aL, 1998). For the next construct we have used another type of linker, the 15-mer (Gly-Gly-Gly-Gly-Ser)3 (Huston et aL, 1988). D2-$(Gly_4Ser)_3$-Fc protein was generated and it contains Flt-1 domain 2, $(Gly_4Ser)_3$ linker and the Fc region of human IgG1 heavy chain..

[0086] D2-$(Gly_4Ser)_3$ was further characterized in HUVECs proliferation assay. Biological activity of D2-$(Gly_4Ser)_3$-Fc as measured by inhibition of HUVEC proliferation was similar to that of D2-9Gly-Fc and D2-9Gly-Ex3/CH3 (Fig. 6).

[0087] The D2-$(Gly_4Ser)_3$-Fc construct was further characterized by Western blot and compared to D2-9Gly-Fc (Fig. 9). Both constructs are present mostly in a dimer form and the monomer forms were detected after separation of reduced samples.

Example 6

Role of 9Gly or VEGF Ex3 in Flt-1 (D2) constructs

[0088] In order to investigate the role of 9Gly linker or VEGF dimerizing sequence Ex3 on soluble receptor VEGF binding, three other constructs were generated: D2-9Gly-CH3, D2-CH3 and D2-Ex3/CH3 (Fig. 8). All three constructs were generated and like all the previous constructs were also put under control of CMV promoter. Their VEGF blocking activity was assessed in HUVECs proliferation assay (Fig. 9).

[0089] The HUVEC proliferation assay of proteins containg the CH3 region of IgG1 has shown that D2-9Gly-CH3 (without Ex3) and protein D2-Ex3/CH3 (without 9Gly linker) had similar VEGF blocking potency as compared to the parental D2-9Gly-Ex3/CH3 protein. However, protein D2-CH3 appeared to be the weakest VEGF inhibitor from all of them (Fig. 9).

[0090] The Flt-1 ELISA data of conditioned media from transfected 293 cells has shown similar Flt 1 levels for D2-9Gly-Ex3/CH3, D2-9Gly-CH3 and D2-Ex3/CH3 and D2-CH3 (70-90 ng/ml) and a little higher (~150 ng/ml) for the least active form of D2-CH3. Western blot of D2-9Gly-CH3 and D2-CH3 constructs (Fig.10) is showing a prevalence of dimer forms in non-reduced conditions.

Example 7

D2-9Gly-Fc binds VEGF better than all constructs

[0091] VEGF binding assay allows us to compare the relative VEGF binding affinities of our soluble VEGF receptors in a cell free system.

[0092] Briefly, conditioned media containing known concentrations of soluble receptor (ranging in concentrations from 0.29 - 150 pM) were serially diluted and mixed with 10 pM VEGF. The amount of unbound VEGF was then measured by ELISA. D2-9Gly-Fc binds VEGF with higher affinity at receptor concentrations from 0.001 to ~ 0.2 pM than all other constructs. D2-CH3 has the lowest affinity to bind VEGF (Fig. 11).

References

[0093]

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Table of sequences

SEQ ID NO	Clone Name	Length	Type
1	FLT1D29GLYFC	1077	DNA
2	FLT1D29GLYFC	358	Protein
3	FLT1D2DEL9GLYFC	1050	DNA
4	FLT1D2DEL9GLYFC	349	Protein
5	FLT1D29GLYEX3	426	DNA
6	FLT1D29GLYEX3	141	Protein
7	FLTID29GLYEX3CH3	744	DNA
8	FLT1D29GLYEX3CH3	247	Protein
9	IGg1 HEAVY	1434	DNA

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SEQ ID NO	Clone Name	Length	Type
10	IgG1 HEAVY	477	Protein
11	VEGF	648	DNA
12	VEGF	215	Protein
13	VEGF exon 3 (EX3)	14	Protein
14	FLT-1	5777	DNA
15	FLT-1	1338	Protein
16	KDR	5830	DNA
17	KDR	1356	Protein
18	D2-CH3	675	DNA
19	D2-CH3	224	Protein
20	D2-EX3-CH3	717	DNA
21	D2-EX3-CH3	238	Protein
22	D2-9GLY-CH3	702	DNA
23	D2-9GLY-CH3	233	Protein
24	D2(G4S)3-FC	1095	DNA
25	D2(G4S)3-FC	364	Protein
26	random linker	9	Protein
27	linker	9	Protein
28	linker	9	Protein
29	linker	9	Protein
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31	linker	7	Protein
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Pechan, Peter
Wadsworth, Samuel

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gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccggtgga     360
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gaccctgagg tcaagttcaa ctggtacgtg gacggcgtgg aggtgcataa tgccaagaca     600
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caccaggact ggctgaatgg caaggagtac aagtgcaagg tctccaacaa agccctccca     720
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ctcaccgtgg acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat    1020
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            20                  25                  30
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile
        35                  40                  45
Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe
    50                  55                  60
Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser
65                  70                  75                  80
Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu
                85                  90                  95
Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr
            100                 105                 110
Leu Thr His Arg Gln Thr Gly Gly Gly Gly Gly Gly Gly Gly Gly Pro
        115                 120                 125
Lys Ser Cys Asp Lys Thr His Thr Cys Pro Pro Cys Pro Ala Pro Glu
    130                 135                 140
Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro Lys Pro Lys Asp
145                 150                 155                 160
Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys Val Val Val Asp
                165                 170                 175
Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp Tyr Val Asp Gly
            180                 185                 190
Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu Glu Gln Tyr Asn
        195                 200                 205
Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu His Gln Asp Trp
    210                 215                 220
Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn Lys Ala Leu Pro
225                 230                 235                 240
Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly Gln Pro Arg Glu
                245                 250                 255
Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn
            260                 265                 270
Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile
        275                 280                 285
Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr
    290                 295                 300
Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys
305                 310                 315                 320
Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser Cys
                325                 330                 335
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actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact     180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt     240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa     300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aacccctaaa     360
tcttgtgaca aaactcacac atgcccaccg tgcccagcac ctgaactcct gggggggaccg     420
tcagtcttcc tcttcccccc aaaacccaag gacaccctca tgatctcccg gacccctgag     480
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gtcacatgcg tggtggtgga cgtgagccac gaagaccctg aggtcaagtt caactggtac 540
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acgtaccgtg tggtcagcgt cctcaccgtc ctgcaccagg actggctgaa tggcaaggag 660
tacaagtgca aggtctccaa caaagccctc ccagccccca tcgagaaaac catctccaaa 720
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gactccgacg gctccttctt cctctacagc aagctcaccg tggacaagag caggtggcag 960
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Cys Leu Leu Leu Thr Gly Ser Gly Arg Pro Phe Val Glu Met Tyr Ser
            20                  25                  30
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile
        35                  40                  45
Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe
    50                  55                  60
Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser
65                  70                  75                  80
Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu
                85                  90                  95
Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr
            100                 105                 110
Leu Thr His Arg Gln Thr Pro Lys Ser Cys Asp Lys Thr His Thr Cys
        115                 120                 125
Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu
    130                 135                 140
Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
145                 150                 155                 160
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
                165                 170                 175
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
            180                 185                 190
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
        195                 200                 205
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
    210                 215                 220
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
225                 230                 235                 240
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
                245                 250                 255
Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
            260                 265                 270
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
        275                 280                 285
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
    290                 295                 300
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
305                 310                 315                 320
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
                325                 330                 335

His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
            340                 345
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<211> 426
<212> DNA
<213> Artificial Sequence

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<223> Recombinant fusion protein or sequence encoding same

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actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact     180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt     240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa     300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccggtgga     360
ggtggaggtg gaggtggagg tccttcctgt gtgcccctga tgcgatgcgg gggctgctgc     420
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Cys Leu Leu Leu Thr Gly Ser Gly Arg Pro Phe Val Glu Met Tyr Ser
            20                  25                  30
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile
        35                  40                  45
Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe
    50                  55                  60
Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser
65                  70                  75                  80
Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu
                85                  90                  95
Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr
            100                 105                 110
Leu Thr His Arg Gln Thr Gly Gly Gly Gly Gly Gly Gly Gly Gly Pro
        115                 120                 125
Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn
    130                 135                 140
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<223> Recombinant fusion protein or sequence encoding same

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actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact     180
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agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa     300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccggtgga     360
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            20                  25                  30
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile
        35                  40                  45
Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe
    50                  55                  60
Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser
65                  70                  75                  80
Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu
                85                  90                  95
Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr
            100                 105                 110
Leu Thr His Arg Gln Thr Gly Gly Gly Gly Gly Gly Gly Gly Gly Pro
        115                 120                 125
Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn Gln Pro Arg
    130                 135                 140
Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu Leu Thr Lys
145                 150                 155                 160
Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr Pro Ser Asp
                165                 170                 175
Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn Asn Tyr Lys
            180                 185                 190
Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe Leu Tyr Ser
        195                 200                 205
Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn Val Phe Ser
    210                 215                 220
Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr Gln Lys Ser
225                 230                 235                 240
Leu Ser Leu Ser Pro Gly Lys
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<212> DNA
<213> Homo sapiens

<400> 9

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gtggacaaga gagttgagcc caaatcttgt gacaaaactc acacatgccc accgtgccca     780
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cctgaggtca agttcaactg gtacgtggac ggcgtggagg tgcataatgc caagacaaag     960
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accgtggaca agagcaggtg gcagcagggg aacgtcttct catgctccgt gatgcatgag    1380
gctctgcaca accactacac gcagaagagc ctctccctgt ccccgggtaa atga          1434
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<213> Homo sapiens

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

Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu
        275                 280                 285
Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys
    290                 295                 300
Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys
305                 310                 315                 320
Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu
                325                 330                 335
Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys
            340                 345                 350
Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys
        355                 360                 365
Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser
    370                 375                 380
Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys
385                 390                 395                 400
Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln
                405                 410                 415
Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly
            420                 425                 430
Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln
        435                 440                 445
Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn
    450                 455                 460
His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
465                 470                 475
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<210> 11
<211> 648

<212> DNA
<213> Homo sapiens

<400> 11

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gtgaagttca tggatgtcta tcagcgcagc tactgccatc caatcgagac cctggtggac     180
atcttccagg agtaccctga tgagatcgag tacatcttca agccatcctg tgtgcccctg     240
atgcgatgcg ggggctgctg caatgacgag ggcctggagt gtgtgcccac tgaggagtcc     300
aacatcacca tgcagattat gcggatcaaa cctcaccaag gccagcacat aggagagatg     360
agcttcctac agcacaacaa atgtgaatgc agaccaaaga aagatagagc aagacaagaa     420
aaaaaatcag ttcgaggaaa gggaaagggg caaaaacgaa agcgcaagaa atcccggtat     480
aagtcctgga gcgttccctg tgggccttgc tcagagcgga gaaagcattt gtttgtacaa     540
gatccgcaga cgtgtaaatg ttcctgcaaa aacacagact cgcgttgcaa ggcgaggcag     600
cttgagttaa acgaacgtac ttgcagatgt gacaagccga ggcggtga                  648
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<210> 12
<211> 215
<212> PRT
<213> Homo sapiens

<400> 12

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

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
            100                 105                 110
Gln Gly Gln His Ile Gly Glu Met Ser Phe Leu Gln His Asn Lys Cys
        115                 120                 125
Glu Cys Arg Pro Lys Lys Asp Arg Ala Arg Gln Glu Lys Lys Ser Val
    130                 135                 140
Arg Gly Lys Gly Lys Gly Gln Lys Arg Lys Arg Lys Lys Ser Arg Tyr
145                 150                 155                 160
Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His
                165                 170                 175
Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr
            180                 185                 190
Asp Ser Arg Cys Lys Ala Arg Gln Leu Glu Leu Asn Glu Arg Thr Cys
        195                 200                 205
Arg Cys Asp Lys Pro Arg Arg
    210                 215
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gcatcgcgaa agtgtatcca cagggacctg gcggcacgaa atatcctctt atcggagaag    3420
aacgtggtta aaatctgtga ctttggcttg gcccgggata tttataaaga tccagattat    3480
gtcagaaaag gagatgctcg cctccctttg aaatggatgg ccccagaaac aatttttgac    3540
```

```
agagtgtaca caatccagag tgacgtctgg tcttttggtg ttttgctgtg ggaaatattt  3600
tccttaggtg cttctccata tcctggggta aagattgatg aagaattttg taggcgattg  3660
aaagaaggaa ctagaatgag ggcccctgat tatactacac cagaaatgta ccagaccatg  3720
ctggactgct ggcacgggga gcccagtcag agacccacgt tttcagagtt ggtggaacat  3780
ttgggaaatc tcttgcaagc taatgctcag caggatggca aagactacat tgttcttccg  3840
atatcagaga ctttgagcat ggaagaggat tctggactct ctctgcctac ctcacctgtt  3900
tcctgtatgg aggaggagga agtatgtgac cccaaattcc attatgacaa cacagcagga  3960
atcagtcagt atctgcagaa cagtaagcga aagagccggc ctgtgagtgt aaaaacattt  4020
gaagatatcc cgttagaaga accagaagta aaagtaatcc cagatgacaa ccagacggac  4080
agtggtatgg ttcttgcctc agaagagctg aaaactttgg aagacagaac caaattatct  4140
ccatcttttg gtggaatggt gcccagcaaa agcagggagt ctgtggcatc tgaaggctca  4200
aaccagacaa gcggctacca gtccggatat cactccgatg acacagacac caccgtgtac  4260
tccagtgagg aagcagaact tttaaagctg atagagattg gagtgcaaac cggtagcaca  4320
gcccagattc tccagcctga ctcggggacc acactgagct ctcctcctgt ttaaaaggaa  4380
gcatccacac cccaactccc ggacatcaca tgagaggtct gctcagattt tgaagtgttg  4440
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ctcggactgc agggagccag tcttctaggc atatcctgga agaggcttgt gacccaagaa  4560
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cggggtttct ggttttagaa ggttgcgtgt tcttcgagtt gggctaaagt agagttcgtt  5040
gtgctgtttc tgactcctaa tgagagttcc ttccagaccg ttagctgtct ccttgccaag  5100
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gcacaaacca gcttctggtt tcttctggaa tgaataccct catatctgtc ctgatgtgat  5280
atgtctgaga ctgaatgcgg gaggttcaat gtgaagctgt gtgtggtgtc aaagtttcag  5340
gaaggatttt acccttttgt tcttcccccc gtccccaacc cactctcacc ccgcaaccca  5400
tcagtatttt agttatttgg cctctactcc agtaaacctg attgggtttg ttcactctct  5460
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ttatttagac ttttaacata tagagctatt tctactgatt tttgcccttg ttctgtcctt  5580
tttttcaaaa aagaaaatgt gtttttttgtt tggtaccata gtgtgaaatg ctgggaacaa  5640
tgactataag acatgctatg gcacatatat ttatagtctg tttatgtaga aacaaatgta  5700
atatattaaa gccttatata taatgaactt tgtactattc acattttgta tcagtattat  5760
gtagcataac aaaggtcata atgctttcag caattgatgt cattttatta aagaacattg  5820
aaaaacttga                                                         5830
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<210> 17

<211> 1356

<212> PRT

<213> Homo sapiens

<400> 17

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Met Gln Ser Lys Val Leu Leu Ala Val Ala Leu Trp Leu Cys Val Glu
 1               5                  10                  15
Thr Arg Ala Ala Ser Val Gly Leu Pro Ser Val Ser Leu Asp Leu Pro
            20                  25                  30
Arg Leu Ser Ile Gln Lys Asp Ile Leu Thr Ile Lys Ala Asn Thr Thr
        35                  40                  45
Leu Gln Ile Thr Cys Arg Gly Gln Arg Asp Leu Asp Trp Leu Trp Pro
    50                  55                  60
Asn Asn Gln Ser Gly Ser Glu Gln Arg Val Glu Val Thr Glu Cys Ser
65                  70                  75                  80
Asp Gly Leu Phe Cys Lys Thr Leu Thr Ile Pro Lys Val Ile Gly Asn
                85                  90                  95
Asp Thr Gly Ala Tyr Lys Cys Phe Tyr Arg Glu Thr Asp Leu Ala Ser
            100                 105                 110
Val Ile Tyr Val Tyr Val Gln Asp Tyr Arg Ser Pro Phe Ile Ala Ser
```

```
                115                 120                 125
Val Ser Asp Gln His Gly Val Val Tyr Ile Thr Glu Asn Lys Asn Lys
    130                 135                 140
Thr Val Val Ile Pro Cys Leu Gly Ser Ile Ser Asn Leu Asn Val Ser
145                 150                 155                 160
Leu Cys Ala Arg Tyr Pro Glu Lys Arg Phe Val Pro Asp Gly Asn Arg
                165                 170                 175
Ile Ser Trp Asp Ser Lys Lys Gly Phe Thr Ile Pro Ser Tyr Met Ile
            180                 185                 190
Ser Tyr Ala Gly Met Val Phe Cys Glu Ala Lys Ile Asn Asp Glu Ser
        195                 200                 205
Tyr Gln Ser Ile Met Tyr Ile Val Val Val Val Gly Tyr Arg Ile Tyr
    210                 215                 220
Asp Val Val Leu Ser Pro Ser His Gly Ile Glu Leu Ser Val Gly Glu
225                 230                 235                 240
Lys Leu Val Leu Asn Cys Thr Ala Arg Thr Glu Leu Asn Val Gly Ile
                245                 250                 255
Asp Phe Asn Trp Glu Tyr Pro Ser Ser Lys His Gln His Lys Lys Leu
            260                 265                 270
Val Asn Arg Asp Leu Lys Thr Gln Ser Gly Ser Glu Met Lys Lys Phe
        275                 280                 285
Leu Ser Thr Leu Thr Ile Asp Gly Val Thr Arg Ser Asp Gln Gly Leu
    290                 295                 300
Tyr Thr Cys Ala Ala Ser Ser Gly Leu Met Thr Lys Lys Asn Ser Thr
305                 310                 315                 320
Phe Val Arg Val His Glu Lys Pro Phe Val Ala Phe Gly Ser Gly Met
                325                 330                 335
Glu Ser Leu Val Glu Ala Thr Val Gly Glu Arg Val Arg Ile Pro Ala
            340                 345                 350
Lys Tyr Leu Gly Tyr Pro Pro Pro Glu Ile Lys Trp Tyr Lys Asn Gly
        355                 360                 365
Ile Pro Leu Glu Ser Asn His Thr Ile Lys Ala Gly His Val Leu Thr
    370                 375                 380
Ile Met Glu Val Ser Glu Arg Asp Thr Gly Asn Tyr Thr Val Ile Leu
385                 390                 395                 400
Thr Asn Pro Ile Ser Lys Glu Lys Gln Ser His Val Val Ser Leu Val
                405                 410                 415
Val Tyr Val Pro Pro Gln Ile Gly Glu Lys Ser Leu Ile Ser Pro Val
            420                 425                 430
Asp Ser Tyr Gln Tyr Gly Thr Thr Gln Thr Leu Thr Cys Thr Val Tyr
        435                 440                 445
Ala Ile Pro Pro Pro His His Ile His Trp Tyr Trp Gln Leu Glu Glu
    450                 455                 460
Glu Cys Ala Asn Glu Pro Ser Gln Ala Val Ser Val Thr Asn Pro Tyr
465                 470                 475                 480
Pro Cys Glu Glu Trp Arg Ser Val Glu Asp Phe Gln Gly Gly Asn Lys
                485                 490                 495
Ile Glu Val Asn Lys Asn Gln Phe Ala Leu Ile Glu Gly Lys Asn Lys
            500                 505                 510
Thr Val Ser Thr Leu Val Ile Gln Ala Ala Asn Val Ser Ala Leu Tyr
        515                 520                 525
Lys Cys Glu Ala Val Asn Lys Val Gly Arg Gly Glu Arg Val Ile Ser
    530                 535                 540
Phe His Val Thr Arg Gly Pro Glu Ile Thr Leu Gln Pro Asp Met Gln
545                 550                 555                 560
Pro Thr Glu Gln Glu Ser Val Ser Leu Trp Cys Thr Ala Asp Arg Ser
                565                 570                 575
Thr Phe Glu Asn Leu Thr Trp Tyr Lys Leu Gly Pro Gln Pro Leu Pro
            580                 585                 590
Ile His Val Gly Glu Leu Pro Thr Pro Val Cys Lys Asn Leu Asp Thr
        595                 600                 605
Leu Trp Lys Leu Asn Ala Thr Met Phe Ser Asn Ser Thr Asn Asp Ile
    610                 615                 620
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Leu Ile Met Glu Leu Lys Asn Ala Ser Leu Gln Asp Gln Gly Asp Tyr
625 630 635 640
Val Cys Leu Ala Gln Asp Arg Lys Thr Lys Lys Arg His Cys Val Val
645 650 655
Arg Gln Leu Thr Val Leu Glu Arg Val Ala Pro Thr Ile Thr Gly Asn
660 665 670
Leu Glu Asn Gln Thr Thr Ser Ile Gly Glu Ser Ile Glu Val Ser Cys
675 680 685
Thr Ala Ser Gly Asn Pro Pro Pro Gln Ile Met Trp Phe Lys Asp Asn
690 695 700
Glu Thr Leu Val Glu Asp Ser Gly Ile Val Leu Lys Asp Gly Asn Arg
705 710 715 720
Asn Leu Thr Ile Arg Arg Val Arg Lys Glu Asp Glu Gly Leu Tyr Thr
725 730 735
Cys Gln Ala Cys Ser Val Leu Gly Cys Ala Lys Val Glu Ala Phe Phe
740 745 750
Ile Ile Glu Gly Ala Gln Glu Lys Thr Asn Leu Glu Ile Ile Ile Leu
755 760 765
Val Gly Thr Ala Val Ile Ala Met Phe Phe Trp Leu Leu Leu Val Ile
770 775 780
Ile Leu Arg Thr Val Lys Arg Ala Asn Gly Gly Glu Leu Lys Thr Gly
785 790 795 800
Tyr Leu Ser Ile Val Met Asp Pro Asp Glu Leu Pro Leu Asp Glu His
805 810 815
Cys Glu Arg Leu Pro Tyr Asp Ala Ser Lys Trp Glu Phe Pro Arg Asp
820 825 830
Arg Leu Lys Leu Gly Lys Pro Leu Gly Arg Gly Ala Phe Gly Gln Val
835 840 845
Ile Glu Ala Asp Ala Phe Gly Ile Asp Lys Thr Ala Thr Cys Arg Thr
850 855 860
Val Ala Val Lys Met Leu Lys Glu Gly Ala Thr His Ser Glu His Arg
865 870 875 880
Ala Leu Met Ser Glu Leu Lys Ile Leu Ile His Ile Gly His His Leu
885 890 895
Asn Val Val Asn Leu Leu Gly Ala Cys Thr Lys Pro Gly Gly Pro Leu
900 905 910
Met Val Ile Val Glu Phe Cys Lys Phe Gly Asn Leu Ser Thr Tyr Leu
915 920 925
Arg Ser Lys Arg Asn Glu Phe Val Pro Tyr Lys Thr Lys Gly Ala Arg
930 935 940
Phe Arg Gln Gly Lys Asp Tyr Val Gly Ala Ile Pro Val Asp Leu Lys
945 950 955 960
Arg Arg Leu Asp Ser Ile Thr Ser Ser Gln Ser Ser Ala Ser Ser Gly
965 970 975
Phe Val Glu Glu Lys Ser Leu Ser Asp Val Glu Glu Glu Glu Ala Pro
980 985 990
Glu Asp Leu Tyr Lys Asp Phe Leu Thr Leu Glu His Leu Ile Cys Tyr
995 1000 1005
Ser Phe Gln Val Ala Lys Gly Met Glu Phe Leu Ala Ser Arg Lys Cys
1010 1015 1020
Ile His Arg Asp Leu Ala Ala Arg Asn Ile Leu Leu Ser Glu Lys Asn
1025 1030 1035 1040
Val Val Lys Ile Cys Asp Phe Gly Leu Ala Arg Asp Ile Tyr Lys Asp
1045 1050 1055
Pro Asp Tyr Val Arg Lys Gly Asp Ala Arg Leu Pro Leu Lys Trp Met
1060 1065 1070
Ala Pro Glu Thr Ile Phe Asp Arg Val Tyr Thr Ile Gln Ser Asp Val
1075 1080 1085
Trp Ser Phe Gly Val Leu Leu Trp Glu Ile Phe Ser Leu Gly Ala Ser
1090 1095 1100
Pro Tyr Pro Gly Val Lys Ile Asp Glu Glu Phe Cys Arg Arg Leu Lys
1105 1110 1115 1120
Glu Gly Thr Arg Met Arg Ala Pro Asp Tyr Thr Thr Pro Glu Met Tyr

```
                1125                1130                1135
Gln Thr Met Leu Asp Cys Trp His Gly Glu Pro Ser Gln Arg Pro Thr
            1140                1145                1150
Phe Ser Glu Leu Val Glu His Leu Gly Asn Leu Leu Gln Ala Asn Ala
        1155                1160                1165
Gln Gln Asp Gly Lys Asp Tyr Ile Val Leu Pro Ile Ser Glu Thr Leu
    1170                1175                1180
Ser Met Glu Glu Asp Ser Gly Leu Ser Leu Pro Thr Ser Pro Val Ser
1185                1190                1195                1200
Cys Met Glu Glu Glu Glu Val Cys Asp Pro Lys Phe His Tyr Asp Asn
                1205                1210                1215
Thr Ala Gly Ile Ser Gln Tyr Leu Gln Asn Ser Lys Arg Lys Ser Arg
            1220                1225                1230
Pro Val Ser Val Lys Thr Phe Glu Asp Ile Pro Leu Glu Glu Pro Glu
        1235                1240                1245
Val Lys Val Ile Pro Asp Asp Asn Gln Thr Asp Ser Gly Met Val Leu
    1250                1255                1260
Ala Ser Glu Glu Leu Lys Thr Leu Glu Asp Arg Thr Lys Leu Ser Pro
1265                1270                1275                1280
Ser Phe Gly Gly Met Val Pro Ser Lys Ser Arg Glu Ser Val Ala Ser
                1285                1290                1295
Glu Gly Ser Asn Gln Thr Ser Gly Tyr Gln Ser Gly Tyr His Ser Asp
            1300                1305                1310
Asp Thr Asp Thr Thr Val Tyr Ser Ser Glu Glu Ala Glu Leu Leu Lys
        1315                1320                1325
Leu Ile Glu Ile Gly Val Gln Thr Gly Ser Thr Ala Gln Ile Leu Gln
    1330                1335                1340
Pro Asp Ser Gly Thr Thr Leu Ser Ser Pro Pro Val
1345                1350                1355
```

<210> 18
<211> 675
<212> DNA
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 18

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atggtcagct actgggacac cggggtcctg ctgtgcgcgc tgctcagctg tctgcttctc      60
acaggatctg gtagaccttt cgtagagatg tacagtgaaa tccccgaaat tatacacatg     120
actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact     180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt     240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa     300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aacccagccc     360
cgagaaccac aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc     420
agcctgacct gcctggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc     480
aatgggcagc cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc     540
ttcttcctct acagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc     600
tcatgctccg tgatgcatga ggctctgcac aaccactaca cgcagaagag cctctccctg     660
tctccgggta aatag                                                      675
```

<210> 19
<211> 224
<212> PRT
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 19

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Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
 1               5                  10                  15
Cys Leu Leu Leu Thr Gly Ser Gly Arg Pro Phe Val Glu Met Tyr Ser
            20                  25                  30
Glu Ile Pro Glu Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile
        35                  40                  45
Pro Cys Arg Val Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe
    50                  55                  60
Pro Leu Asp Thr Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser
65                  70                  75                  80
Arg Lys Gly Phe Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu
                85                  90                  95
Leu Thr Cys Glu Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr
            100                 105                 110
Leu Thr His Arg Gln Thr Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu
        115                 120                 125
Pro Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys
    130                 135                 140
Leu Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser
145                 150                 155                 160
Asn Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp
                165                 170                 175
Ser Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser
            180                 185                 190
Arg Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala
        195                 200                 205
Leu His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
    210                 215                 220
```

<210> 20
<211> 717
<212> DNA
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 20

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atggtcagct actgggacac cggggtcctg ctgtgcgcgc tgctcagctg tctgcttctc   60
acaggatctg gtagaccttt cgtagagatg tacagtgaaa tccccgaaat tatacacatg   120
actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact   180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt   240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa   300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aacccttcc    360
tgtgtgcccc tgatgcgatg cgggggctgc tgcaatcagc cccgagaacc acaggtgtac   420
accctgcccc catcccggga tgagctgacc aagaaccagg tcagcctgac ctgcctggtc   480
aaaggcttct atcccagcga catcgccgtg gagtgggaga gcaatgggca gccggagaac   540
aactacaaga ccacgcctcc cgtgctggac tccgacggct ccttcttcct ctacagcaag   600
ctcaccgtgg acaagagcag gtggcagcag gggaacgtct tctcatgctc cgtgatgcat   660
gaggctctgc acaaccacta cacgcagaag agcctctccc tgtctccggg taaatag      717
```

<210> 21
<211> 238
<212> PRT
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 21

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser

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

<210> 22
<211> 702
<212> DNA
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 22

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atggtcagct actgggacac cggggtcctg ctgtgcgcgc tgctcagctg tctgcttctc   60
acaggatctg gtagaccttt cgtagagatg tacagtgaaa tccccgaaat tatacacatg   120
actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact   180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt   240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa   300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccggtgga   360
ggtggaggtg gaggtggagg tcagccccga gaaccacagg tgtacaccct gccccccatcc  420
cgggatgagc tgaccaagaa ccaggtcagc ctgacctgcc tggtcaaagg cttctatccc   480
agcgacatcg ccgtggagtg ggagagcaat gggcagccgg agaacaacta caagaccacg   540
cctcccgtgc tggactccga cggctccttc ttcctctaca gcaagctcac cgtggacaag   600
agcaggtggc agcaggggaa cgtcttctca tgctccgtga tgcatgaggc tctgcacaac   660
cactacacgc agaagagcct ctccctgtct ccgggtaaat ag                      702
```

<210> 23
<211> 233
<212> PRT
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 23

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

<210> 24
<211> 1095
<212> DNA
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 24

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atggtcagct actgggacac cggggtcctg ctgtgcgcgc tgctcagctg tctgcttctc      60
acaggatctg gtagaccttt cgtagagatg tacagtgaaa tccccgaaat tatacacatg     120
actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacat cactgttact     180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt     240
agaaagggct tcatcatatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa     300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccggtgga     360
ggtggatcgg gtggaggtgg atcgggtgga ggtggatcgc ctaagagctg cgacaaaact     420
cacacatgcc caccgtgccc agcacctgaa ctcctggggg gaccgtcagt cttcctcttc     480
cccccaaaac ccaaggacac cctcatgatc tcccggaccc ctgaggtcac atgcgtggtg     540
gtggacgtga gccacgaaga ccctgaggtc aagttcaact ggtacgtgga cggcgtggag     600
gtgcataatg ccaagacaaa gccgcgggag gagcagtaca acagcacgta ccgtgtggtc     660
agcgtcctca ccgtcctgca ccaggactgg ctgaatggca aggagtacaa gtgcaaggtc     720
tccaacaaag ccctcccagc ccccatcgag aaaaccatct ccaaagccaa agggcagccc     780
cgagaaccac aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc     840
agcctgacct gcctggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc     900
aatgggcagc cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc     960
ttcttcctct acagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc    1020
tcatgctccg tgatgcatga ggctctgcac aaccactaca cgcagaagag cctctccctg    1080
tctccgggta aatag                                                     1095
```

<210> 25
<211> 364
<212> PRT
<213> Artificial Sequence

<220>
<223> Recombinant fusion protein or sequence encoding same

<400> 25

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

1. A fusion protein of the formula X-Y-Z, wherein:

X comprises the Ig-like domain 2 of VEGF-R1 but lacks the Ig-like domains 1 and 3 of VEGF-R1, with said Ig-like domain 2 of VEGF-R1 being covalently linked to moiety Z via moiety Y; and
Y consists of a 5 - 25 amino acid residue polypeptide; and

Z is a CH3 region of an IgG heavy chain molecule or an Fc portion of an antibody molecule.

2. The fusion protein of claim 1, wherein X is the Ig-like domain 2 of VEGF-R1 (FLT-1).

3. The fusion protein of claim 1, wherein the polypeptide Y is flexible.

4. The fusion protein of claim 1, wherein the polypeptide Y is selected from the group consisting of gly_9 (SEQ ID NO: 27), glu_9 (SEQ ID NO: 28), seq (SEQ ID NO: 29), $gly_5cyspro_2cys$ (SEQ ID NO: 30), $(gly_4ser)_3$ (SEQ ID NO: 31), SerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 32), Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn (SEQ ID NO: 13), Gly-Asp-Leu-Ile-Tyr-Arg-Asn-Gln-Lys(SEQ ID NO:26), and Gly_9ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 34).

5. The fusion protein of claim 1, wherein Z is an IgG1 CH3 region.

6. The fusion protein of claim 1, wherein Z is an IgG2 CH3 region.

7. The fusion protein of claim 1, wherein the Fc is an IgG Fc.

8. The fusion protein of claim 7, wherein the IgG Fc is IgG1 Fc.

9. A composition comprising the fusion protein of any preceding claim which further comprises one or more pharmaceutically acceptable excipients or carriers.

10. The composition of claim 9, wherein said composition is a liquid formulation.

11. The composition of claim 9, wherein said composition is a lyophilized formulation.

12. A composition comprising one or more fusion proteins of any one of claims 1 - 8, wherein said composition comprises multimers of said fusion proteins.

13. The composition of claim 12, wherein said composition consists essentially of a single species of fusion protein which forms homomultimers.

14. The composition of claim 12, wherein said composition consists essentially of fusion proteins, wherein the X moiety in said fusion proteins in the composition are heterogeneous.

15. The composition of claim 12, which further comprises one or more pharmaceutically acceptable excipients or carriers.

16. The compositions of claim 15, wherein said composition is a liquid formulation.

17. The composition of claim 15, wherein said composition is a lyophilized formulation.

18. A method of multimerizing a polypeptide X, the method comprising:

linking a polypeptide X to a polypeptide Z via a polypeptide Y to form polypeptide XYZ, wherein:
X comprises the Ig-like domain 2 of VEGF-R1 but lacks the Ig-like domains 1 and 3 of VEGF-R1, with said Ig-like domain 2 of VEGF-R1 being covalently linked to polypeptide Z via polypeptide Y;
Y consists of a 5 - 25 amino acid residue polypeptide; and
Z is a CH3 region of an IgG heavy chain molecule or an Fc portion of an antibody molecule; **whereby polypeptide XYZ multimerizes.**

19. The method of claim 18, wherein the step of linking comprises constructing a nucleic acid molecule which encodes each of X, Y, and Z as a single open reading frame, wherein said polypeptide XYZ is expressed from the nucleic acid construct in a host cell.

20. The method of claim 18, wherein X is the Ig-like domain 2 of VEGF-R1 (Flt-1).

21. A nucleic acid molecule which encodes a fusion protein according to claim 1.

22. A nucleic acid molecule according to claim 21, wherein Z is an Fc portion of an antibody molecule.

23. The nucleic acid of claim 21 or 22, wherein X is the Ig-like domain 2 of VEGF-R1 (FIt-1).

24. The nucleic acid of claim 23, wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 8, 21, and 23.

25. The nucleic acid of claim 23, wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2 and 25.

26. The fusion protein of claim 1, wherein X is the Ig-like domain 2 of VEGF-R1 (Flt1) and the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 8, 21, and 23.

27. The fusion protein of claim 1, wherein X is the Ig-like domain 2 of VEGF-R1 (Flt-1) and the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2 and 25.

28. A mammalian cell which comprises the nucleic acid of any one of claims 21 - 25.

29. The mammalian cell of claim 28 which is a human cell.

30. The mammalian cell of claim 29 which is selected from the group consisting of a fibroblast, a hepatocyte, an endothelial cell, a keratinocyte, a hematopoietic cell, a synoviocyte, an epithelial cell, a retinal cell and a stem cell.

31. An *in vitro* method comprising:

delivering the nucleic acid of any one of claims 21 - 25 to an isolated mammalian cell to form a cell which expresses said fusion protein.

32. The method of claim 31, wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

33. The method of claim 31 or 32 further comprising:

using the cell which expresses said fusion protein for the manufacture of a composition for delivery to a mammal.

34. The mammalian cell of any one of claims 28-30, for use in treating a mammal

35. The nucleic acid of any one of claims 21-25, for use in treating a mammal, whereby said fusion protein is expressed in the mammal.

36. The nucleic acid of claim 35 wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

37. The mammalian cell of claim 34, or the nucleic acid of claim 35, wherein the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy.

38. The mammalian cell of claim 34, or the nucleic acid of claim 35, wherein the mammal has cancer.

39. The mammalian cell of claim 34, or the nucleic acid of claim 35, wherein the mammal has rheumatoid arthritis.

40. The mammalian cell of claim 34, or the nucleic acid of claim 35, wherein the mammal has asthma.

41. The mammalian cell of claim 34, or the nucleic acid of claim 35, wherein the mammal has osteoarthritis.

42. The fusion protein of any one of claims 1 - 8, for use in treating a mammal.

43. The fusion protein of claim 42, wherein the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy.

44. The fusion protein of claim 42, wherein the mammal has cancer.

45. The fusion protein of claim 42, wherein the mammal has rheumatoid arthritis.

46. The fusion protein of claim 42, wherein the mammal has asthma.

47. The fusion protein of claim 42, wherein the mammal has osteoarthritis.

48. The fusion protein of claim 42, wherein the the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

49. A vector which comprises the nucleic acid of any one of claims 21-25.

50. The vector of claim 49, wherein the vector is a viral vector that is selected from the group consisting of an adenovirus vector, an adeno-associated virus vector, a retrovirus vector, and a lentivirus vector.

51. The vector of claim 50, wherein the vector is an adeno-associated virus vector.

52. The vector of any one of claims 49-51, for use in treating a mammal.

53. The vector of claim 52, wherein the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy.

54. The vector of claim 52, wherein the mammal has cancer.

55. The vector of claim 52, wherein the mammal has rheumatoid arthritis.

56. The vector of claim 52, wherein the mammal has asthma.

57. The vector of claim 52, wherein the mammal has osteoarthritis.

Patentansprüche

1. Fusionsprotein der Formel X-Y-Z, wobei:

X die Ig-ähnliche Domäne 2 von VEGF-R1 umfasst, aber nicht die Ig-ähnlichen Domänen 1 und 3 von VEGF-R1 enthält, wobei die Ig-ähnliche Domäne 2 von VEGF-R1 kovalent an die Einheit Z über die Einheit Y gebunden ist; und
Y aus einem 5 bis 25 Aminosäurerest-Polypeptid besteht; und
Z eine CH3-Region eines IgG-Schwere-Kette-Moleküls oder ein Fc-Teil eines Antikörpermoleküls ist.

2. Fusionsprotein nach Anspruch 1, wobei X die Ig-ähnliche Domäne 2 von VEGF-R1 (FLT-1) ist.

3. Fusionsprotein nach Anspruch 1, wobei das Polypeptid Y flexibel ist.

4. Fusionsprotein nach Anspruch 1, wobei das Polypeptid Y aus der Gruppe ausgewählt ist, welche aus gly_9 (SEQ ID Nr.: 27), glu_9 (SEQ ID Nr.: 28), ser_9 (SEQ ID Nr.: 29), $gly_5scyspro_2cys$ (SEQ ID Nr.: 30), $(gly_4ser)_3$ (SEQ ID Nr.: 31), SerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID Nr.: 32), Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn (SEQ ID Nr.: 13), Gly-Asp-Leu-Ile-Tyr-Arg-Asn-Gln-Lys (SEQ ID Nr.: 26) und Gly_9ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID Nr.: 34) besteht.

5. Fusionsprotein nach Anspruch 1, wobei Z eine IgG1-CH3-Region ist.

6. Fusionsprotein nach Anspruch 1, wobei Z eine IgG2-CH3-Region ist.

7. Fusionsprotein nach Anspruch 1, wobei das Fc ein IgG-Fc ist.

8. Fusionsprotein nach Anspruch 7, wobei das IgG-Fc IgG1-Fc ist.

9. Zusammensetzung, umfassend das Fusionsprotein nach einem vorhergehenden Anspruch, welche ferner einen oder mehrere pharmazeutisch verträgliche Exzipienten oder Träger umfasst.

10. Zusammensetzung nach Anspruch 9, wobei die Zusammensetzung eine flüssige Formulierung ist.

11. Zusammensetzung nach Anspruch 9, wobei die Zusammensetzung eine lyophilisierte Formulierung ist.

12. Zusammensetzung, umfassend ein oder mehrere Fusionsproteine nach einem der Ansprüche 1 bis 8, wobei die Zusammensetzung Multimere der Fusionsproteine umfasst.

13. Die Zusammensetzung nach Anspruch 12, wobei die Zusammensetzung im Wesentlichen aus einer einzigen Spezies von Fusionsprotein, welche Homomultimere bildet, besteht.

14. Zusammensetzung nach Anspruch 12, wobei die Zusammensetzung im Wesentlichen aus Fusionsproteinen besteht, wobei die Einheit X in den Fusionsproteinen in der Zusammensetzung heterogen ist.

15. Zusammensetzung nach Anspruch 12, welche ferner einen oder mehrere pharmazeutisch verträgliche Exzipienten oder Träger umfasst.

16. Zusammensetzung nach Anspruch 15, wobei die Zusammensetzung eine flüssige Formulierung ist.

17. Zusammensetzung nach Anspruch 15, wobei die Zusammensetzung eine lyophilisierte Formulierung ist.

18. Verfahren zur Multimerisierung eines Polypeptids X, wobei das Verfahren umfasst:

das Binden eines Polypeptids X an ein Polypeptid Z über ein Polypeptid Y, um ein Polypeptid XYZ zu bilden, wobei:

X die Ig-ähnliche Domäne 2 von VEGF-R1 umfasst, aber nicht die Ig-ähnlichen Domänen 1 und 3 von VEGF-R1 enthält, wobei die Ig-ähnliche Domäne 2 von VEGF-R1 kovalent an das Polypeptid Z über das Polypeptid Y gebunden ist;
Y aus einem 5 bis 25 Aminosäurerest-Polypeptid besteht; und
Z eine CH3-Region eines IgG-Schwere-Kette-Moleküls oder ein Fc-Teil eines Antikörpermoleküls ist;

wobei das Polypeptid XYZ multimerisiert.

19. Verfahren nach Anspruch 18, wobei der Schritt des Bindens das Aufbauen eines Nukleinsäuremoleküls umfasst, das jedes von X, Y und Z als einen einzelnen offenen Leserahmen kodiert, wobei das Polypeptid XYZ von dem Nukleinsäurekonstrukt in einer Wirtzelle exprimiert wird.

20. Verfahren nach Anspruch 18, wobei X die Ig-ähnliche Domäne 2 von VEGF-R1 (Flt-1) ist.

21. Nukleinsäuremolekül, welches ein Fusionsprotein gemäß Anspruch 1 kodiert.

22. Nukleinsäuremolekül gemäß Anspruch 21, wobei Z ein Fc-Teil eines Antikörpermoleküls ist.

23. Nukleinsäure nach Anspruch 21 oder 22, wobei X die Ig-ähnliche Domäne 2 von VEGF-R1 (Flt-1) ist.

24. Nukleinsäure nach Anspruch 23, wobei das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 8, 21 und 23 besteht.

25. Nukleinsäure nach Anspruch 23, wobei das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 2 und 25 besteht.

26. Fusionsprotein nach Anspruch 1, wobei X die Ig-ähnliche Domäne 2 von VEGF-R1 (Flt-1) ist und das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 8, 21 und 23 besteht.

27. Fusionsprotein nach Anspruch 1, wobei X die Ig-ähnliche Domäne 2 von VEGF-R1 (Flt-1) ist und das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 2 und 25 besteht.

28. Säugerzelle, welche die Nukleinsäure nach einem der Ansprüche 21 bis 25 umfasst.

29. Säugerzelle nach Anspruch 28, welche eine humane Zelle ist.

30. Säugerzelle nach Anspruch 29, welche aus der Gruppe ausgewählt ist, die aus einem Fibroblast, einem Hepatozyten, einer Endothelzelle, einem Keratinozyten, einer hämopoetischen Zelle, einem Synoviozyten, einer Epithelzelle, einer Retinazelle und einer Stammzelle besteht.

31. *In vitro*-Verfahren, umfassend:

Zuführen der Nukleinsäure nach einem der Ansprüche 21 bis 25 zu einer isolierten Säugerzelle, um eine Zelle zu bilden, welche das Fusionsprotein exprimiert.

32. Verfahren nach Anspruch 31, wobei das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 2, 8, 21, 23 und 25 besteht.

33. Verfahren nach Anspruch 31 oder 32, ferner umfassend:

das Verwenden der Zelle, welche das Fusionsprotein exprimiert, zur Herstellung einer Zusammensetzung zum Zuführen zu einem Säuger.

34. Säugerzelle nach einem der Ansprüche 28 bis 30 zur Verwendung in der Behandlung eines Säugers.

35. Nukleinsäure nach einem der Ansprüche 21 bis 25 zur Verwendung in der Behandlung eines Säugers, wobei das Fusionsprotein in dem Säuger exprimiert wird.

36. Nukleinsäure nach Anspruch 35, wobei das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 2, 8, 21, 23 und 25 besteht.

37. Säugerzelle nach Anspruch 34 oder Nukleinsäure nach Anspruch 35, wobei der Säuger eine mit dem Alter in Zusammenhang stehende feuchte Makuladegeneration oder Retinopathia diabetica haemorrhagica proliferans aufweist.

38. Säugerzelle nach Anspruch 34 oder Nukleinsäure nach Anspruch 35, wobei der Säuger Krebs hat.

39. Säugerzelle nach Anspruch 34 oder Nukleinsäure nach Anspruch 35, wobei der Säuger rheumatoide Arthritis aufweist.

40. Säugerzelle nach Anspruch 34 oder Nukleinsäure nach Anspruch 35, wobei der Säuger Asthma hat.

41. Säugerzelle nach Anspruch 34 oder Nukleinsäure nach Anspruch 35, wobei der Säuger Osteoarthritis aufweist.

42. Fusionsprotein nach einem der Ansprüche 1 bis 8 zur Verwendung in der Behandlung eines Säugers.

43. Fusionsprotein nach Anspruch 42, wobei der Säuger eine mit dem Alter in Zusammenhang stehende feuchte Makuladegeneration oder Retinopathia diabetica haemorrhagica proliferans aufweist.

44. Fusionsprotein nach Anspruch 42, wobei der Säuger Krebs hat.

45. Fusionsprotein nach Anspruch 42, wobei der Säuger rheumatoide Arthritis aufweist.

46. Fusionsprotein nach Anspruch 42, wobei der Säuger Asthma hat.

47. Fusionsprotein nach Anspruch 42, wobei der Säuger Osteoarthritis aufweist.

48. Fusionsprotein nach Anspruch 42, wobei das Fusionsprotein eine Sequenz umfasst, welche aus der Gruppe ausgewählt ist, die aus SEQ ID Nr.: 2, 8, 21, 23 und 25 besteht.

49. Vektor, welcher die Nukleinsäure nach einem der Ansprüche 21 bis 25 umfasst.

50. Vektor nach Anspruch 49, wobei der Vektor ein viraler Vektor ist, welcher aus der Gruppe ausgewählt ist, die aus einem Adenovirusvektor, einem Adeno-assoziierten Virusvektor, einem Retrovirusvektor und einem Lentivirusvektor besteht.

51. Vektor nach Anspruch 50, wobei der Vektor ein Adeno-assoziierter Virusvektor ist.

52. Vektor nach einem der Ansprüche 49 bis 51 zur Verwendung in der Behandlung eines Säugers.

53. Vektor nach Anspruch 52, wobei der Säuger eine mit dem Alter in Zusammenhang stehende feuchte Makuladegeneration oder Retinopathia diabetica haemorrhagica proliferans aufweist.

54. Vektor nach Anspruch 52, wobei der Säuger Krebs hat.

55. Vektor nach Anspruch 52, wobei der Säuger rheumatoide Arthritis aufweist.

56. Vektor nach Anspruch 52, wobei der Säuger Asthma hat.

57. Der Vektor nach Anspruch 52, wobei der Säuger Osteoarthritis aufweist.

Revendications

1. Protéine de fusion de formule X-Y-Z, dans laquelle :

X comprend le domaine 2 de type Ig du VEGF-R1 mais ne possède pas les domaines 1 et 3 de type Ig du VEGF-R1, avec ledit domaine 2 de type Ig du VEGF-R1 étant lié de manière covalente à la fraction Z via la fraction Y ;
et
Y est constitué par un polypeptide de 5 à 25 résidus d'acides aminés ; et
Z est une région CH3 d'une molécule de chaîne lourde d'IgG ou une partie Fc d'une molécule d'anticorps.

2. Protéine de fusion selon la revendication 1, dans laquelle X est le domaine 2 de type Ig du VEGF-R1 (FLT-1).

3. Protéine de fusion selon la revendication 1, dans laquelle le polypeptide Y est flexible.

4. Protéine de fusion selon la revendication 1, dans laquelle le polypeptide Y est choisi dans le groupe constitué par gly_9 (SEQ ID NO : 27), glu_9 (SEQ ID NO : 28), ser_9 (SEQ ID NO : 29), $gly_5cyspro_2cys$ (SEQ ID NO : 30), $(gly_4ser)_3$ (SEQ ID NO : 31), SerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO : 32), Pro Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn (SEQ ID NO : 13), Gly-Asp-Leu-Ile-Tyr-Arg-Asn-Gln-Lys (SEQ ID NO : 26), et Gly_9ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO : 34).

5. Protéine de fusion selon la revendication 1, dans laquelle Z est une région CH3 d'une IgG1.

6. Protéine de fusion selon la revendication 1, dans laquelle Z est une région CH3 d'une IgG2.

7. Protéine de fusion selon la revendication 1, dans laquelle le Fc est un Fc d'une IgG.

8. Protéine de fusion selon la revendication 7, dans laquelle le Fc de l'IgG est un Fc d'IgG1.

9. Composition comprenant la protéine de fusion selon l'une quelconque des revendications précédentes, qui comprend en outre un ou plusieurs excipients ou vecteurs pharmaceutiquement acceptables.

10. Composition selon la revendication 9, ladite composition étant une formulation liquide.

11. Composition selon la revendication 9, ladite composition étant une formulation lyophilisée.

12. Composition comprenant une ou plusieurs protéines de fusion selon l'une quelconque des revendications 1 à 8, ladite composition comprenant des multimères desdites protéines de fusion.

13. Composition selon la revendication 12, ladite composition étant essentiellement constituée par une espèce unique de protéine de fusion qui forme des homomultimères.

14. Composition selon la revendication 12, ladite composition étant essentiellement constituée par des protéines de fusion, la fraction X dans lesdites protéines de fusion dans la composition étant hétérogène.

15. Composition selon la revendication 12, qui comprend en outre un ou plusieurs excipients ou vecteurs pharmaceutiquement acceptables.

16. Composition selon la revendication 15, ladite composition étant une formulation liquide.

17. Composition selon la revendication 15, ladite composition étant une formulation lyophilisée.

18. Procédé de multimérisation d'un polypeptide X, le procédé comprenant :

la liaison d'un polypeptide X à un polypeptide Z via un polypeptide Y pour former un polypeptide XYZ, dans lequel :

X comprend le domaine 2 de type Ig du VEGF-R1 mais ne possède pas les domaines 1 et 3 de type Ig du VEGF-R1, avec ledit domaine 2 de type Ig du VEGF-R1 étant lié de manière covalente à la fraction Z via la fraction Y ;
Y est constitué par un polypeptide de 5 à 25 résidus d'acides aminés ; et
Z est une région CH3 d'une molécule de chaîne lourde d'IgG ou une partie Fc d'une molécule d'anticorps,

moyennant quoi le polypeptide XYZ se multimérise.

19. Procédé selon la revendication 18, dans lequel l'étape de liaison comprend la construction d'une molécule d'acide nucléique qui code pour chacun de X, Y et Z comme un cadre de lecture ouvert unique, ledit polypeptide XYZ étant exprimé par la construction d'acide nucléique dans une cellule hôte.

20. Procédé selon la revendication 18, dans lequel X est le domaine 2 de type Ig du VEGF-1 (Flt-1).

21. Molécule d'acide nucléique qui code pour une protéine de fusion selon la revendication 1.

22. Molécule d'acide nucléique selon la revendication 21, dans laquelle Z est une partie Fc d'une molécule d'anticorps.

23. Acide nucléique selon la revendication 21 ou 22, dans lequel X est le domaine 2 de type Ig du VEGF-R1 (Flt-1).

24. Acide nucléique selon la revendication 23, dans lequel la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO : 8, 21 et 23.

25. Acide nucléique selon la revendication 23, dans lequel la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO : 2 et 25.

26. Protéine de fusion selon la revendication 1, dans laquelle X est le domaine 2 de type Ig du VEGF-R1 (Flt-1) et la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO 8, 21 et 23.

27. Protéine de fusion selon la revendication 1, dans laquelle X est le domaine 2 de type Ig du VEGF-R1 (Flt-1) et la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO : 2 et 25.

28. Cellule de mammifère qui comprend l'acide nucléique selon l'une quelconque des revendications 21 à 25.

29. Cellule de mammifère selon la revendication 28, qui est une cellule humaine.

30. Cellule de mammifère selon la revendication 29, qui est choisie dans le groupe constitué par un fibroblaste, un hépatocyte, une cellule endothéliale, un kératinocyte, une cellule hématopoïétique, un synoviocyte, une cellule épithéliale, une cellule rétinienne et une cellule souche.

31. Procédé *in vitro* qui comprend :

la délivrance de l'acide nucléique selon l'une quelconque des revendications 21 à 25 à une cellule de mammifère isolée pour former une cellule qui exprime ladite protéine de fusion.

32. Procédé selon la revendication 31, dans lequel la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO : 2, 8, 21, 23 et 25.

33. Procédé selon la revendication 31 ou 32, qui comprend en outre :

l'utilisation de la cellule qui exprime ladite protéine de fusion pour la fabrication d'une composition destinée à être délivrée à un mammifère.

34. Cellule de mammifère selon l'une quelconque des revendications 28 à 30, destinée à être utilisée pour traiter un mammifère.

35. Acide nucléique selon l'une quelconque des revendications 21 à 25, destiné à être utilisé pour traiter un mammifère, moyennant quoi ladite protéine de fusion est exprimée chez ledit mammifère.

36. Acide nucléique selon la revendication 35, dans lequel la protéine de fusion comprend une séquence choisie dans le groupe constitué par SEQ ID NO : 2, 8, 21, 23 et 25.

37. Cellule de mammifère selon la revendication 34 ou acide nucléique selon la revendication 35, le mammifère présentant une dégénérescence maculaire liée à l'âge humide ou une rétinopathie diabétique prolifératives.

38. Cellule de mammifère selon la revendication 34 ou acide nucléique selon la revendication 35, le mammifère ayant le cancer.

39. Cellule de mammifère selon la revendication 34 ou acide nucléique selon la revendication 35, le mammifère souffrant de polyarthrite rhumatoïde.

40. Cellule de mammifère selon la revendication 34 ou acide nucléique selon la revendication 35, le mammifère souffrant d'asthme.

41. Cellule de mammifère selon la revendication 34 ou acide nucléique selon la revendication 35, le mammifère souffrant d'ostéoarthrite.

42. Protéine de fusion selon l'une quelconque des revendications 1 à 8, destinée à être utilisée dans le traitement d'un mammifère.

43. Protéine de fusion selon la revendication 42, le mammifère présentant une dégénérescence maculaire liée à l'âge humide ou une rétinopathie diabétique prolifératives.

44. Protéine de fusion selon la revendication 42, le mammifère ayant le cancer.

45. Protéine de fusion selon la revendication 42, le mammifère souffrant de polyarthrite rhumatoïde.

46. Protéine de fusion selon la revendication 42, le mammifère souffrant d'asthme.

47. Protéine de fusion selon la revendication 42, le mammifère souffrant d'ostéoarthrite.

48. Protéine de fusion selon la revendication 42, la protéine de fusion comprenant une séquence choisie dans le groupe constitué par SEQ ID NO : 2, 8, 21, 23 et 25.

49. Vecteur qui comprend l'acide nucléique selon l'une quelconque des revendications 21 à 25.

50. Vecteur selon la revendication 49, dans lequel le vecteur est un vecteur viral qui est choisi dans le groupe constitué par un vecteur adénoviral, un vecteur de virus adéno-associé, un vecteur rétroviral, et un vecteur lentiviral.

51. Vecteur selon la revendication 50, dans lequel le vecteur est un vecteur de virus adéno-associé.

52. Vecteur selon l'une quelconque des revendications 49 à 51, destiné à être utilisé pour traiter un mammifère.

53. Vecteur selon la revendication 52, le mammifère présentant une dégénérescence maculaire liée à l'âge humide ou une rétinopathie diabétique prolifération.

54. Vecteur selon la revendication 52, le mammifère ayant le cancer.

55. Vecteur selon la revendication 52, le mammifère souffrant de polyarthrite rhumatoïde.

56. Vecteur selon la revendication 52, le mammifère souffrant d'asthme.

57. Vecteur selon la revendication 52, le mammifère souffrant d'ostéoarthrite.

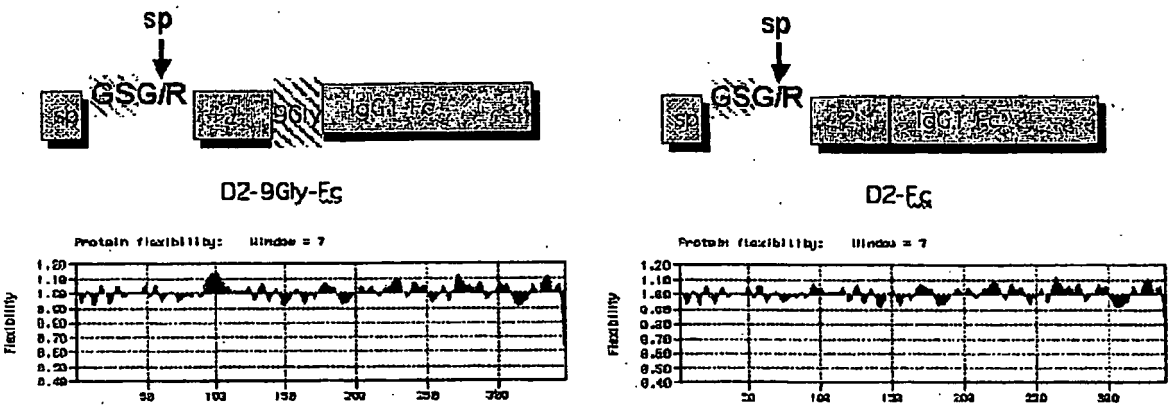
sp
GSG/R
sp
9Gly
IgG1 Fc
D2-9Gly-Fc
Protein flexibility: Window = 7
Flexibility
sp
GSG/R
sp
IgG1 Fc
D2-Fc
Protein flexibility: Window = 7
Flexibility

FIG 1

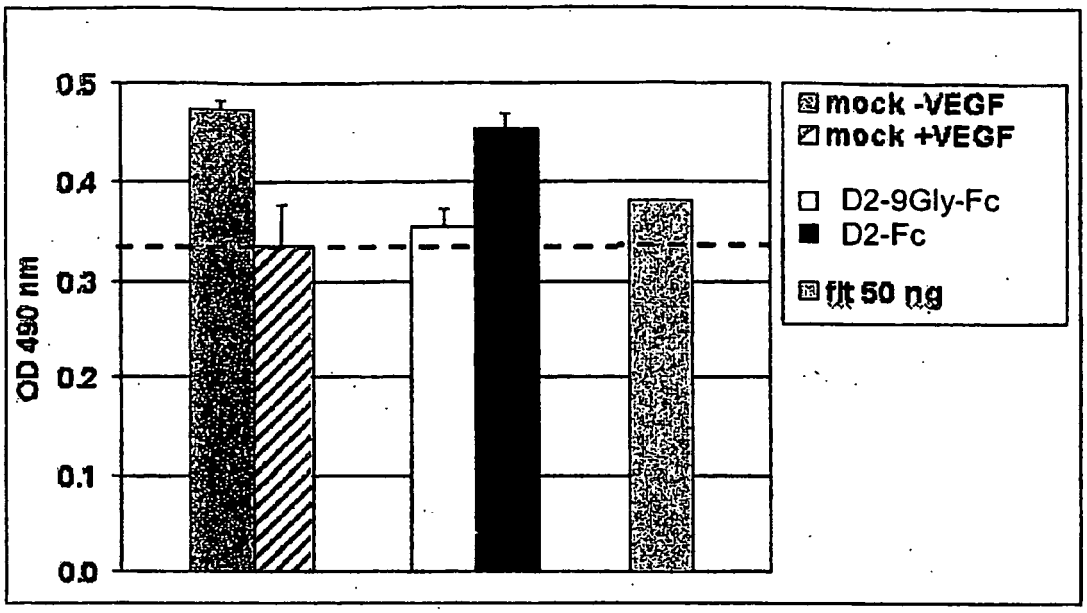
0.5
0.4
0.3
0.2
0.1
0.0
OD 490 nm
mock -VEGF
mock +VEGF
D2-9Gly-Fc
D2-Fc
flt 50 ng

FIG 2

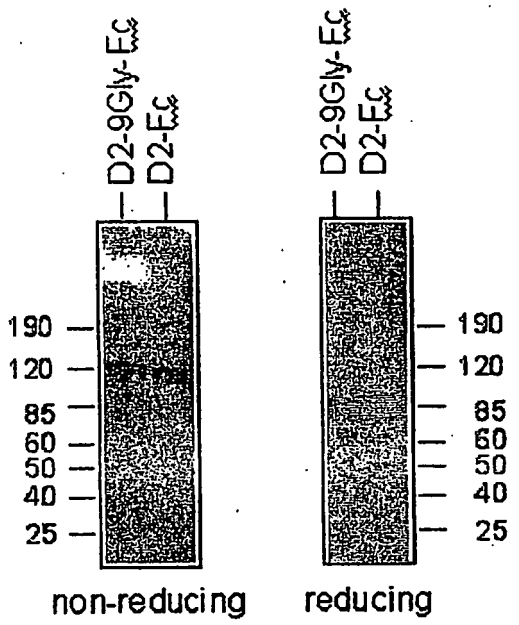
D2-9Gly-Fc
D2-Fc
190
120
85
60
50
40
25
non-reducing
D2-9Gly-Fc
D2-Fc
190
120
85
60
50
40
25
reducing

FIG 3

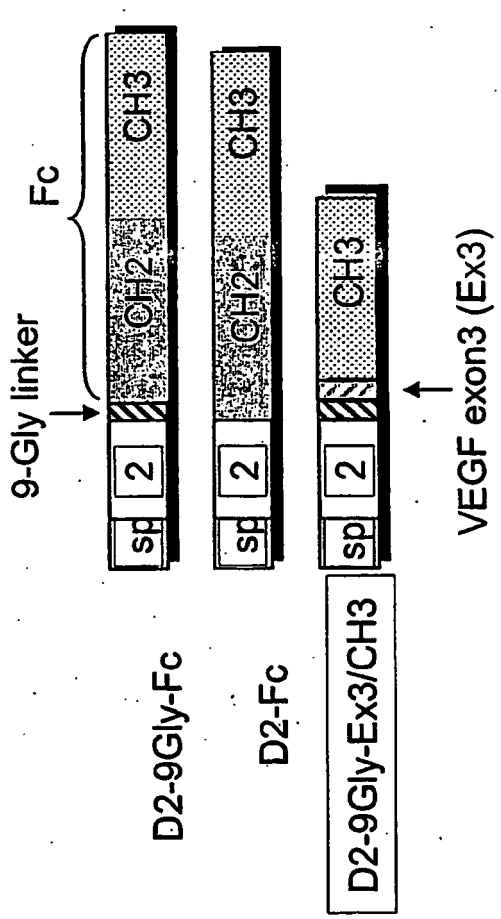
9-Gly linker
Fc
CH3
CH2
2
sp
D2-9Gly-Fc
D2-Fc
D2-9Gly-Ex3/CH3
VEGF exon3 (Ex3)

Fig. 4

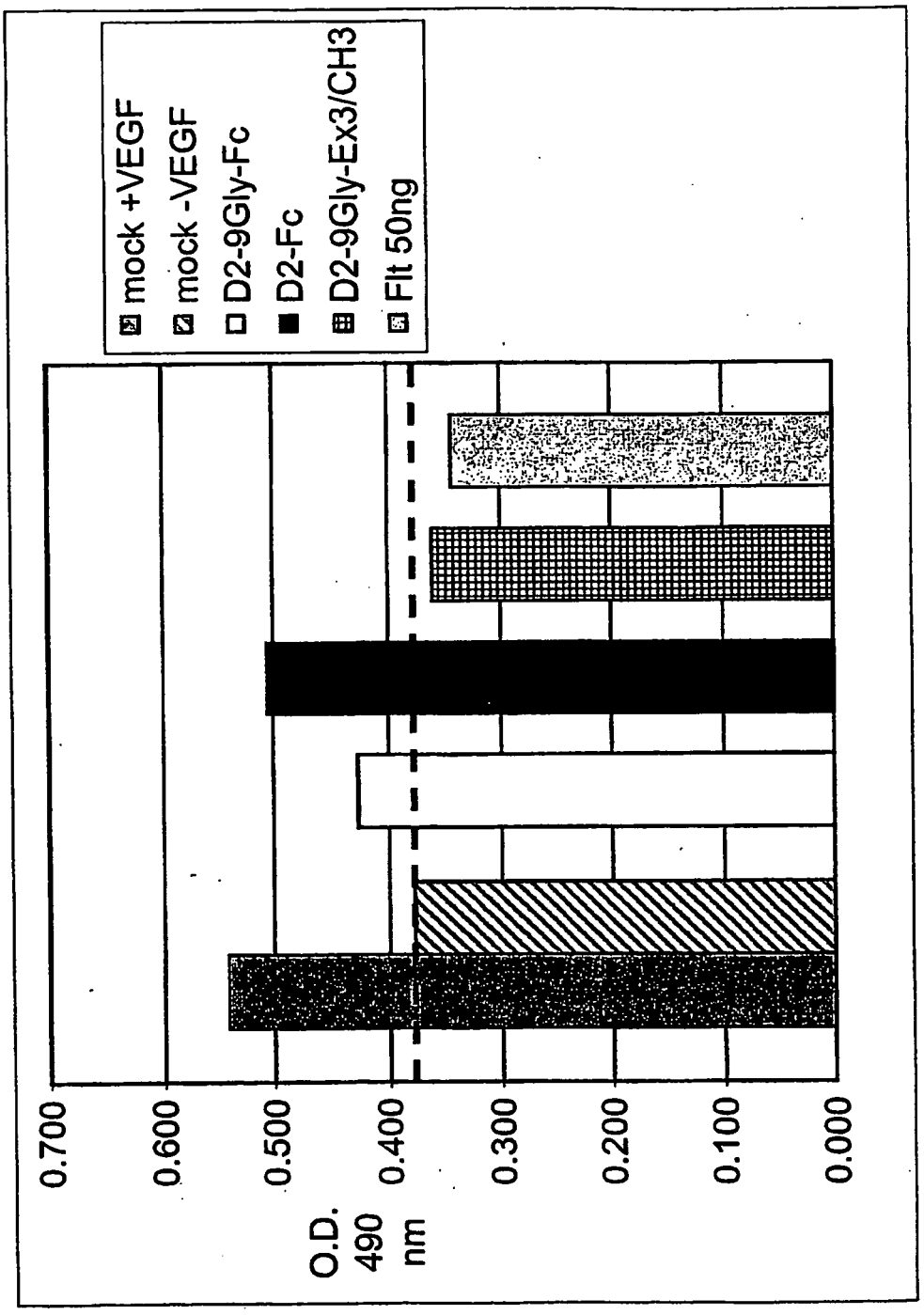
mock +VEGF
mock -VEGF
D2-9Gly-Fc
D2-Fc
D2-9Gly-Ex3/CH3
Flt 50ng
0.700
0.600
0.500
0.400
0.300
0.200
0.100
0.000
O.D.
490
nm

Fig. 5

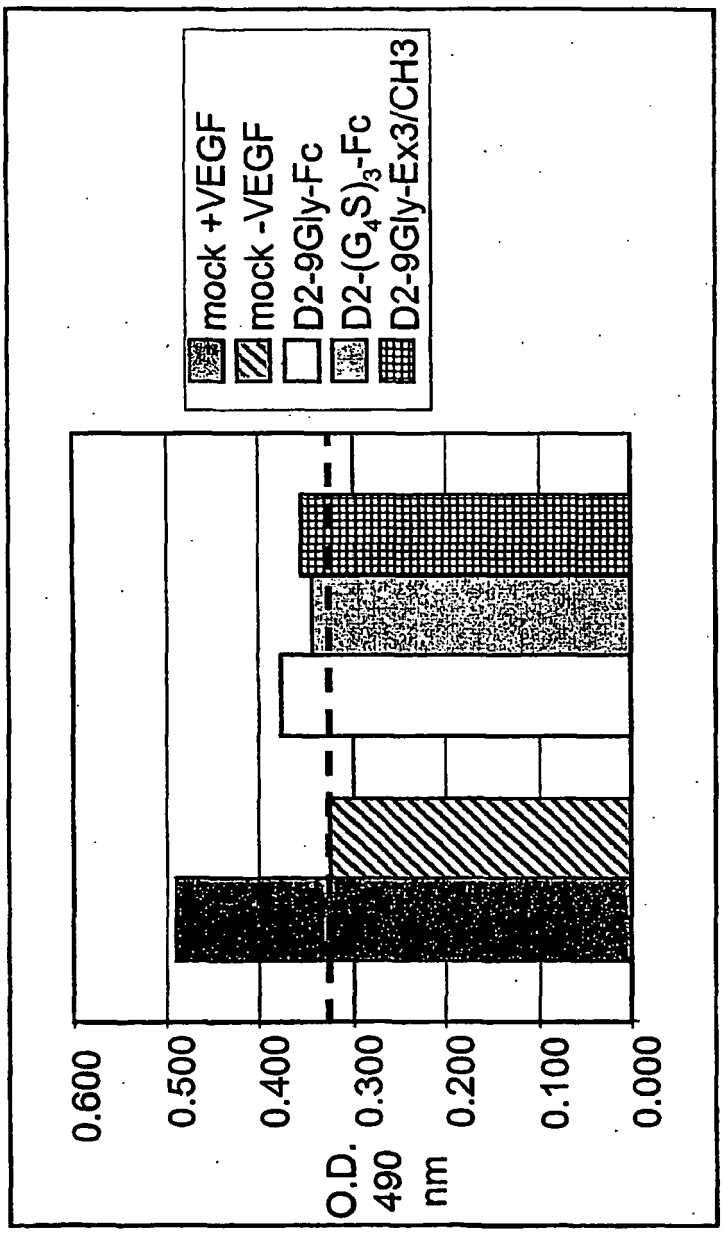
mock +VEGF
mock -VEGF
D2-9Gly-Fc
D2-(G4S)3-Fc
D2-9Gly-Ex3/CH3
0.600
0.500
0.400
0.300
0.200
0.100
0.000
O.D. 490 nm

Fig. 6

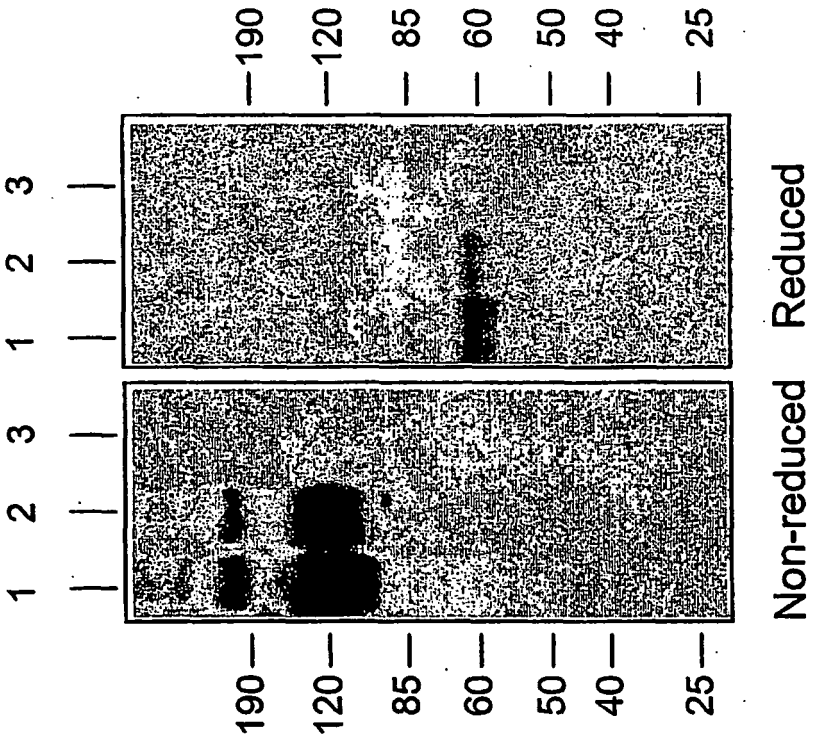

Fig.7

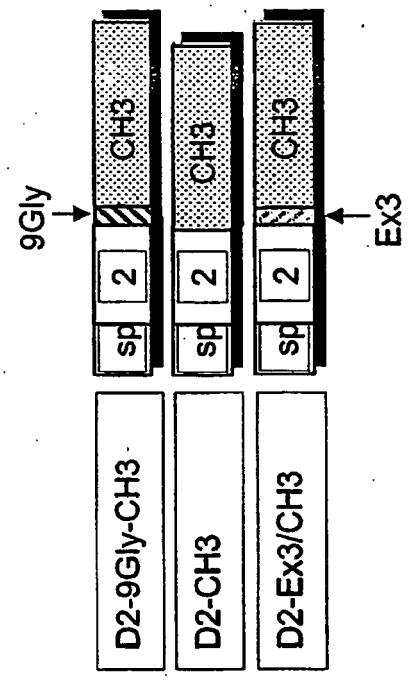

Fig. 8

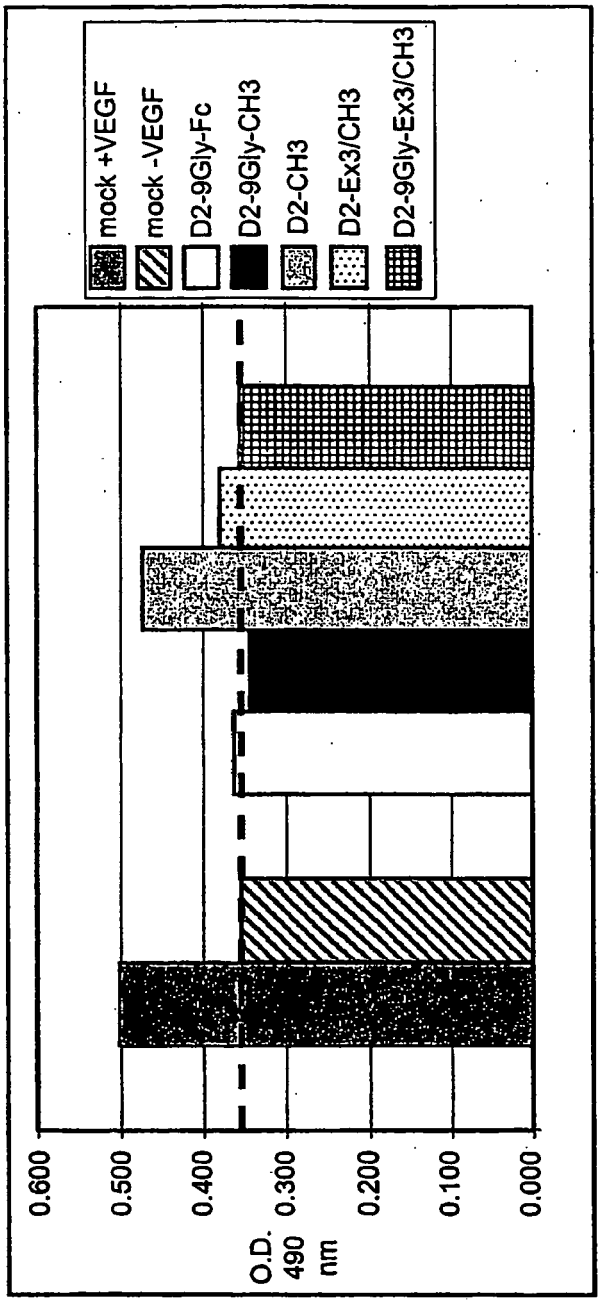
mock +VEGF
mock -VEGF
D2-9Gly-Fc
D2-9Gly-CH3
D2-CH3
D2-Ex3/CH3
D2-9Gly-Ex3/CH3
0.600
0.500
0.400
0.300
0.200
0.100
0.000
O.D.
490
nm

Fig. 9

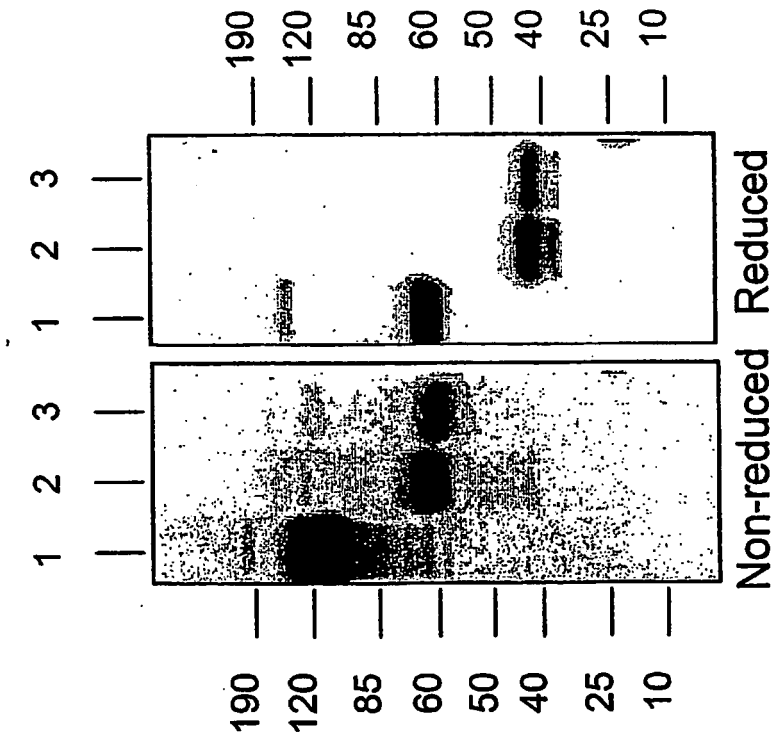
190
120
85
60
50
40
25
10
1 2 3
Reduced
Non-reduced
1 2 3
190
120
85
60
50
40
25
10

Fig.10

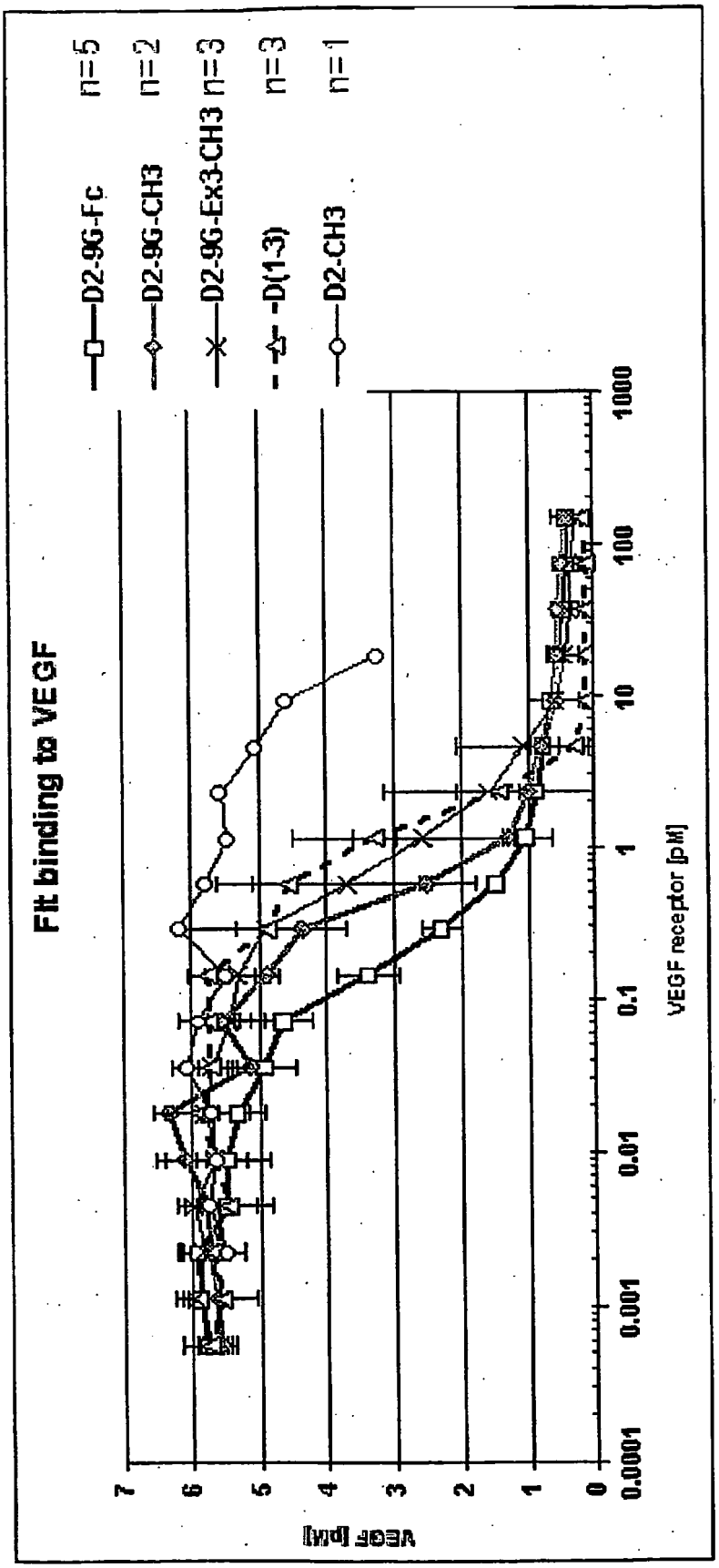
Fit binding to VEGF
D2-9G-Fc n=5
D2-9G-CH3 n=2
D2-9G-Ex3-CH3 n=3
-D(1-3) n=3
D2-CH3 n=1
VEGF [pM]
VEGF receptor [pM]
0.0001
0.001
0.01
0.1
1
10
100
1000
0
1
2
3
4
5
6
7

Fig. 11

BIAcore binding kinetics for sFlt-1 molecules

Sensorgrams:

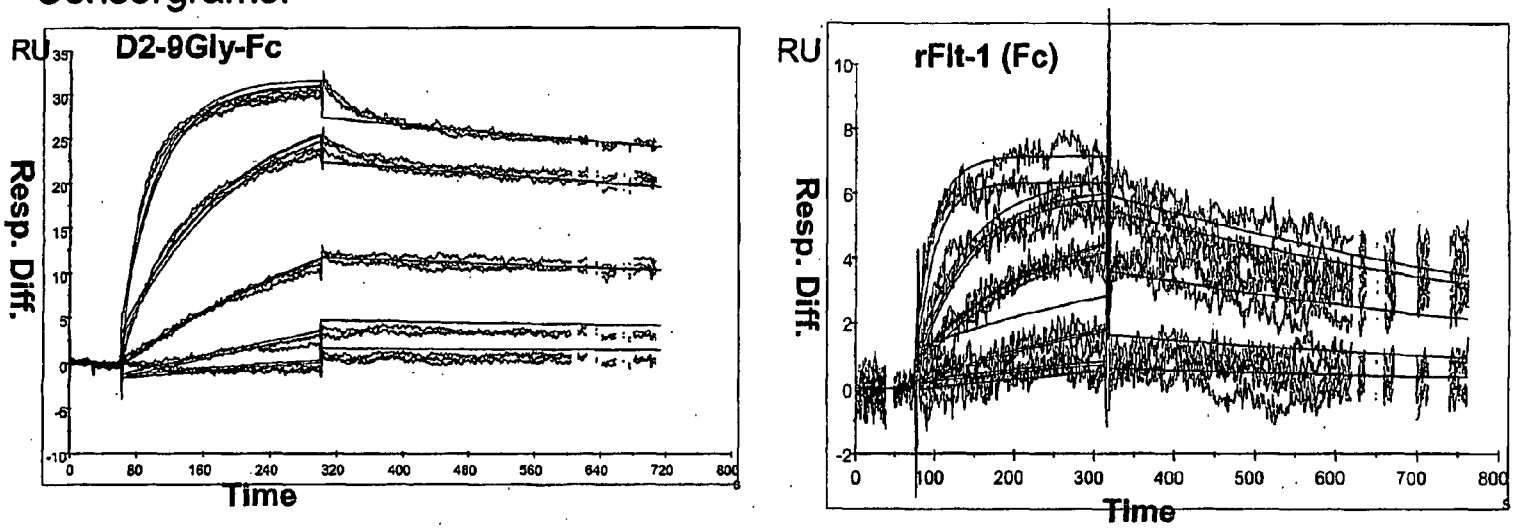

Table:

sFlt-1 molecule	K_a (1/Ms)	K_d (1/s)	K_D (nM)
D2-9Gly-Fc	1.44e6	3.15e-4	2.19e-10
rFlt-1(Fc)(R&D)	2.71e6	1.22e-3	4.48e-10

The binding kinetics of the soluble Flt-1 were measured by surface plasmon resonance with a BIAcore instrument. sFlt-1 was immobilized onto a sensor chip, and VEGF165 was injected at concentrations ranging from 0.2 to 15 nM. The sensorgrams were evaluated using the BIA Evaluation program, the rate constants K_a and K_d were determined and the dissociation constant (K_D) calculated from the ratio of Kd/Ka = K_D. The lower K_D value means better affinity.

FIG 12

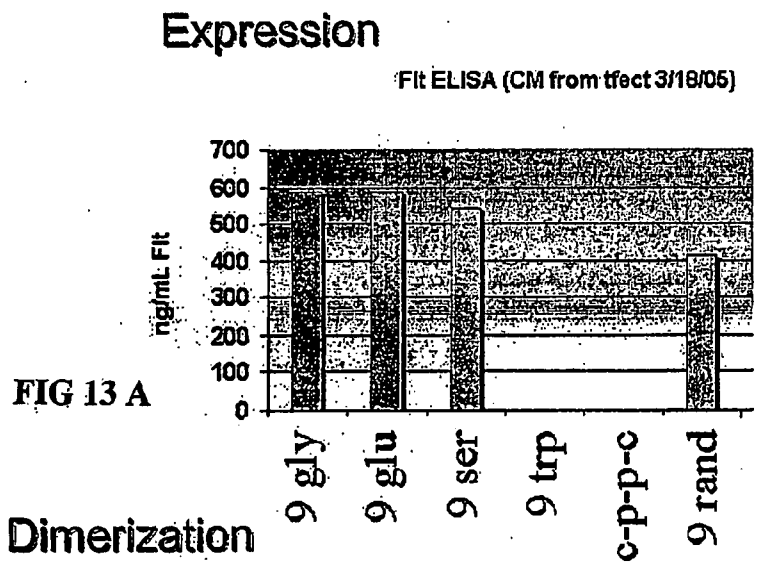

FIG 13 A

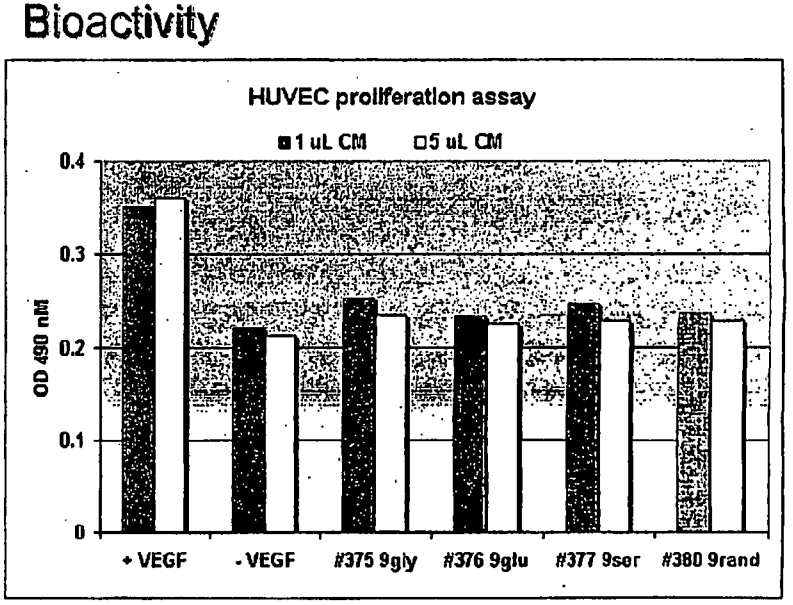

FIG 13 B

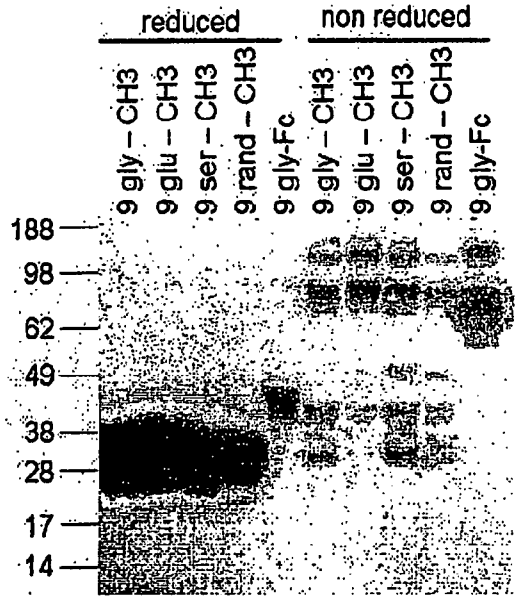

FIG 13 C

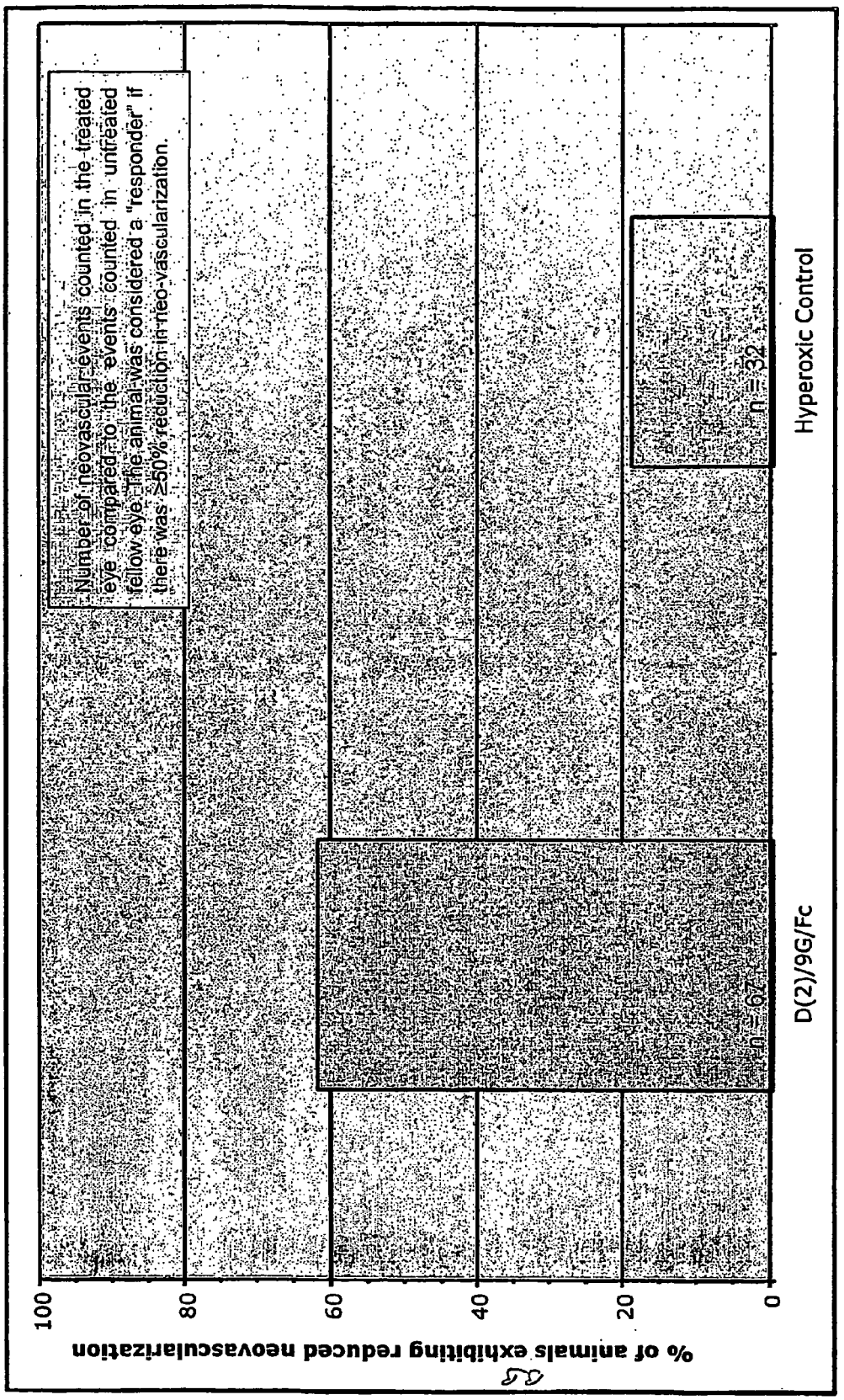
Mouse OIR model
% of animals exhibiting reduced neovascularization
100
80
60
40
20
0
Number of neovascular events counted in the treated eye compared to the events counted in untreated fellow eye. The animal was considered a "responder" if there was ≥50% reduction in neo-vascularization.
n = 67
n = 32
D(2)/9G/Fc
Hyperoxic Control

FIG 14

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

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