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(54) Titre : POLYPEPTIDES HYBRIDES CAPABLES D'ACTIVER DES RECEPTEURS
(54) Title: FUSION POLYPEPTIDES CAPABLE OF ACTIVATING RECEPTORS

(57) Abrégé/Abstract:
A fusion polypeptide comprising $(A)_X$-M-$(A')_Y$, wherein A and A' are each polypeptides capable of binding a target receptor. The fusion polypeptides of the invention form multimeric proteins which activate the target receptor. A and A~ may be each be an antibody or fragment derived form an antibody specific for a target receptor. The antibody or fragment may be the same or different ScFv fragments, and/or a ligand or ligand fragment, or derivative, capable of binding the target protein. M is a multimerizing component, and X and Y are independently a number between 1-10.

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(54) Title: FUSION POLYPEPTIDES CAPABLE OF ACTIVATING RECEPTORS

(57) Abstract: A fusion polypeptide comprising $(A)_x$-M-$(A')_Y$, wherein A and A' are each polypeptides capable of binding a target receptor. The fusion polypeptides of the invention form multimeric proteins which activate the target receptor. A and A' may be each be an antibody or fragment derived form an antibody specific for a target receptor. The antibody or fragment may be the same or different ScFv fragments, and/or a ligand or ligand fragment, or derivative, capable of binding the target protein. M is a multimerizing component, and X and Y are independently a number between 1-10.

FUSION POLYPEPTIDES CAPABLE OF ACTIVATING RECEPTORS

Field of the Invention

[0001] This invention relates to multimeric fusion proteins capable of activating a target receptor, methods of producing such fusion polypeptides, and methods for treating, diagnosing, or monitoring diseases or conditions in which activation of the target receptor is desired.

Description of Related Art

[0002] The clustering of soluble Eph ligand domains to create multimers capable of activating their cognate receptors is described in US Patent 5,747,033. US Patent 6,319,499 recites a method of activating an erythropoietin receptor with an antibody.

BRIEF SUMMARY OF THE INVENTION

[0003] The present invention provides multimeric fusion polypeptides capable of activating a target receptor requiring multimerization to be activated. The polypeptides of the invention are useful for treating conditions in which activation of a target receptor is desirable, as well as having a variety of *in vitro* and *in vivo* diagnostic and prognostic uses. The polypeptides of the invention may be monospecific or bispecific tetramers exhibiting improved capacity to activate a target receptor relative to, for example, a target-specific antibody or the natural ligand.

[0004] Accordingly, in a first aspect the invention provides an isolated nucleic acid molecule which encodes a fusion polypeptide $(A)_x$–M–$(A')_y$, wherein A is a polypeptide specific for a target receptor, M is a multimerizing component, A' is a polypeptide specific for the same target receptor as A, and X and Y are independently a number between 1-10.

[0005] In a first embodiment, A and A' are antibodies or antibody fragments specific to the target receptor, and are the same antibody or antibody fragment specific to a target receptor. In another embodiment, A and A' are different antibodies or antibody fragments specific to the same target receptor. Preferably, A and A' are single chain Fv (ScFv) fragments. When the fusion polypeptide is intended as a human therapeutic, the invention encompasses humanized antibody or antibody fragments.

[0006] In a second embodiment, A and A' are ligands or ligand fragments specific for the same target receptor. In a more specific embodiment, A and A' are different ligands or ligand fragments specific to the same target receptor. In another specific embodiment, A and A' are the same ligand or ligand fragment.

[0007] In a third embodiment, A is an antibody or antibody fragment specific to the target receptor, and A' is a ligand or ligand fragment specific to the same target receptor. In preferred embodiments, A is an antibody or antibody fragment to a Tie receptor (Tie-1 or Tie-2), and A' is the fibrinogen domain of a Tie receptor.

[0008] In specific embodiments, M is a multimerizing component which multimerizes with a multimerizing component on another fusion polypeptide to form a multimer of the fusion

polypeptides. In a preferred embodiment, M is the Fc domain of IgG or the heavy chain of IgG. The Fc domain of IgG may be selected from the isotypes IgG1, IgG2, IgG3, and IgG4, as well as any allotype within each isotype group.

[0009] In a second aspect the invention provides a fusion polypeptide comprising $(A)_x$–M–$(A')_y$, wherein A, M, A', X and Y are as described above.

[0010] In a first embodiment, A and A' are antibodies or antibody fragments specific to the target receptor, and are the same antibody or antibody fragment specific to a target receptor. In another embodiment, A and A' are different antibodies or antibody fragments specific to the same target receptor. Preferably, A and A' are single chain Fv (ScFv) fragments.

[0011] In a second embodiment, A and A' are ligands or ligand fragments specific for the same target receptor. In a more specific embodiment, A and A' are the same or different ligands or ligand fragments specific to the same target receptor.

[0012] In a third embodiment, A is an antibody or antibody fragment specific to the target receptor, and A' is a ligand or ligand fragment specific to the same target receptor.

[0013] In a third aspect, the invention provides an activating dimeric fusion polypeptide comprising two fusion polypeptides of the invention, e.g., a dimer formed from two polypeptides of $(A)_x$–M–$(A')_y$ as defined above. The activating dimers of the invention are capable of binding to and clustering four or more receptors, leading to receptor activation, as compared with the ability of an antibody to cluster no more than two receptors.

[0014] In one embodiment, the components of the fusion polypeptides of the invention are connected directly to each other. In other embodiments, a spacer sequence may be included between one or more components, which may comprise one or more molecules, such as amino acids. For example, a spacer sequence may include one or more amino acids naturally connected to a domain-containing component. A spacer sequence may also include a sequence used to enhance expression of the fusion polypeptide, provide restriction sites, allow component domains to form optimal tertiary and quaternary structures and/or to enhance the interaction of a component with its target receptor. In one embodiment, the fusion polypeptide of the invention comprises one or more peptide sequences between one or more components which is(are) between 1-25 amino acids. Further embodiments may include a signal sequence at the beginning or amino-terminus of an fusion polypeptide of the invention. Such a signal sequence may be native to the cell, recombinant, or synthetic.

[0015] The components of the fusion polypeptide of the invention may be arranged in a variety of configurations. For example, described from the beginning or amino-terminus of the fusion polypeptide, $(A)_x$-M-$(A')_y$, $(A)_x$-$(A')_y$-M, M-$(A)_x$-$(A')_y$, $(A')_Y$-M-$(A)_X$, $(A')_Y$-$(A)_X$-M, M-$(A')_Y$-$(A)_X$, $(A)_x$-M-$(A')_y$, $(A)_x$-$(A')_y$-M, M-$(A)_x$-$(A')_y$, etc., wherein X = 1-10 and Y = 1-10. In an even more specific embodiment, X = 1, and Y = 1 or X = 2 and Y = 2.

[0016] In a fourth aspect, the invention features a vector comprising a nucleic acid sequence of the invention. The invention further features an expression vector comprising a nucleic acid of

the invention, wherein the nucleic acid molecule is operably linked to an expression control sequence. Also provided is a host-vector system for the production of the fusion polypeptides of the invention which comprises the expression vector of the invention which has been introduced into a host cell or organism, including, but not limited to, transgenic animals, suitable for expression of the fusion polypeptides.

[0017] In a fifth aspect, the invention features a method of producing a fusion polypeptide of the invention, comprising culturing a host cell transfected with a vector comprising a nucleic acid sequence of the invention, under conditions suitable for expression of the polypeptide from the host cell, and recovering the fusion polypeptide so produced.

[0018] In a sixth aspect, the invention features therapeutic methods for the treatment of a target receptor-related disease or condition, comprising administering a therapeutically effective amount of an activating dimer of the invnetion to a subject in need thereof, wherein the target receptor is activated, and the disease or condition is ameliorated or inhibited.

[0019] Accordingly, in a seventh aspect, the invention features pharmaceutical compositions comprising an activating dimer of the invention with a pharmaceutically acceptable carrier. Such pharmaceutical compositions may comprise dimeric proteins or encoding nucleic acids.

[0020] Other objects and advantages will become apparent from a review of the ensuing detailed description.

DETAILED DESCRIPTION

[0021] Before the present methods are described, it is to be understood that this invention is not limited to particular methods, and experimental conditions described, as such methods and conditions may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims.

[0022] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described.

Definitions

[0023] As used herein, the term "target receptor-related condition or disease" generally encompasses a condition of a mammalian host, particularly a human host, which is associated with a particular target receptor. Thus, treating a target receptor-related condition will encompass the treatment of a mammal, in particular, a human, who has symptoms reflective of decreased target receptor activation, or who is expected to have such decreased levels in response to a disease, condition or treatment regimen. Treating an target receptor-related condition or disease encompasses the treatment of a human subject wherein enhancing the

activation of a target receptor with an activating dimer of the invention results in amelioration of an undesirable symptom resulting from the target receptor-related condition or disease. As used herein, an "target receptor-related condition" also includes a condition in which it is desirable to alter, either transiently, or long-term, activation of a particular target receptor.

Target Receptors

[0024] Examples of target receptors are members of the Eph family (e.g. EphA1, EphA2, EphA3, EphA4, EphA5, EphA6, EphA7, EphA8, EphB1, EphB2, EphB3, EphB4, EphB5, EphB6), Tie receptors (e.g. Tie-1 or Tie-2). Suitable ligands or fragments thereof include the soluble domain of an ephrin (e.g. ephrin-A1, ephrin-A2, ephrin-A3, ephrin-A4, ephrin-A5, ephrin-B1, ephrin-B2, ephrin-B3), and the fibrinogen domain of an angiopoietin (e.g. angiopoietin-1 (ang-1), ang-2, ang-3, and/or ang-4).

[0025] Suitable target receptors are receptors that are activated when multimerized. This class of receptors includes, but is not limited to, those that possess an integral kinase domain. Within this class of integral kinase receptors are those that form homodimers, or clusters of the same receptor, such as Tie-1, Tie-2, EGFR, FGFR, the Trk family and the Eph family of receptors, and those that form heterodimers, or clusters, such as the VEGF receptors VEGFR1, VEGFR2, the PDGF receptors PDGFRα and PDGFRβ, and the TGF-β family receptors. Suitable target receptors also include, but are not limited to, the class of receptors with associated kinases. These receptors include those that form homodimers, or clusters, such as the growth hormone receptor, EPOR and the G-CSF receptor CD114, and those that form heterodimers, or clusters, such as the GM-CSF receptors GMRα and GMRβ

Target Receptor-Specific Antibodies and Ligands

[0026] In specific embodiments, the activating dimers of the invention comprise one or more immunoglobulin binding domains isolated from antibodies generated against a selected target receptor. The term "immunoglobulin" or "antibody" as used herein refers to a mammalian, including human, polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen, which, in the case of the present invention, is an target receptor or portion thereof. If the intended activating dimer will be used as a mammalian therapeutic, immunoglobulin binding regions should be derived from the corresponding mammalian immunoglobulins. If the activating dimer is intended for non-therapeutic use, such as for diagnostics and ELISAs, the immunoglobulin binding regions may be derived from either human or non-human mammals, such as mice. The human immunoglobulin genes or gene fragments include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant regions, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD,

and IgE, respectively. Within each IgG class, there are different isotypes (eg. IgG_1, IgG_2, etc.). Typically, the antigen-binding region of an antibody will be the most critical in determining specificity and affinity of binding.

[0027] An exemplary immunoglobulin (antibody) structural unit of human IgG, comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one light chain (about 25 kD) and one heavy chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100-110 or more amino acids primarily responsible for antigen recognition. The terms "variable light chain" (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0028] Antibodies exist as intact immunoglobulins, or as a number of well-characterized fragments produced by digestion with various peptidases, e.g., $F(ab)'_2$, Fab', etc. Thus, the terms immunoglobulin or antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) (ScFv) or those identified using phase display libraries (see, for example, McCafferty et al. (1990) Nature 348:552-554). In addition, the target receptor-binding domain component of the fusion polypeptides of the invention include the variable regions of the heavy (V_H) or the light (V_L) chains of immunoglobulins, as well as target receptor-binding portions thereof. Methods for producing such variable regions are described in Reiter, et al. (1999) J. Mol. Biol. 290:685-698.

[0029] Methods for preparing antibodies are known to the art. See, for example, Kohler & Milstein (1975) Nature 256:495-497; Harlow & Lane (1988) Antibodies: a Laboratory Manual, Cold Spring Harbor Lab., Cold Spring Harbor, NY). The genes encoding the heavy and light chains of an antibody of interest can be cloned from a cell, e.g., the genes encoding a monoclonal antibody can be cloned from a hybridoma and used to produce a recombinant monoclonal antibody. Gene libraries encoding heavy and light chains of monoclonal antibodies can also be made from hybridoma or plasma cells. Random combinations of the heavy and light chain gene products generate a large pool of antibodies with different antigenic specificity. Techniques for the production of single chain antibodies or recombinant antibodies (US Patent No. 4,946,778; US Patent No. 4,816,567) can be adapted to produce antibodies used in the fusion polypeptides, activating dimers and methods of the instant invention. Also, transgenic mice, or other organisms such as other mammals, may be used to express human or humanized antibodies. Alternatively, phage display technology can be used to identify antibodies, antibody fragments, such as variable domains, and heteromeric Fab fragments that specifically bind to selected antigens. Phage display is of particular value to isolate weakly binding antibodies or fragments thereof from unimmunized animals which, when combined with other weak binders in accordance with the invention described herein, create strongly binding activating dimers.

[0030] Screening and selection of preferred immunoglobulins (antibodies) can be conducted by

a variety of methods known to the art. Initial screening for the presence of monoclonal antibodies specific to an target receptor may be conducted through the use of ELISA-based methods or phage display, for example. A secondary screen is preferably conducted to identify and select a desired monoclonal antibody for use in construction of the fusion polypeptides of the invention. Secondary screening may be conducted with any suitable method known to the art.

Nucleic Acid Construction and Expression

[0031] Individual components of the fusion polypeptides of the invention may be produced from nucleic acids molecules using molecular biological methods known to the art. Nucleic acid molecules are inserted into a vector that is able to express the fusion polypeptides when introduced into an appropriate host cell. Appropriate host cells include, but are not limited to, bacterial, yeast, insect, and mammalian cells. Any of the methods known to one skilled in the art for the insertion of DNA fragments into a vector may be used to construct expression vectors encoding the fusion polypeptides of the invention under control of transcriptional/translational control signals. These methods may include *in vitro* recombinant DNA and synthetic techniques and *in vivo* recombinations (See Sambrook et al. Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory; Current Protocols in Molecular Biology, Eds. Ausubel, et al., Greene Publ. Assoc., Wiley-Interscience, NY).

[0032] Expression of the nucleic acid molecules of the invention may be regulated by a second nucleic acid sequence so that the molecule is expressed in a host transformed with the recombinant DNA molecule. For example, expression of the nucleic acid molecules of the invention may be controlled by any promoter/enhancer element known in the art.

[0033] Immunoglobulin-derived components. The nucleic acid constructs include regions which encode binding domains derived from an anti-target receptor antibodies. In general, such binding domains will be derived from V_H or V_L chain variable regions. After identification and selection of antibodies exhibiting the desired binding characteristics, the variable regions of the heavy chains and/or light chains of each antibody is isolated, amplified, cloned and sequenced. Modifications may be made to the V_H and V_L nucleotide sequences, including additions of nucleotide sequences encoding amino acids and/or carrying restriction sites, deletions of nucleotide sequences encoding amino acids, or substitutions of nucleotide sequences encoding amino acids.

[0034] The invention encompasses antibodies or antibody fragments which are humanized or chimeric. "Humanized" or chimeric forms of non-human (e.g., murine) antibodies are immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab') 2 or other antigen-binding subsequences of antibodies) that contain minimal sequences required for antigen binding derived from non-human immunoglobulin. They have the same or similar binding specificity and affinity as a mouse or other nonhuman antibody that provides the starting

material for construction of a chimeric or humanized antibody. Chimeric antibodies are antibodies whose light and heavy chain genes have been constructed, typically by genetic engineering, from immunoglobulin gene segments belonging to different species. For example, the variable (V) segments of the genes from a mouse monoclonal antibody may be joined to human constant (C) segments, such as IgG1 and IgG4. Human isotype IgG1 is preferred. A typical chimeric antibody is thus a hybrid protein consisting of the V or antigen-binding domain from a mouse antibody and the C or effector domain from a human antibody. Humanized antibodies have variable region framework residues substantially from a human antibody (termed an acceptor antibody) and complementarity determining regions (CDR regions) substantially from a mouse antibody, (referred to as the donor immunoglobulin). See, Queen et al., Proc. Natl. Acad Sci. USA 86:10029-10033 (1989) and WO 90/07861, U.S. 5,693,762, 5,693,761, 5,585,089, 5,530,101 and 5,225,539. The constant region(s), if present, are also substantially or entirely from a human immunoglobulin. The human variable domains are usually chosen from human antibodies whose framework sequences exhibit a high degree of sequence identity with the murine variable region domains from which the CDRs were derived. The heavy and light chain variable region framework residues can be derived from the same or different human antibody sequences. The human antibody sequences can be the sequences of naturally occurring human antibodies or can be consensus sequences of several human antibodies. See WO 92/22653. Certain amino acids from the human variable region framework residues are selected for substitution based on their possible influence on CDR conformation and/or binding to antigen. Investigation of such possible influences is by modeling, examination of the characteristics of the amino acids at particular locations, or empirical observation of the effects of substitution or mutagenesis of particular amino acids. For example, when an amino acid differs between a murine variable region framework residue and a selected human variable region framework residue, the human framework amino acid should usually be substituted by the equivalent framework amino acid from the mouse antibody when it is reasonably expected that the amino acid: (1) noncovalently binds antigen directly; (2) is adjacent to a CDR region; (3) otherwise interacts with a CDR region (e.g. is within about 6 A of a CDR region), or (4) participates in the V_L-V_H interface. Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. These amino acids can be substituted with amino acids from the equivalent position of the mouse donor antibody or from the equivalent positions of more typical human immunoglobulins. Other candidates for substitution are acceptor human framework amino acids that are unusual for a human immunoglobulin at that position. The variable region frameworks of humanized immunoglobulins usually show at least 85% sequence identity to a human variable region framework sequence or consensus of such sequences.

[0035] Fully human antibodies may be made by any method known to the art. For example, US 6,596,541 describes a method of generating fully human antibodies. Briefly, initially a

transgenic animal such as a mouse is generated that produces hybrid antibodies containing human variable regions (VDJ/VJ) and mouse constant regions. This is accomplished by a direct, in situ replacement of the mouse variable region (VDJ/VJ) genes with their human counterparts. The mouse is then exposed to human antigen, or an immunogenic fragment thereof. The resultant hybrid immunoglobulin loci will undergo the natural process of rearrangements during B-cell development to produce hybrid antibodies having the desired specificity. The antibody of the invention is selected as described above. Subsequently, fully-human antibodies are made by replacing the mouse constant regions with the desired human counterparts. Fully human antibodies can also be isolated from mice or other transgenic animals such as cows that express human transgenes or minichromosomes for the heavy and light chain loci. (Green (1999) J Immunol Methods. 231:11-23 and Ishida et al (2002) Cloning Stem Cells. 4:91-102) Fully human antibodies can also be isolated from humans to whom the protein has been administered. Fully human antibodies can also be isolated from immune compromised mice whose immune systems have been regenerated by engraftment with human stem cells, splenocytes, or peripheral blood cells (Chamat et al (1999) J Infect Dis. 180:268-77). To enhance the immune response to the protein of interest one can knockout the gene encoding the protein of interest in the human-antibody-transgenic animal.

[0036] Receptor-binding domains. In accordance with the invention, the nucleic acid constructs include components which encode binding domains derived from target receptor ligands. After identification of a ligand's target receptor -binding domain exhibiting desired binding characteristics, the nucleic acid that encodes such domain is used in the nucleic acid constructs. Such nucleic acids may be modified, including additions of nucleotide sequences encoding amino acids and/or carrying restriction sites, deletions of nucleotide sequences encoding amino acids, or substitutions of nucleotide sequences encoding amino acids.

[0037] The nucleic acid constructs of the invention are inserted into an expression vector or viral vector by methods known to the art, wherein the nucleic acid molecule is operatively linked to an expression control sequence. Also provided is a host-vector system for the production of the fusion polypeptides and activating dimers of the invention, which comprises the expression vector of the invention, which has been introduced into a suitable host cell. The suitable host cell may be a bacterial cell such as *E. coli*, a yeast cell, such as *Pichia pastoris,* an insect cell, such as *Spodoptera frugiperda*, or a mammalian cell, such as a COS, CHO, 293, BHK or NS0 cell.

[0038] The invention further encompasses methods for producing the activating dimers of the invention by growing cells transformed with an expression vector under conditions permitting production of the fusion polypeptides and recovery of the activating dimers formed from the fusion polypeptides. Cells may also be transduced with a recombinant virus comprising the nucleic acid construct of the invention.

[0039] The activating dimers may be purified by any technique, which allows for the subsequent

formation of a stable dimer. For example, and not by way of limitation, the activating dimers may be recovered from cells either as soluble polypeptides or as inclusion bodies, from which they may be extracted quantitatively by 8M guanidinium hydrochloride and dialysis. In order to further purify the activating dimers, conventional ion exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography or gel filtration may be used. The activating dimers may also be recovered from conditioned media following secretion from eukaryotic or prokaryotic cells.

Screening and Detection Methods

[0040] The activating dimers of the invention may also be used in *in vitro* or *in vivo* screening methods where it is desirable to detect and/or measure target receptor levels. Screening methods are well known to the art and include cell-free, cell-based, and animal assays. *In vitro* assays can be either solid state or soluble. target receptor detection may be achieved in a number of ways known to the art, including the use of a label or detectable group capable of identifying an activating dimer which is bound to an target receptor. Detectable labels are well developed in the field of immunoassays and may generally be used in conjunction with assays using the activating dimer of the invention.

Therapeutic Methods

[0041] The ability of the activating dimers of the invention to exhibit high affinity binding for their receptors makes them therapeutically useful for efficiently activating their receptors. Thus, it certain instances it may be to increase the effect of endogenous ligands for target receptors, such as, for example, the ephrins. For example, in the area of nervous system trauma, certain conditions may benefit from an increase in ephrin responsiveness. It may therefore be beneficial to increase the binding affinity of an ephrin in patients suffering from such conditions through the use of the compositions described herein.

[0042] The invention herein further provides for the development of an activating dimer described herein as a therapeutic for the treatment of patients suffering from disorders involving cells, tissues or organs which express the Tie-2 receptor. Such molecules may be used in a method of treatment of the human or animal body, or in a method of diagnosis.

[0043] The target receptor known as Tie-2 receptor has been identified in association with endothelial cells and, as was previously demonstrated, blocking of agonists of the receptor such as Tie-2 ligand 1 (Ang1) has been shown to prevent vascularization. Accordingly, activating dimers of the invention wherein the target receptor is Tie-2 may be useful for the induction of vascularization in diseases or disorders where such vascularization is indicated. Such diseases or disorders would include wound healing, ischemia and diabetes. The ligands may be tested in animal models and used therapeutically as described for other agents, such as vascular endothelial growth factor (VEGF), another endothelial cell-specific factor that is angiogenic.

Ferrara et al. U.S. Patent No. 5,332,671 issued July 26, 1994. Ferrara et al. describe *in vitro* and *in vivo* studies that may be used to demonstrate the effect of an angiogenic factor in enhancing blood flow to ischemic myocardium, enhancing wound healing, and in other therapeutic settings wherein neoangiogenesis is desired. According to the invention, such a Tie-2 specific activating dimer may be used alone or in combination with one or more additional pharmaceutically active compounds such as, for example, VEGF or basic fibroblast growth factor (bFGF).

Methods of Administration

[0044] Methods known in the art for the therapeutic delivery of agents such as proteins or nucleic acids can be used for the therapeutic delivery of an activating dimer or a nucleic acid encoding an activating dimer of the invention for activating target receptors in a subject, e.g., cellular transfection, gene therapy, direct administration with a delivery vehicle or pharmaceutically acceptable carrier, indirect delivery by providing recombinant cells comprising a nucleic acid encoding an activating dimer of the invention.

[0045] Various delivery systems are known and can be used to administer the activating dimer of the invention, *e.g.*, encapsulation in liposomes, microparticles, microcapsules, recombinant cells capable of expressing the compound, receptor-mediated endocytosis (see, *e.g.*, Wu and Wu, 1987, J. Biol. Chem. 262:4429-4432), construction of a nucleic acid as part of a retroviral or other vector, etc. Methods of introduction can be enteral or parenteral and include but are not limited to intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, pulmonary, intranasal, intraocular, epidural, and oral routes. The compounds may be administered by any convenient route, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, etc.) and may be administered together with other biologically active agents. Administration can be systemic or local. In addition, it may be desirable to introduce the pharmaceutical compositions of the invention into the central nervous system by any suitable route, including intraventricular and intrathecal injection; intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir, such as an Ommaya reservoir. Pulmonary administration can also be employed, *e.g.*, by use of an inhaler or nebulizer, and formulation with an aerosolizing agent.

[0046] In a specific embodiment, it may be desirable to administer the pharmaceutical compositions of the invention locally to the area in need of treatment; this may be achieved, for example, and not by way of limitation, by local infusion during surgery, topical application, *e.g.*, by injection, by means of a catheter, or by means of an implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, fibers, or commercial skin substitutes.

[0047] In another embodiment, the active agent can be delivered in a vesicle, in particular a liposome (see Langer (1990) Science 249:1527-1533). In yet another embodiment, the active

agent can be delivered in a controlled release system. In one embodiment, a pump may be used (see Langer (1990) *supra*).

Pharmaceutical Compositions

[0048] The present invention also provides pharmaceutical compositions comprising an activating dimer of the invention and a pharmaceutically acceptable carrier. The term "pharmaceutically acceptable" means approved by a regulatory agency of the Federal or a state government or listed in the U.S. Pharmacopeia or other generally recognized pharmacopeia for use in animals, and more particularly in humans. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the therapeutic is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Suitable pharmaceutical excipients include starch, glucose, lactose, sucrose, gelatin, malt, rice, flour, chalk, silica gel, sodium stearate, glycerol monostearate, talc, sodium chloride, dried skim milk, glycerol, propylene, glycol, water, ethanol and the like. The composition, if desired, can also contain minor amounts of wetting or emulsifying agents, or pH buffering agents. These compositions can take the form of solutions, suspensions, emulsion, tablets, pills, capsules, powders, sustained-release formulations and the like. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, etc. Examples of suitable pharmaceutical carriers are described in "Remington's Pharmaceutical Sciences" by E.W. Martin.

[0049] In a preferred embodiment, the composition is formulated in accordance with routine procedures as a pharmaceutical composition adapted for intravenous administration to human beings. Where necessary, the composition may also include a solubilizing agent and a local anesthetic such as lidocaine to ease pain at the site of the injection. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration.

[0050] The active agents of the invention can be formulated as neutral or salt forms. Pharmaceutically acceptable salts include those formed with free amino groups such as those derived from hydrochloric, phosphoric, acetic, oxalic, tartaric acids, etc., and those formed with free carboxyl groups such as those derived from sodium, potassium, ammonium, calcium, ferric hydroxides, isopropylamine, triethylamine, 2-ethylamino ethanol, histidine, procaine, etc.

Kits

[0051] The invention also provides a pharmaceutical pack or kit comprising one or more containers filled with at least one activating dimer of the invention. Optionally associated with such container(s) can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects (a) approval by the agency of manufacture, use or sale for human administration, (b) directions for use, or both.

Transgenic Animals

[0052] The invention includes transgenic non-human animals expressing a fusion polypeptide of the invention. A transgenic animal can be produced by introducing nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. Any of the regulatory or other sequences useful in expression vectors can form part of the transgenic sequence. A tissue-specific regulatory sequence(s) can be operably linked to the transgene to direct expression of the transgene to particular cells. A transgenic non-human animal expressing an fusion polypeptide of the invention is useful in a variety of applications, including as a means of producing such fusion proteins.

EXAMPLES

[0053] The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the methods and compositions of the invention, and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is average molecular weight, temperature is in degrees Centigrade, and pressure is at or near atmospheric.

Example 1. Production of Anti-Tie-2 Hybridomas

[0054] Five 8-weeks old Balb/c mice were first immunized with purified human Tie-2-Fc (hTie2-Fc); each mouse was injected subcutaneously with 200 μl emulsion containing 100 μg purified hTie2-Fc protein and 100 μl Freund's complete adjuvant. Fifteen days after the primary injection, each mouse received subcutaneous injection of 200 μl emulsion containing 100 μg purified hTie2-Fc in 100 μl PBS and 100 μl Freund's incomplete adjuvant. This injection was repeated for the five mice seven days later. One mouse was used for generation of hybridomas against hTie-2. Each of the four remaining mice were given subcutaneous injections of 200 μl emulsion each containing 100 μg purified rat Tie-2-Fc (rTie2-Fc) in 100 μl PBS and 100 μl

Freund's incomplete adjuvant six months after the primary injection of hTie2-Fc. Eleven days later, the immune response of the mice to rTie2-Fc was boosted by subcutaneous injection of 200 μl of emulsion containing 100 μg purified rTie2-Fc in 100 μl PBS and 100 μl Freund's incomplete adjuvant for each mouse. Mouse sera were collected from tail veins three days after the injection, then the antibody titers against rTie2-Fc were determined by ELISA. The two mice with the highest titers were given a final boost by tail vein injection of 100 μg purified rTie2-Fc in 100 μl PBS. The mice were sacrificed three days later and their spleen cells were collected for fusion with Sp2/0-Ag14 cells.

[0055] To generate hybridomas, mouse spleen cells were fused with Sp2/0-Ag14 myeloma cells using polyethylene glycol (PEG). Briefly, after the spleens were aseptically removed from the mice, one tip of each spleen was cut open and spleen cells collected. The spleen cells were washed twice with D-MEM and cell numbers were counted using a hemocytometer. 2×10^8 spleen cells were combined with 3×10^7 Sp2/0-Ag14 cells that were in log growth stage. The cell mix was washed with 30 mls D-MEM. 1 ml 50% PEG at 37^0C was slowly added to the cell pellet while stirring. D-MEM was added to the mix to bring the volume to 10 mls. The cells were spun down at 400 x g for 10 minutes. After removal of supernatant, the cells were gently resuspended in 20 mls growth medium containing 60% D-MEM with 4.5 g/L glucose, 20% FCS, 10% NCTC109 medium, 10% hybridoma cloning factor, 1 mM oxaloacetate, 2 mM glutamine, 0.2 units/ml insulin, and 3 μM glycine. The cells were transferred to two T225 flasks, each containing 100 mls of the growth medium and were put into a tissue culture incubator. On the next day, 1 x HAT was added to the medium to select against the myeloma cells that were not fused. Nine days after the fusion, the cultures were replenished with fresh medium. Human IgG was added to the cultures at 1 mg/ml. On the tenth day after the fusion, 2.6×10^7 fused cells were stained sequentially with 1 μg/ml biotin-rTie2-Fc for one hour and 2.5 μg/ml phycoerythrin (PE)-conjugated streptavidin for 45 minutes in growth medium at room temperature. As a control, 1×10^6 fused cells were stained with 2.5 μg/ml PE-streptavidin for 45 minutes at room temperature. The cells were washed with 10 ml PBS after each stain. After staining, the cells were resuspended in PBS with 0.1% FCS and were analyzed by flow cytometry on a MoFlo (Cytomation). A population of cells (4% total cells) stained with both biotin rTie2-Fc and PE-streptavidin exhibited fluorescence higher than the unstained cells and the cells stained with PE-streptavidin alone. The cells in this 4% gate were cloned by sorting single cells into two 96-well plates containing 200 μl growth medium per well. The cells were cultured for 10 days before splitting into two sets of 96-well plates. Cells in one set of plate were processed for RT-PCR of mouse IgG heavy chain variable region following by sequencing. The clones were grouped into 14 bins, with members of each bin having identical sequence in their heavy chain variable region. Conditioned medium of hybridoma cells in each bin was tested for its ability to stimulate phosphorylation of rTie-2 in cultured rat aortic endothelial cells (RAECs).

[0056] Antibodies from two hybridomas, B2 and A12A, were chosen for further study because they were active in phosphorylation of Tie-2 in RAECs, and did not compete for binding to rTie-2 as determined by BIAcore analysis. In addition, these antibodies did not block binding of derivatives of angiopoietin-1 (Ang1) and angiopoietin-2 (Ang2), the natural ligands of Tie-2.

Example 2. Construction of ScFvs (B2 and A12A).

[0057] Generally, antibody variable regions from hybridomas expressing antibodies specific for rTie-2 were cloned by first determining the DNA sequence of RT-PCR products using primers specific for mouse antibody variable regions, then using specific primers based on the determined sequence in order to amplify DNA fragments encoding ScFvs. The ScFv DNA fragments were cloned such that they could be cassette exchanged with multiple plasmids to yield all combinations of activating dimers. For example, one amplified ScFv fragment could be fused to a signal sequence at the N-terminus and to a coding sequence for the IgG Fc domain at the C-terminus, or it could be fused to the C-terminus of an IgG Fc coding sequence such that the 3' end of the ScFv coding sequence contained a translation stop codon.

[0058] The B2 hybridoma was found to express an antibody capable of inducing phosphorylation of the Tie-2 receptor in RAECs. Total RNA was isolated from this hybridoma using the promega SV96 Total RNA Isolation System (Promega) and variable heavy cDNA was synthesized using the Qiagen One-Step* RT-PCR system (Qiagen) with heavy chain primers from the Ratner primer set (Wang et al. (2000) J. Immunol. Methods 233:167) that included equimolar amounts of the 5' primers (SEQ ID NO:1-7) and the 3' primer (SEQ ID NO:8). Similarly, the light chain variable regions were amplified from cDNA using equimolar amounts of the light chain-specific primers (SEQ ID NO:9 and 10). The amplified variable region fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and the DNA sequences were determined. Based on the determined variable region sequences for the B2 antibody, the variable heavy sequence was PCR amplified using the pCR2.1-TOPO cloned variable region as template and an equimolar mix of 5' and 3' primers (SEQ ID NO: 19 and SEQ ID NO: 20). The variable light sequence was PCR amplified using a similar strategy. The pCR2.1-TOPO cloned variable region was used as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:21 and SEQ ID NO:22). The variable regions were joined by a (G4S)3 linker; ScFv genes were assembled and PCR amplified using an equimolar mix of the above specific variable heavy and variable light PCR products and an equimolar mix of 5' B2 heavy primer (SEQ ID NO:19) and the 3' light primer (SEQ ID NO:22). PCR product was cloned into Invitrogen pCR2.1-TOPO (Invitrogen) to yield pRG1039. The sequence was confirmed before sub-cloning the 744bp AscI/SrfI to fuse the ScFv gene to the N-terminus of a DNA encoding the human IgG1 Fc fragment (hFc), or the 753bp AscI/NotI restriction fragments to fuse the same ScFv to the C-terminus of a DNA encoding hFc.

[0059] The A12A hybridoma was also found to express an antibody capable of inducing

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phosphorylation of the Tie-2 receptor in RAECs. Total RNA was isolated from this hybridoma using the Quick Prep* mRNA purification kit (Amersham Pharmacia Biotech) and variable heavy cDNA was synthesized using the Qiagen One-Step* RT-PCR system, with equimolar amounts of primers from the from the Wright primer set (Morrison et al. (1995) Antibody Engineering, second edition, Borrebaeck, C.K.A. editor 267-293) that included the 5' heavy chain primers (SEQ ID NO: 11-13) and the 3' primer (SEQ ID NO:8). Similarly, the light chain variable regions were amplified from cDNA with equimolar amounts of the 5' heavy chain primers (SEQ ID NO: 14-18) and the 3' primer (SEQ ID NO:10). The amplified variable region fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and the DNA sequences were determined.

[0060] Based on the determined variable region sequences for the A12A antibody, the variable heavy sequence was PCR amplified using the pCR2.1-TOPO cloned variable region as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:23 and SEQ ID NO:24). The variable light sequence was PCR amplified using a similar strategy. The pCR2.1-TOPO cloned variable region was used as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:25 and SEQ ID NO:26). The variable regions were joined by a $(G_4S)_3$ linker; ScFv genes were assembled and PCR amplified using an equimolar mix of the above specific variable heavy and variable light PCR products and an equimolar mix of 5' A12A heavy primer (SEQ ID NO:23) and the 3' light primer (SEQ ID NO:26). PCR product was cloned into Invitrogen pCR2.1-TOPO to yield pRG1090. The sequence was confirmed before sub-cloning the 747bp AscI/SrfI to fuse the ScFv gene to the N-terminus of a DNA encoding the hFc fragment.

Example 3. Construction of Monospecific and Bispecific Activating Dimers

[0061] The general scheme for constructing both monspecific and bispecific tetravalent activating dimers was based on the ability of either the B2 or A12A ScFv genes to be inserted between the murine ROR1 signal sequence (SEQ ID NO:27) and the gene encoding hFc (nucleotides 85 to 765 of GenBank accession # X70421) when cut with one set of restriction enzymes, or after the hFc gene if cut with a different set of enzymes. This design of the ScFv genes allowed the exchange of ScFv cassettes among plasmids to obtain different combinations of ScFv and hFc using standard known methods. All constructs have an optional three amino acid linker (spacer) between the cleavage site of the signal peptide and the start of the ScFv gene, resulting from engineering a restriction site onto the 5' end of the ScFv genes. Similarly, fusion to the amino terminus of the hFc gene was facilitated by a three amino acid sequence (Gly-Pro-Gly), and fusion to the carboxy terminus of the hFc gene was facilitated by an eight amino acid sequence consisting of the residues Gly_4-Ser-Gly-Ala-Pro (SEQ ID NO:32) As a consequence of the terminal restriction site linkers on the ScFv genes, all constructs that have a carboxy terminal ScFv end with the amino acids Gly-Pro-Gly.

[0062] Two types of svFc-based chimeric molecules were constructed to assess the ability of ScFv-based molecules to activate the rTie-2 receptor. One type of molecule used a single

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ScFv fused to both the N-terminus and the C-terminus of hFc, the consequence of which was a monospecific tetravalent molecule capable of binding rTie-2. This molecule was expected to be capable of simultaneously binding four rTie-2 molecules. The plasmid pTE586 encodes the gene for $ScFv_{B2}$-Fc-$ScFv_{B2}$ (SEQ ID NO: 29) whose secretion is directed by the mROR1 signal peptide. The expression of $ScFv_{B2}$-Fc-$ScFv_{B2}$ in pTE586 was directed by the CMV-MIE promoter when transfected into CHO cells. This protein was easily purified by Protein A-Sepaharose affinity chromatography.

[0063] Construction of an ScFv-Fc-ScFv molecule wherein the two ScFv domains are derived from two different non-competing anti-rTie-2 antibodies would yield a molecule capable of clustering more than four receptors, in contrast to the $ScFv_{B2}$-Fc-$ScFv_{B2}$ described above, which can cluster only four receptors. It was determined by BIAcore analysis that the binding of the B2 antibody did not block binding of A12A to rTie-2, and A12A binding first did not block binding of B2. Consequently, ScFv molecules made from these antibodies should be capable of clustering more than four receptors. To construct a bispecific tetravalent ScFv-based molecule, the $ScFv_{A12A}$ gene was used in combination with the $ScFv_{B2}$ gene to yield $ScFv_{A12A}$-Fc-$ScFv_{B2}$ (SEQ ID NO: 28). The plasmid pTE585 encodes the gene for $ScFv_{A12A}$-Fc-$ScFv_{B2}$ and has the mROR1 signal peptide and CMV-MIE promoter when transfected into CHO cells. Both $ScFv_{B2}$-Fc- $ScFv_{B2}$ and $ScFv_{A12A}$-Fc- $ScFv_{B2}$ were expressed in CHO cells, and purified by Protein A-Sepharose affinity chromatography.

Example 4. Assays

[0064] Antibodies to rTie-2, and chimeric molecules related to these antibodies, were evaluated for their ability to induce phosphorylation of Tie-2 in cultured rat aortic endothelial cells. Confluent RAECs, between passage 3 and 6 (Vec Technologies), were grown in MCDB-131 media (Vec Technologies) on 0.2% gelatin coated T-75 flasks. Cells were starved for 2 hrs. in serum-free DME-Hi glucose medium (Irvine Scientific) prior to incubation at 37°C for 5 min. in 1.5 ml serum-free DME-Hi glucose medium with 0.1 % BSA and the challenge molecule. The challenge medium was then removed and cells were lysed in 20 mM Tris, pH 7.6, 150 mMNaCl, 50 mM NaF, 1 mM Na orthovanadate, 5 mM benzamidine, 1mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, with 10μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF. Tie-2 was immunoprecipitated by incubating the lysates at 4°C for 16 hrs. with 5 μg anti-Tie-2 mouse monoclonal antibody KP-m33, 10 μg biotinylated anti-mouse IgG (Jackson Laboratories), and 100 μl of neutravidin beads (Pierce). Beads were collected by centrifugation, washed 3 times with RIPA buffer, and bound proteins were eluted with 40 μl of 5X Laemmli buffer with 10% B-mercaptoethanol by heating at 100°C for 5 min. After SDS-gel electrophoresis on a 4-12% Tris/glycine polyacrylamide gel (Novex), proteins were transferred to PVDF membranes and probed with mouse anti-phophotyrosine monoclonal antibody 4G10 (Upstate) then detected using goat anti-mouse IgG-HRP conjugate (Pierce) followed by ECL reagent (Amersham). The

ability to induce Tie-2 phosphorylation in RAECs was determined for each activating dimer. Activity was evaluated by comparison to the level of stimulation obtained with FD1-Fc-FD1 (BA1) – a chimeric protein shown to be as active as Ang1 in binding and activation Tie-2 (Davis et al. (2003) Nature Struct. Biol. 10:38-44) (FD1 or FD2 = human fibrinogen domain of Ang1 or Ang2, respectively). Maximum stimulation (ECmax) of Tie-2 in RAECs was observed when BA1 was used at about 0.5 to 1.0 μg/ml, and phosphorylation levels in mock treated cells were low. Similarly, the ECmax of $ScFv_{B2}$-Fc- $ScFv_{B2}$, $ScFv_{A12A}$-Fc- $ScFv_{B2}$, $ScFv_{B2}$-Fc-FD1, and $ScFv_{B2}$-Fc-FD2 were about 0.5 to 1.0 μg/ml. In all cases, the ScFv-based molecules were capable of inducing a higher phosphorylation signal than observed for the related native antibodies isolated from hybridoma conditioned media.

[0065] Purified $ScFv_{B2}$-Fc- $ScFv_{B2}$ and $ScFv_{A12A}$-Fc- $ScFv_{B2}$ were characterized for their ability to bind rTie-2 and induce phosphorylation. Binding to rTie-2 was determined by BIAcore analysis. Both the monospecific and the dispecific activating dimers were found to have significantly higher affinity for rTie-2 than FD1-Fc-FD1. In addition, both $ScFv_{B2}$-Fc- $ScFv_{B2}$ and $ScFv_{A12A}$-Fc- $ScFv_{B2}$ were able to stimulate phosphorylation of rTie-2 in RAECs comparable to FD1-Fc-FD1.

Example 5. Construction of ScFv/Ligand Activating Dimers

[0066] Bispecific tetravalent molecules were constructed to include both Tie-2 specific ScFv and FD1 or FD2. The chimeric molecules were made by fusing the gene encoding $ScFv_{B2}$ to the N-terminus of hFc and the gene encoding Ang1 FD (Phe283 to Phe498 of GenBank accession # Q15389) or Ang2 FD (Phe281 to Phe496 of GenBank accession # O15123) to the C-terminus. Plasmid pTE514 encodes the gene for $ScFv_{B2}$-Fc-FD1 (SEQ ID NO: 30) and contained the mROR1 signal peptide and CMV-MIE promoter. Plasmid pTE614 encodes the gene for $ScFv_{B2}$-Fc-FD2 (SEQ ID NO: 31) and contained the mROR1 signal peptide and CMV-MIE promoter. Similar to $ScFv_{B2}$-Fc-$ScFv_{B2}$ and $ScFv_{A12A}$-Fc-$ScFv_{B2}$ the proteins expressed from pTE514 and pTE614 had a Gly-Ala-Pro linker between the mROR1 signal peptide and the $ScFv_{B2}$, a Gly-Pro-Gly linker between the N-terminal $ScFv_{B2}$ and hFc and a Gly_4-Ser-Gly-Ala-Pro linker (SEQ ID NO:32) between the C-terminus of hFc and the N-terminus of the Ang FDs. Both $ScFv_{B2}$-Fc-FD1 and $ScFv_{B2}$-Fc-FD2 were expressed and purified as described above.

[0067] Purified $ScFv_{B2}$-Fc-FD1 and $ScFv_{B2}$-Fc-FD2 were characterized for their ability to bind rTie-2 and induce phosphorylation as described in above. As determined by BIAcore analysis, the chimeric activating dimer $ScFv_{B2}$-Fc-FD1was found to have significantly higher affinity for rTie-2 (2 nM) than FD1-Fc-FD1 (0.04 nM). Moreover, both $ScFv_{B2}$-Fc-FD1 and $ScFv_{B2}$-Fc-FD2 were able to stimulate phosphorylation of rTie-2 in RAECs comparable to FD1-Fc-FD1.

Example 6. Construction of Fully Human Activating Dimers

[0068] Bispecific tetravalent molecules are formed from dimerized fusion constructs of the

invention which include either two ScFvs derived from human antibodies specific for hTie-2 or one ScFv derived from a human antibody specific for hTie-2 and human FD1 or FD2. Human ScFvs specific for hTie-2 are obtained by methods known to the art and as described above. In one embodiment, human ScFvs are obtained recombinantly as described in Reiter et al. (1999) J. Mol. Biol. 290:685-698 and Gilliland et al. (1996) Tissue Antigens 47(1):1-20.

Example 7. Construction of ScFvs (1-1F11 and 2-1G3).

[0069] Anti-rTie-1 hybridomas were produced following the procedures described above for the production of anti-rTie-2 hybridomas. Briefly, mice were immunized three times with purified rat Tie-1-Fc protein and Freund's adjuvant. Spleen cells from the mouse with the highest anti-Tie-1 antibody titer were fused with Sp2/0-Ag14 myeloma cells using polyethylene glycol (PEG). After fusion, the cells were cultured in two T225 flasks. HAT was added to the cultures on the next day. Nine days after the fusion, the cultures were replenished with fresh medium. Human IgG was added to the cultures at 1 mg/ml. On the tenth day after the fusion, the HAT-resistant cells were stained sequentially with 1 μg/ml biotin-rat Tie-1-Fc for one hour and 2.5 μg/ml phycoerythrin (PE)-conjugated streptavidin for 45 minutes in growth medium at room temperature. After staining, the cells were analyzed by flow cytometery. Cells that bound rTie1-Fc were cloned by sorting single cells into 96-well plates. The 96-well plate cultures were split into two sets ten days after sorting. RT-PCR of mouse IgG heavy chain variable region followed by sequencing were performed on one set of the 96-well plate cultures. Clones with unique IgG heavy chain variable region sequences were identified and expanded for the production of anti-rTie-1 antibodies. Antibodies were tested for binding rTie-1 protein and two clones, 1-1F11 and 1-2G3, were chosen for more detailed study.

[0070] The 1-1F11 hybridoma was found to express an antibody capable of inducing phosphorylation of the Tie-1 receptor in RAECs. Messenger RNA was isolated and variable heavy cDNA synthesized as described above with heavy chain primers from the Wright primer set (Morrison et al. (1995) Antibody Engineering, second edition, Borrebaeck, C.K.A. editor 267-293) that included the 5' heavy chain primers (SEQ ID NO:35-37) and the 3' primer (SEQ ID NO:33). Similarly, the light chain variable regions were amplified from cDNA with equimolar amounts of the 5' light chain primers (SEQ ID NO:38-41) and the 3' primer (SEQ ID NO:34). The amplified variable region fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and DNA sequences determined. Based on the determined variable region sequences for the 1-1F11 antibody, the variable heavy sequence was PCR amplified using the pCR2.1-TOPO cloned variable region as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:42 and SEQ ID NO:43). The variable light sequence was PCR amplified using a similar strategy. The pCR2.1-TOPO cloned variable region was used as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:44 and SEQ ID NO:45). The variable regions were joined by a $(G_4S)_3$ linker; ScFv genes were assembled and PCR amplified using an equimolar mix of the above

specific variable heavy and variable light PCR products and an equimolar mix of 5' heavy primer (SEQ ID NO:42) and the 3' light primer (SEQ ID NO:45). PCR product was cloned into Invitrogen pCR2.1-TOPO (Invitrogen) to yield pRG1192. The sequence was confirmed before sub-cloning the 747bp AscI/SrfI to fuse the ScFv gene to the N-terminus of a DNA encoding the human IgG1 Fc fragment (hFc), or the 756bp AscI/NotI restriction fragments to fuse the same ScFv to the C-terminus of a DNA encoding hFc.

[0071] The 2-1G3 hybridoma was also found to express an antibody capable of inducing phosphorylation of the Tie-2 receptor in RAECs. Messenger RNA was isolated and variable heavy cDNA synthesized as described above with equimolar amounts of primers from the from the Wright primer set (Morrison et al. (1995) *supra*) that included the 5' heavy chain primers (SEQ ID NO:35-37) and the 3' primer (SEQ ID NO:33). Similarly, the light chain variable regions were amplified from cDNA with equimolar amounts of the 5' heavy chain primers (SEQ ID NO:38-41) and the 3' primer (SEQ ID NO:34). The amplified variable region fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and the DNA sequences were determined.

[0072] Based on the determined variable region sequences for the 2-1G3 antibody, the variable heavy sequence was PCR amplified using the pCR2.1-TOPO cloned variable region as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:46 and SEQ ID NO:47). The variable light sequence was PCR amplified using a similar strategy. The pCR2.1-TOPO cloned variable region was used as template and an equimolar mix of 5' and 3' primers (SEQ ID NO:48 and SEQ ID NO:49). The variable regions were joined by a (G4S)3 linker; ScFv genes were assembled and PCR amplified using an equimolar mix of the above specific variable heavy and variable light PCR products and an equimolar mix of 5' 2-1G3 heavy primer (SEQ ID NO:46) and the 3' light primer (SEQ ID NO:49). PCR product was cloned into Invitrogen pCR2.1-TOPO (Invitrogen) to yield pRG1198. The sequence was confirmed before sub-cloning the 738bp AscI/SrfI to fuse the ScFv gene to the N-terminus of a DNA encoding the hFc fragment or the 747bp AscI/NotI restriction fragments to fuse the same ScFv to the C-terminus of a DNA encoding hFc.

Example 8. Construction of Monospecific and Bispecific Activating Dimers

[0073] Two types of ScFv-based chimeric molecules were constructed to assess the ability of ScFv-based molecules to activate the rTie-1 receptor. One type of molecule used a single ScFv fused to both the N-terminus and the C-terminus of hFc, the consequence of which was a monospecific tetravalent molecule capable of binding rTie-1. This molecule should be capable of simultaneously binding four rTie-1 molecules. The plasmid pTE778 encodes the gene for $ScFv_{1\text{-}1F11}$-Fc-$ScFv_{1\text{-}1F11}$ (SEQ ID NO:50) and contains the mROR1 signal peptide and CMV-MIE promoter. The protein was expressed and purified as described above.

[0074] Construction of an ScFv-Fc-ScFv molecule where the two ScFv domains are derived from two different non-competing anti-rTie-1 antibodies is expected to yield a molecule capable

of clustering more than four receptors, in contrast to the $ScFv_{1\text{-}1F11}$-Fc-$ScFv_{1\text{-}1F11}$ described above, which can cluster only four receptors. It was determined by BIAcore analysis that the binding of the 1-1F11 antibody did not block binding of 2-1G3 to rTie-1, and 1-1F11 binding first did not block binding of 2-1G3. Consequently, ScFv molecules made from these antibodies should be capable of clustering more than four receptors. To construct a bispecific tetravalent ScFv-based molecule, the $ScFv_{2\text{-}1G3}$ gene was used in combination with the $ScFv_{1\text{-}1F11}$ gene to yield $ScFv_{2\text{-}1G3}$-Fc-$ScFv_{1\text{-}1F11}$ (SEQ ID NO:51). Both constructs were expressed and purified as described above.

SEQUENCE LISTING

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<220>
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<400> 8
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<220>
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<400> 13
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<400> 14
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<400> 15
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<400> 17
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tgaggttcct tgacc 75

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<220>
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<400> 21
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cagtctccaa aatcc 75

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<220>
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<400> 22
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<213> Artificial Sequence

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<212> DNA
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<400> 24
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agtggtgcct tggcc 75

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<400> 25
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cagtctccag cttcc 75

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<400> 26
cgatgcggcc gctcagcccg ggccccgttt gatttccagc ttggtgcctc cacc 54

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<213> Mus musculus

<400> 27
Met His Arg Pro Arg Arg Arg Gly Thr Arg Pro Pro Pro Leu Ala Leu
1 5 10 15
Leu Ala Ala Leu Leu Leu Ala Ala Arg Gly Ala Asp Ala
20 25

<210> 28
<211> 762
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 28
Met His Arg Pro Arg Arg Arg Gly Thr Arg Pro Pro Pro Leu Ala Leu
1 5 10 15
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20 25 30
Glu Val Lys Leu Gln Glu Ser Gly Ala Glu Leu Met Lys Pro Gly Ala
35 40 45
Ser Val Lys Ile Ser Cys Lys Ala Thr Gly Tyr Thr Phe Ser Ser Tyr
50 55 60
Trp Ile Glu Trp Val Lys Gln Arg Pro Gly His Gly Leu Glu Trp Ile
65 70 75 80
Gly Glu Ile Leu Pro Gly Ser Gly Ser Thr Asn Tyr Asn Glu Lys Phe
85 90 95
Lys Gly Lys Ala Thr Phe Thr Ala Asp Thr Phe Ser Asn Thr Ala Tyr
100 105 110
Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys
115 120 125
Ala Arg Phe Asp Gly Tyr Leu Pro Phe Asp His Trp Gly Gln Gly Thr
130 135 140
Thr Leu Thr Val Ser Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
145 150 155 160
Gly Gly Gly Gly Ser Asp Ile Val Leu Thr Gln Ser Pro Ala Ser Leu
165 170 175
Ala Val Ser Leu Gly Gln Arg Ala Thr Ile Ser Cys Arg Ala Ser Lys
180 185 190
Ser Val Ile Thr Ser Gly Tyr Ser Tyr Met His Trp Tyr Gln Gln Lys

195 200 205

Pro Gly Gln Pro Pro Gln Leu Leu Ile Tyr Leu Ala Ser Asn Leu Glu
210 215 220
Ser Gly Val Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Phe
225 230 235 240
Thr Leu Asn Ile His Pro Val Glu Glu Glu Asp Ala Ala Thr Tyr Tyr
245 250 255
Cys His His Ser Arg Glu Leu Pro Arg Thr Phe Gly Gly Gly Thr Lys
260 265 270
Leu Glu Ile Lys Arg Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro
275 280 285
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
290 295 300
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
305 310 315 320
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
325 330 335
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
340 345 350
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
355 360 365
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
370 375 380
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
385 390 395 400
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp
405 410 415

Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
420 425 430
Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
435 440 445
Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
450 455 460
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
465 470 475 480
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
485 490 495
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly Gly Gly Ser
500 505 510
Gly Ala Pro Gln Val Lys Leu Glu Glu Ser Gly Pro Gly Leu Val Lys
515 520 525
Pro Ser Gln Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile
530 535 540
Thr Ser Asp Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asn Lys
545 550 555 560
Leu Glu Trp Met Gly Tyr Ile Asn Tyr Ser Gly Ile Thr Ser Tyr Asn
565 570 575
Pro Ser Leu Lys Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn
580 585 590
Gln Phe Phe Leu Gln Leu Asn Ser Val Thr Ala Glu Asp Thr Ala Thr
595 600 605
Tyr Tyr Cys Ala Arg Tyr Tyr Gly Ser Ser Tyr Asn Tyr Tyr Gly Met
610 615 620
Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly
625 630 635 640
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met
645 650 655
Thr Gln Ser Pro Lys Ser Met Ser Met Ser Val Gly Glu Arg Val Thr
660 665 670
Leu Asn Cys Lys Ala Ser Glu Asn Val Gly Thr Tyr Ile Ser Trp Tyr

675 680 685
Gln Gln Lys Pro Asp Gln Ser Pro Lys Leu Leu Ile Tyr Gly Ala Ser
690 695 700
Asn Arg Tyr Pro Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Ala
705 710 715 720
Thr Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Leu Ala
725 730 735
Asp Tyr His Cys Gly Gln Gly Tyr Thr Tyr Leu Thr Phe Gly Ala Gly
740 745 750
Thr Lys Leu Glu Leu Lys Arg Gly Pro Gly
755 760

<210> 29
<211> 761
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 29
Met His Arg Pro Arg Arg Arg Gly Thr Arg Pro Pro Pro Leu Ala Leu
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Leu Ala Ala Leu Leu Leu Ala Ala Arg Gly Ala Asp Ala Gly Ala Pro
20 25 30
Gln Val Lys Leu Glu Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
35 40 45
Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr Ser Asp
50 55 60
Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asn Lys Leu Glu Trp
65 70 75 80
Met Gly Tyr Ile Asn Tyr Ser Gly Ile Thr Ser Tyr Asn Pro Ser Leu
85 90 95
Lys Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln Phe Phe
100 105 110
Leu Gln Leu Asn Ser Val Thr Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
115 120 125
Ala Arg Tyr Tyr Gly Ser Ser Tyr Asn Tyr Tyr Gly Met Asp Tyr Trp
130 135 140
Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
145 150 155 160
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met Thr Gln Ser
165 170 175
Pro Lys Ser Met Ser Met Ser Val Gly Glu Arg Val Thr Leu Asn Cys
180 185 190
Lys Ala Ser Glu Asn Val Gly Thr Tyr Ile Ser Trp Tyr Gln Gln Lys
195 200 205
Pro Asp Gln Ser Pro Lys Leu Leu Ile Tyr Gly Ala Ser Asn Arg Tyr
210 215 220
Pro Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Ala Thr Asp Phe
225 230 235 240
Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Leu Ala Asp Tyr His
245 250 255
Cys Gly Gln Gly Tyr Thr Tyr Leu Thr Phe Gly Ala Gly Thr Lys Leu
260 265 270
Glu Leu Lys Arg Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys
275 280 285
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
290 295 300
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys

305 310 315 320
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
325 330 335
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
340 345 350
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
355 360 365
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
370 375 380
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
385 390 395 400
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
405 410 415
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
420 425 430
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
435 440 445
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
450 455 460
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
465 470 475 480
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
485 490 495
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly Gly Gly Ser Gly
500 505 510
Ala Pro Gln Val Lys Leu Glu Glu Ser Gly Pro Gly Leu Val Lys Pro
515 520 525
Ser Gln Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr
530 535 540
Ser Asp Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asn Lys Leu
545 550 555 560
Glu Trp Met Gly Tyr Ile Asn Tyr Ser Gly Ile Thr Ser Tyr Asn Pro
565 570 575
Ser Leu Lys Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln
580 585 590
Phe Phe Leu Gln Leu Asn Ser Val Thr Ala Glu Asp Thr Ala Thr Tyr
595 600 605
Tyr Cys Ala Arg Tyr Tyr Gly Ser Ser Tyr Asn Tyr Tyr Gly Met Asp
610 615 620
Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Gly
625 630 635 640
Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met Thr
645 650 655
Gln Ser Pro Lys Ser Met Ser Met Ser Val Gly Glu Arg Val Thr Leu
660 665 670
Asn Cys Lys Ala Ser Glu Asn Val Gly Thr Tyr Ile Ser Trp Tyr Gln
675 680 685
Gln Lys Pro Asp Gln Ser Pro Lys Leu Leu Ile Tyr Gly Ala Ser Asn
690 695 700
Arg Tyr Pro Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Ala Thr
705 710 715 720
Asp Phe Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Leu Ala Asp
725 730 735
Tyr His Cys Gly Gln Gly Tyr Thr Tyr Leu Thr Phe Gly Ala Gly Thr
740 745 750
Lys Leu Glu Leu Lys Arg Gly Pro Gly
755 760

<210> 30
<211> 730

<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 30

Met	His	Arg	Pro	Arg	Arg	Arg	Gly	Thr	Arg	Pro	Pro	Pro	Leu	Ala	Leu
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Leu	Ala	Ala	Leu	Leu	Leu	Ala	Ala	Arg	Gly	Ala	Asp	Ala	Gly	Ala	Pro
		20					25					30			
Gln	Val	Lys	Leu	Glu	Glu	Ser	Gly	Pro	Gly	Leu	Val	Lys	Pro	Ser	Gln
	35					40					45				
Ser	Leu	Ser	Leu	Thr	Cys	Thr	Val	Thr	Gly	Tyr	Ser	Ile	Thr	Ser	Asp
50					55					60					
Tyr	Ala	Trp	Asn	Trp	Ile	Arg	Gln	Phe	Pro	Gly	Asn	Lys	Leu	Glu	Trp
65				70					75					80	
Met	Gly	Tyr	Ile	Asn	Tyr	Ser	Gly	Ile	Thr	Ser	Tyr	Asn	Pro	Ser	Leu
			85					90					95		
Lys	Ser	Arg	Ile	Ser	Ile	Thr	Arg	Asp	Thr	Ser	Lys	Asn	Gln	Phe	Phe
		100					105					110			
Leu	Gln	Leu	Asn	Ser	Val	Thr	Ala	Glu	Asp	Thr	Ala	Thr	Tyr	Tyr	Cys
	115					120					125				
Ala	Arg	Tyr	Tyr	Gly	Ser	Ser	Tyr	Asn	Tyr	Tyr	Gly	Met	Asp	Tyr	Trp
130					135					140					
Gly	Gln	Gly	Thr	Ser	Val	Thr	Val	Ser	Ser	Gly	Gly	Gly	Gly	Ser	Gly
145				150					155					160	
Gly	Gly	Gly	Ser	Gly	Gly	Gly	Gly	Ser	Asp	Ile	Val	Met	Thr	Gln	Ser
			165					170					175		
Pro	Lys	Ser	Met	Ser	Met	Ser	Val	Gly	Glu	Arg	Val	Thr	Leu	Asn	Cys
		180					185					190			
Lys	Ala	Ser	Glu	Asn	Val	Gly	Thr	Tyr	Ile	Ser	Trp	Tyr	Gln	Gln	Lys
	195					200					205				
Pro	Asp	Gln	Ser	Pro	Lys	Leu	Leu	Ile	Tyr	Gly	Ala	Ser	Asn	Arg	Tyr
210					215					220					
Pro	Gly	Val	Pro	Asp	Arg	Phe	Thr	Gly	Ser	Gly	Ser	Ala	Thr	Asp	Phe
225				230					235					240	
Thr	Leu	Thr	Ile	Ser	Ser	Leu	Gln	Ala	Glu	Asp	Leu	Ala	Asp	Tyr	His
			245					250					255		
Cys	Gly	Gln	Gly	Tyr	Thr	Tyr	Leu	Thr	Phe	Gly	Ala	Gly	Thr	Lys	Leu
		260					265					270			
Glu	Leu	Lys	Arg	Gly	Pro	Gly	Asp	Lys	Thr	His	Thr	Cys	Pro	Pro	Cys
	275					280					285				
Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe	Leu	Phe	Pro	Pro
290					295					300					
Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro	Glu	Val	Thr	Cys
305				310					315					320	
Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val	Lys	Phe	Asn	Trp
			325					330					335		
Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr	Lys	Pro	Arg	Glu
		340					345					350			
Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr	Val	Leu
	355					360					365				
His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys	Lys	Val	Ser	Asn
370					375					380					
Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser	Lys	Ala	Lys	Gly
385				390					395					400	
Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro	Ser	Arg	Asp	Glu
			405					410					415		
Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val	Lys	Gly	Phe	Tyr
		420					425					430			

Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
435 440 445
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
450 455 460
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
465 470 475 480
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
485 490 495
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly Gly Gly Ser Gly
500 505 510
Ala Pro Phe Arg Asp Cys Ala Asp Val Tyr Gln Ala Gly Phe Asn Lys
515 520 525
Ser Gly Ile Tyr Thr Ile Tyr Ile Asn Asn Met Pro Glu Pro Lys Lys
530 535 540
Val Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp Thr Val Ile Gln
545 550 555 560
His Arg Glu Asp Gly Ser Leu Asp Phe Gln Arg Gly Trp Lys Glu Tyr
565 570 575
Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu
580 585 590
Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met Leu Arg Ile Glu
595 600 605
Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln Tyr Asp Arg Phe
610 615 620
His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr Leu Lys Gly His
625 630 635 640
Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu His Gly Ala Asp
645 650 655
Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met Cys Lys Cys Ala
660 665 670
Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro Ser Asn
675 680 685
Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His Gly Lys Leu Asn
690 695 700
Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser Tyr Ser Leu Arg Ser
705 710 715 720
Thr Thr Met Met Ile Arg Pro Leu Asp Phe
725 730

<210> 31
<211> 730
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 31
Met His Arg Pro Arg Arg Arg Gly Thr Arg Pro Pro Pro Leu Ala Leu
1 5 10 15
Leu Ala Ala Leu Leu Leu Ala Ala Arg Gly Ala Asp Ala Gly Ala Pro
20 25 30
Gln Val Lys Leu Glu Glu Ser Gly Pro Gly Leu Val Lys Pro Ser Gln
35 40 45
Ser Leu Ser Leu Thr Cys Thr Val Thr Gly Tyr Ser Ile Thr Ser Asp
50 55 60
Tyr Ala Trp Asn Trp Ile Arg Gln Phe Pro Gly Asn Lys Leu Glu Trp
65 70 75 80
Met Gly Tyr Ile Asn Tyr Ser Gly Ile Thr Ser Tyr Asn Pro Ser Leu
85 90 95
Lys Ser Arg Ile Ser Ile Thr Arg Asp Thr Ser Lys Asn Gln Phe Phe

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                100                 105                 110
Leu Gln Leu Asn Ser Val Thr Ala Glu Asp Thr Ala Thr Tyr Tyr Cys
        115                 120                 125
Ala Arg Tyr Tyr Gly Ser Ser Tyr Asn Tyr Tyr Gly Met Asp Tyr Trp
    130                 135                 140
Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
145                 150                 155                 160
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Val Met Thr Gln Ser
                165                 170                 175
Pro Lys Ser Met Ser Met Ser Val Gly Glu Arg Val Thr Leu Asn Cys
            180                 185                 190
Lys Ala Ser Glu Asn Val Gly Thr Tyr Ile Ser Trp Tyr Gln Gln Lys
        195                 200                 205
Pro Asp Gln Ser Pro Lys Leu Leu Ile Tyr Gly Ala Ser Asn Arg Tyr
    210                 215                 220
Pro Gly Val Pro Asp Arg Phe Thr Gly Ser Gly Ser Ala Thr Asp Phe
225                 230                 235                 240
Thr Leu Thr Ile Ser Ser Leu Gln Ala Glu Asp Leu Ala Asp Tyr His
                245                 250                 255
Cys Gly Gln Gly Tyr Thr Tyr Leu Thr Phe Gly Ala Gly Thr Lys Leu
            260                 265                 270
Glu Leu Lys Arg Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro Cys
        275                 280                 285
Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro Pro
    290                 295                 300
Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr Cys
305                 310                 315                 320
Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn Trp
                325                 330                 335
Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg Glu
            340                 345                 350
Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val Leu
        355                 360                 365
His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser Asn
    370                 375                 380
Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys Gly
385                 390                 395                 400
Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp Glu
                405                 410                 415
Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe Tyr
            420                 425                 430
Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu Asn
        435                 440                 445
Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe Phe
    450                 455                 460
Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly Asn
465                 470                 475                 480
Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr Thr
                485                 490                 495
Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly Gly Gly Ser Gly
            500                 505                 510
Ala Pro Phe Arg Asp Cys Ala Glu Val Phe Lys Ser Gly His Thr Thr
        515                 520                 525
Asn Gly Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr Glu Glu Ile Lys
    530                 535                 540
Ala Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp Thr Ile Ile Gln
545                 550                 555                 560
Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr Trp Lys Glu Tyr
                565                 570                 575
Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp Leu Gly Asn Glu
            580                 585                 590
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Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val Leu Lys Ile His
        595                 600                 605
Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu Tyr Glu His Phe
    610                 615                 620
Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile His Leu Lys Gly Leu
625                 630                 635                 640
Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln Pro Gly Asn Asp
                645                 650                 655
Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile Cys Lys Cys Ser
            660                 665                 670
Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys Gly Pro Ser Asn
        675                 680                 685
Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn Thr Asn Lys Phe Asn
    690                 695                 700
Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser Gly Tyr Ser Leu Lys Ala
705                 710                 715                 720
Thr Thr Met Met Ile Arg Pro Ala Asp Phe
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<210> 32
<211> 8
<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

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<220>
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<400> 41
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<210> 42
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<210> 43
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<220>
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<210> 44
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tcc 63

<210> 45
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<210> 47
<211> 63
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<400> 47
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acc 63

<210> 48
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<210> 49
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<211> 734
<212> PRT
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35 40 45
Glu Trp Val Ala Tyr Ile Ser Ser Gly Ser Ser Asn Ile Tyr Tyr Ala
50 55 60
Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Pro Lys Asn
65 70 75 80
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85 90 95

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Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly
115 120 125
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165 170 175
Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val Tyr Gly Ala Thr
180 185 190
Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
195 200 205
Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Ser Glu Asp Phe Gly
210 215 220
Ser Tyr Tyr Cys Gln His Phe Trp Gly Thr Pro Trp Thr Leu Gly Gly
225 230 235 240
Gly Thr Lys Leu Glu Ile Lys Arg Gly Pro Gly Asp Lys Thr His Thr
245 250 255
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
260 265 270
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
275 280 285
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
290 295 300
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
305 310 315 320
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
325 330 335
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340 345 350
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
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Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
370 375 380
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385 390 395 400
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405 410 415
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
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Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
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465 470 475 480
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485 490 495
Leu Val Gln Pro Gly Gly Ser Arg Lys Leu Ser Cys Ala Ala Ser Gly
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515 520 525
Lys Gly Leu Glu Trp Val Ala Tyr Ile Ser Ser Gly Ser Ser Asn Ile
530 535 540
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545 550 555 560
Pro Lys Asn Thr Leu Phe Leu Gln Met Thr Ser Leu Arg Ser Glu Asp
565 570 575
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580 585 590
Tyr Ala Ile Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser
595 600 605
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610 615 620
Ile Gln Met Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu
625 630 635 640
Thr Val Thr Ile Thr Cys Arg Ala Ser Glu Asn Ile Tyr Ser Asn Leu
645 650 655
Ala Trp Tyr Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val Tyr
660 665 670
Gly Ala Thr Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly Ser
675 680 685
Gly Ser Gly Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Ser Glu
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35 40 45
Glu Trp Val Ala Thr Ile Ser Gly Gly Gly Ser Tyr Thr Tyr Tyr Pro
50 55 60
Asp Ser Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Ala Lys Asn
65 70 75 80
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85 90 95
Tyr Tyr Cys Ala Arg Arg Arg Tyr Asp Pro Tyr Ala Met Asp Tyr Trp
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Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly Gly Ser Gly
115 120 125
Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met Thr Gln Thr
130 135 140
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145 150 155 160
Arg Ala Ser Gln Asp Ile Ser Asn Tyr Leu Asn Trp Tyr Gln Gln Lys
165 170 175
Pro Asp Gly Thr Val Lys Leu Leu Ile Tyr Tyr Ile Ser Arg Leu His
180 185 190
Ser Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly Thr Asp Tyr
195 200 205
Ser Leu Thr Ile Ser Asn Leu Glu Gln Glu Asp Ile Ala Thr Tyr Phe
210 215 220
Cys Gln Gln Gly Asn Thr Leu Pro Tyr Thr Phe Gly Gly Gly Thr Lys
225 230 235 240
Leu Glu Ile Lys Arg Gly Pro Gly Asp Lys Thr His Thr Cys Pro Pro

245 250 255
Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe Leu Phe Pro
260 265 270
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr
275 280 285
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Lys Phe Asn
290 295 300
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg
305 310 315 320
Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val Leu Thr Val
325 330 335
Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser
340 345 350
Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Ala Lys
355 360 365
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Asp
370 375 380
Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
385 390 395 400
Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
405 410 415
Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser Phe
420 425 430
Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
435 440 445
Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
450 455 460
Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly Gly Gly Ser
465 470 475 480
Gly Ala Pro Asp Val Gln Leu Val Glu Ser Gly Gly Gly Leu Val Gln
485 490 495
Pro Gly Gly Ser Arg Lys Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe
500 505 510
Ser Ser Phe Gly Met His Trp Val Arg Gln Ala Pro Glu Lys Gly Leu
515 520 525
Glu Trp Val Ala Tyr Ile Ser Ser Gly Ser Ser Asn Ile Tyr Tyr Ala
530 535 540
Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Pro Lys Asn
545 550 555 560
Thr Leu Phe Leu Gln Met Thr Ser Leu Arg Ser Glu Asp Thr Ala Met
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580 585 590
Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly
595 600 605
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met
610 615 620
Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr
625 630 635 640
Ile Thr Cys Arg Ala Ser Glu Asn Ile Tyr Ser Asn Leu Ala Trp Tyr
645 650 655
Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val Tyr Gly Ala Thr
660 665 670
Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
675 680 685
Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Ser Glu Asp Phe Gly
690 695 700
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<212> PRT
<213> Artificial Sequence

<220>
<223> Synthetic

<400> 52

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			20					25					30		
Ser	Ser	Phe	Gly	Met	His	Trp	Val	Arg	Gln	Ala	Pro	Glu	Lys	Gly	Leu
		35					40					45			
Glu	Trp	Val	Ala	Tyr	Ile	Ser	Ser	Gly	Ser	Ser	Asn	Ile	Tyr	Tyr	Ala
	50					55					60				
Asp	Thr	Val	Lys	Gly	Arg	Phe	Thr	Ile	Ser	Arg	Asp	Asn	Pro	Lys	Asn
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Thr	Leu	Phe	Leu	Gln	Met	Thr	Ser	Leu	Arg	Ser	Glu	Asp	Thr	Ala	Met
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Tyr	Tyr	Cys	Ala	Arg	Gly	Tyr	Asp	Tyr	Asp	Arg	Gly	Tyr	Tyr	Ala	Ile
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	130					135					140				
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145					150					155					160
Ile	Thr	Cys	Arg	Ala	Ser	Glu	Asn	Ile	Tyr	Ser	Asn	Leu	Ala	Trp	Tyr
				165					170					175	
Gln	Gln	Lys	Gln	Gly	Lys	Ser	Pro	Gln	Leu	Leu	Val	Tyr	Gly	Ala	Thr
			180					185					190		
Asn	Leu	Ala	Asp	Gly	Val	Pro	Ser	Arg	Phe	Ser	Gly	Ser	Gly	Ser	Gly
		195					200					205			
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225					230					235					240
Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	Gly	Pro	Gly	Asp	Lys	Thr	His	Thr
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Cys	Pro	Pro	Cys	Pro	Ala	Pro	Glu	Leu	Leu	Gly	Gly	Pro	Ser	Val	Phe
			260					265					270		
Leu	Phe	Pro	Pro	Lys	Pro	Lys	Asp	Thr	Leu	Met	Ile	Ser	Arg	Thr	Pro
		275					280					285			
Glu	Val	Thr	Cys	Val	Val	Val	Asp	Val	Ser	His	Glu	Asp	Pro	Glu	Val
	290					295					300				
Lys	Phe	Asn	Trp	Tyr	Val	Asp	Gly	Val	Glu	Val	His	Asn	Ala	Lys	Thr
305					310					315					320
Lys	Pro	Arg	Glu	Glu	Gln	Tyr	Asn	Ser	Thr	Tyr	Arg	Val	Val	Ser	Val
				325					330					335	
Leu	Thr	Val	Leu	His	Gln	Asp	Trp	Leu	Asn	Gly	Lys	Glu	Tyr	Lys	Cys
			340					345					350		
Lys	Val	Ser	Asn	Lys	Ala	Leu	Pro	Ala	Pro	Ile	Glu	Lys	Thr	Ile	Ser
		355					360					365			
Lys	Ala	Lys	Gly	Gln	Pro	Arg	Glu	Pro	Gln	Val	Tyr	Thr	Leu	Pro	Pro
	370					375					380				
Ser	Arg	Asp	Glu	Leu	Thr	Lys	Asn	Gln	Val	Ser	Leu	Thr	Cys	Leu	Val

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405 410 415
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
420 425 430
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
435 440 445
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
450 455 460
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly
465 470 475 480
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485 490 495
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500 505 510
Glu Pro Lys Lys Val Phe Cys Asn Met Asp Val Asn Gly Gly Gly Trp
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Trp Lys Glu Tyr Lys Met Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp
545 550 555 560
Leu Gly Asn Glu Phe Ile Phe Ala Ile Thr Ser Gln Arg Gln Tyr Met
565 570 575
Leu Arg Ile Glu Leu Met Asp Trp Glu Gly Asn Arg Ala Tyr Ser Gln
580 585 590
Tyr Asp Arg Phe His Ile Gly Asn Glu Lys Gln Asn Tyr Arg Leu Tyr
595 600 605
Leu Lys Gly His Thr Gly Thr Ala Gly Lys Gln Ser Ser Leu Ile Leu
610 615 620
His Gly Ala Asp Phe Ser Thr Lys Asp Ala Asp Asn Asp Asn Cys Met
625 630 635 640

Cys Lys Cys Ala Leu Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys
645 650 655
Gly Pro Ser Asn Leu Asn Gly Met Phe Tyr Thr Ala Gly Gln Asn His
660 665 670
Gly Lys Leu Asn Gly Ile Lys Trp His Tyr Phe Lys Gly Pro Ser Tyr
675 680 685
Ser Leu Arg Ser Thr Thr Met Met Ile Arg Pro Leu Asp Phe
690 695 700

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<212> PRT
<213> Artificial Sequence

<220>
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35 40 45
Glu Trp Val Ala Tyr Ile Ser Ser Gly Ser Ser Asn Ile Tyr Tyr Ala
50 55 60
Asp Thr Val Lys Gly Arg Phe Thr Ile Ser Arg Asp Asn Pro Lys Asn

65 70 75 80
Thr Leu Phe Leu Gln Met Thr Ser Leu Arg Ser Glu Asp Thr Ala Met
85 90 95
Tyr Tyr Cys Ala Arg Gly Tyr Asp Tyr Asp Arg Gly Tyr Tyr Ala Ile
100 105 110
Asp Tyr Trp Gly Gln Gly Thr Ser Val Thr Val Ser Ser Gly Gly Gly
115 120 125
Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Asp Ile Gln Met
130 135 140
Thr Gln Ser Pro Ala Ser Leu Ser Ala Ser Val Gly Glu Thr Val Thr
145 150 155 160
Ile Thr Cys Arg Ala Ser Glu Asn Ile Tyr Ser Asn Leu Ala Trp Tyr
165 170 175
Gln Gln Lys Gln Gly Lys Ser Pro Gln Leu Leu Val Tyr Gly Ala Thr
180 185 190
Asn Leu Ala Asp Gly Val Pro Ser Arg Phe Ser Gly Ser Gly Ser Gly
195 200 205
Thr Gln Tyr Ser Leu Lys Ile Asn Ser Leu Gln Ser Glu Asp Phe Gly
210 215 220
Ser Tyr Tyr Cys Gln His Phe Trp Gly Thr Pro Trp Thr Leu Gly Gly
225 230 235 240
Gly Thr Lys Leu Glu Ile Lys Arg Gly Pro Gly Asp Lys Thr His Thr
245 250 255
Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
260 265 270
Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
275 280 285
Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
290 295 300
Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
305 310 315 320
Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser Val
325 330 335
Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
340 345 350
Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
355 360 365
Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
370 375 380
Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
385 390 395 400
Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly
405 410 415
Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp
420 425 430
Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
435 440 445
Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His
450 455 460
Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys Gly Gly
465 470 475 480
Gly Gly Ser Gly Ala Pro Phe Arg Asp Cys Ala Glu Val Phe Lys Ser
485 490 495
Gly His Thr Thr Asn Gly Ile Tyr Thr Leu Thr Phe Pro Asn Ser Thr
500 505 510
Glu Glu Ile Lys Ala Tyr Cys Asp Met Glu Ala Gly Gly Gly Gly Trp
515 520 525
Thr Ile Ile Gln Arg Arg Glu Asp Gly Ser Val Asp Phe Gln Arg Thr
530 535 540
Trp Lys Glu Tyr Lys Val Gly Phe Gly Asn Pro Ser Gly Glu Tyr Trp
545 550 555 560

Leu Gly Asn Glu Phe Val Ser Gln Leu Thr Asn Gln Gln Arg Tyr Val
565 570 575
Leu Lys Ile His Leu Lys Asp Trp Glu Gly Asn Glu Ala Tyr Ser Leu
580 585 590
Tyr Glu His Phe Tyr Leu Ser Ser Glu Glu Leu Asn Tyr Arg Ile His
595 600 605
Leu Lys Gly Leu Thr Gly Thr Ala Gly Lys Ile Ser Ser Ile Ser Gln
610 615 620
Pro Gly Asn Asp Phe Ser Thr Lys Asp Gly Asp Asn Asp Lys Cys Ile
625 630 635 640
Cys Lys Cys Ser Gln Met Leu Thr Gly Gly Trp Trp Phe Asp Ala Cys
645 650 655
Gly Pro Ser Asn Leu Asn Gly Met Tyr Tyr Pro Gln Arg Gln Asn Thr
660 665 670
Asn Lys Phe Asn Gly Ile Lys Trp Tyr Tyr Trp Lys Gly Ser Gly Tyr
675 680 685
Ser Leu Lys Ala Thr Thr Met Met Ile Arg Pro Ala Asp Phe
690 695 700

What is claimed is:

1. A Tie2-binding protein comprising fusion polypeptide A-M-A', wherein:

(a) A is a Tie2-binding fragment of an antibody that binds a Tie2 receptor on the surface of a cell;

(b) A' is a fibrinogen domain of Ang1 or Ang2 or Tie2-binding fragment thereof, that binds to the same Tie2 receptor as the Tie2-binding fragment of A; and

(c) M is a multimerizing component;

wherein A and A' do not compete for binding to the Tie2 receptor, and wherein exposing a cell bearing a Tie2 receptor to the Tie2-binding protein results in clustering of four or more Tie2 receptors of the cell and also results in phosphorylation and activation of the Tie2 receptor of the cell.

2. The Tie2-binding protein of claim 1, wherein A is a single chain Fv (scFV) fragment.

3. The Tie2-binding protein of claim 1, wherein A' is a fibrinogen domain of Ang1.

4. The Tie2-binding protein of claim 1, wherein A' is a fibrinogen domain of Ang2.

5. The Tie2-binding protein of claim 1, wherein M is an Fc domain of an IgG.

6. The Tie2-binding protein of claim 2, wherein the scFv comprises a variable region of SEQ ID NO:28.

7. A nucleic acid molecule having a nucleotide sequence encoding the fusion polypeptide according to any one of claims 1 to 6.

8. A vector comprising the nucleic acid molecule of claim 7.

9. A host cell that produces the fusion polypeptide according to any one of claims 1 to 6.

10. A Tie1-binding protein comprising fusion polypeptide A-M-A', wherein:

(a) A is a Tie1-binding fragment of an antibody that binds a Tie1 receptor on the surface of a cell;

(b) A' is a fibrinogen domain of Ang1 or Ang2, or a Tie1-binding fragment thereof, that binds to the same Tie1 receptor as the Tie1-binding fragment of A; and;

(c) M is a multimerizing component;

wherein A and A' do not compete for binding to the Tie1 receptor, and wherein exposing a cell bearing a Tie1 receptor to the Tie1-binding protein results in clustering four or more Tie receptors of the cell and also results in phosphorylation and activation of the Tie1 receptor of the cell.

11. The Tie1-binding protein of claim 10, wherein A is a single chain Fv (scFV) fragment.

12. The Tie1-binding protein of claim 10, wherein A' is a fibrinogen domain of Ang1.

13. The Tie1-binding protein of claim 10, wherein A' is a fibrinogen domain of Ang2.

14. The Tie1-binding protein of claim 10, wherein M is an Fc domain of an IgG.

15. The Tie1-binding protein of claim 11, wherein the scFv comprises a variable region of SEQ ID NO:51.

16. A nucleic acid molecule having a nucleotide sequence encoding the fusion polypeptide according to any one of claims 10 to 15.

17. A vector comprising the nucleic acid molecule of claim 16.

18. A host cell that produces the fusion polypeptide according to any one of claims 10 to 15.

19. A nucleic acid encoding a fusion protein, wherein the fusion protein is selected from the group consisting of SEQ ID NO:28, SEQ ID NO:29, SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:50, and SEQ ID NO:51.

20. An expression vector encoding the fusion protein of claim 19.

21. An isolated host cell comprising the expression vector of claim 20.

22. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:28.

23. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:29.

24. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:30.

25. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:31.

26. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:50.

27. The nucleic acid of claim 19, encoding the fusion protein of SEQ ID NO:51.