

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
23 March 2006 (23.03.2006)

PCT

(10) International Publication Number
WO 2006/031689 A2(51) International Patent Classification:
C07K 14/47 (2006.01)(21) International Application Number:
PCT/US2005/032320(22) International Filing Date:
13 September 2005 (13.09.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/608,887 13 September 2004 (13.09.2004) US
60/658,209 4 March 2005 (04.03.2005) US(71) Applicant (for all designated States except US): **GENZYME CORPORATION** [US/US]; One Mountain Road, Framingham, MA 01701 (US).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

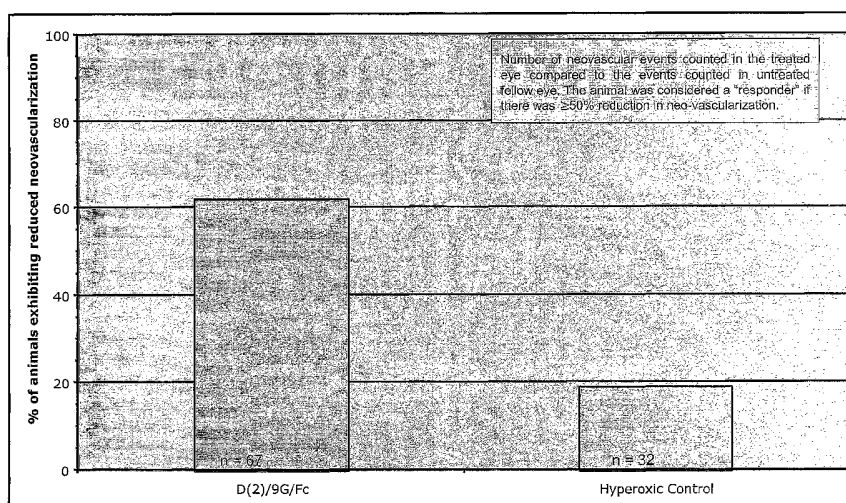
Published:

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

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

(54) Title: MULTIMERIC CONSTRUCTS

Mouse OIR model



(57) **Abstract:** Multimeric fusion proteins of an Ig-like domain of Flt-1 are rendered functional by inclusion of a linker moiety. Vectors encoding the fusion proteins and host cells expressing the fusion proteins can be used therapeutically to block neovascularization in individuals with pathological conditions related to neovascularization. Such conditions include age-related macular degeneration, cancer, psoriasis, proliferative diabetic retinopathy, asthma, uveitis, osteoarthritis, and rheumatoid arthritis. The same means of multimerization used for an Ig-like domain of Flt-1, *i.e.*, a linker and a multimerization domain, can be used for other polypeptides, including extracellular receptors, antibody variable regions, cytokines, chemokines, and growth factors.

MULTIMERIC CONSTRUCTS

FIELD OF THE INVENTION

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

BACKGROUND OF THE INVENTION

- [02] 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.
- [03] 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.
- [04] 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.

BRIEF SUMMARY OF THE INVENTION

- [05] According to one embodiment of the invention 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.
- [06] Another embodiment of the invention 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 residues. Z is a CH3 region of an IgG heavy chain molecule.
- [07] Yet another aspect of the invention 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.
- [08] A fusion protein of the formula X-Y-Z is also provided. 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.
- [09] Still another aspect of the invention 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.

- [10] Yet another embodiment of the invention 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.
- [11] In one embodiment of the invention 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.
- [12] In another embodiment of the invention 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.
- [13] In another embodiment of the invention 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.

- [14] Still another embodiment of the invention 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.
- [15] Another aspect of the invention 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.
- [16] These and other embodiments of the invention which will be described in more detail below provide the art with methods and agents for treating disease related to vascular proliferation and inflammation. The agents may provide increased stability and bioavailability relative to natural forms of the proteins.

BRIEF DESCRIPTION OF THE DRAWINGS

- [17] 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 IgG1-Fc residues (244 aa). The arrow represents the signal peptidase cleavage site as predicted using the SignalP V2.0 program (Nielsen et al., 1997).

- [18] 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 μ l 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[®] AQueous reagent (Promega). The data represent the means of the average values of OD₄₉₀ of two experiments each assayed in triplicates.
- [19] 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.
- [20] 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.
- [21] 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.

- [22] Fig. 6. HUVECs proliferation assay comparing D2-(Gly₄Ser)₃-Fc protein activity with D2-9Gly-Fc and D2-9Gly-Ex3/CH3.
- [23] 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-(G₄S)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.
- [24] 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.
- [25] 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.
- [26] 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).
- [27] 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).

- [28] 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 K_a and K_d were determined and the dissociation constant (KD) calculated from the ratio of $K_d/K_a = KD$. The lower KD value means better affinity.
- [29] 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.
- [30] 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

- [31] 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.

- [32] 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 threonine 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-1 α , IL-1 β , 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-1 α , MIP-1 β , TGF- β , TNF α , 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, I-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.

- [33] 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).

- [34] Multimerization domains can be used as are known in the art. Sequences of the Fc portion of IgG1 or IgG2 lambda 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 lambda heavy chain protein sequence, see Genbank accession no Y14737 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.
- [35] 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 refractive index in an aqueous layer close to a sensor chip surface. Any method known in the art can be used to detect multimerization.
- [36] Linker moieties according to the invention 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, 5-9 amino acid residues. Examples of useful linkers include: gly₉ (SEQ ID

NO: 27), glu₉ (SEQ ID NO: 28), ser₉ (SEQ ID NO: 29), gly₅cyspro₂cys (SEQ ID NO: 30), (gly₄ser)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 Gly₉ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (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.

- [37] Fusion proteins according to the 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.

- [38] 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.
- [39] 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.
- [40] 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.

- [41] 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, osteoarthritis, uveitis, asthma, and cancer. The treatments will improve symptoms and/or markers of disease and/or disease severity.
- [42] 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.
- [43] Combinations of protein and nucleic acid treatments can be used. For example, a fusion protein according to the 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 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.

- [44] 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)).
- [45] 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.

- [46] 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.
- [47] 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 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).
- [48] 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 hemagglutination of red blood cells, oncogenicity, DNA and protein amino acid compositions and homologies, and antigenic relationships.

- [49] 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.
- [50] 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, incorporated herein by reference). 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. Virol.* 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.

- [51] 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.
- [52] 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 invention may be the GeneSwitchTM technology (Valentis, Inc., Woodlands, TX) described in Abruzzese et al., *Hum. Gene Ther.* 1999 10:1499-507, the disclosure of which is hereby incorporated herein by reference. The partially deleted adenoviral expression system is further described in WO99/57296, the disclosure of which is hereby incorporated by reference herein.

- [53] 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 76, Rep 68, Rep 52, and Rep 40) and the cap gene, which codes for AAV replication, rescue, transcription and integration, while the cap proteins 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).
- [54] 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 a 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).

- [55] 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.
- [56] 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.

- [57] Retrovirus vectors are a common tool for gene delivery (Miller, *Nature* (1992) 357:455-460). The ability of retrovirus vectors to deliver an unarranged, 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.
- [58] 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.
- [59] 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 pol 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 virion proteins.

- [60] Lentiviruses are complex retroviruses which, in addition to the common retroviral genes gag, pol 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, the disclosures of which are hereby incorporated herein by reference.
- [61] 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 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.

- [62] 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, the disclosures of which are hereby incorporated herein by reference.
- [63] In addition, 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; the disclosures of which are hereby incorporated herein by reference.
- [64] 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.

- [65] 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.
- [66] 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) 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.
- [67] 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 spirit and scope of the invention as set forth in the appended claims.

EXAMPLES

Example 1

- [68] 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).
- [69] We analyzed the amino acid sequences of D2-9Gly-Fc and D2-Fc proteins using the Protein Analysis Toolbox of the sequence analysis program MacVector 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

- [70] We tested an isolated Flt-1 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

- [71] 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.
- [72] 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

- [73] Domain 2 of Flt-1 has been shown to be essential for VEGF₁₆₅ binding. However, it was demonstrated that Flt-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).
- [74] 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.

- [75] 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).
- [76] The protein D2-Fc did not show efficient inhibitory activity in the HUVEC proliferation assay (Fig. 5) and by implication did not bind to VEGF₁₆₅ 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 VEGF₁₆₅ efficiently.

Example 5

Using linker (Gly₄Ser)₃ in Flt-1 D2 construct

- [77] 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-(Gly₄Ser)₃-Fc protein was generated and it contains Flt-1 domain 2, (Gly₄Ser)₃ linker and the Fc region of human IgG1 heavy chain..
- [78] D2-(Gly₄Ser)₃-Fc was further characterized in HUVECs proliferation assay. Biological activity of D2-(Gly₄Ser)₃-Fc as measured by inhibition of HUVEC proliferation was similar to that of D2-9Gly-Fc and D2-9Gly-Ex3/CH3 (Fig. 6).

- [79] The D2-(Gly₄Ser)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

- [80] 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).
- [81] The HUVEC proliferation assay of proteins containing 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).
- [82] 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

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

- [84] 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 to bind VEGF 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).

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We claim:

1. A fusion protein of the formula X-Y-Z, wherein
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;
and
Y consists essentially of a 5 - 25 amino acid residue polypeptide, and
Z is a CH3 region of an IgG heavy chain molecule.
2. The fusion protein of claim 1 wherein X comprises an extracellular receptor and said receptor is selected from the group consisting of a tyrosine kinase receptor and a serine threonine kinase receptor.
3. The fusion protein of claim 1 wherein X comprises an extracellular receptor and said receptor is a VEGF receptor.
4. The fusion protein of claim 1 wherein X is the IgG-like domain 2 of VEGF-R1 (FLT-1).
5. The fusion protein of claim 1 wherein the polypeptide Y is flexible.
6. The fusion protein of claim 1 wherein the polypeptide Y is selected from the group consisting of gly₉ (SEQ ID NO: 27), glu₉ (SEQ ID NO: 28), ser₉ (SEQ ID NO: 29), gly₃cyspro₂cys (SEQ ID NO: 30), (gly₄ser)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 Gly₉ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 34).
7. The fusion protein of claim 1 wherein Z is an IgG1 CH3 region.
8. The fusion protein of claim 7 wherein Z is an IgG2 CH3 region.
9. A composition comprising the fusion protein of claim 1 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 claim 1, 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 wherein the X moiety is an extracellular receptor, and said
receptor is selected from the group consisting of a tyrosine kinase receptor and a serine
threonine kinase receptor.
16. The composition of claim 12 wherein the X moiety is an extracellular receptor, and said
receptor is a VEGF receptor or a portion thereof.
17. The composition of claim 12 wherein the X moiety is IgG-like domain 2 of VEGF-R1
(FLT-1).
18. The composition of claim 12 wherein Y is a flexible polypeptide.
19. The composition of claim 12 wherein Y is a polypeptide selected from the group
consisting of gly₉ (SEQ ID NO: 27), glu₉ (SEQ ID NO: 28), ser₉ (SEQ ID NO: 29),
gly₅cyspro₂cys (SEQ ID NO: 30), (gly₄ser)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
Gly₉ProSerCysValProLeuMetArgCysGlyGlyCysCysAsn (SEQ ID NO: 34).
20. The composition of claim 12 wherein Z is a CH₃ region and said region is an IgG1 CH₃
region.
21. The composition of claim 12 which further comprises one or more pharmaceutically
acceptable excipients or carriers.

22. The composition of claim 21 wherein said composition is a liquid formulation.
23. The composition of claim 21 wherein said composition is a lyophilized formulation.
24. A polypeptide of the formula X-Y-Z, wherein
- 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; and
- Z is** a CH3 region of an IgG heavy chain molecule.
25. The polypeptide of claim 24 wherein the linker moiety is an oligomer of from 10-100 ethylene glycol monomer residues
26. A fusion protein of the formula X-Y-Z, wherein
- 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; and
- Y consists essentially of** a 5 - 25 amino acid residue polypeptide, and
- Z is** an Fc portion of an antibody molecule.
27. The fusion protein of claim 26, wherein the Fc is an IgG Fc.
28. The fusion protein of claim 27, wherein the IgG Fc is IgG1 Fc.
29. A composition comprising the fusion protein of claim 26 which further comprises one or more pharmaceutically acceptable excipients or carriers.
30. The composition of claim 29 wherein Z is an Fc portion of an antibody molecule which is an IgG Fc.
31. The composition of claim 29 wherein Z is an Fc portion of an antibody molecule which is an IgG1 Fc.
32. A fusion protein of the formula X-Y-Z, wherein
- 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; and
- Y consists essentially of** a linker moiety which provides the spatial separation of 5-25 amino acid residues, and

Z is an Fc portion of an antibody molecule.

33. The fusion protein of claim 32, wherein the Fc is an IgG Fc.
34. The fusion protein of claim 33, wherein the IgG Fc is IgG1 Fc.
35. The fusion protein of claim 32 wherein the linker moiety is an oligomer of from 10-100 ethylene glycol monomer residues.
36. A composition comprising the fusion protein of claim 32 which further comprises one or more pharmaceutically acceptable excipients or carriers.
37. The composition of claim 36 wherein Z is an Fc portion of an antibody molecule which is an IgG Fc.
38. The composition of claim 36 wherein Z is an Fc portion of an antibody molecule which is an IgG1 Fc.
39. A method of multimerizing a polypeptide X comprising:

linking a polypeptide X to a polypeptide Z via a polypeptide Y to form polypeptide XYZ, wherein

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, and

Z is a CH3 region of an IgG heavy chain molecule;

whereby polypeptide XYZ multimerizes.

40. The method of claim 39 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.
41. A method of multimerizing a polypeptide X comprising:

linking a polypeptide X to a polypeptide Z via a moiety Y to form polymer XYZ, wherein

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, and

Z is a CH3 region of an IgG heavy chain molecule;

whereby polypeptide XYZ multimerizes.

42. The method of claim 41 wherein the linker moiety consists essentially of an oligomer of from 10-100 ethylene glycol monomer residues.
43. A nucleic acid molecule which encodes a fusion protein according to claim 1.
44. A nucleic acid molecule which encodes a fusion protein according to claim 26.
45. The nucleic acid of claim 43 or 44 wherein X is Ig-like domain 2 of VEGF-R1 (Flt-1).
46. The nucleic acid of claim 43 wherein X is 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: 8, 21, and 23.
47. The nucleic acid of claim 44 wherein X is 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.
48. The fusion protein of claim 1 or 26 wherein X is Ig-like domain 2 of VEGF-R1 (Flt-1).
49. The fusion protein of claim 1 wherein X is 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: 8, 21, and 23.
50. The fusion protein of claim 26 wherein X is 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.
51. A mammalian cell which comprises the nucleic acid of claim 43 or 44.
52. The mammalian cell of claim 51 which is a human cell.
53. The mammalian cell of claim 52 which is selected from the group consisting of a fibroblast, a hepatocyte, an endothelial cell, a keratinocyte, a hematopoietic cell, synoviocyte, epithelial cell, retinal cell, and a stem cell..

54. An *in vitro* method comprising:
delivering the nucleic acid of claim 43 or 44 to an isolated mammalian cell to form a cell which expresses said fusion protein.
55. The method of claim 54 wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.
56. The method of claim 54 further comprising:
delivering the cell which expresses said fusion protein to a mammal.
57. A method comprising:
delivering the mammalian cell of claim 51 to a mammal.
58. A method comprising:
delivering the nucleic acid of claim 43 or 44 to a mammal, whereby said fusion protein is expressed in the mammal.
59. The method of claim 58 wherein the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.
60. The method of claim 57 wherein the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy.
61. The method of claim 58 wherein the mammal has cancer.
62. The method of claim 58 wherein the mammal has rheumatoid arthritis.
63. The method of claim 58 wherein the mammal has asthma.
64. The method of claim 58 wherein the mammal has osteoarthritis.
65. A method comprising:
delivering the fusion protein of claim 1 or 26 to a mammal.
66. The method of claim 65 wherein the mammal has wet age-related macular degeneration or proliferative diabetic retinopathy.
67. The method of claim 65 wherein the mammal has cancer.
68. The method of claim 65 wherein the mammal has rheumatoid arthritis.
69. The method of claim 65 wherein the mammal has asthma.
70. The method of claim 65 wherein the mammal has osteoarthritis.
71. The method of claim 65 wherein the the fusion protein comprises a sequence selected from the group consisting of SEQ ID NO: 2, 8, 21, 23, and 25.

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7	FLT1D29GLYVEG	744	DNA
8	FLT1D29GLYVEG	247	Protein
9	iGg1 HEAVY	1434	DNA
10	IgG1 HEAVY	477	Protein
11	VEGF	648	DNA
12	VEGF	215	Protein
13	VEGF exon 3	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
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<212> PRT

<213> Homo sapiens

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Pro	Gly	Arg	Ser	Leu	Arg	Leu	Ser	Cys	Ala	Ala	Ser	Gly	Phe	Thr	Phe
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Ser	Asn	Tyr	Gly	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Gly	Lys	Gly	Leu
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Thr	Leu	Tyr	Met	Gln	Met	Asn	Ser	Leu	Arg	Ala	Glu	Asp	Thr	Ala	Val
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Leu	Tyr	Ser	Leu	Ser	Ser	Val	Val	Thr	Val	Pro	Ser	Ser	Ser	Leu	Gly
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Thr	Gln	Thr	Tyr	Ile	Cys	Asn	Val	Asn	His	Lys	Pro	Ser	Asn	Thr	Lys
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Val	Asp	Lys	Arg	Val	Glu	Pro	Lys	Ser	Cys	Asp	Lys	Thr	His	Thr	Cys
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 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
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<212> DNA

<213> Homo sapiens

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<400> 12

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 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

00025GENZ SEQ

Thr Glu Glu Ser Asn Ile Thr Met Gln Ile Met Arg Ile Lys Pro His
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 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
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 Lys Ser Trp Ser Val Pro Cys Gly Pro Cys Ser Glu Arg Arg Lys His
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 Leu Phe Val Gln Asp Pro Gln Thr Cys Lys Cys Ser Cys Lys Asn Thr
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 Leu His Leu Gln Cys Arg Gly Glu Ala Ala His Lys Trp Ser Leu Pro
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 Glu Met Val Ser Lys Glu Ser Glu Arg Leu Ser Ile Thr Lys Ser Ala
 65 70 75 80
 Cys Gly Arg Asn Gly Lys Gln Phe Cys Ser Thr Leu Thr Leu Asn Thr
 85 90 95
 Ala Gln Ala Asn His Thr Gly Phe Tyr Ser Cys Lys Tyr Leu Ala Val
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 Pro Thr Ser Lys Lys Lys Glu Thr Glu Ser Ala Ile Tyr Ile Phe Ile
 115 120 125
 Ser Asp Thr Gly Arg Pro Phe Val Glu Met Tyr Ser Glu Ile Pro Glu
 130 135 140
 Ile Ile His Met Thr Glu Gly Arg Glu Leu Val Ile Pro Cys Arg Val
 145 150 155 160
 Thr Ser Pro Asn Ile Thr Val Thr Leu Lys Lys Phe Pro Leu Asp Thr
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 Leu Ile Pro Asp Gly Lys Arg Ile Ile Trp Asp Ser Arg Lys Gly Phe
 180 185 190
 Ile Ile Ser Asn Ala Thr Tyr Lys Glu Ile Gly Leu Leu Thr Cys Glu
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 Ala Thr Val Asn Gly His Leu Tyr Lys Thr Asn Tyr Leu Thr His Arg
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 Gln Thr Asn Thr Ile Ile Asp Val Gln Ile Ser Thr Pro Arg Pro Val
 225 230 235 240
 Lys Leu Leu Arg Gly His Thr Leu Val Leu Asn Cys Thr Ala Thr Thr
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 Pro Leu Asn Thr Arg Val Gln Met Thr Trp Ser Tyr Pro Asp Glu Lys
 260 265 270
 Asn Lys Arg Ala Ser Val Arg Arg Arg Ile Asp Gln Ser Asn Ser His
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 Ala Asn Ile Phe Tyr Ser Val Leu Thr Ile Asp Lys Met Gln Asn Lys
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 Asp Lys Gly Leu Tyr Thr Cys Arg Val Arg Ser Gly Pro Ser Phe Lys
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 Tyr Arg Leu Ser Met Lys Val Lys Ala Phe Pro Ser Pro Glu Val Val
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Gly	Asn	Tyr	Thr	Ile	Leu	Leu	Ser	Ile	Lys	Gln	Ser	Asn	Val	Phe	Lys
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Lys	Ala	Val	Ser	Ser	Phe	Pro	Asp	Pro	Ala	Leu	Tyr	Pro	Leu	Gly	Ser
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00025GENZ SEQ

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00025GENZ SEQ

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Arg	Gln	Leu	Thr 660	Val	Leu	Glu	Arg	Val 665	Ala	Pro	Thr	Ile	Thr 670	Gly	Asn
Leu	Glu	Asn 675	Gln	Thr	Thr	Ser	Ile 680	Gly	Glu	Ser	Ile	Glu 685	Val	Ser	Cys
Thr	Ala	Ser 690	Gly	Asn	Pro	Pro 695	Pro	Gln	Ile	Met	Trp 700	Phe	Lys	Asp	Asn
Glu 705	Thr	Leu	Val	Glu	Asp 710	Ser	Gly	Ile	Val	Leu 715	Lys	Asp	Gly	Asn	Arg 720
Asn	Leu	Thr	Ile	Arg 725	Arg	Val	Arg	Lys	Glu 730	Asp	Glu	Gly	Leu	Tyr 735	Thr
Cys	Gln	Ala	Cys 740	Ser	Val	Leu	Gly	Cys 745	Ala	Lys	Val	Glu	Ala 750	Phe	Phe
Ile	Ile	Glu 755	Gly	Ala	Gln	Glu	Lys 760	Thr	Asn	Leu	Glu	Ile 765	Ile	Ile	Leu
Val	Gly 770	Thr	Ala	Val	Ile	Ala 775	Met	Phe	Phe	Trp	Leu 780	Leu	Leu	Val	Ile
Ile 785	Leu	Arg	Thr	Val	Lys 790	Arg	Ala	Asn	Gly	Gly 795	Glu	Leu	Lys	Thr	Gly 800
Tyr	Leu	Ser	Ile	Val 805	Met	Asp	Pro	Asp	Glu 810	Leu	Pro	Leu	Asp	Glu 815	His
Cys	Glu	Arg	Leu 820	Pro	Tyr	Asp	Ala	Ser 825	Lys	Trp	Glu	Phe	Pro 830	Arg	Asp
Arg	Leu	Lys 835	Leu	Gly	Lys	Pro	Leu 840	Gly	Arg	Gly	Ala	Phe 845	Gly	Gln	Val
Ile	Glu 850	Ala	Asp	Ala	Phe	Gly 855	Ile	Asp	Lys	Thr	Ala 860	Thr	Cys	Arg	Thr
Val 865	Ala	Val	Lys	Met	Leu 870	Lys	Glu	Gly	Ala	Thr 875	His	Ser	Glu	His	Arg 880
Ala	Leu	Met	Ser	Glu 885	Leu	Lys	Ile	Leu	Ile 890	His	Ile	Gly	His	His 895	Leu
Asn	Val	Val	Asn 900	Leu	Leu	Gly	Ala	Cys 905	Thr	Lys	Pro	Gly	Gly 910	Pro	Leu
Met	Val	Ile 915	Val	Glu	Phe	Cys	Lys 920	Phe	Gly	Asn	Leu	Ser 925	Thr	Tyr	Leu
Arg	Ser 930	Lys	Arg	Asn	Glu	Phe 935	Val	Pro	Tyr	Lys	Thr 940	Lys	Gly	Ala	Arg
Phe 945	Arg	Gln	Gly	Lys	Asp 950	Tyr	Val	Gly	Ala	Ile 955	Pro	Val	Asp	Leu	Lys 960
Arg	Arg	Leu	Asp	Ser 965	Ile	Thr	Ser	Ser	Gln 970	Ser	Ser	Ala	Ser	Ser 975	Gly
Phe	Val	Glu	Glu 980	Lys	Ser	Leu	Ser	Asp 985	Val	Glu	Glu	Glu	Glu 990	Ala	Pro
Glu	Asp	Leu	Tyr	Lys	Asp	Phe	Leu	Thr 1000	Leu	Glu	His	Leu	Ile 1005	Cys	Tyr
Ser	Phe 1010	Gln	Val	Ala	Lys	Gly 1015	Met	Glu	Phe	Leu	Ala 1020	Ser	Arg	Lys	Cys
Ile 1025	His	Arg	Asp	Leu	Ala 1030	Ala	Arg	Asn	Ile	Leu 1035	Leu	Ser	Glu	Lys	Asn 1040
Val	Val	Lys	Ile	Cys 1045	Asp	Phe	Gly	Leu	Ala 1050	Arg	Asp	Ile	Tyr	Lys 1055	Asp
Pro	Asp	Tyr	Val 1060	Arg	Lys	Gly	Asp	Ala 1065	Arg	Leu	Pro	Leu	Lys 1070	Trp	Met
Ala	Pro	Glu	Thr 1075	Ile	Phe	Asp	Arg 1080	Val	Tyr	Thr	Ile	Gln 1085	Ser	Asp	Val
Trp	Ser	Phe	Gly	Val	Leu	Leu	Trp 1095	Glu	Ile	Phe	Ser	Leu	Gly	Ala	Ser
Pro 1105	Tyr	Pro	Gly	Val	Lys 1110	Ile	Asp	Glu	Glu	Phe 1115	Cys	Arg	Arg	Leu	Lys 1120
Glu	Gly	Thr	Arg	Met	Arg	Ala	Pro	Asp	Tyr	Thr	Thr	Pro	Glu	Met	Tyr

00025GENZ SEQ

```

      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

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

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

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acaggatctg gtagaccttt cgtagagatg tacagtgaat tccccgaaat tatacacatg      120
actgaaggaa gggagctcgt cattccctgc cgggttacgt cacctaacaat cactgttact      180
ttaaaaaagt ttccacttga cactttgatc cctgatggaa aacgcataat ctgggacagt      240
agaaagggtc tcatcataatc aaatgcaacg tacaaagaaa tagggcttct gacctgtgaa      300
gcaacagtca atgggcattt gtataagaca aactatctca cacatcgaca aaccagccc      360
cgagaaccac aggtgtacac cctgccccca tcccgggatg agctgaccaa gaaccaggtc      420
agcctgacct gcctgggtcaa aggcttctat cccagcgaca tcgccgtgga gtgggagagc      480
aatgggcagc cggagaacaa ctacaagacc acgcctcccg tgctggactc cgacggctcc      540
ttcttctctc acagcaagct caccgtggac aagagcaggt ggcagcaggg gaacgtcttc      600
tcatgtccg 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

00025GENZ SEQ

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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actgaaggaa	gggagctcgt	cattccctgc	cggggtacgt	cacctaacat	cactgttact	180
ttaaaaaagt	ttccacttga	cactttgatc	cctgatggaa	aacgcataat	ctgggacagt	240
agaaagggct	tcatcataatc	aaatgcaacg	tacaaagaaa	tagggcttct	gacctgtgaa	300
gcaacagtca	atgggcattt	gtataagaca	aactatctca	cacatcgaca	aacccttcc	360
tgtgtgcccc	tgatgcatg	cgggggctgc	tgcaatcagc	cccgagaacc	acagggtgtac	420
accctgcccc	catcccggga	tgagctgacc	aagaaccagg	tcagcctgac	ctgcctggtc	480
aaaggcttct	atcccagcga	catcgccgtg	gagtgggaga	gcaatgggca	gccggagAAC	540
aactacaaga	ccacgcctcc	cgtgctggac	tccgacggct	ccttcttctc	ctacagcaag	600
ctcaccgtgg	acaagagcag	gtggcagcag	gggaacgtct	tctcatgctc	cgatgatcat	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
-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----

00025GENZ SEQ

1	Cys	Leu	Leu	Leu	5	Thr	Gly	Ser	Gly	Arg	10	Pro	Phe	Val	Glu	Met	15	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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actgaaggaa	gggagctcgt	cattccctgc	cggttacgt	cacctaacaat	cactgttact	180
ttaaaaaagt	ttccacttga	cactttgatc	cctgatggaa	aacgcataat	ctgggacagt	240
agaaagggtc	tcatcataat	aaatgcaacg	tacaaagaaa	tagggcttct	gacctgtgaa	300
gcaacagtca	atgggcattt	gtataagaca	aactatctca	cacatcgaca	aaccgggtgga	360
ggtggaggtg	gaggtggagg	tcagccccga	gaaccacagg	tgtacaccct	gcccccatcc	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

00025GENZ SEQ

Met Val Ser Tyr Trp Asp Thr Gly Val Leu Leu Cys Ala Leu Leu Ser
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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 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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actgaaggaa	gggagctcgt	cattccctgc	cgggttacgt	cacctaakat	cactgttact	180
ttaaaaaagt	ttccacttga	cactttgatc	cctgatggaa	aacgcataat	ctgggacagt	240
agaaagggtc	tcatacatatc	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
ccccaaaac	ccaaggacac	cctcatgatc	tcccggacc	ctgaggtcac	atgcgtggtg	540
gtggacgtga	gccacgaaga	ccctgaggtc	aagttcaact	ggtacgtgga	cggcgtggag	600
gtgcataatg	ccaagacaaa	gccgcgggag	gagcagtaca	acagcacgta	ccgtgtgggtc	660
agcgtcctca	ccgtcctgca	ccaggactgg	ctgaatggca	aggagtacaa	gtgcaagggtc	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	cgacgggtcc	960
ttcttctctc	acagcaagct	caccgtggac	aagagcaggt	ggcagcaggg	gaacgtcttc	1020
tcattgctccg	tgatgcatga	ggctctgcac	aaccactaca	cgcagaagag	cctctccctg	1080
tctccgggta	aatag					1095

<210> 25

<211> 364

00025GENZ SEQ

<212> PRT

<213> Artificial Sequence

<220>

<223> Recombinant fusion protein or sequence encoding same

<400> 25

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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 Ser Gly Gly Gly Ser
 115     120     125
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
Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln
 325     330     335
Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
 340     345     350
Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 355     360

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<210> 26

<211> 9

<212> PRT

<213> Artificial Sequence

<220>

<223> linker of fusion protein

00025GENZ SEQ

<400> 26
Gly Asp Leu Ile Tyr Arg Asn Gln Lys
1 5

<210> 27
<211> 9
<212> PRT
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<220>
<223> linker moiety for fusion proteins

<400> 27
Gly Gly Gly Gly Gly Gly Gly Gly Gly
1 5

<210> 28
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> linker moiety for fusion proteins

<400> 28
Glu Glu Glu Glu Glu Glu Glu Glu Glu
1 5

<210> 29
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> linker moiety for fusion proteins

<400> 29
Ser Ser Ser Ser Ser Ser Ser Ser Ser
1 5

<210> 30
<211> 9
<212> PRT
<213> Artificial Sequence

<220>
<223> linker moiety for fusion proteins

<400> 30
Gly Gly Gly Gly Gly Cys Pro Pro Cys
1 5

<210> 31
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> linker moiety for fusion proteins

<400> 31
Gly Gly Gly Gly Ser Ser Ser
1 5

00025GENZ SEQ

<210> 32
 <211> 13
 <212> PRT
 <213> Artificial Sequence

<220>
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<400> 32
 Ser Cys Val Pro Leu Met Arg Cys Gly Gly Cys Cys Asn
 1 5 10

<210> 33
 <211> 9
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> linker moiety for fusion proteins

<400> 33
 Gly Asp Leu Ile Tyr Arg Asn Gln Lys
 1 5

<210> 34
 <211> 23
 <212> PRT
 <213> Artificial Sequence

<220>
 <223> linker moiety for fusion proteins

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 1 5 10 15
 Arg Cys Gly Gly Cys Cys Asn
 20

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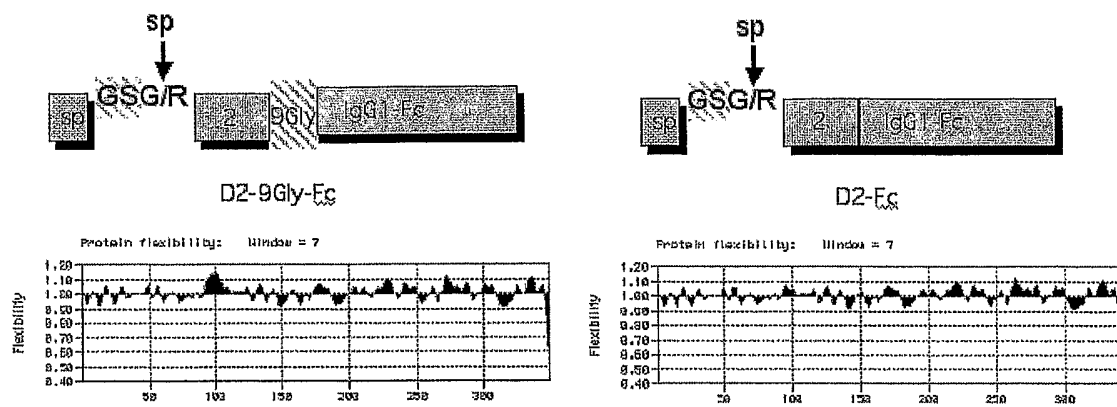


FIG 1

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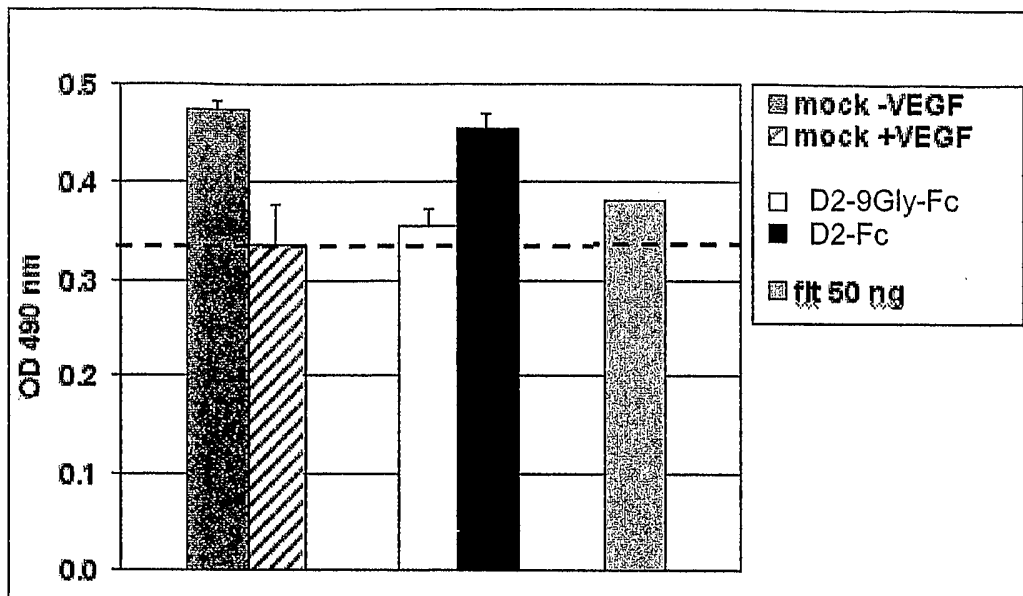
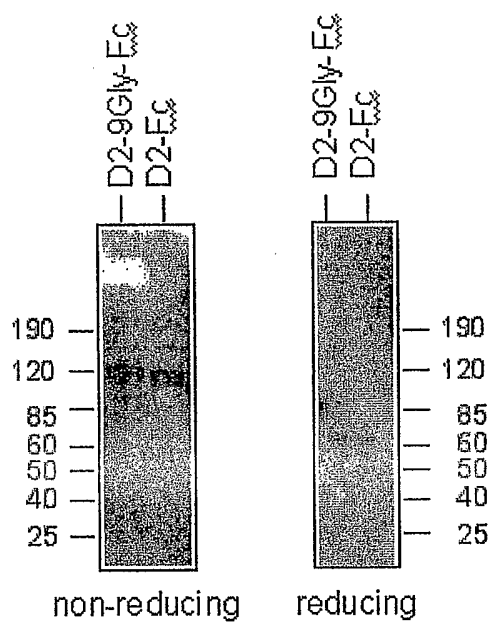


FIG 2

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**FIG 3**

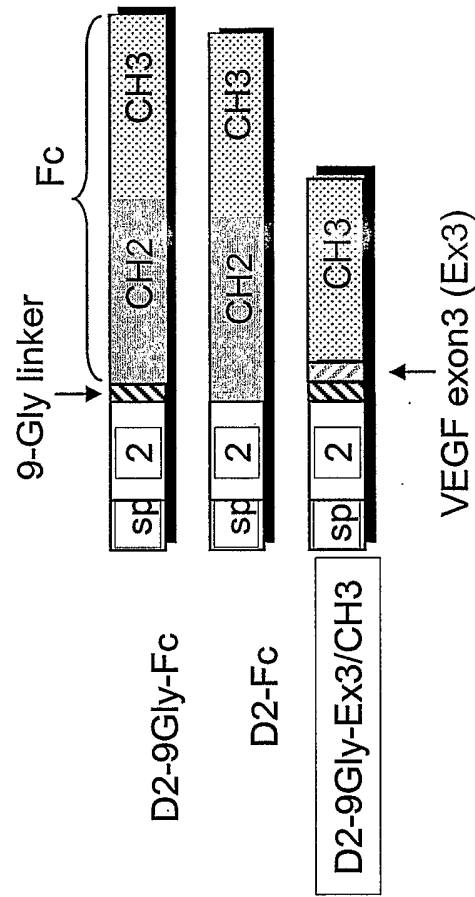


Fig. 4

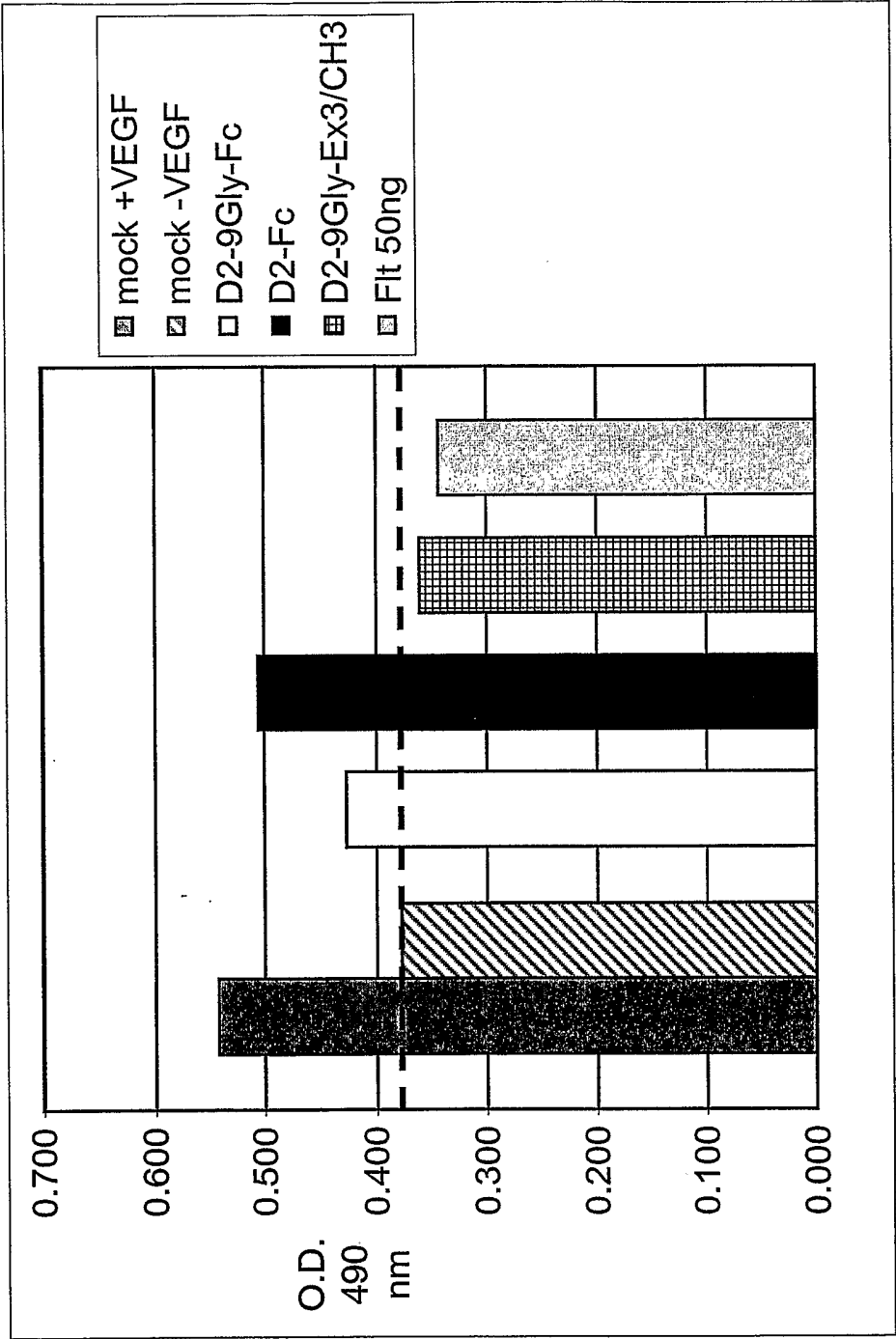


Fig. 5

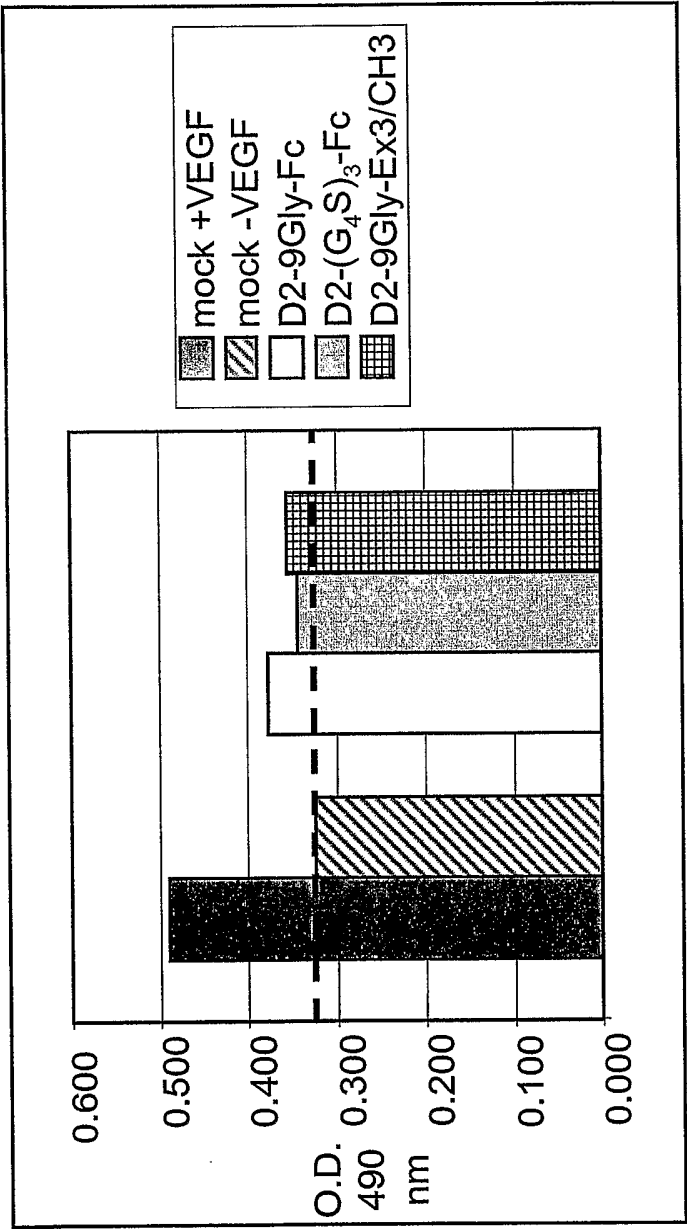


Fig. 6

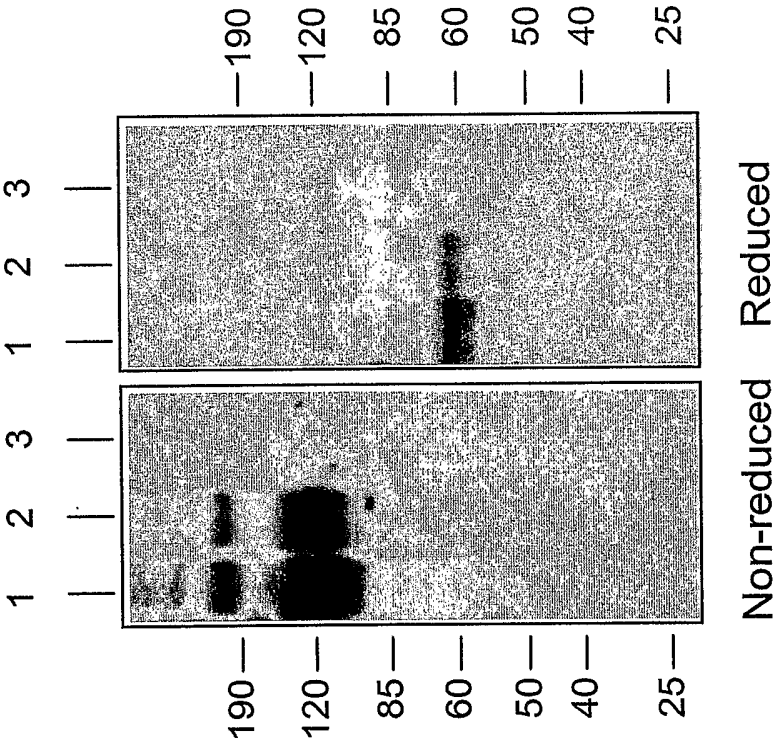


Fig.7

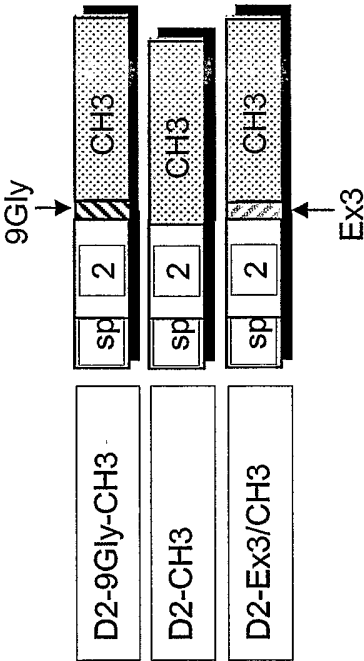


Fig. 8

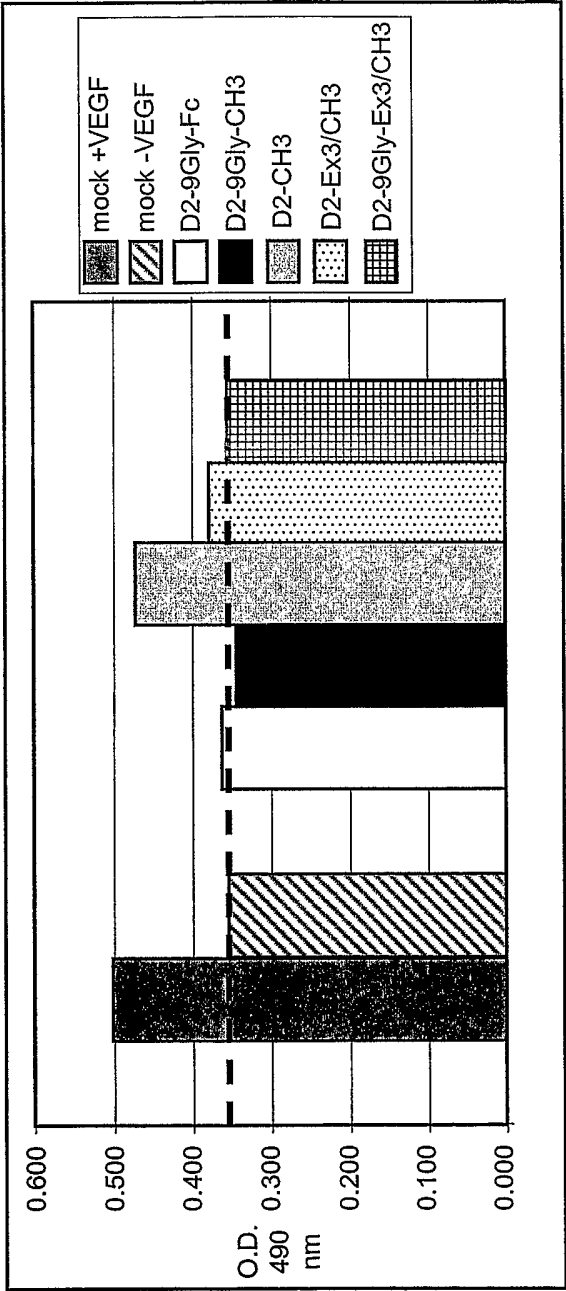


Fig. 9

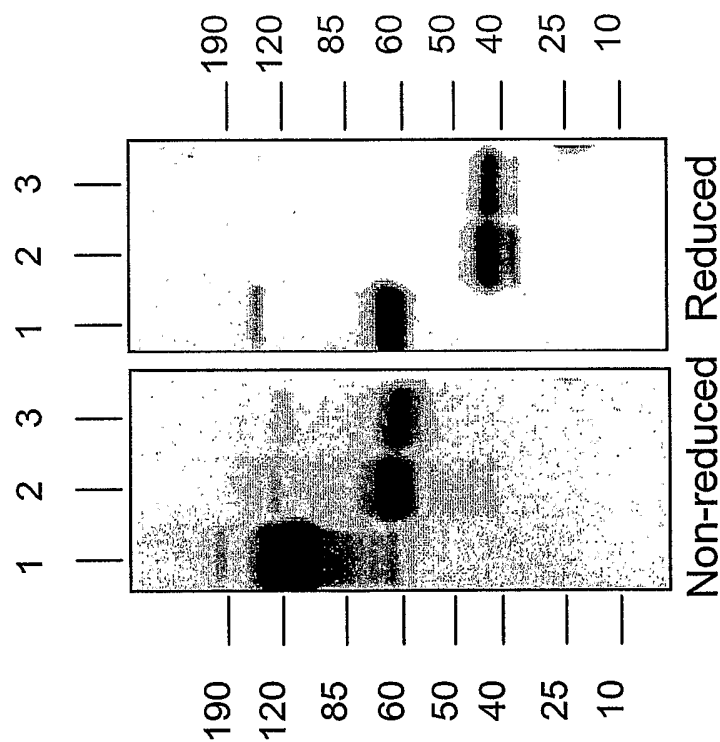


Fig.10

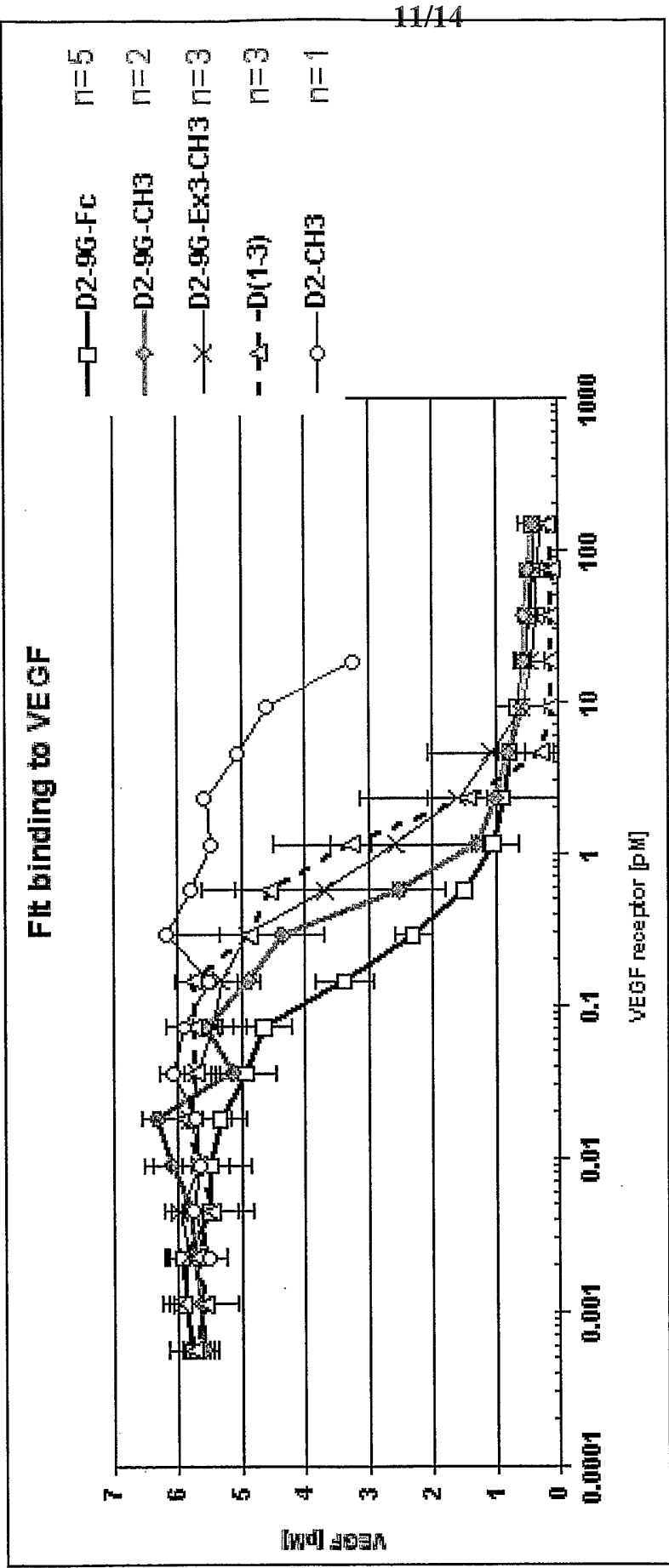


Fig. 11

BIAcore binding kinetics for sFlt-1 molecules

Sensorgrams:

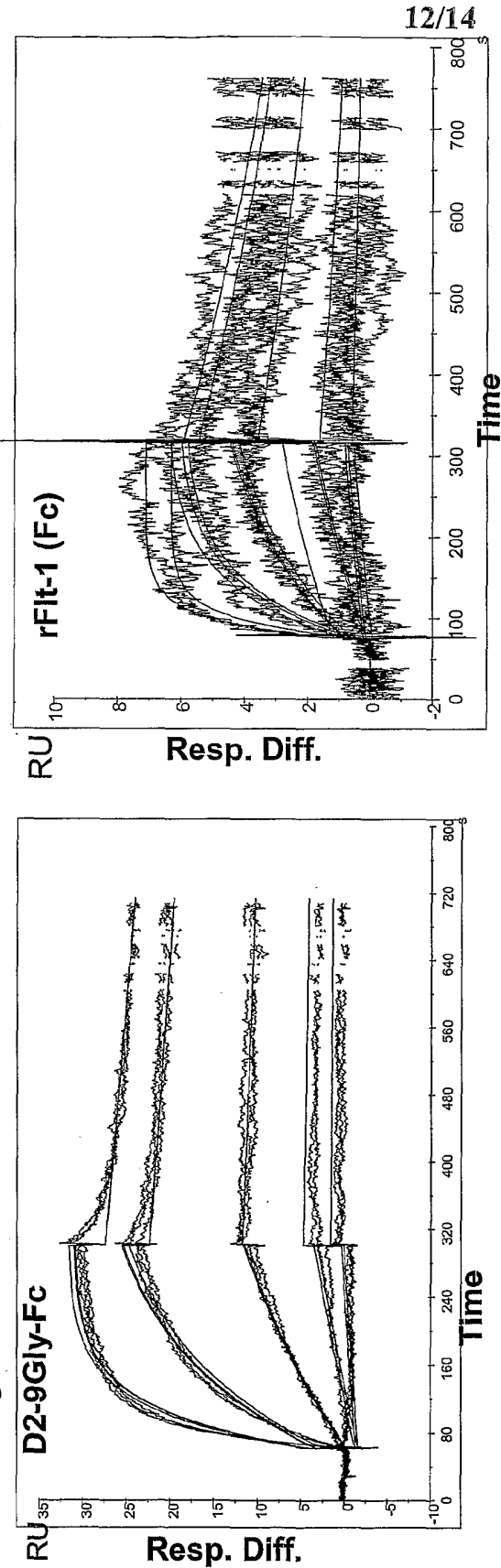


Table:

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 $K_d/K_a = K_D$. The lower K_D value means better affinity.

FIG 12

Bioactivity

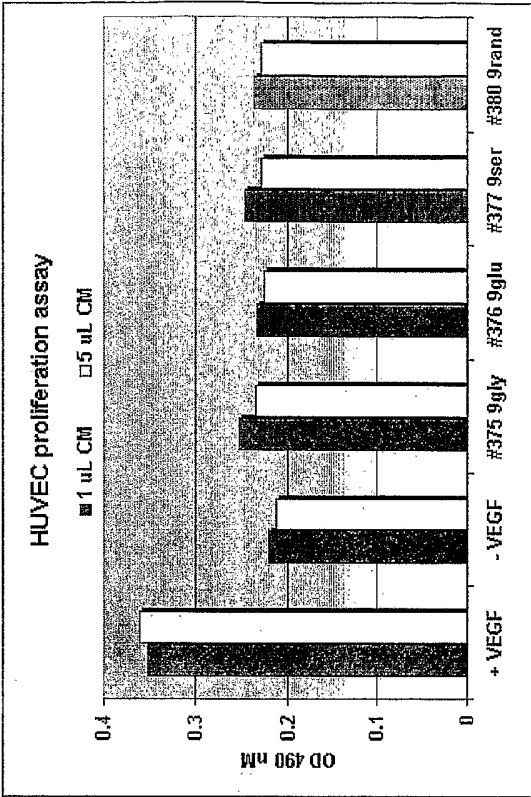


FIG 13 B

Expression

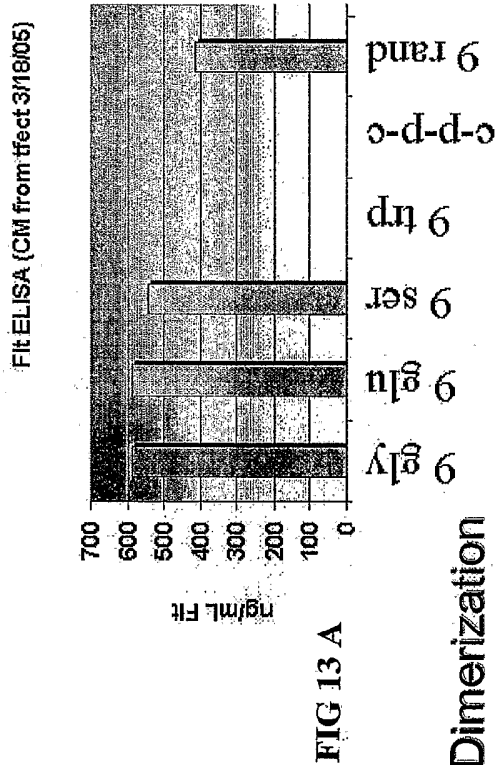


FIG 13 A

Dimerization

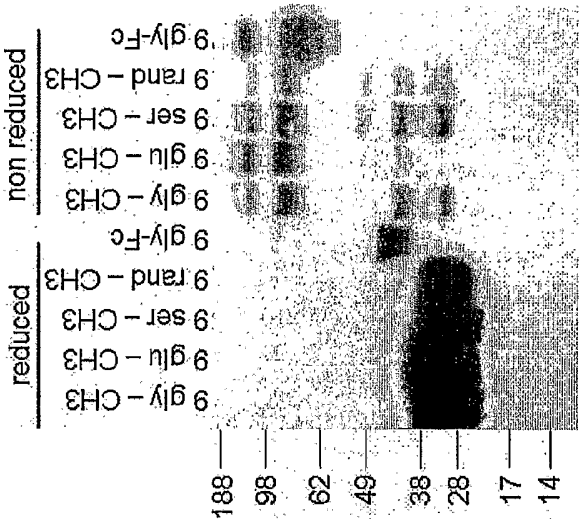
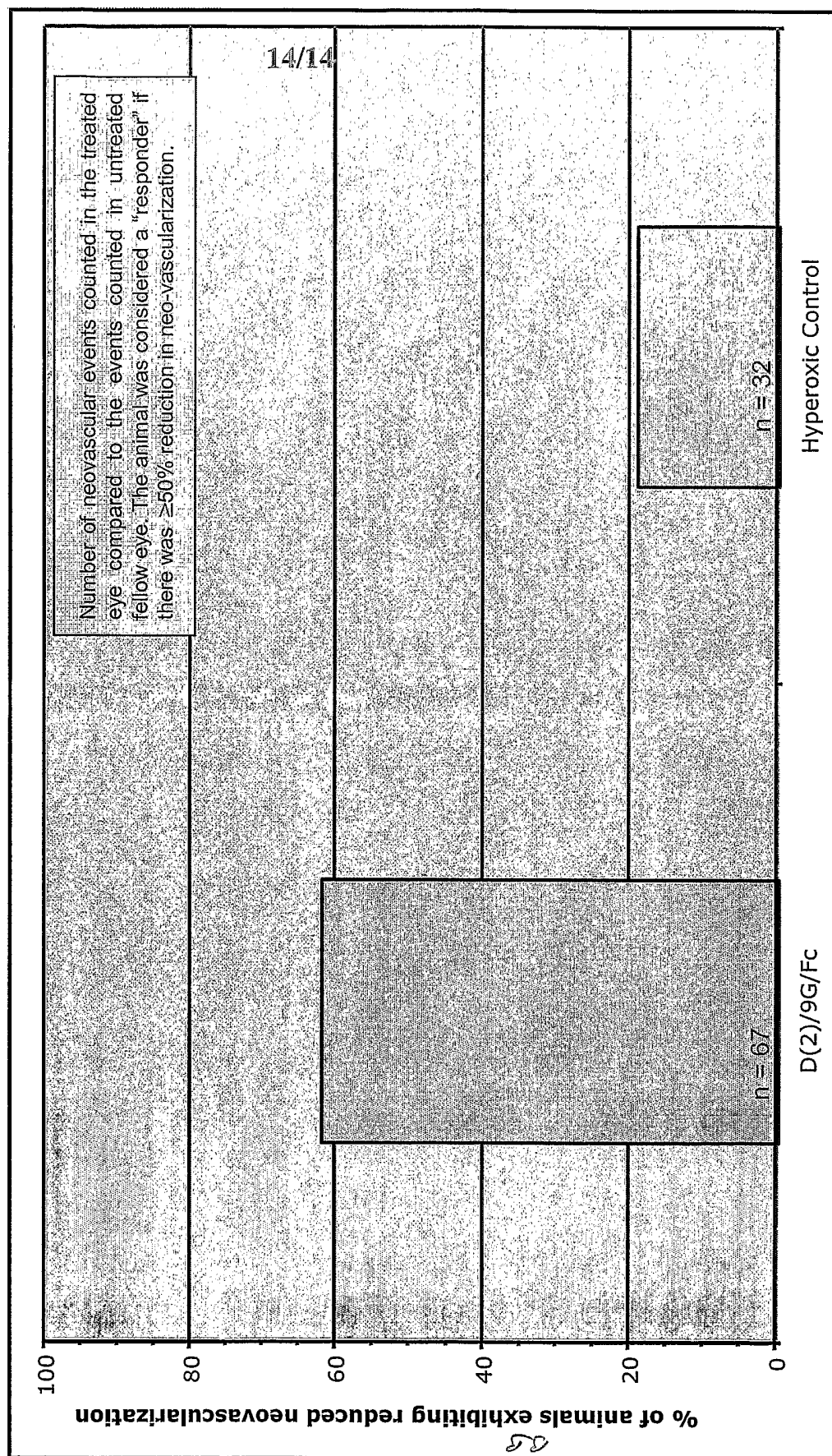


FIG 13 C

Mouse OIR model

**FIG 14**