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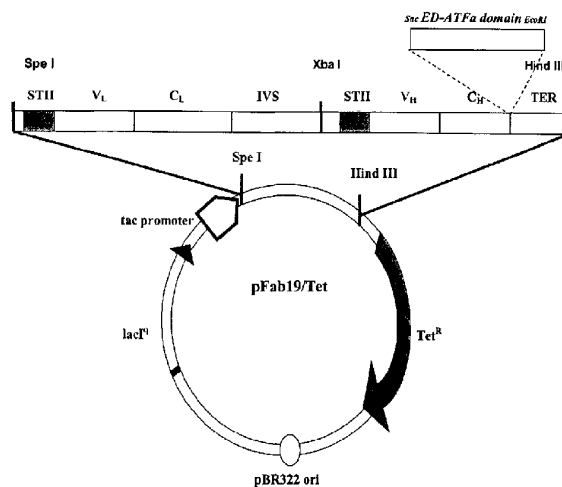
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(54) Title: MULTIMERIC PROTEINS AND METHODS OF MAKING AND USING SAME



(57) Abstract: The invention provides multimerization polypeptides capable of conferring formation of multimers when the multimerization polypeptide is linked to a molecule, such as a heterologous polypeptide sequence.



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

Multimeric Proteins and Methods of Making and Using Same

Related Applications

This application claims priority to application serial no. 60/306,746, filed July 19,
 5 2001, and application serial no. 60/335,425, filed November 30, 2001.

Field of the Invention

The invention relates to engineered polypeptide sequences that mediate formation
 of oligomers (e.g., dimers, trimers, tetramers, hexamers, pentamers, and higher order
 10 oligomeric forms) between molecules attached thereto.

Background

Several ways of making multimeric recombinant antibodies have been reported,
 15 which include the mini-antibody (Pack *et al.*, *Biotechnology* 11:1271 (1993); Pack *et al.*,
Biochemistry 31:1579 (1992); Pack *et al.* *J. Mol. Biol.* 246:28 (1995); Rheinacker *et al.*,
J. Immunol. 157:2989 (1996); and Pluckthun and Pack, *Immunotechnology* 3:83 (1997)),
 diabody-triabody-tetradody (Hudson PJ, *Curr. Opin. Biotech.* 9:395 (1998); Hiades *et al.*,
FEBS Lett. 409:437 (1997); Kortt *et al.*, *Protein Engineering* 10:423 (1997); and Gall *et*
 20 *al.*, *FEBS Lett.* 453:164 (1999)), protein A-fusion protein (Ito and Kurosawa, *J. Biol.*
Chem. 268:20668 (1993)), streptavidin-fusion protein (Kipriyanov *et al.*, *Protein*
Engineering 9:203 (1996)), disulfide-linked fragments (Carter *et al.*, *Biotechnology*
 10:163 (1992)), fragments joined with chemically attached spacers (Cook and Wood, *J.*
Immunol. Methods 171:227 (1994)), or post purification assembly of scFv multimers by
 25 denaturation and renaturation (Whitlow *et al.*, *Protein Engineering* 7:1017 (1994) and
 U.S. Patent No. 5,869,620).

Multimeric antibodies made by fusion with protein A or streptavidin may not be
 suitable for human use since protein A and streptavidin are highly immunogenic in
 humans. Linking antibody fragments by a disulfide bond can lead to a dimeric
 30 recombinant antibody. It has been reported that single chain antibody scFv-based
 molecules can sometimes be made in multimeric forms by changing the length of linker
 between the VH and VL domains in scFv. When the linker is longer than 3 but shorter
 than 12 amino acid residues, scFv can form dimers, called "diabodies;" when the linker is
 less than 2 residues, or when no linker residue is used, scFv can form either trimers or

than 12 amino acid residues, scFv can form dimers, called "diabodies;" when the linker is less than 2 residues, or when no linker residue is used, scFv can form either trimers or tetramers, called "triabodies" and "tetrabodies." Since these multimeric scFv molecules are structurally constrained, little affinity improvement resulted from higher valency.

- 5 Triabodies have identical or lower affinities than the diabodies, and tetrabodies have less than one fold higher affinity than the diabodies (Kortt *et al.*, *Protein Engineering* 10:423 (1997); and Gall *et al.*, *FEBS Lett.* 453:164 (1999)).

Summary

- 10 In a first aspect, the present invention provides a multimeric protein comprising an isolated multimerization polypeptide, wherein said isolated multimerization polypeptide comprises SEQ ID NO: 154 or a fragment thereof that is at least 11 amino acids in length.

In a second aspect, the present invention provides a multimeric protein comprising an isolated multimerization polypeptide having 70% or greater identity to SEQ ID NO: 154.

- 15 In a third aspect, the present invention provides a homo- or hetero-dimer, trimer, tetramer or higher order polypeptide oligomer comprising at least one multimeric protein of the invention.

In a fourth aspect, the present invention provides a multimeric protein comprising an isolated multimerization polypeptide, wherein said isolated multimerization polypeptide comprises at least 28 contiguous amino acids of SEQ ID NO: 154.

- 20 In a fifth aspect, the present invention provides a pharmaceutical formulation comprising a multimeric protein of the first, second or third aspect.

- In a sixth aspect, the present invention provides a method of treating respiratory syncytial virus infection in a subject said method comprising step of administering to a subject in the treatment a multimeric protein of the first, second or third aspect or a
25 pharmaceutical formulation of the invention.

In a seventh aspect, the present invention provides a method of treating human rhinovirus infection in a subject said method comprising step of administering to a subject in the treatment a multimeric protein of the first, second or third aspect or a pharmaceutical formulation of the invention.

- 30 In an eighth aspect, the present invention provides a method of treating asthma in a subject said method comprising step of administering to a subject in the treatment a

multimeric protein of the first, second or third aspect or a pharmaceutical formulation of the invention.

In a ninth aspect, the present invention provides use of a multimeric protein of the first, second or third aspect in the proviso of a medicament or the treatment of respiratory
5 syncytial virus, human rhinovirus, or asthma.

Unless the context clearly requires otherwise, throughout the description and the claims, the words "comprise", "comprising", and the like are to be construed in an inclusive sense as opposed to an exclusive or exhaustive sense; that is to say, in the sense of "including, but not limited to".

10 The invention includes polypeptide sequences capable of conferring multimer formation. In one embodiment, a multimerization polypeptide is selected from the amino acid sequences set forth in SEQ ID NOs: 1 to 7; SEQ ID NOs: 9 to 37; and SEQ ID NOs: 154 to 163. Multimerization polypeptides also include sequences having various amounts of sequence identity to the sequences disclosed herein, so long as the polypeptide is
15 capable of multimerization. In various embodiments, a polypeptide has 70% or greater, 75% or greater, 80% or greater, 85% or greater, 90% or greater, 95% or greater identity to a multimerization polypeptide, for example, as set forth in any one of SEQ ID NOs: 1 to 7; SEQ ID NOs: 9 to 37; and SEQ ID NOs: 154 to 163.

Subsequences, modified forms, e.g., sequences having amino acid substitutions,
20 additions or deletions of the multimerization polypeptides capable of multimerization are also included. In one embodiment, a modified form has one or more amino acid substitutions, provided that all of positions a or d of a seven residue repeat sequence (a.b.c.d.e.f.g), are either leucine, isoleucine or valine, said substituted polypeptide being capable of conferring multimerization. In another embodiment, a modified form has one
25 or more amino acid substitutions, provided that one or more of positions a or d of a seven residue repeat sequence (a.b.c.d.e.f.g), are either leucine, isoleucine or valine, said substituted polypeptide being capable of conferring multimerization. In one aspect, at least one of positions a or d of a seven residue repeat sequence (a.b.c.d.e.f.g), is an amino acid other than leucine, isoleucine or valine. In another aspect, the polypeptide has 1 to 5
30 amino acid substitutions.

In still another embodiment, a modified form has one or more amino acid substitutions in positions b, c, e, f or g, provided that said substituted polypeptide is

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capable of conferring multimerization. In one aspect, the polypeptide has 1 to 5 amino acid substitutions. _____

Multimerization polypeptides are of various lengths. In various embodiments, the polypeptide has a sequence at least 11 amino acids in length, at least 15 amino acids in length, at least 18 amino acids in length, at least 22 amino acids in length, at least 27 amino acids in length, at least 31 amino acids in length. In additional embodiments, the polypeptide has a sequence less than about 125 amino acids in length, less than about 100 amino acids in length, less than about 75 amino acids in length, less than about 50 amino acids in length.

Multimerization polypeptides including subsequences and modified forms thereof may be fused to any molecule thereby conferring multimer formation. In one embodiment, the molecule comprises a polypeptide sequence, e.g., a heterologous polypeptide. In various aspects, the multimerization polypeptide is fused to the amino or carboxy terminus of the heterologous polypeptide, to form a chimeric polypeptide. Chimeric polypeptides have a sequence length typically from about 18-30, 30-50, 50-75, 75-100, 100-150, 150-200, 200-250, 250-500 or 500-1000 amino acids, but may be less or greater. Particular heterologous polypeptides are selected, for example, from: a binding protein (e.g., an antigen binding polypeptide), enzyme, receptor, ligand, nucleic acid binding protein, growth regulatory factor, differentiative factor, and chemotactic factor. In various aspects, the antigen binding polypeptide comprises at least one antibody variable domain, such as a human or humanized variable domain. In additional aspects, the antigen binding polypeptide includes a single chain antibody, Fab, Fab', (Fab')₂, or Fv antibody subsequence. In additional aspects, the antigen binding polypeptide comprises a multispecific or multifunctional antibody. In one particular aspect, the antigen binding polypeptide binds to ICAM-1 or an epitope thereof, e.g., the antigen binding polypeptide inhibits human rhinovirus infection of a cell that expresses ICAM-1.

Multimers form hetero- or homo-dimer, -trimer, -tetramer, -pentamer or higher order oligomer. Binding between the monomeric substituents of the multimer may be measured by dissociation, or K_D of the monomers. Various exemplary K_D are 1×10^{-7} or less, 1×10^{-8} or less, or 1×10^{-9} .

In additional embodiments, linkers are included between the multimeric polypeptides and the molecule to which it is attached. In one embodiment, a linker comprises a polypeptide sequence, such as a human or humanized amino acid sequence. Linkers can be of any size. For example, a polypeptide linker can be an amino acid

sequence from about 5 to 20 amino acids, from about 10 to 30 amino acids, from about 25 to 50 amino acids, from about 30 to 60 amino acids, from about 50 to 75 amino acids, or greater or less in length. In particular aspects, a linker includes an amino acid sequence set forth in any of SEQ ID NO:43 (D30), SEQ ID NO:44 (D35), SEQ ID NO:45 (ED), SEQ ID NO:46 (EDC) or SEQ ID NO:47 (D63), or a subsequence thereof.

The invention also provides pharmaceutical formulations that include the multimers. In one embodiment, a pharmaceutical formulation includes a multimer fused to a molecule. In one aspect, the molecule comprises a heterologous polypeptide sequence.

The invention further provides nucleic acids encoding the multimeric polypeptides alone, and in combination with heterologous polypeptides, as well as linked sequences. Expression cassettes, vectors and cells (e.g., bacterial, fungal, animal, plant, and insect), including the nucleic acids are included.

The invention additionally provides methods of producing a multimerization polypeptide having one or more seven residue repeat sequence, (a.b.c.d.e.f.g), that confers formation of a multimer. In one embodiment, a method includes modifying a polypeptide comprising a seven residue repeat sequence, (a.b.c.d.e.f.g), wherein one or more of positions a or d are replaced with either leucine or isoleucine, thereby producing a multimerization polypeptide that confers multimer formation. In another embodiment, a method includes modifying a seven residue repeat sequence, (a.b.c.d.e.f.g), wherein positions a or d are replaced with valine and either of leucine or isoleucine, thereby producing a multimerization polypeptide that confers dimer formation.

In various aspects, the modified polypeptide forms a trimer, tetramer or pentamer, whereas the unmodified polypeptide forms a dimer; the modified polypeptide forms a tetramer or pentamer, whereas the unmodified polypeptide forms a dimer or trimer; the unmodified polypeptide forms a trimer or tetramer or pentamer. In another aspect, the modified polypeptide has increased or decreased multimer stability in comparison to unmodified polypeptide.

The invention further provides methods of producing a chimeric polypeptide that forms a multimer. In one embodiment, a method includes producing a chimeric polypeptide comprising a multimerization polypeptide comprising a seven residue repeat sequence, (a.b.c.d.e.f.g), wherein one or more of positions a and d are either leucine or

isoleucine, fused to a heterologous polypeptide thereby producing chimeric polypeptide that forms a trimer; a tetramer; or a pentamer.

The invention moreover provides methods of producing a molecule that forms a multimer. In one embodiment, a method includes producing a molecule including a
 5 multimerization polypeptide comprising a seven residue repeat sequence, **(a.b.c.d.e.f.g)**, wherein positions **a** or **d** are valine and either of leucine or isoleucine, fused to the molecule thereby producing a molecule that forms a multimer; e.g., a trimer or tetramer or pentamer.

Further provided are methods of identifying a multimerization polypeptide
 10 including a seven residue repeat sequence, **(a.b.c.d.e.f.g)**. In one embodiment, a method includes: incubating a polypeptide comprising a seven residue repeat sequence, **(a.b.c.d.e.f.g)**, wherein one or more of positions **a** and **d** are either leucine, isoleucine or valine, under conditions allowing formation of homo- or hetero-multimers; and assaying for the presence of homo- or hetero-multimers of the polypeptide, wherein formation of a
 15 homo- or hetero-multimer identifies a multimerization polypeptide comprising a seven residue repeat sequence, **(a.b.c.d.e.f.g)**.

Also provided are methods of inhibiting RSV infection of a cell (e.g., in a subject). In one embodiment, a method includes contacting RSV or a cell susceptible to RSV infection with an amount of a hetero- or homo-dimer, -trimer, -tetramer, -pentamer
 20 or higher order oligomer with a multimer effective to inhibit RSV infection of the cell. Additionally provided are methods of inhibiting RSV infection, inhibiting RSV progression or treating RSV infection of a subject. In another embodiment, a method includes administering to a subject having or at risk of having RSV infection an amount of a multimer antibody, such as a hetero- or homo-dimer, -trimer, -tetramer or higher
 25 order oligomer of an antibody, effective to inhibit, inhibit progression or treat RSV infection of the subject. In various aspects, the subject has or is at risk of having asthma; is a new born or between the ages of 1 to 5, 5 to 10 or 10 to 18; the cell is an epithelial cell; the multimer comprises a chimeric polypeptide, for example, an antibody such as a humanized antibody. In additional aspects, the antibody is administered locally; via
 30 inhalation or intranasally.

Further provided are methods of treating the common cold. In one embodiment, a method includes administering to a subject having or at risk of having a common cold an amount of an antibody, or a hetero- or homo-dimer, -trimer, -tetramer or higher order

oligomer of the antibody, effective to treat the common cold in the subject. In various aspects, the treatment comprises inhibiting infection by HRV, progression of HRV infection or a symptom of HRV infection; the antibody is humanized; the antibody is administered locally; the antibody is administered via inhalation or intranasally; the
 5 subject has or is at risk of having asthma; and the subject is a new born or between the ages of 1 to 5, 5 to 10 or 10 to 18.

Brief Description Of The Drawings

10 **Figure 1** shows an expression vector ("Fab vector") for expressing exemplary chimeric polypeptide comprising a multimerization domain (ATF α), a linker (ED) and an antibody sequence (V_L, V_H, C_L and C_H), as described in Examples 1- 3.

Figures 2A-2B show characterization of purified CFY196. **A)** Purified CFY196 runs as a single peak on the HPLC size exclusion column; **B)** Coomassie Blue stained
 15 SDS-PAGE of purified CFY196, which is shown as two components, light chain (LC) and composite heavy chain (CHC) bands (lane 1). Molecular weight markers are shown in lane 2.

Figures 3A-3C show processed sedimentation velocity data from **A)** CFY 195 (peak is at 5.5 S); **B)** CFY192B; and **C)** CFY196 (peaks at 6.55). Note the narrower peak
 20 for CFY196.

Figure 4 shows protection of HeLa cells from infection by HRV14 with monovalent Fab and multivalent Fab-ATF α domain chimeric proteins. Chimeric proteins are denoted as follows: CFY193B, Fab-ED-ATF α LL; CFY196, Fab-ED-ATF α (1)LI+S; CFY192B, Fab-ED-ATF α (2)LI; and CFY195, Fab-ED-ATF α II.

25 **Figure 5** shows protection of HeLa cells from HRV15 infection with monovalent Fab19, bivalent monoclonal antibody RR/1, bivalent CFY202, trimeric CFY193B, and tetrameric CFY196.

Figures 6A-6B show a bispecific multimeric protein. **A)** Fab moieties illustrated with different hatching have different specificities or functions. A linker sequence is
 30 illustrated as a hexagon. **B)** This polypeptide is produced from one tricistronic RNA molecule. The coding sequence for the first polypeptide chain translated from this message is illustrated by the hatched box, representing the anti-CD-3 light chain (LC-1). The second DNA fragment encodes the central chimeric polypeptide consisting of the

anti-CD-3 heavy chain (HC-1) linked to a hinge derived from IgD, followed by a dimerization domain, a second hinge, and the anti-CD19 light chain (LC-2). The third RNA (empty box) would encode the anti-CD19 heavy chain (HC-2). The restriction sites are as indicated.

5 **Figure 7A-7B** show improvement in tetramerization of ATFaIL by substituting amino acids at solvent exposed positions: **A)** CFY1971; **B)** CFY1972; and **C)** CFY197.

Figure 8 shows the results of a competition ELISA. The data is presented as a percent of inhibition of tracer antibody binding to ICAM-1.

10 **Detailed Description**

The invention is based, at least in part, on the identification of peptide sequences that confer multimerization, referred to herein as "multimerization polypeptides," "multimerization domains" or "multimerization devices." Invention multimerization polypeptides confer oligomer formation. For example, a multimerization polypeptide, 15 when fused to a second molecule, such as a heterologous polypeptide sequence, facilitates the formation of dimers, trimers, tetramers, pentamers or higher order oligomers or mixtures thereof among the polypeptides. Such invention multimerization polypeptides are useful in a variety of diagnostic or therapeutic applications. For example, fusing a multimerization polypeptide to a binding protein, such as an antigen 20 binding polypeptide (e.g., antibody) can be used to increase the number of antigen binding sites via oligomer formation. Increasing the number of antigen binding sites can in turn increase antibody avidity for the antigen, which is useful in any therapeutic or diagnostic antibody application, particularly where it is desirable or advantageous to increase antibody avidity. A fully human or humanized multimerized antibody has 25 decreased or absent immunogenicity in humans in comparison to non-humanized antibody.

Multimerization polypeptides attached to molecules allow multimers to form between any molecule. In other words, the oligomer may comprise, for example, two or more molecules of the same protein (e.g., a homo-dimer, -trimer, -tetramer or higher 30 oligomer) or a mixture of two or more different (i.e., non-identical) proteins (e.g. a hetero-dimer, -trimer, -tetramer or higher oligomer). For example, oligomeric antibodies may comprise the same antibody or two or more different antibodies, each of which have two or more functions or activities (e.g., bind to two or more epitopes). Such hetero-

oligomers, also referred to as multifunctional oligomers (e.g., bifunctional, trifunctional, tetrafunctional, etc., as appropriate) due to the multiple functions of the proteins that comprise the oligomer, are useful in a variety of diagnostic and therapeutic applications. For example, a multifunctional oligomer comprising two or more different antibodies is

5 useful in applications in which it is desired to utilize a multifunctional antibody. In particular, a multifunctional antibody may include an antibody that binds to a cell surface receptor and an antibody that mediates the complement cascade so that cells bearing the receptor are targeted for killing. The specific functionality of the oligomer can be determined by the skilled artisan depending upon the application.

10 Thus, in accordance with the invention, there are provided multimerization polypeptides that confer multimer formation. In one embodiment, a multimerization polypeptide is selected from any of the amino acid sequences set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163. In other embodiments, a multimerization polypeptide has 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%,

15 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or greater identity to the amino acid sequences set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163, provided that the multimerization polypeptide is capable of conferring multimerization. In additional embodiments, a multimerization polypeptide confers formation of hetero- or homo-dimers, trimers, tetramers, pentamers, hexamers, higher

20 order oligomers and mixtures thereof. In yet additional embodiments, a multimerization polypeptide is a sequence that is made fully human or humanized based on the sequence of a human peptide that can form homo- or hetero-dimers, trimers, tetramers, or higher order oligomers.

Multimerization polypeptides and nucleic acids encoding multimerization

25 polypeptides when not fused to heterologous polypeptides are distinct from known wild type leucine zipper sequences. Thus, the multimerization polypeptides of the invention do not include wild type leucine zipper domain sequences known in the art, such as those present in naturally occurring GCN4, ATF α , ATF-7 (100% identical to the coiled-coil domain in ATF α), ATF-2, cyclic AMP response element binding protein Pa (CREB-Pa),

30 JUN-D and C-JUN. The multimerization polypeptides of the invention are also distinct from the multimerization device described in WO 96/37621, wild type sequences derived from human p53, PF4, TSP-4, COMP, thrombospondin, dTAF $_{II}$ 42, dTAF $_{II}$ 31, dTAF $_{II}$ 62, dTAF $_{II}$ 80, histone 3 and histone 4.

Invention multimerization polypeptides may be of any length provided that they are capable of conferring multimerization. In specific embodiments, a multimerization polypeptide has a sequence at least 11 amino acids in length, at least 15 amino acids in length, at least 18 amino acids in length; at least 22 amino acids in length; at least 25
 5 amino acids in length; at least 29 amino acids in length; and at least 31 amino acids in length. In additional specific embodiments, a multimerization polypeptide has a sequence less than about 100 amino acids in length; less than about 75 amino acids in length; and less than about 50 amino acids in length.

The invention also provides chimeric polypeptides that include a multimerization
 10 polypeptide fused to a heterologous polypeptide. Such chimeric polypeptides form oligomers (multimers) via the multimerization polypeptide. In one embodiment, a chimeric polypeptide includes a multimerization polypeptide selected from any of the amino acid sequences set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163. In other embodiments, a chimeric polypeptide includes a
 15 multimerization polypeptide having 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or greater identity to an amino acid sequence set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163, provided that the chimeric polypeptide forms multimers. In additional embodiments, a chimeric polypeptide forms hetero- or homo-dimers, trimers, tetramers, pentamers,
 20 hexamers, higher order oligomers and mixtures thereof.

As used herein, the term "multimer" and grammatical variations thereof refers to formation of an oligomeric complex between two or more distinct molecules. When used in reference to a polypeptide, e.g., "polypeptide multimer," this means that the polypeptide forms a higher order oligomer with itself (homo-multimer) or with other
 25 molecules (hetero-multimer). A polypeptide that confers multimerization means an amino acid sequence that can confer the formation of a dimer, trimer, tetramer, pentamer, hexamers or any higher order oligomer with itself (homo-oligomer) or with one or more different proteins (hetero-oligomer) under appropriate conditions. For example, a chimeric polypeptide comprising a multimerization polypeptide fused to a heterologous
 30 polypeptide is able to form dimers, trimers, tetramers, pentamers, hexamers, or higher order oligomers with itself or with different proteins having domains capable of interacting with the multimerization polypeptide portion. Multimers therefore additionally include monovalent or oligomeric (e.g., dimer, trimer, tetramer, etc.)

chimeric polypeptides either joined directly or indirectly through covalent or non-covalent binding.

The multimerization polypeptide may be directly linked to the heterologous polypeptide via a covalent bond, such as a chemical cross linking agent. Alternatively, multimerization polypeptide can be connected to the heterologous polypeptide via a linker sequence (e.g., a peptide sequence such as the antibody hinge sequence). Although optional, inclusion of a linker ensures that the multimerization polypeptide does not block function of the heterologous polypeptide and that the heterologous polypeptide does not block multimerization function. A linker thus allows each antigen-binding domain in the multimer enough flexibility to bind antigen.

One specific example of a linker is an immunoglobulin hinge sequence (e.g., IgG or IgD). As with the multimerization domains, linker amino acid sequences may be fully human, humanized or non-human amino acid sequences, unmodified or modified as set forth herein. A fully human or humanized linker sequence may be particularly useful for therapeutic purposes.

Invention compositions comprising a chimeric polypeptide that include a multimerization polypeptide fused to a heterologous polypeptide are distinct from the known native (i.e., naturally occurring) and recombinant proteins that contain a multimerization polypeptide sequence. For example, scFv fused to yeast based protein GCN4-LI domain, which forms tetravalent scFv (Pack *et al.*, *J. Mol. Biol.* 246:28 (1995)) and scFv fused to wild type leucine zipper domains from jun and fos (U.S. Patent No. 5,910,573) are distinct from chimeric polypeptide that includes a multimerization polypeptide fused to a heterologous polypeptide. Invention chimeric polypeptides may include multimerization sequences as known in the art, provided that the chimeric polypeptides form multimers and are distinct from known proteins that contain the multimerization sequences known in the art.

As used herein, the term "heterologous," when used in reference to a polypeptide, means that the polypeptide is not normally contiguous with the other polypeptide in its natural environment. Thus, an invention chimeric polypeptide including a multimerization polypeptide fused to a heterologous polypeptide means that the multimerization polypeptide does not exist fused with the heterologous polypeptide in normal cells. In other words, a chimeric polypeptide including a multimerization polypeptide fused to a heterologous polypeptide is a molecule that does not normally

exist in nature, i.e., such a molecule is produced by the hand of man, e.g., artificially produced through recombinant DNA technology.

The term "fusion," when used in reference to two or more molecules (e.g., polypeptides) means that the molecules are covalently attached or linked. Any method of attachment of the molecules is contemplated. Thus, the chemical nature of the linkage between the multimer and the molecule to which it is attached is not limited. A particular example for the linkage of two protein sequences is an amide bond or equivalent.

The term "chimera," and grammatical variations thereof, when used in reference to a protein, means that the protein is comprised of one or more amino acid residues from two or more different proteins (i.e., non-identical proteins). A particular example of a chimera containing amino acid residues from two or more different proteins is a polypeptide comprising a multimerization polypeptide and a heterologous polypeptide. A particular example of a chimera containing amino acid residues from three or more different proteins is a polypeptide comprising a multimerization polypeptide, a linker (e.g. hinge) and a heterologous polypeptide.

Although not wishing to be bound by theory, amino acid sequences that confer multimerization mediate protein-protein binding via Van der Waals' forces, hydrophobic interactions, hydrogen bonding or charge-charge bonds. Molecules may also form an oligomer if they are covalently linked to each other. For example, two distinct proteins, chemically synthesized, in vitro translated or isolated or purified from a cell or a sample, may be chemically cross-linked together via a non-amide bond to form an oligomer. Thus, two molecules that exist as separate entities and that do not form oligomers through non-covalent interaction but are joined together via covalent bonds are also considered to be a multimer. Accordingly, an oligomer or a multimer of the invention (e.g., hetero- or homo-dimer, trimer, tetramer, pentamer, hexamers, etc.) may be formed through covalent bonding, non-covalent bonding or mixtures thereof.

The coiled-coil domain is composed of interacting, amphipathic α helices characterized by a seven-residue repeat sequence (a heptad repeat), **a.b.c.d.e.f.g**, with hydrophobic residues predominant at positions **a** and **d** (positions one and four), and polar residues generally elsewhere (Harbury *et al.*, *Science* 262:1401 (1993)). The leucine zipper domains are coiled-coil domains that typically have leucine at the **d** position of the heptad repeats. Naturally occurring coiled-coils are typically made up of multiple heptad repeats, for example, three or more sequences, (**a.b.c.d.e.f.g**)₁ - (**a.b.c.d.e.f.g**)₂ -

(a.b.c.d.e.f.g)₃, etc. The designation “(a.b.c.d.e.f.g)_n” merely refers to two or more additional half (3-4 amino acids) or full length (7 amino acids) heptad repeat sequences, where each half or full (a.b.c.d.e.f.g.) repeat need not have the identical amino acid sequence (see, e.g., Tables 3A and 3B).

5 Generally the nature of the hydrophobic residues at the **a** and **d** positions of the repeat sequence determines its particular multimeric status. Furthermore, with a given pair of **a** and **d** residues, the multimerization status may vary and can be affected by the sequence at **b**, **c**, **e**, **f** and **g** positions. These rules even apply to the coiled-coil sequences having only 29% to 50% identity with the coiled-coil domain in yeast protein GCN4.

10 When leucine or isoleucine are at positions **a** and **d** as disclosed herein, dimer, trimer or tetramer or pentamer form. Specifically, tetramers are likely to form from coiled-coil sequences that have leucine at position **a** and isoleucine at position **d**, as shown by ATFα-LI, ATF1-LI, ATF2-LI, CJUN-LI, JUND-LI and CREB-LI domains. Coiled-coil sequences with isoleucine at position **a** and leucine at position **d** may form
15 dimers, such as CREB-IL, CJUN-IL and ATF2-IL, tetramers such as ATFα-IL, or dimers such as ATF1-IL. With leucine at both positions **a** and **d** either trimers or tetramers form. For example, ATFα-LL, ATF1-LL, CJUN-LL form trimer, and ATF2-LL and CREB-LL both form tetramers. Coiled-coils with isoleucine at both positions **a** and **d** have a tendency to form trimers; for example, ATFα-II and ATF1-II form trimer. These multiple
20 forms exist because amino acids outside the hydrophobic core, positions **b**, **c**, **e**, **f**, and **g**, can modulate the multimerization state of the coiled coil by creating a network of inter- and intra- helical hydrogen bonds and salt bridges. This network of hydrophilic bonds changes the relative orientation of the helices as it tends toward optimal geometry, resulting in the observed effect on multimerization state. Accordingly, it is possible in a
25 context dependent manner to change one or a few residues at individual **b**, **c**, **e**, **f** and **g** positions to modulate the multimerization state.

When valine is paired with either isoleucine or leucine at **a** and **d** position, the resulting multimerization polypeptides tend to be unstable, but can confer mixtures of dimer and tetramer formation. For example, the coiled-coil domain of JUN-D, amino
30 acids 249-280, which has 48% identity to ATFα domain (Table 3B) was modified in accordance with the invention. JUND-LI forms trimer and JUND-LL forms a dimer (Table 8). Similarly, the coiled-coil domain of C-JUN, amino acids 277-308, has 39%

identity to ATF α domain. C-JUN-LI forms tetramer, C-JUN-LL forms trimer and C-JUN-IL forms dimer (Table 8).

Generally, multimerization domains with two or more (a.b.c.d.e.f.g.) heptad repeat sequences will tolerate amino acids other than leucine, isoleucine or valine at the a and d positions. For example, in a four heptad repeat sequence, one or more of the a and d positions may be amino acids other than leucine, isoleucine or valine. For example, a single hydrophilic residue may be inserted at an a or d position in a sequence including at least three heptad repeats to modulate the multimerization state. Thus, the invention includes multimerization polypeptides in which the a and d positions in a given heptad repeat sequence may be amino acids other than leucine, isoleucine or valine. Furthermore, the strength of the binding between the monomers that comprise the multimer, also referred to herein as stability or tightness, may be altered by modifying (a.b.c.d.e.f.g.) heptad repeat sequences. For example, replacing an entire interacting layer of a and d residues with residues that can interact through hydrogen bonds, for example, a and d can be replaced by glutamine: CFY196Q: L S S I E K K Q E E Q T S Q L I Q I S N E L T L I R N E L A Q S (SEQ ID NO:37). Thus, the invention includes multimerization polypeptides in which the a, b, c, d, e, f and g positions may be modified to alter the multimerization state as well as increase or decrease the strength of binding between the multimers.

Each of the multimeric domains derived from coiled-coil sequences may exist as mixtures of monomer, dimer, trimer, tetramer or higher oligomer. The major multimeric form, which constitutes over 50% of the mass, is the designated multimer form. For example, if a multimer is designated a tetramer, at least 50% of the mass is in the tetrameric form.

Specific examples of multimerization polypeptides of the invention include, for example, SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163. Additional multimerization polypeptides may be identified by assaying putative coiled-coil sequences, composed of one or more heptad repeats (a.b.c.d.e.f.g.) for oligomer formation using routine detection assays as set forth herein or known in the art (see, e.g., Example 6). A sequence including a heptad repeat, (a.b.c.d.e.f.g.), wherein one or more positions a and d are either leucine, isoleucine or valine can be incubated under conditions allowing formation of a hetero- or homo-oligomer.

For example, the leucine zipper domain of cAMP response element binding protein-Pa (CREB-Pa, amino acids 393-424) is 74% identical to ATF α leucine zipper domain sequence (Table 3C). Variants of CREB-Pa leucine zipper domain, CREB-LL and CREB-LI, formed tetramers and CREB-IL formed dimer (Table 8). ATF-1 leucine zipper domain (amino acids 238-269) is 29% identical to ATF α leucine zipper domain sequence. Variants of ATF-1 leucine zipper domain, ATF1-LI form tetramers; ATF1-IL, ATF1-LL and ATF1-II all form trimers (Table 8).

Additional multimerization domains can be produced by first identifying a wild type coiled-coil domain. For example, five leucine zipper domains were identified by searching the public database (Table 3B). These domains show 32% to 77% identity to the ATF α leucine zipper domain, and less than 50% identity to GCN4 leucine zipper domain (Table 3C). The wild type residues at positions **a** and **d** were replaced with leucine or isoleucine (Table 3D). Additional coiled-coil domains that may be modified in accordance with the invention, include, for example, three and four helix bundles such as lung surfactant D protein, tetranectin, and mannose binding protein; and ROP, cytochrome B562, and tetrabrachion stalk, respectively.

Other multimerization domains can therefore be identified by comparison to sequence databases, and mutating the sequence as set forth herein. For example, one or more coiled-coil forming sequences may be selected from the protein database (e.g., GEN-BANK, SWISS-PROT) and isoleucine, leucine or valine may be introduced into one or more **a** or **d** positions of the heptad repeats to alter the multimerization status of the sequence. The multimerization state may be determined using the assays described herein (Example 6). Optionally, the heptad repeat may be substituted with one or more point mutations, additions, or deletions at positions **b**, **c**, **e**, **f** and **g** where the additional mutations are selected to modulate or stabilize the multimeric state that is formed, that is, to alter the type of multimer formed, e.g., trimer vs. tetramer, or to modulate the binding strength between the monomers that form the multimer. Thus, one or more positions **b**, **c**, **e**, **f** and **g** may be modified alone or in combination with modifications at one or more **a** or **d** positions.

Specific examples of such mutations include those that make additional interhelical hydrogen bonds or salt bridges, for example, between **e** and **g** positions of a four helix bundle. The mutations can also be selected by considering the effect of change

on the network of hydrophilic bonds on the surface of the domain. Inspection of the hydrophilic bonding network is generally predictive but multimer status can be confirmed using the multimerization assays described herein (Example 6).

If the multimerization state has been increased by the modification, for example, the modified sequence forms a trimer or tetramer or higher order oligomer instead of a dimer, it may be desired to substitute one or more hydrophobic residues at positions **b**, **c**, **e**, **f** and **g** to hydrophilic residues to facilitate proper hydrophobic interface formation (Example 12) to increase tightness or stability of the multimer. Because helix formation is at least in part dependent on the residues immediately preceding and following the helix, one or more residues may be added at the helix's N or C terminus, or at the N or C terminus of the series of heptad repeat sequences to improve stability or tightness of the multimer. The phrase to increase or improve "tightness" or "stability" means that the multimer formed is less likely to dissociate into its constituent monomers. A general approximation of multimer tightness or stability can be assessed by the narrowness or broadness of the peak formed in sedimentation velocity studies. Tightness or stability can also be assayed, for example, by denaturing the multimer formed while simultaneously monitoring circular dichroism. A specific example of such a modification is the addition of a terminal serine (Example 11).

Thus, the invention provides methods of producing a multimerization polypeptide comprising one or more seven residue coiled-coil, or leucine zipper repeat sequence, (**a.b.c.d.e.f.g**). In one embodiment a method includes producing a polypeptide comprising a coiled-coil sequence, (**a.b.c.d.e.f.g**), wherein positions **a** and **d** are replaced with either leucine or isoleucine, thereby producing a multimerization polypeptide that confers dimer, trimer, tetramer or pentamer formation. In another embodiment, a method includes producing a coiled-coil sequence, (**a.b.c.d.e.f.g**), wherein positions **a** or **d** are replaced with valine and either of leucine or isoleucine or valine, thereby producing a multimerization polypeptide that confers dimer and tetramer formation. In yet another embodiment, a method includes synthesizing a coiled-coil sequence, (**a.b.c.d.e.f.g**), wherein positions **a** and **d** are either leucine or isoleucine or valine to produce a multimerization polypeptide that confers dimer, trimer or tetramer formation. In still another embodiment, a method includes synthesizing a coiled-coil sequence, (**a.b.c.d.e.f.g**), wherein positions **a** or **d** are valine and either of leucine or isoleucine to produce a multimerization polypeptide that confers dimer and tetramer formation. In

additional embodiments, where there are multiple (a.b.c.d.e.f.g), repeats, positions a or d are replaced such that they are predominantly (e.g., greater than 50%) either leucine, isoleucine or valine. In further embodiments, one or more positions b, c, e, f and g are replaced, e.g., one or more hydrophobic residues are substituted with hydrophilic residues. In yet further embodiments, one or more amino acids are added to the N- or C-terminus of the multimerization domain or flanking a heptad repeat within the domain.

The invention also provides methods of identifying multimerization polypeptides comprising a coiled-coil sequence, (a.b.c.d.e.f.g), wherein positions a or d are either leucine, isoleucine or valine. In one embodiment, a method includes, incubating a polypeptide comprising a coiled-coil sequence, (a.b.c.d.e.f.g), wherein positions a or d are either leucine, isoleucine or valine, under conditions allowing formation of homo- or hetero-multimers, and assaying for the presence of homo- or hetero-multimers of the polypeptide. Formation of a homo- or hetero-multimer identifies the polypeptide as a multimerization polypeptide. In various aspects, where there are multiple (a.b.c.d.e.f.g), repeats, positions a or d are predominantly either leucine, isoleucine or valine. In another aspect, the polypeptide comprises a heterologous polypeptide fused to a polypeptide comprising the coiled-coil sequence, (a.b.c.d.e.f.g). In another aspect, the polypeptide contains a linker between the heterologous polypeptide and the polypeptide comprising the coiled-coil sequence, (a.b.c.d.e.f.g). In yet another aspect, the homo- or hetero-multimer that is detected is a dimer, trimer, tetramer, pentamer, hexamer, or higher order oligomer.

The terms "protein," "polypeptide" and "peptide" are used interchangeably herein to refer to two or more contiguous amino acids, also referred to as "residues," covalently linked through an amide bond or equivalent. Proteins are of unlimited length and may be comprised of L- or D-amino acids as well as mixtures thereof. Amino acids may be linked by non-natural and non-amide chemical bonds including, for example, those formed with glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, or N,N'-dicyclohexylcarbodiimide (DCC). Non-amide bonds include, for example, ketomethylene, aminomethylene, olefin, ether, thioether and the like (see, e.g., Spatola (1983) in Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, Vol. 7, pp 267-357, "Peptide and Backbone Modifications," Marcel Decker, NY).

Polypeptides may have one or more cyclic structures such as an end-to-end amide bond between the amino and carboxy- terminus of the molecule or intra- or inter-

molecular disulfide bond. Polypeptides may be modified *in vitro* or *in vivo*, e.g., post-translationally modified to include, for example, sugar residues, phosphate groups, ubiquitin, fatty acids or lipids. Polypeptides further include amino acid structural and functional analogues, for example, peptidomimetics having synthetic or non-natural amino acids or amino acid analogues.

The term "antibody" refers to a protein that binds to other molecules (antigens) via heavy and light chain variable domains, V_H and V_L , respectively. Antibodies include IgG, IgD, IgA, IgM and IgE. The antibodies may be intact immunoglobulin molecules, two full length heavy chains linked by disulfide bonds to two full length light chains, as well as subsequences (i.e. fragments) of immunoglobulin molecules, with or without constant region, that bind to an epitope of an antigen, or subsequences thereof (i.e. fragments) of immunoglobulin molecules, with or without constant region, that bind to an epitope of an antigen. Antibodies may comprise full length heavy and light chain variable domains, V_H and V_L , individually or in any combination.

Polypeptide sequences can be made using recombinant DNA technology of polypeptide-encoding nucleic acids via cell expression or *in vitro* translation, or chemical synthesis of polypeptide chains using methods known in the art. Antibodies and subsequences can be expressed from recombinantly produced antibody-encoding nucleic acid, such as a polynucleotide isolated from hybridoma cells or selected from a library of naturally occurring or synthetic antibody genes (see, e.g., Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1989; Harlow and Lane, *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1999; Fitzgerald *et al.*, *J.A.C.S.* 117:11075 (1995); Gram *et al.*, *Proc. Natl. Acad. Sci. USA* 89:3576 (1992)). Polypeptide sequences can also be produced by a chemical synthesizer (see, e.g., Applied Biosystems, Foster City, CA).

The term "multifunctional" means that the composition referred to has two or more activities or functions (e.g., bifunctional, trifunctional, tetrafunctional, etc.). For example, a multifunctional polypeptide has two or more of antigen binding, enzyme activity, ligand or receptor binding, toxin, etc.). Similarly, a multifunctional oligomer comprises a mixture of two or more polypeptides each having at least one function or activity, such as antigen binding and enzyme activity, ligand or receptor binding, toxin, etc.

An antibody that binds to a particular antigen, and which also has an attached polypeptide with enzyme activity (e.g., luciferase, acetyltransferase, galactosidase, peroxidase, etc.) is one particular example of a bifunctional antibody. Candidate functions for multifunctional oligomers other than antigen binding and in addition to enzyme activity include, for example, detectable domains such as immunoglobulin, T7 and polyhistidine amino acid sequences, toxins (e.g., ricin, cholera, pertussis), cell surface proteins such as receptors, ligands (substrates, agonists and antagonists), adhesion proteins (e.g., streptavidin, avidin, lectins), growth factors, differentiative factors, chemotactic factors and proenzymes.

Multifunctional multimers further include multispecific (e.g., bispecific, trispecific, tetraspecific, etc.) compositions. The term "multispecific" means an antigen binding polypeptide (e.g., an antibody) that binds to different antigenic epitopes. The different epitopes may be present on the same antigen or on different antigens. For example, a multispecific antibody oligomer comprises a mixture of two or more antibodies each having different epitope binding specificity. Multispecific oligomers may be comprised of individual antigen binding polypeptides each of which have distinct variable domains. For example, one of the antigen binding polypeptides of the oligomer may have two variable domains each of which recognize a different epitope.

Multifunctional polypeptides can be produced through chemical crosslinking of the selected molecules (which have been produced by synthetic means or by expression of nucleic acid that encodes the polypeptides) or through recombinant DNA technology combined with in vitro, or cellular expression of the polypeptide, and subsequent oligomerization. Multispecific antibodies can be similarly produced through recombinant technology and expression, fusion of hybridomas that produce antibodies with different epitopic specificities, or expression of multiple nucleic acid encoding antibody variable chains with different epitopic specificities in a single cell.

A specific example of a bispecific antibody is illustrated in Figures 6A and 6B. The CREB-IL multimerization polypeptide is positioned at the center of a molecule linking Fab from two different antibodies producing a tetravalent binding antibody. Use of multimeric polypeptides conferring trimer or tetramer formation would produce hexavalent and octavalent molecules, respectively. The Fab moieties in the bispecific protein can have different specificities. A flexible linker (e.g., hinge) sequence can be

used to join the Fab portion of each protein. The exemplary bispecific antibody polypeptide may be produced from one tricistronic RNA molecule (Figure 6B).

As used herein, the term "subsequence" or "fragment" means a portion of the full length molecule. For example, a subsequence of a multimerization polypeptide is one or more amino acids less in length than full length polypeptide (e.g. one or more internal or terminal amino acid deletions from either amino or carboxy-termini). Subsequences therefore can be any length up to the full length molecule.

Specific examples of antibody subsequences include, for example, chimeric polypeptides in which a heterologous domain comprises an Fab, Fab', (Fab')₂, Fv, or single chain antibody (SCA) fragment (e.g., scFv). Subsequences include portions, which retain at least part of the function or activity of full length sequence. For example, an antibody subsequence will retain the ability to selectively bind to an antigen even though the binding affinity of the antibody subsequence may be greater or less than the binding affinity of the full length antibody.

For antibody subsequences, pepsin or papain digestion of whole antibodies can be used to generate antibody fragments. In particular, an Fab fragment consists of a monovalent antigen-binding fragment of an antibody molecule, and can be produced by digestion of a whole antibody molecule with the enzyme papain, to yield a fragment consisting of an intact light chain and a portion of a heavy chain. An (Fab')₂ fragment of an antibody can be obtained by treating a whole antibody molecule with the enzyme pepsin, without subsequent reduction. An Fab' fragment of an antibody molecule can be obtained from (Fab')₂ by reduction with a thiol reducing agent, which yields a molecule consisting of an intact light chain and a portion of a heavy chain. Two Fab' fragments are obtained per antibody molecule treated in this manner.

An Fv fragment is a fragment containing the variable region of a light chain V_L and the variable region of a heavy chain V_H expressed as two chains. The association may be non-covalent or may be covalent, such as a chemical cross-linking agent or an intermolecular disulfide bond (Inbar *et al.*, *Proc. Natl. Acad. Sci. USA* 69:2659 (1972); Sandhu, *Crit. Rev. Biotech.* 12:437 (1992)).

A single chain antibody ("SCA") is a genetically engineered or enzymatically digested antibody containing the variable region of a light chain V_L and the variable region of a heavy chain, linked by a flexible linker, such as a polypeptide sequence, in either V_L-linker-V_H orientation or in V_H-linker-V_L orientation. Alternatively, a single

chain Fv fragment can be produced by linking two variable domains via a disulfide linkage between two cysteine residues. Methods for producing scFv antibodies are described, for example, by Whitlow *et al.*, In: Methods: A Companion to Methods in Enzymology 2:97 (1991); U.S. Patent No. 4,946,778; and Pack *et al.*, *Bio/Technology* 11:1271 (1993). Other methods of producing antibody subsequences, such as separation of heavy chains to form monovalent light-heavy chain fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, provided that the subsequences bind to the antigen to which the intact antibody binds.

As used herein, the term "bind" or "binding" means that the compositions referred to have affinity for each other. "Specific binding" is where the binding is selective between two molecules. A particular example of specific binding is that which occurs between an antibody and an antigen. Typically, specific binding can be distinguished from non-specific when the dissociation constant (K_D) is less than about 1×10^{-5} M or less than about 1×10^{-6} M or 1×10^{-7} M. Specific binding can be detected, for example, by ELISA, immunoprecipitation, coprecipitation, with or without chemical crosslinking, two-hybrid assays and the like. Appropriate controls can be used to distinguish between "specific" and "non-specific" binding.

Full length antibodies, subsequences (e.g., single chain forms) or modified forms, fully human, humanized or non-human may be present as heteromeric or homomeric dimers, trimers, tetramers, pentamers, hexamers or any higher order oligomer that retains at least a part of the antigen binding activity of the monomer. Antibody multimers include oligomeric (e.g., dimer, trimer, tetramer, etc.) combinations of different antibodies that are multispecific (e.g., bispecific, trispecific, tetraspecific, etc.) or multifunctional (e.g., bifunctional, trifunctional, tetrafunctional, etc.).

The invention further provides linker polypeptide sequences. The invention linker sequences can be fused to a heterologous polypeptide to form a chimeric polypeptide. In one embodiment, a linker comprises a portion of a hinge region from an immunoglobulin. In one aspect, a linker is derived from or modeled after a human or humanized immunoglobulin. In particular aspects, a linker sequence comprises a portion of an immunoglobulin hinge region selected from an amino acid sequence set forth in any of (SEQ ID NO:43 (D30), SEQ ID NO:44 (D35), SEQ ID NO:45 (ED), SEQ ID NO:46 (EDC) or SEQ ID NO:47 (D63)). In additional particular aspects, a linker sequence comprises an amino acid sequence from about 2 to 20 amino acids, from about 5 to 10

amino acids, from about 10 to 30 amino acids, from about 25 to 50 amino acids, from about 30 to 60 amino acids, from about 50 to 75 amino acids. In further particular aspects, a linker sequence comprises an amino acid sequence from about 2 to 20 amino acids, from about 5 to 10 amino acids, from about 10 to 30 amino acids, from about 25 to 50 amino acids, from about 30 to 60 amino acids, from about 50 to 75 amino acids, from about 75 to 100 amino acids and which includes an amino acid sequence set forth in any of (SEQ ID NO:43 (D30), SEQ ID NO:44 (D35), SEQ ID NO:45 (ED), SEQ ID NO:46 (EDC) or SEQ ID NO:47 (D63)).

As used herein, "linker" or "spacer" refers to a molecule or group of molecules that connects two or more molecules to each other. A flexible linker between two molecules joined to each other allows enough free rotation of one or more of the molecules so that the molecules do not block each others' function. For example, a linker such as an amino acid sequence located between a multimerization polypeptide and a heterologous polypeptide of a chimeric polypeptide, e.g., a humanized antibody, allows the antibody to bind to antigen without significant steric interference from other multimers within the oligomer.

Thus, the invention also provides chimeric polypeptides including a multimerization polypeptide fused to a heterologous polypeptide that further include a linker polypeptide between the multimerization polypeptide and heterologous polypeptide. Also provided are chimeric polypeptides comprising linker sequences fused to a heterologous sequence.

Polypeptides of the invention, including multimerization polypeptides, chimeric polypeptides oligomers, and linkers, include modified forms such as sequences having one or more amino acid substitutions, additions or deletions (i.e., subsequences), provided the modification does not destroy function. The term "modification" therefore denotes an alteration of the molecule that does not destroy an activity or function of the modified molecule. A modified multimerization polypeptide will therefore retain, for example, at least in part, the ability to form oligomers, even if the oligomers formed are less stable due to decreased affinity, e.g., form dimers instead of trimers, form trimers instead of tetramers, etc. A modified heterologous polypeptide will retain at least a part of one or more functions or activities associated with the polypeptide (e.g., protein binding activity, enzyme activity, ligand activity, nucleic acid binding activity, growth regulatory activity, cell differentiative activity, or chemotactic activity). For example, a

chimeric polypeptide that includes a modified antibody sequence, such as an antibody subsequence or antibody having one or more amino acid additions or insertions will retain, at least in part, antigen binding capability.

Modifications therefore include amino acid additions, insertions, deletions and substitutions, for example. An example of an addition is where one or more amino acids are added to the N- or C-terminal end multimerization polypeptide. An example of an insertion is where an amino acid is inserted into the sequence. An example of a deletion is where one or more amino acids are deleted from the N- or C-terminal end, or internally within the sequence.

Modifications may improve an activity or function of the modified molecule or provide a distinct functionality. For example, affinity of a multimerization polypeptide may be increased by addition of all or a portion of a heptad repeat sequence (e.g., a half turn). Addition of a heptad repeat sequence, or a portion of a heptad repeat sequence may also change the oligomer from forming dimer to trimer or tetramer formation.

Exemplary multimerization polypeptides are set forth, for example, in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163, each contain 4 and one-half heptad repeat sequences (see Table 3A). These and other heptad containing sequences may therefore be modified. For example, full length (7 amino acids) or half (3 to 4 amino acids of the motif) heptad repeat sequences may be added.

Alternatively, it may be desirable to shorten the multimerization polypeptide, in which case one or more amino acid residues may be deleted from a multimerization polypeptide without destroying multimerization function. Thus, multimerization polypeptides of the invention, including exemplary multimerization polypeptides set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; and SEQ ID NOs:154 to 163, may be modified to delete half (3 to 4 amino acids) or full (7 amino acids) length heptad repeat, for example.

Exemplary amino acid substitutions include conservative amino acid substitutions. The term "conservative substitution" means the replacement of one amino acid by a biologically or chemically similar residue. Biologically similar means that the substitution is compatible with biological activity, e.g., for a multimerization polypeptide, oligomer formation. Particular examples of conservative substitutions include the substitution of one hydrophobic residue, such as isoleucine, valine, leucine or methionine for another, or the substitution of one polar residue for another, such as the substitution of

arginine for lysine, glutamic for aspartic acids, or glutamine for asparagine, serine for threonine, and the like.

In one embodiment, a multimerization polypeptide has 1-3, 3-5 or 5-10 amino acid substitutions, provided that positions **a** (one) or **d** (four) of a coiled-coil heptad repeat sequence, (**a.b.c.d.e.f.g**), are either leucine, isoleucine or valine, and that the substituted polypeptide be capable of multimerization. In one aspect, where there are multiple heptad repeat sequences, positions **a** and **d** are predominantly either leucine, isoleucine or valine. In another aspect, a multimerization polypeptide has 1-3, 3-5 or 5-10 amino acid substitutions at positions **b**, **c**, **e**, **f** or **g**, e.g., to modulate the type of multimer that forms or to modulate the stability or tightness of the multimer formed. In yet another aspect, one or more of the amino acid substitutions are conservative amino acid substitutions. In still another aspect, the substitution is with a human amino acid.

Modifications also include derivatized sequences, for example, amino acids in which free amino groups form amine hydrochlorides, p-toluene sulfonyl groups, carbobenzoxy groups; the free carboxy groups from salts, methyl and ethyl esters; free hydroxyl groups that form O-acyl or O-alkyl derivatives, as well as naturally occurring amino acid derivatives, for example, 4-hydroxyproline, for proline, 5-hydroxylysine for lysine, homoserine for serine, ornithine for lysine, etc. Also included are modifications that confer covalent bonding, for example, a disulfide linkage between two cysteine residues thereby producing a cyclic polypeptide. Modifications can be produced using any of a variety of methods well known in the art (e.g., PCR based site-directed, deletion and insertion mutagenesis, chemical modification and mutagenesis, chemical cross-linking, etc.).

Modifications also include addition of functional entities such as tags (e.g., polyhistidine, T7, immunoglobulin, etc.), gold particles, covalently or non-covalently attached to the multimerization polypeptide, chimeric polypeptide or oligomers. Thus, the invention provides modified polypeptides having one or more activities (e.g., retain at least part of multimer activity, antigen binding activity, etc.) of unmodified polypeptide. Modifications include radioactive and non-radioactive detectable labels attached to or incorporated into the molecule.

The term "identical" or "identity" means that two or more referenced entities are the same. Thus, where two polypeptide sequences are identical, they have the same amino acid sequence. "Areas of identity" means that a portion of two or more referenced

entities are the same. Thus, where two polypeptide sequences are identical over one or more parts of their sequence, they share identity in these areas. The term "substantial identity" means that the identity is structurally or functionally significant. That is, the identity is such that the molecules are structurally identical or perform the same function (e.g., biological function) even though the molecules differ. Due to variation in the amount of sequence conservation between structurally and functionally related proteins, the amount of sequence identity for molecules having substantial identity will depend upon the type of region/domain and its function. For nucleic acid sequences, 50% sequence homology and above may constitute substantial identity. Substantial homology for proteins can be significantly less, for example, as little as 30% sequence identity, but typically is more, e.g., 50%, 60%, 75%, 85% or more.

The extent of identity between two sequences can be ascertained using various computer programs and mathematical algorithms known in the art. Such algorithms that calculate percent sequence identity (homology) generally account for sequence gaps and mismatches over the comparison region. For example, a BLAST (e.g., BLAST 2.0) search algorithm (see, e.g., Altschul *et al.*, *J. Mol. Biol.* 215:403 (1990), publicly available through NCBI at <http://www.ncbi.nlm.nih.gov>) has exemplary search parameters as follows: Mismatch -2; gap open 5; gap extension 2. For polypeptide sequence comparisons, a BLASTP algorithm is typically used in combination with a scoring matrix, such as PAM100, PAM 250, BLOSUM 62 and the like.

As used herein, the term "isolated," when used as a modifier of invention compositions (e.g., multimerization polypeptides, chimeras, linkers, antibodies, subsequences, modified forms, nucleic acids encoding same, cells, vectors, etc.), means that the compositions are made by the hand of man and are separated from their naturally occurring *in vivo* environment. Generally, compositions so separated are substantially free of one or more materials with which they normally associate with in nature, for example, one or more protein, nucleic acid, lipid, carbohydrate, cell membrane. An "isolated" polypeptide can also be "substantially pure" when free of most or all of the materials with which it normally is associated in nature. Thus, an isolated polypeptide that also is substantially pure does not include polypeptides or polynucleotides present among millions of other sequences, such as antibodies of an antibody library or nucleic acids in a genomic or cDNA library, for example. Purity can be at least about 60% or more by mass. The purity can also be about 70% or 80% or more, and can be greater, for

example, 90% or more. Purity can be determined by any appropriate method, including, for example, UV spectroscopy, mass spectroscopy, chromatography (*e.g.*, HPLC, gas phase), gel electrophoresis (*e.g.*, silver or coomassie staining) and sequence analysis (nucleic acid and peptide).

5 The invention also provides nucleic acids encoding invention polypeptides, including multimerization polypeptides, chimeras, linkers, subsequences, modified forms and multimers thereof. In various embodiments, a nucleic acid encodes a polypeptide set forth in SEQ ID NOs:1 to 7; SEQ ID NOs:9 to 37; or SEQ ID NOs:154 to 163. In additional embodiments, a nucleic acid encodes a chimeric polypeptide comprising a
10 sequence set forth in any of (SEQ ID NOs:1 to 36 and SEQ ID NOs:154 to 163), fused to a heterologous domain. In another embodiment, a nucleic acid sequence encodes (SEQ ID NO:43 (D30), SEQ ID NO:44 (D35), SEQ ID NO:45 (ED), SEQ ID NO:46 (EDC) or SEQ ID NO:47 (D63)).

As used herein, a "nucleic acid" refers to at least two or more ribo- or deoxy-
15 ribonucleic acid base pairs that are linked through a phosphodiester bond or equivalent. Nucleic acids include polynucleotides and polynucleosides. Nucleic acids include single, double or triple, circular or linear molecules. A nucleic acid molecule may belong exclusively or in a mixture to any group of nucleotide-containing molecules, as exemplified by, but not limited to, the following groups of nucleic acid molecules: RNA,
20 DNA, cDNA, genomic nucleic acids, non-genomic nucleic acids, naturally occurring and non naturally occurring nucleic acids and synthetic nucleic acids. This includes, by way of example, nucleic acids associated with any organelle, such as the mitochondria, ribosomal RNA, and nucleic acid molecules comprised chimerically of one or more components that are not naturally occurring along with naturally occurring components.

25 Additionally, a "nucleic acid molecule" may contain in part one or more non-nucleotide-based components as exemplified by, but not limited to, amino acids and sugars. Thus, by way of example, but not limitation, a ribozyme that is in part nucleotide-based and in part protein-based is considered a "nucleic acid molecule."

Nucleic acids can be of any length. Nucleic acid lengths typically range from
30 about 20 to 10 Kb, 10 to 5Kb, 1 to 5 Kb or less, 1000 to about 500 base pairs or less in length. Nucleic acids can also be shorter, for example, 100 to about 500 base pairs, or from about 12 to 25, 25 to 50, 50 to 100, 100 to 250, or about 250 to 500 base pairs in length.

As a result of the degeneracy of the genetic code, nucleic acids include sequences and subsequences degenerate with respect to nucleic acids that encode (SEQ ID NO:1 to 7, SEQ ID NOs:9 to 37, SEQ ID NOs:154 to 163, SEQ ID NO:43 (D30), SEQ ID NO:44 (D35), SEQ ID NO:45 (ED), SEQ ID NO:46 (EDC) or SEQ ID NO:47 (D63)). Nucleic acids also include sequences set forth in Tables 1, 2 and 4, sequences complementary thereto and subsequences thereof. Such nucleic acids are useful for hybridization to detect the presence or an amount of chimeric polypeptide in a sample (*in vitro*, cell, culture medium, tissue or organ, serum, in a subject, etc.).

Nucleic acids of the invention can be produced using various standard cloning and chemical synthesis techniques. Such techniques include, but are not limited to: 1) nucleic acid amplification, *e.g.*, polymerase chain reaction (PCR), with genomic DNA or cDNA targets, using primers (*e.g.*, a degenerate primer mixture) capable of annealing to antibody sequence; 2) chemical synthesis of nucleic acid sequences which can then be cloned into a plasmid, propagated amplified and purified and; 3) computer searches of databases for related sequences. Purity of nucleic acids can be determined through sequencing, gel electrophoresis and the like.

The invention further provides expression cassettes comprising a nucleic acid encoding a chimeric polypeptide operably linked to an expression control element. As used herein, the term "operably linked" refers to a physical or a functional relationship between the elements referred to that permit them to operate in their intended fashion. Thus, an expression control element "operably linked" to a nucleic acid means that the control element modulates transcription and as appropriate, translation of the transcript.

There need not be physical linkage to nucleic acid in order to control expression. Thus, physical linkage is not required for the elements to be operably linked. For example, a minimal element can be linked to a nucleic acid encoding a chimeric polypeptide. A second element that controls expression of an operably linked nucleic acid encoding a protein that functions "in trans" to bind to the minimal element can influence expression of the chimeric polypeptide. Because the second element regulates expression of chimeric polypeptide, the second element is operably linked to the nucleic acid encoding the chimeric polypeptide.

The term "expression control element" refers to nucleic acid that influences expression of an operably linked nucleic acid. Promoters and enhancers are particular non-limiting examples of expression control elements. A "promotor sequence" is a DNA

regulatory region capable of initiating transcription of a downstream (3' direction) coding sequence. The promoter sequence includes a minimum number of bases necessary to initiate transcription. Enhancers also regulate gene expression but can function a distance from the transcription start site of the gene to which it is operably linked. Enhancers also
5 function at either 5' or 3' ends of the gene, as well as within the gene (e.g., in introns or coding sequences).

An expression control element can confer expression in a manner that is "constitutive," such that transcription of the operably linked nucleic acid occurs without the presence of a signal or stimuli. Expression control elements can confer expression in
10 a manner that is "regulatable," that is, a signal or stimuli increases or decreases expression of the operably linked nucleic acid. A regulatable element that increases expression of the operably linked nucleic acid in response to a signal or stimuli is also referred to as an "inducible element." A regulatable element that decreases expression of the operably linked nucleic acid in response to a signal or stimuli is referred to as a
15 "repressible element" (*i.e.*, the signal decreases expression such that when the signal, is removed or absent, expression is increased).

Expression control elements include elements active in a particular tissue or cell type, referred to herein as a "tissue-specific expression control elements." Tissue-specific expression control elements are typically active in specific cell or tissue because
20 they are recognized by transcriptional activator proteins, or other regulators of transcription, that are unique to a specific cell or tissue type.

Expression control elements additionally include elements that confer expression at a particular stage of the cell cycle or differentiation. Accordingly, the invention further includes expression control elements that confer constitutive, regulatable, tissue-specific,
25 cell cycle specific, and differentiation stage specific expression.

Expression control elements include full-length nucleic acid sequences, such as native promoter and enhancer elements, as well as subsequences or nucleotide variants thereof (e.g., substituted/mutated or other forms that differ from native sequences) which retain all or part of full-length or non-variant control element function (confer regulation,
30 e.g., retain some amount of inducibility in response to a signal or stimuli).

For bacterial systems, constitutive promoters such as T7 and the like, as well as inducible promoters such as pL of bacteriophage λ , plac, ptrp, ptac (ptrp-lac hybrid promoter) may be used. In insect cell systems, constitutive or inducible promoters (*e.g.*,

- ecdysone) may be used. In yeast, constitutive or inducible promoters may be used (see, e.g., Ausubel *et al.*, In: Current Protocols in Molecular Biology, Vol. 2, Ch. 13, ed., Greene Publish. Assoc. & Wiley Interscience (1988); Grant *et al.*, In: Methods in Enzymology, 153, 516-544 (1987), eds. Wu & Grossman, 31987, Acad. Press, N.Y.;
- 5 Glover, DNA Cloning, Vol. II, Ch. 3, IRL Press, Wash., D.C. (1986); Bitter In: Methods in Enzymology, 152, 673-684 (1987), eds. Berger & Kimmel, Acad. Press, N.Y.; and, Strathern *et al.*, The Molecular Biology of the Yeast *Saccharomyces* Cold Spring Harbor Press, Vols. I and II (1982)). A constitutive yeast promoter such as ADH or LEU2 or an inducible promoter such as GAL may be used (R. Rothstein In: DNA Cloning. A
- 10 Practical Approach, Vol.11, Ch. 3, ed. D.M. Glover, IRL Press, Wash., D.C. (1986)).
- For mammalian cells, constitutive promoters of viral or other origins may be used. For example, SV40, or viral long terminal repeats (LTRs) and the like, or inducible promoters derived from the genome of mammalian cells (e.g., metallothionein IIA promoter; heat shock promoter, steroid/thyroid hormone/retinoic acid response elements)
- 15 or from mammalian viruses (e.g., the adenovirus late promoter; the inducible mouse mammary tumor virus LTR) can be used for expression.
- The invention also provides transformed cells and progeny thereof into which nucleic acids encoding invention polypeptides have been introduced by means of recombinant DNA techniques in vitro, *ex vivo* or *in vivo*. The transformed cells and
- 20 progeny thereof can be propagated and the introduced nucleic acid transcribed, or encoded protein expressed. It is understood that a progeny cell may not be identical to the parental cell, since there may be mutations that occur during replication.
- Transformed cells include but are not limited to prokaryotic and eukaryotic cells such as bacteria, fungi, plant, insect, and animal (e.g., mammalian, including human) cells. The
- 25 cells may be present in culture, in a cell, tissue or organ *ex vivo* or present in a subject.
- The term "transformed" means a genetic change in a cell following incorporation of nucleic acid (e.g., a transgene) exogenous to the cell. Thus, a "transformed cell" is a cell into which, or a progeny of which a nucleic acid molecule has been introduced by means of recombinant DNA techniques. Cell transformation may be carried out as
- 30 described herein or using techniques known in the art. Accordingly, methods of producing cells containing the nucleic acids and cells expressing the chimeric polypeptides of the invention are also provided.

Typically cell transformation employs a vector. The term "vector," refers to, *e.g.*, a plasmid, virus, such as a viral vector, or other vehicle known in the art that can be manipulated by insertion or incorporation of a nucleic acid, for genetic manipulation (*i.e.*, "cloning vectors"), or can be used to transcribe or translate the inserted nucleic acid (*i.e.*, "expression vectors"). Such vectors are useful for introducing nucleic acids, including a nucleic acid that encodes a chimeric polypeptide operably linked with an expression control element, and expressing the encoded protein *in vitro* (*e.g.*, in solution or in solid phase), in cells or *in vivo*.

A vector generally contains at least an origin of replication for propagation in a cell. Control elements, including expression control elements as set forth herein, present within a vector, are included to facilitate transcription and translation. The term "expression control element" is intended to include, at a minimum, one or more components whose presence can influence expression, and can include components other than or in addition to promoters or enhancers, for example, leader sequences and fusion partner sequences, internal ribosome binding sites (IRES) elements for the creation of multigene, or polycistronic, messages, splicing signal for introns, maintenance of the correct reading frame of the gene to permit in-frame translation of mRNA, polyadenylation signal to provide proper polyadenylation of the transcript of a gene of interest, stop codons, etc.

Vectors can include a selection marker. As is known in the art, "selection marker" means a gene that allows for the selection of cells containing the gene. "Positive selection" refers to a process whereby only cells that contain the selection marker will survive upon exposure to the positive selection. Drug resistance is one example of a positive selection marker; cells containing the marker will survive in culture medium containing the selection drug whereas cells which do not contain the marker will die. Such markers include drug resistance genes such as *neo*, which confers resistance to G418, *hygr*, which confers resistance to hygromycin, or *puro* which confers resistance to puromycin, among others. Other positive selection marker genes include genes that allow identification or screening of cells containing the marker. These genes include genes for fluorescent proteins (GFP), the lacZ gene, the alkaline phosphatase gene, and surface markers such as CD8, among others.

Vectors can contain negative selection markers. "Negative selection" refers to a process whereby cells containing a negative selection marker are killed upon exposure to

an appropriate negative selection agent. For example, cells which contain the herpes simplex virus-thymidine kinase (*HSV-tk*) gene (Wigler *et al.*, *Cell* 11:223 (1977)) are sensitive to the drug gancyclovir (GANC). Similarly, the *gpt* gene renders cells sensitive to 6-thioxanthine.

5 Additional selection systems may be used, including, but not limited to the hypoxanthine-guanine phosphoribosyltransferase gene (Szybalska *et al.*, *Proc. Natl. Acad. Sci. USA* 48:2026 (1962)), and the adenine phosphoribosyltransferase (Lowy *et al.*, *Cell* 22:817 (1980)) genes. Additional selectable genes have been described, namely *trpB*, which allows cells to utilize indole in place of tryptophan; *hisD*, which allows cells
10 to utilize histinol in place of histidine (Hartman *et al.*, *Proc. Natl. Acad. Sci. USA* 85:8047 (1988)); and ODC (ornithine decarboxylase), which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue (1987) In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory, ed.).

15 Vectors included are those based on viral vectors, such as retroviral, adeno-associated virus, adenovirus, reovirus, lentivirus, rotavirus genomes, simian virus 40 (SV40) or bovine papilloma virus, etc., modified for introducing and expressing a nucleic acid in a cell (Cone *et al.*, *Proc. Natl. Acad. Sci. USA* 81:6349 (1984); Eukaryotic Viral Vectors, Cold Spring Harbor Laboratory, Gluzman ed., 1982; Sarver *et al.*, *Mol. Cell. Biol.* 1:486 (1981)). Additional viral vectors useful for expression include parvovirus, rotavirus, Norwalk virus, coronaviruses, paramyxo and rhabdoviruses, togavirus (e.g., sindbis virus and semliki forest virus) and vesicular stomatitis virus.

Mammalian expression systems further include vectors specifically designed for *in vivo* and *ex vivo* expression. Such systems include adeno-associated virus (AAV)
25 vectors (U.S. Patent No. 5,604,090) which have been shown to provide expression of Factor IX in humans and in mice at levels sufficient for therapeutic benefit (Kay *et al.*, *Nat. Genet.* 24:257 (2000); Nakai *et al.*, *Blood* 91:4600 (1998)). Adenoviral vectors (U.S. Patent Nos. 5,700,470, 5,731,172 and 5,928,944), herpes simplex virus vectors (U.S. Patent No. 5,501,979) and retroviral (e.g., lentivirus vectors are useful for infecting
30 dividing as well as non-dividing cells and foamy viruses) vectors (U.S. Patent Nos. 5,624,820, 5,693,508, 5,665,577, 6,013,516 and 5,674,703 and WIPO publications WO92/05266 and WO92/14829) and papilloma virus vectors (e.g., human and bovine papilloma virus) have all been employed in gene therapy (U.S. Patent No. 5,719,054).

Vectors also include cytomegalovirus (CMV) based vectors (U.S. Patent No. 5,561,063). Vectors that efficiently deliver genes to cells of the intestinal tract have been developed and also may be used (see, e.g., U.S. Patent Nos. 5,821,235, 5,786,340 and 6,110,456).

In yeast, vectors that facilitate integration of foreign nucleic acid sequences into a
5 chromosome, via homologous recombination, for example, are known in the art and can be used. Yeast artificial chromosomes (YAC) are typically used when the inserted nucleic acids are too large for more conventional vectors (e.g., greater than about 12 kb).

Introduction of nucleic acid encoding humanized antibody and humanized
antibody into target cells can also be carried out by conventional methods known in the
10 art such as osmotic shock (e.g., calcium phosphate), electroporation, microinjection, cell fusion, etc. Introduction of nucleic acid and polypeptide *in vitro*, *ex vivo* and *in vivo* can also be accomplished using other techniques. For example, a polymeric substance, such as polyesters, polyamine acids, hydrogel, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, protamine sulfate, or lactide/glycolide
15 copolymers, polylactide/glycolide copolymers, or ethylenevinylacetate copolymers. A nucleic acid can be entrapped in microcapsules prepared by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethylcellulose or gelatin-microcapsules, or poly (methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system. Colloidal dispersion systems include macromolecule
20 complexes, nano-capsules, microspheres, beads, and lipid-based systems, including oil-in-water emulsions, micelles, mixed micelles, and liposomes.

The use of liposomes for introducing various compositions into cells, including nucleic acids, is known to those skilled in the art (see, e.g., U.S. Patent Nos. 4,844,904, 5,000,959, 4,863,740, and 4,975,282). A carrier comprising a natural polymer, or a
25 derivative or a hydrolysate of a natural polymer, described in WO 94/20078 and U.S. Patent No. 6,096,291, is suitable for mucosal delivery of molecules, such as polypeptides and polynucleotides. Piperazine based amphiphilic cationic lipids useful for gene therapy also are known (see, e.g., U.S. Patent No. 5,861,397). Cationic lipid systems also are known (see, e.g., U.S. Patent No. 5,459,127). Accordingly, viral and non-viral vector
30 means of delivery into cells or tissue, *in vitro*, *in vivo* and *ex vivo* are included.

The invention further provides kits comprising one or more compositions of the invention, including pharmaceutical formulations, packaged into suitable packaging material. In one embodiment, a kit includes a chimeric polypeptide or hetero- or homo-

oligomer thereof. In another embodiment, a kit includes a nucleic acid encoding a chimeric polypeptide. In additional embodiments, the nucleic acids further include an expression control element conferring expression in a cell; an expression vector; a viral expression vector; an adeno-associated virus expression vector; an adenoviral expression
5 vector; and a retroviral expression vector.

In additional embodiments, a kit includes a label or packaging insert including instructions for expressing a chimeric polypeptide (e.g., humanized antibody) or a nucleic acid encoding a chimeric polypeptide in cells *in vitro*, *in vivo*, or *ex vivo*. In yet
10 additional embodiments, a kit includes a label or packaging insert including instructions for treating a subject (e.g., a subject having or at risk of having asthma) with a chimeric polypeptide (e.g., humanized antibody) or a nucleic acid encoding a chimeric polypeptide *in vivo*, or *ex vivo*.

As used herein, the term "packaging material" refers to a physical structure housing the components of the kit. The packaging material can maintain the components
15 sterily, and can be made of material commonly used for such purposes (e.g., paper, corrugated fiber, glass, plastic, foil, ampules, etc.). The label or packaging insert can include appropriate written instructions, for example, practicing a method of the invention, e.g., treating HRV or RSV infection or the common cold. Kits of the invention therefore can additionally include instructions for using the kit components in a method
20 of the invention.

Instructions can include instructions for practicing any of the methods of the invention described herein. Thus, invention pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration to a subject. Instructions may additionally include indications of a satisfactory clinical
25 endpoint or any adverse symptoms that may occur, or additional information required by the Food and Drug Administration for use on a human subject.

The instructions may be on "printed matter," e.g., on paper or cardboard within the kit, on a label affixed to the kit or packaging material, or attached to a vial or tube containing a component of the kit. Instructions may comprise voice recording or video
30 tape and additionally be included on a computer readable medium, such as a disk (floppy diskette or hard disk), optical CD such as CD- or DVD-ROM/RAM, magnetic tape, electrical storage media such as RAM and ROM and hybrids of these such as magnetic/optical storage media.

Invention kits can additionally include a buffering agent, a preservative, or a protein/nucleic acid stabilizing agent. The kit can also include control components for assaying for activity, e.g., a control sample or a standard. Each component of the kit can be enclosed within an individual container or in a mixture and all of the various
5 containers can be within single or multiple packages. For example, an invention composition can be packaged into a hand pump container or pressurized (e.g., aerosol) container for spraying the composition into the throat or nasal or sinus passages of a subject.

The molecules of the invention, including multimerization polypeptides, chimeras, multimeric forms, modified forms, and nucleic acids encoding the
10 polypeptides, can be incorporated into pharmaceutical compositions. Such pharmaceutical compositions are useful for administration to a subject *in vivo* or *ex vivo*, and for providing therapy for a physiological disorder or condition treatable with a polypeptide of the invention.

Pharmaceutical compositions include "pharmaceutically acceptable" and "physiologically acceptable" carriers, diluents or excipients. As used herein the terms "pharmaceutically acceptable" and "physiologically acceptable" include solvents (aqueous or non-aqueous), solutions, emulsions, dispersion media, coatings, isotonic and absorption promoting or delaying agents, compatible with pharmaceutical administration.
15 Such formulations can be contained in a liquid; emulsion, suspension, syrup or elixir, or solid form; tablet (coated or uncoated), capsule (hard or soft), powder, granule, crystal, or microbead. Supplementary active compounds (e.g., preservatives, antibacterial, antiviral and antifungal agents) can also be incorporated into the compositions.

Pharmaceutical compositions can be formulated to be compatible with a particular
25 local or systemic route of administration. Thus, pharmaceutical compositions include carriers, diluents, or excipients suitable for administration by particular routes.

Specific non-limiting examples of routes of administration for compositions of the invention are inhalation or intranasal delivery. Additional routes include parenteral, e.g., intravenous, intradermal, subcutaneous, oral, transdermal (topical), transmucosal, and
30 rectal administration.

Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents;

antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide.

Pharmaceutical compositions for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. Fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Antibacterial and antifungal agents include, for example, parabens, chlorobutanol, phenol, ascorbic acid and thimerosal. Isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride can be included in the composition. Including an agent which delays absorption, for example, aluminum monostearate and gelatin can prolong absorption of injectable compositions.

Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of above ingredients followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle containing a basic dispersion medium and other ingredients as above. In the case of sterile powders for the preparation of sterile injectable solutions, methods of preparation include, for example, vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays, inhalation devices (e.g., aspirators) or suppositories. For

transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

Invention polypeptides and nucleic acids encoding them can be prepared with carriers that protect against rapid elimination from the body, such as a controlled release formulation or a time delay material such as glyceryl monostearate or glyceryl stearate. The compositions can also be locally or systemically delivered using implants and microencapsulated delivery systems to achieve sustained delivery or for controlled release.

Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to cells or tissues using antibodies or viral coat proteins) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

Additional pharmaceutical formulations appropriate for the compositions for administration in the methods of the invention are known in the art (see, e.g., Remington's Pharmaceutical Sciences (1990) 18th ed., Mack Publishing Co., Easton, PA; The Merck Index (1996) 12th ed., Merck Publishing Group, Whitehouse, NJ; and Pharmaceutical Principles of Solid Dosage Forms, Technomic Publishing Co., Inc., Lancaster, Pa., (1993)).

The pharmaceutical formulations can be packaged in dosage unit form for ease of administration and uniformity of dosage. "Dosage unit form" as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the pharmaceutical carrier or excipient.

Multimeric antibodies of the invention include antibodies that protect against virus infection of cells. For example, antibodies that bind ICAM-1 that have been fused with a multimerization polypeptide form multimers that can protect cells from HRV infection (Figures 6 and 7). In particular, CFY196 (Fab-ATF α (1)LH+S), CFY197 (Fab-ATF α (3)IL) and CFY193B (Fab-ATF α (2)LL) demonstrated the greatest protective efficacy against HRV infection.

As respiratory syncytial virus (RSV) also utilizes ICAM-1 as a co-receptor for cell infection, antibody or ligand that binds ICAM-1 and that have been fused with a multimerization polypeptide to form multimers can also protect cells from RSV infection. Thus, in another embodiment, the invention provides antibody and ligand multimers that protect against RSV infection of cells.

As used herein, the term "protective efficacy" is the amount of an antibody which can protect 50% of susceptible cells from infection (i.e. EC_{50}) under experimental conditions (see, e.g., Example 6). For example, for RSV, protective efficacy in EC_{50} is the amount of antibody that protects 50% of cells from RSV infection. Thus, an antibody having a protective efficacy 5 times greater than another antibody (e.g., non-humanized) can be used in an amount 5 fold less than the other antibody while still providing the same degree of protection from infection.

Multimeric antibodies typically exhibit greater protective efficacy than a monomeric counterpart. In one embodiment, an antibody multimer has a protective efficacy at least 2 to 5 times greater than the antibody monomer. In another embodiment, an antibody multimer has a protective efficacy at least 5 to 10 times greater than antibody monomer. In yet another embodiment, an antibody multimer has a protective efficacy at least 10 to 20 times greater than the antibody monomer. In still another embodiment, an antibody has a protective efficacy at least 20 to 30 times greater or more than the antibody monomer, for example, 30 to 50 times, 50 to 100 times, or 100 to 1000 times or more.

Chimeric polypeptides of the invention include multimerization polypeptide fused to antibody that binds to ICAM-1. Although not wishing to be bound by theory, it is believed that antibody binding to ICAM-1 inhibits viral binding or the ability to infect or penetrate the cell thereby inhibiting viral infection or proliferation. Such antibodies are therefore useful for inhibiting pathogens such as respiratory syncytial and other viruses (e.g., HRV and coxsackie A virus), bacteria, fungi and protozoa (e.g., malaria) that bind to ICAM-1. Thus, the multimerized antibodies are useful for inhibiting HRV and RSV infection as well as for inhibiting any microorganism or other pathogen in which ICAM-1 receptor participates. Accordingly, the invention provides multimerized antibodies (including fully or partially humanized forms) that inhibit pathogen infection of cells where infection is mediated, at least in part, by binding to ICAM-1, and methods for

inhibiting pathogen infection of cells where infection is mediated, at least in part, by binding to ICAM-1.

In one embodiment, a method includes contacting a virus or cell with an amount of multimerized antibody that binds to ICAM-1 sufficient to inhibit viral infection of the cell. In one aspect, the multimerized antibody is humanized. In another embodiment, a method includes administering to a subject an amount of multimerized antibody that binds to ICAM-1 sufficient to inhibit viral infection of the subject. In various aspects, the virus is RSV, coxsackie A virus and HRV. In yet another embodiment, a method includes administering to a subject an amount of multimerized antibody that binds to ICAM-1 sufficient to inhibit infection of the subject by a pathogen. In still another embodiment, a method includes administering to a subject an amount of multimerized antibody that binds to ICAM-1 sufficient to ameliorate a symptom of the infection, *e.g.*, a symptom of the common cold.

As used herein, the term "ameliorate," when used in reference to a condition such as a symptom of a disease means to reduce one or more symptoms of the condition. For example, symptoms associated with the common cold include fever, headache, chills, sneezing, coughing, congestion, sore throat, runny nose, sore muscles, general malaise, etc. Thus, to ameliorate the common cold or a pathogen associated with the common cold (*e.g.*, HRV) means to reduce one or more of fever, headache, chills, sneezing, coughing, congestion, sore throat, runny nose, sore muscles, general malaise, etc.

In addition to inhibiting a symptom, replication or progression of pathogens that function directly or indirectly through ICAM-1, invention multimerized antibodies can be used to treat undesirable conditions, such as diseases or disorders in which ICAM-1 plays a role. For example, LFA-1 interaction with ICAM-1 participates in inflammation. An invention antibody may be used to inhibit this interaction thereby modulating (*e.g.*, decrease) local or systemic inflammation. Thus, in another embodiment, a method includes administering to a subject enough multimerized antibody to reduce or prevent inflammation.

Furthermore, ICAM-1 plays a role in other immune response pathways, cancer and metastasis. Thus, an invention antibody may be used to modulate immune response in order to reduce or prevent organ transplant rejection or autoimmune diseases or cancer or metastasis. Accordingly, the invention provides multimerized antibodies that modulate immune responsiveness (*e.g.*, inflammation) and other cellular processes in

which ICAM-1 participates, as well as methods for modulating immune response pathways and other cellular processes in which ICAM-1 participates.

The invention also provides methods for inhibiting infection (e.g., prophylaxis), inhibiting progression or treating a pathogenic infection of a subject. For example,
 5 blocking RSV from the upper respiratory tract will prevent the entry of RSV, and therefore prevent RSV from invading the lower respiratory tract. ICAM-1 binding molecules may be delivered intra-nasally or intra-orally as sprays or drops to a subject.

Thus, in one embodiment, a method includes administering to a subject having or at risk of having an RSV infection an amount of multimerized antibody sufficient to
 10 inhibit infection, inhibit progression or to treat RSV infection of the subject. In another embodiment, a method includes administering to a subject having or at risk of having an coxackie A virus or HRV infection an amount of multimerized antibody sufficient to inhibit infection, inhibit progression or to treat coxackie A virus or HRV infection of the subject. In still another embodiment, a method includes administering to a subject having
 15 or at risk of having malaria an amount of multimerized antibody sufficient to inhibit infection, inhibit progression or to treat malaria of the subject.

The invention further provides methods of decreasing or inhibiting (i.e., ameliorating) one or more symptoms of a pathogen infection (e.g., caused by RSV, coxackie A virus, HRV or malaria). In one embodiment, a method includes
 20 administering to a subject having one or more symptoms associated with RSV, coxackie A virus, HRV or malaria an amount of a multimerized antibody sufficient to decrease or inhibit one or more symptoms associated with, RSV, coxackie A virus, HRV or malaria in the subject. Symptoms decreased or inhibited include, for example, for RSV, one or more of pneumonia, fever, bronchitis, and upper respiratory tract infection; for coxackie
 25 A virus, one or more of fever, headache, chills, sneezing, coughing, congestion, sore throat, etc; for malaria, one or more of fever, chill, enlarged liver, anemia.

In another embodiment, a method includes administering to a subject having pneumonia, fever, bronchitis, or upper respiratory tract infection an amount of a multimerized antibody sufficient to decrease or inhibit one or more symptoms of
 30 pneumonia, fever, bronchitis, or upper respiratory tract infection in the subject. In one aspect, the humanized antibody is administered locally. In another aspect, the multimerized antibody is administered via inhalation or intranasally. In yet another aspect, the subject has or is at risk of having asthma.

The methods of the invention may be practiced prior to infection (i.e. prophylaxis) or after infection, before or after acute or chronic symptoms of the infection or physiological condition or disorder develops (e.g., before organ transplantation). Administering a composition prior to or immediately following development of symptoms may lessen the severity of the symptoms in the subject. Administering a composition prior to development of symptoms in the subject may decrease contagiousness of the subject thereby decreasing the likelihood of other subjects becoming infected from the infected subject.

The term "subject" refers to animals, typically mammalian animals, such as a non-human primate (apes, gibbons, chimpanzees, orangutans, macaques), a domestic animal (dogs and cats), a farm animal (horses, cows, goats, sheep, pigs), experimental animal (mouse, rat, rabbit, guinea pig) and humans. Human subjects include adults, and children, for example, newborns and older children, for example, between the ages of 1 and 5, 5 and 10 and 10 and 18. Human subjects may include those having or at risk of having a viral infection, such as HRV or RSV, and which develops one or more symptoms of the infection. Human subjects include those having or at risk of having asthma, including asthmatics suffering from chronic asthma prior to or following suffering an acute asthma attack. Subjects include disease model animals (e.g., such as mice and non-human primates) for testing *in vivo* efficacy of humanized antibodies of the invention (e.g., an HRV or RSV animal model, an asthma animal model, an organ transplant model, an autoimmune disorder model, cancer model, etc.).

Unless otherwise defined, 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 methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described herein.

All publications, patents and other references cited herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control.

As used herein, the singular forms "a", "and," and "the" include plural referents unless the context clearly indicates otherwise. Thus, for example, reference to "a polypeptide" includes a plurality of polypeptides (e.g., multimerization, chimeric,

heterologous polypeptides) and reference to "a cell" can include reference to one or more such cells, and so forth.

A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, the following examples are intended to
 5 illustrate but not limit the scope of invention described in the claims.

Examples

Example 1

10 This example describes the design of multimerization domains, linker sequences and chimeric polypeptides.

ATF α amino acids 181-211 constitute four and one-half heptad-repeats (31 amino acids). Since all the residues at the **d** position (position four) of each repeat are leucines, this heptad-repeat sequence is also referred to as a leucine zipper domain. ATF α amino
 15 acids 181-211 has 45% identity to the GCN4 leucine zipper domain.

Variants of ATF α amino acids 181-211 were produced by replacing position **a** and **d** (positions one and four in each of the heptad repeats) with each of the three hydrophobic, apolar residues: leucine, isoleucine and valine. (Table 3A). ATF α variants that have leucine and isoleucine at either **a** or **d** position form trimers or tetramers;
 20 tetramer forming sequences include ATF α -LI and ATF α -IL, trimer forming sequences include ATF α -LL and ATF α -II. ATF α -VL forms a mixture of dimer and tetramer; all variants with valine at **a** or **d** gave a high proportion of dimer.

The ATF α -LI domain forms tetramer when fused with either an antibody Fab fragment or with a reporter protein, thioredoxin.

25 Exemplary linker sequences were based on the human immunoglobulin D hinge sequence, which is the longest known flexible hinge among human immunoglobulins. The human IgD hinge has a total of 63 amino acid residues, and the 57th residue is a cysteine (Padlan, *Mol. Immunol.* 31:169 (1994)).

Five versions of the IgD hinge are listed in Table 5: D30, D35, ED, EDC and
 30 D63. D30 contains the first 30 residues of the IgD hinge, D35 contains the first 35 residues, ED contains all of the 56 amino acid residues before the cysteine, EDC contains all of the 56 amino acid residues in ED plus the cysteine, and D63 contains the complete hinge of 63 amino acids.

When purified tetramers prepared using ATF α -LI were compared, proteins with D35 were less stable than tetramers formed by D30 and ED, which remained in the tetrameric state over time. The C terminus of D35 ends in two glycines (GRGG). When it is fused with the N terminus of the multimerization domain, the two glycines may
 5 destabilize the α helices of the multimerization domain explaining why D35 tetramers were less stable.

Example 2

This example describes the cloning of an anti-ICAM humanized Fab fragment with antigen binding sites derived from Mab 1A6.

10 An expression vector was assembled to produce Fab 19 and multimeric Fab 19 from a dicistronic operon (Figure 1). Dicistron operons are described in Carter *et al.*, *Biotechnology* 10:163 (1992).

In brief, the V_H and V_L domains are precisely fused on their 5' ends to a gene segment encoding the enterotoxin II (stII) signal sequence. The intervening sequence
 15 (IVS) in the dicistronic gene contains a ribosome entry site, while the 3' end of the gene contains the bacteriophage λ t₀ transcriptional terminator (TER). A unique SacI site was inserted just before the stop codon in the C_H1 domain and a unique EcoRI site just after the stop codon to facilitate the addition of sequences encoding hinge and polymerization domains. The isopropyl-1-thio β -D-galactopyranoside (IPTG)-inducible ptac promoter
 20 was used to drive expression of this dicistronic message.

The gene segment for the light chain fragment (from SpeI to XbaI) was synthesized using PCR. The PCR product was a fusion of two templates, the V_L fragment encoding the variable domain of an humanized anti-ICAM antibody, and the C_L template, whose sequence was derived from human κ 1 light chain constant region (Palm
 25 and Hilschmann, *Z. Physiol. Chem.* 356:167 (1975)).

The V_L template was synthesized using a series of overlapping oligonucleotides (Table 1). Oligonucleotides were first annealed in two groups consisting of: oligonucleotides 1, 2 and 3; and oligonucleotides 4, 5 and 6. Each annealed group was extended with Klenow fragment of DNA polymerase. Annealed and extended products
 30 were pooled as overlapping templates that were fused via PCR with P1 and P2 oligonucleotides (Table 2). The PCR product was directly cloned into the pCR2.1 vector (Invitrogen) and sequenced. The C_L template was derived from oligonucleotides that had

been annealed in four groups (Table 1: Fab 4,5 and 6; Fab 7 and 8; Fab 9 and 10; Fab 11 and 28) and extended with the Klenow fragment of DNA polymerase (Stratagene, Lot# 0800176). The pool of DNA was then amplified using a high-fidelity polymerase mixture (Roche Expand High Fidelity, Lot #85610228), and the flanking DNA primers, Fab 29 and Fab 3S. The PCR product was cloned into the PCR 2.1 TOPO cloning vector. The V_L and C_L domains were fused using oligonucleotides Fab1 and Fab 11 and a 5' SpeI site was added with oligonucleotides Fab26 and P3. The final light chain clones were sequenced in their entirety.

A similar approach was used to clone the gene segment containing the heavy chain and the terminator as an Xba I/Hind III fragment. A C_H template based on the sequence of the C_{H1} domain of human IgG1 (Ellison, *et al. Nucl. Acids Res.* 10:4071 (1982)) was made by annealing and extending four groups of oligonucleotides (Table 1, Fab 16 and 17, Fab 18 and 19, Fab 25 and 21 and Fab 20, 22 and 23) with the C_L. The V_H domain encoding the heavy chain variable region of a humanized anti ICAM antibody, was made by first annealing oligonucleotide 7, 8 and 9; oligonucleotide 10, 11 and 12; and oligonucleotide 13 and p4. Each annealed group was extended with the Klenow fragment of DNA polymerase. The annealed and extended products were pooled as overlapping templates that were fused N-terminally to the Fab 1 oligonucleotide with oligonucleotides Fab13, 12A and 12S, and C terminally to the C_{H1} domain using PCR with oligonucleotides Fab 12s and Fab 24 as primers. This fragment was also completely sequenced. The expression plasmid for the humanized, anti ICAM Fab protein, termed Fab 19, was made by ligating the SpeI/ Xba light chain fragment and the XbaI/Hind III heavy chain fragment into SpeI/ HindIII-digested ptac/Tet to generate pFab19/Tet (Figure 1).

Table 1: Oligonucleotide Sequences

Oligo	Sequence
Oligo 1	ACAAACGCGTACGCTGATATCCAGATGACCCAATCTCCGTC AGCCTGAGCGCCAGTGTGGTGATCGAGTTACCATTA (SEQ ID NO:83)
Oligo 2	GGTTTGTGTTGATACCAAGTGAAGATTACTGATAGATTGGCTGGCG CGGCAAGTAATGGTAACGATCACCAACACTGGCGC (SEQ ID NO:84)
Oligo 3	CTTCACTGGTATCAACAAAAACCGGTAAGCTCCGAACTTCTTAT CTATCAGCCTCTCAGAGCATTAGCGGCGTTCCG (SEQ ID NO:85)
Oligo 4	GAGAGCTGATGGTAAGGGTAAAGTCCGTGCCCGAGCCAGAGCCAGA GAAGCGGCTCGGAACGCCGCTAATGCTCTGAGAG (SEQ ID NO:86)

- Oligo 5** CTTTACCCCTTACCATCAGCTCTCTTCAGCCGGAAGACTTTGCCACCTATT
ATTGTCAGCAGTCTA (SEQ ID NO:87)
- Oligo 6** GGTGCAGCCACAGTGGCTTAATCTCGACCTTGGTACCTTGACCGA
AGGTATACGGCCAGCTATTAGACTGCTGACAATAATAGG (SEQ ID NO:88)
- 5 **Oligo 7** ACAAACGCGTACGCTGAAGTTCAACTTGTGACTCTGGTGGCGGTCTGG
TTCAGCCCGGGGGCTCTCTGCGCCTGTCTTGCAGCAAG (SEQ ID NO:89)
- Oligo 8** CTTACCCGGAGCTTGCCTCACCCAATGGATGTAGGTGTCCTTAATGTTG
AAACCGCTTGCTGCGCAAGACAGGCG (SEQ ID NO:90)
- 10 **Oligo 9** GGCAAGCTCCGGGTAAGGGTCTGGAGTGGGTGGCAGTATCGACCCGGCA
AACGACAACACCATTTACGCTGACAGCGTGAAGGGCCG (SEQ ID NO:91)
- Oligo 10** GGTACGGGTGTTCTTAGAGTCGTCGCTAGAAATAGTAAACGGCCCTCA
CGCTGTCAGCGTAAATGGTGTTCGTTTGCCGGGT (SEQ ID NO:92)
- Oligo 11** GACTCTAAGAACACCGCGTACCTTCAGATGAACTCTCTGCGTGGCAGGACA
CCGCCGTCTACTACTGCACGGCTCTGGCTACTGGTTGCTACTGGGGC (SEQ ID NO:93)
- 15 **Oligo 12** GGTGGAGGCGCTCGAGACGGTGACAAGCGTGCCCTGGCCCCA
GTAGGCAAAACCAAGTAGCCAGAGG (SEQ ID NO:94)
- Oligo 13** CCCGGCAAACGACAACACCATTTACGCTG (SEQ ID NO:95)
- P1** ACAAACGCGTACGCTGATATCC (SEQ ID NO:96)
- P2** GATGGTGCAGCCACAGTGGC (SEQ ID NO:97)
- 20 **P3** GCGATATTCTTTTCATATACTGTTTCCTG (SEQ ID NO:98)
- P4** GGTGGAGGCGCTCGAGACGG (SEQ ID NO:99)
- Fab 1** ATG AAA AAG AAT ATC GCA TTT CTT CTT GCA TCT ATG TTC GTT TTT TCT ATT GCT
ACA AAC GCG TAC GCT (SEQ ID NO:100)
- 25 **Fab 4** ACT GTG GCT GCA CCA TCT GTC TTC ATC TTC CCG CCA TCT GAT GAG CAG TTG
AAA TCT GGA ACT GCC (SEQ ID NO:101)
- Fab 5** GGC CTC TCT GGG ATA GAA GTT ATT CAG CAG GCA CAC AAC AGA GGC AGT TCC
AGA TTT CAA (SEQ ID NO:102)
- 30 **Fab 6** CTT CTA TCC CAG AGA GGC CAA AGT ACA GTG GAA GGT GGA TAA CGC
CCT CCA ATC GGG (SEQ ID NO:103)
- Fab 7** GGC TGT AGG TGC TGT CCT TGC TGT CCT GCT CTG TGA CAC TCT CCT
GAG AGT TAC CCG ATT GGA GGG CG (SEQ ID NO:104)
- 35 **Fab 8** GCA AGG ACA GCA CCT ACA GCC TCA GCA GCA CCC TGA CGC TGA CGC
TGA GCA AGG CAG ACT ACG AGA AAC ACA AAG TCT ACG CCT (SEQ ID NO:105)
- 40 **Fab 9** AAG CTC TTT GTG ACG GGG CTC GAC AGG CCC TGA TGG GTG ACT TCG
CAG GCG TAG ACT TTG TGT TTC (SEQ ID NO:106)
- Fab 10** AGC CCC GTC ACA AAG AGC TTC AAC AGG GGA GAG TGT TAA GCT GAT
CCT CTA CGC CGG ACG CAT CGT (SEQ ID NO:107)
- 45 **Fab 11** CTC TAG ATA CCC TTT TTA CGT GAA CTT GCG TAC TAG GGC CAC GAT
GCG TCC GGC GTA GAG G (SEQ ID NO:108)
- 50 **Fab 12a** GAA ATG CGA TAT TCT TTT TCA TAA AAT CAC CTC AAC CTC TAG ATA CCC (SEQ
ID NO:109)

- Fab 12s** AAA AAG GGT ATC TAG AGG TTG AGG (SEQ ID NO:110)
- Fab 13** GAC TCA ACA AGT TGA ACT TCA GCG TAC GCG TTT GTA GCA A (SEQ ID NO:111)
- 5 **Fab 14** GAA GTT CAA CTT GTT GAG TCT (SEQ ID NO:112)
- Fab 16** CAC GCT TGT CAC CGT CTC GAG CGC CTC CAC CAA GGG CCC AT (SEQ ID NO:113)
- 10 **Fab 17** GTG CCC CCA GAG GTG CTC TTG GAT GAG GGT GCC AGG GGG AAG ACC
GAT GGG CCC TTG GTG GAG CG (SEQ ID NO:114)
- Fab 18** CAC CTC TGG GGG CAC AGC GGC CCT GGG CTG CCT GGT CAA GGA CTA
CTT CCC CGA ACC GGT GAC G (SEQ ID NO:115)
- 15 **Fab 19** GGA CAG CCG GGA AGG TGT GCA CGC CGC TGG TCA GGG CGC CTG AGT
TCC ACG ACA CCG TCA CCG GTT CGG GG (SEQ ID NO:116)
- Fab 20** GAC CTA CAT CTG CAA CGT GAA TCA CAA GCC CAG CAA CAC CAA GGT GGA CAA
GAA AGT TGA GCC CAA ATC TTG TGACAA AAC TCA CAC AGA GCT CTG AGA ATT
20 CCG CGG CAT CGC (SEQ ID NO:117)
- Fab 21** GAT TCA CGT TGC AGA TGT AGG TCT GGG TGC CCA AGC TGC TGG AGG
GCA CGG TCA CCA CGC TGC (SEQ ID NO:118)
- 25 **Fab 22** GGC CCT AGA GTC CCT AAC GCT CGG TTG CCG CCG GGC GTT TTT TAT
TGT TAA CTC ATG TTT GAC AGC TTA TCA TCG ATA AGC TT (SEQ ID NO:119)
- Fab 23** GCG TTA GGG ACT CTA GGG CCG TCG CAT GCC GCG GAA TTC TCA GAG
CTC TGT GTG AGT TTT GTC ACA AGA TTT GGG CTC AAC TTT CTT GTC AC (SEQ ID
30 NO:120)
- Fab 24** AAG CTT ATC GAT GAT AAG C (SEQ ID NO:121)
- Fab 25** GCG GCG TGC ACA CCT TCC CGG CTG TCC TAC AGT CCT CAG GAC TCT
35 ACT CCC TCA GCA GCG TGG TGA CCG TG (SEQ ID NO:122)
- Fab 26** AAC AAT ACT AGT CAG GAA ACA GTA TA (SEQ ID NO:123)
- 40 **Fab 27** ACT AGT ATT GTT ATC CGC TC (SEQ ID NO:124)
- Fab 29** CAT AGA TGC AAG AAG AAA TGC (SEQ ID NO:125)

Example 3

- 45 This example describes cloning of human ATF α leucine zipper domain variants.
This example also describes cloning of the human ATF α leucine zipper domain variants
with attached linker (hinge) sequences.

- The ATF α leucine zipper domain variants (Table 3A) were made by PCR
amplification using two DNA oligonucleotides "A" and "B" that are complementary at
50 the 3' ends as the template (Table 2). The oligonucleotide pairs were annealed, extended
with the Klenow fragment of DNA polymerase, and amplified by PCR using

oligonucleotides P1 and P2 as primers. The resulting PCR products were purified and cloned into the PCR cloning vector, TOPO pCR2.1 (Invitrogen). Plasmid DNA with the cloned insert was isolated and sequenced.

To fuse each cloned ATF α domain variant to a particular hinge sequence, a
 5 junctional oligonucleotide, whose 5' end is complementary to the 3' end of the
 appropriate hinge, was used in a PCR reaction against the ATF α domain variant
 template. This PCR product was then used in combination with the hinge template in
 another PCR reaction to generate the hinge-ATF α domain fragment. By design, the
 hinge-ATF α domain fragment has a SacI site on the N terminus and a EcoRI site on the
 10 C terminus. After sequencing, the hinge-ATF α domain fragment was cloned into the
 expression vector that carries the Fab fragment and was pre-digested with SacI and
 EcoRI, to be fused in-frame with the C terminus of the C₁₁1 domain of Fab (Figure 1).

Table 2: Oligonucleotides for ATF Leucine Zipper Variants

ATF domain	oligonucleotides
ATFa(1)-LI (SEQ ID NOs:126-129):	
A:	CTTAGCTCTATTGAGAAGAACTTGAGGAGATTAC
B:	CTCTCAACTGATTGAGATC
P1:	TTGGGCCAGCTCATTGCGGATAAGGGTCAGTTCATTGC
P2:	TGATCTGAATCAGTTGAG
ATFa(2)-LI (SEQ ID NOs:130-133):	
A:	CTTAGCTCTATTGAGAAGAACTTGAGGAGATTAC
B:	CTCTCAACTGATTGAGATC
P1:	TTGGGCCAGCTCATTGCGGATAAGGGTCAGTTCATTGC
P2:	GGATCTGAATCAGTTGAG
ATFa(3)-LI (SEQ ID NOs:134-137):	
A:	CTTAGCTCTATTGAGAAGAACTTGAGGAGATTAC
B:	CTCTCAACTGCAACAGATC
P1:	TTGGGCCAGCTCATTGCGGATAAGGGTCAGTTCATTGC
P2:	TGATCTGTTGCAGTTGAG
ATFa-LL (SEQ ID NOs:138-141):	
A:	CTTAGCTCTCTTGAGAAGAACTTGAGGAGCTTAC
B:	CTCTCAACTGATTGAGCTTCGG
P1:	ATTGGGCCAGCTCATTACGCAGAAGGGTCAG
P2:	TTCATTCCGAAGCTGAATCAGTTG
P1:	CTTAGCTCTCTTGAGAAGAAAC

P2: CGAATTCATTGGGCCAGCTCATTA

ATFα-IL (SEQ ID NOs:142-145):

5 A: ATTAGCTCTTTAGAGAAGAAAATTGAGGAGCTG
ACCTCTCAAATCCAGCAGCTGCGT
B: TTGGGCAATCTCATTACGAAGAAGGGTGATTTCATT
ACGCAGCTGCTGGATTGAGA
P1: ATTAGCTCTTTAGAGAAGAAA
P2: CGAATTCATTGGGCAATCTCATTACG

10 ATFα-II (SEQ ID NOs:146-149):

A: ATCAGCTCTATTGAGAAGAAAATCGAGGAGATTACCTC
TCAAATCATTC
B: CATTGGGCAATCTCATTACGGATAAGCGTGATTTCATT
CCGAATCTGAATGATTGAGAGGTAATC
P1: ATCAGCTCTATTGAGAAG
P2: CGAATTCATTGGGCAATCTCATTACG

15 ATFα-VL (SEQ ID NOs:150-153):

20 A: GTTAGCTCTCTTGAGAAGAAAGTTGAGGAGCTT
ACCTCTCAAGTGATTACGCTTCGG
B: TTGGGCAACCTCATTACGCAGAAGGGTCACITTCATT
CCGAAGCTGAATCATTGAGAG
P1: GTTAGCTCTCTTGAGAAGAAAG
P2: CGAATTCATTGGGCAACCTCATTACG

25

Table 3A: ATFα Leucine Zipper Domain Variants

	1	2	3	4	5
30 Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c
ATFα(1)-L1:	LSSIEKK	LEEITSQ	LIQISNE	LTIRNE	LAQ (SEQ ID NO:1)
ATFα(2)-L1:	LSSIEKK	LEEITSQ	LIQIRNE	LTIRNE	LAQ (SEQ ID NO:2)
ATFα(3)-L1:	LSSIEKK	LEEITSQ	LQQIRNE	LTIRNE	LAQ (SEQ ID NO:3)
35 ATFα-L1:	LSSLEKK	LEELTSQ	LIQLRNE	LTLLRNE	LAQ (SEQ ID NO:4)
ATFα-IL:	ISSLEKK	IEELTSQ	IQQLRNE	ITLLRNE	IAQ (SEQ ID NO:5)
ATFα-II:	ISSIEKK	IEEITSQ	IIQIRNE	ITLIRNE	IAQ (SEQ ID NO:6)
ATFα-VL:	VSSLEKK	VEELTSQ	VIQLRNE	VTLLRNE	VAQ (SEQ ID NO:7)
40 ATFα-WT:	VSSLEKK	AEELTSQ	NIQLRNE	VTLLSNE	VAQ (SEQ ID NO:8)

Table 3B: Leucine Zipper Domains Homologous to ATFα

	1	2	3	4	5
45 Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c
ATFα-WT:	VSSLEKK	AEELTSQ	NIQLSNE	VTLLRNE	VAQ (SEQ ID NO:8)
CREB-Pa:	VMSLEKK	AEE LTQT	NMQLQNE	VS MLKNE	VAQ (SEQ ID NO:38)
50 JUN-D:	ISRL EKK	VKTLK SQ	NTE LA ST	AS LL R EQ	VAQ (SEQ ID NO:39)

C-JUN: I A R L E E K V K T L K A Q N S E L A S T A N M L R E Q V A Q (SEQ ID NO:40)
 ATF-1: V K C L E N R V A V L E N Q N K T L I E E L K T L K D L Y S N (SEQ ID NO:41)
 5 ATF-2: V Q S L E K K A E D L S S L N G Q L Q S E V T L L R N E V A Q (SEQ ID NO:42)

Table 3C: Sequence Comparison of Wild Type Leucine Zipper Domains (in % identity)

	GCN4	ATF α
10	GCN4	100%
		45%
	ATF α	45%
		100%
15	CREB-Pa	45%
		74%
	ATF1	35%
		29%
20	ATF2	39%
		77%
	C-JUN	29%
		39%
25	JUN-D	32%
		48%

Table 3D: Amino Acid Sequences of Coiled-Coil Domain Based Multimerization Domains

30 ATF1-LI: L K S I E N R L A V I E N Q L K T I E E L K T I K D L L S N (SEQ ID NO:9)
 ATF1-LL: L K S L E N R L A V L E N Q L K T L I E E L K T L K D L L S N (SEQ ID NO:10)
 35 ATF1-IL: I K S L E N R I A V L E N Q I K T L I E E I K T L K D L I S N (SEQ ID NO:11)
 ATF1-II: I K S I E N R I A V I E N Q I K T I I E E I K T I K D L I S N (SEQ ID NO:12)
 ATF1-VL: V K S L E N R V A V L E N Q V K T L I E E V K T L K D L V S N (SEQ ID NO:13)
 40 ATF1-LV: L K S V E N R L A V V E N Q L K T V I E E L K T V K D L L S N (SEQ ID NO:14)
 CJUN-LI: L A R I E E K L K T I K A Q L S E I A S T L N M I R E Q L A Q (SEQ ID NO:15)
 CJUN-LL: L A R L E E K L K T L K A Q L S E L A S T L N M L R E Q L A Q (SEQ ID NO:16)
 45 CJUN-IL: I A R L E E K I K T L K A Q I S E L A S T I N M L R E Q I A Q (SEQ ID NO:17)
 CJUN-II: I A R I E E K I K T I K A Q I S E I A S T I N M I R E Q I A Q (SEQ ID NO:18)
 50 CJUN-VL: V A R L E E K V K T L K A Q V S E L A S T V N M L R E Q V A Q (SEQ ID NO:19)
 CJUN-LV: L A R V E E K L K T V K A Q L S E V A S T L N M V R E Q L A Q (SEQ ID NO:20)

- JUND-LI: LSRIEEKLTIKSQLTEIASTLSLIREQLAQ (SEQ ID NO:21)
- JUND-LL: LSRLEEKLTIKSQLTELASTLSLLREQLAQ (SEQ ID NO:22)
- 5 JUND-IL: ISRLEEKIKTLKSQITELASTISLLREQIAQ (SEQ ID NO:23)
- JUND-II: ISRIEEKIKTLKSQITEIASTISLIREQIAQ (SEQ ID NO:24)
- JUND-VL: VSRLEEKVKTLKSQVTELASTVSLREQVAQ (SEQ ID NO:25)
- 10 JUND-LV: LSRVEEKLTIVKSQLTEVASTLSLVREQLAQ (SEQ ID NO:26)
- CREB-Pa-LI: LMSIEKKLEEITQTLMQIQNELSMIKNELAQ (SEQ ID NO:27)
- 15 CREB-Pa-LL: LMSLEKKLEELTQTLMLQNELSMLKNEIAQ (SEQ ID NO:28)
- CREB-Pa-IL: IMSLEKKIEELTQTIMQLQNEISMLKNEIAQ (SEQ ID NO:29)
- CREB-Pa-II: IMSIEKKIEEITQTIMQIQNEISMIKNEIAQ (SEQ ID NO:30)
- 20 CREB-Pa-VL: VMSLEKKVEELTQTMQLQNEVSMLKNEVAQ (SEQ ID NO:31)
- CREB-Pa-LV: LMSVEKKLEEVTQTLMQVQNELSMVKNELAQ (SEQ ID NO:32)
- 25 ATF2-LI: LQSIEKKLEDISSLLGQIQSELTIRNELAQ (SEQ ID NO:33)
- ATF2-LL: LQSLEKKLEDLSSLLGQLQSELTLLRNELAQ (SEQ ID NO:34)
- ATF2-IL: IQSLEKKIEDLSSLLGQLQSEITLLRNEIAQ (SEQ ID NO:35)
- 30 ATF2-II: IQSIEKKIEDISSLLGQIQSEITLIRNEIAQ (SEQ ID NO:36)

Table 4A: Nucleotide Sequence Encoding ATF α Leucine Zipper Domain Variants

- ATF α (1)-LI:
35 CTTAGCTCTATTGAGAAGAACTTGAGGAGATTACCTCTCAACTGATTCAGATCAGCAATGAA
CTGACCCTTATCCGCAATGAGCTGGCCCAATGA (SEQ ID NO:48)
- ATF α (2)-LI:
40 CTTAGCTCTATTGAGAAGAACTTGAGGAGATTACCTCTCAACTGATTCAGATCCGCAATGAA
CTGACCCTTATCCGCAATGAGCTGGCCCAATGA (SEQ ID NO:49)
- ATF α (3)-LI:
45 CTTAGCTCTATTGAGAAGAACTTGAGGAGATTACCTCTCAACTGCAACAGATCCGCAATGAA
CTGACCCTTATCCGCAATGAGCTGGCCCAATGA (SEQ ID NO:50)
- ATF α -IL:
ATTAGCTCTTTAGAGAAGAAAATTGAGGAGCTGACCTCTCAAATCCAGCAGCTGCGCAATGA
AATCACCCTTCTTCGTAATGAGATTGCCCAATGA (SEQ ID NO:51)
- 50 ATF α -II:
ATCAGCTCTATTGAGAAGAAAATCGAGGAGATTACCTCTCAAATCAATCAGATTGCGCAATGAA
ATCAGCTTATCCGTAATGAGATTGCCCAATGA (SEQ ID NO:52)
- ATF α -LL:
55 CTTAGCTCTTTGAGAAGAACTTGAGGAGCTTACCTCTCAACTGATTCAGCTTCGCAATGAA
CTGACCCTTCTGCGTAATGAGCTGGCCCAATGA (SEQ ID NO:53)

ATFa-VL:
GTTAGCTCTCTTGAGAAGAAAGTTGAGGAGCTTACCTCTCAAGTGATTAGCTTCGCAATGAA
GTGACCCTTCTGCGTAATGAGGTTGCCCAATGA (SEQ ID NO:54)

5 Table 4B: Nucleotide Sequence of Coiled Coil Based Tetramerization Domains

ATF1-LI:
CTTAAATCTATTGAGAACCGGCTTGCCGTTATGAGAACCAACTGAAAACCATCATTGAAGAG
CTTAAGACCATCAAAGACCTTCTGTCTAACTGA (SEQ ID NO:55)

10 ATF1-LL:
CTTAAATCTCTTGAGAACCGGCTTGCCGTTCTTGAGAACCAACTGAAAACCTGATTGAAGAG
CTTAAGACCCTGAAAAGACCTTCTGTCTAACTGA (SEQ ID NO:56)

15 ATF1-IL:
ATTAAATCTCTTGAGAACCGGATCGCGTTCTTGAGAACCAATCAAAAACCTTATTGAAGAG
ATTAAGACCCTGAAAAGACCTTATCTCTAACTGA (SEQ ID NO:57)

20 ATF1-II:
ATTAAATCTATTGAGAACCGGATTGCGGTTATTGAGAACCAATCAAAAACCATCATTGAAGAG
ATTAAGACCATCAAAGACCTTATCTCTAACTGA (SEQ ID NO:58)

25 ATF1-VL:
GTTAAATCTCTTGAGAACCGGTTGCGGTTCTTGAGAACCAAGTTAAAACCTGATTGAAGAG
GTTAAGACCCTTAAAGACCTTGTCTAACTGA (SEQ ID NO:59)

ATF1-LV:
CTTAAATCTGTTGAGAACCGGCTTGCCGTTGTTGAGAACCAACTGAAAACCGTTATTGAAGAG
CTTAAGACCGTTAAAGACCTTCTGTCTAACTGA (SEQ ID NO:60)

30 CJUN-LI:
CTTGCTCGGATTGAAGAGAACTTAAACCATTAAGCGCAACTGTCTGAGATCGCGTCTACC
CTGAACATGATCCGTGAACAACTGGCCAATGA (SEQ ID NO:61)

35 CJUN-LL:
CTTGCTCGGCTTGAAGAGAACTTAAACCTTAAAGCGCAACTGTCTGAGCTGGCGTCTACC
CTGAACATGCTCCGTGAACAACTGGCCAATGA (SEQ ID NO:62)

40 CJUN-IL:
ATTGCTCGGCTTGAAGAGAAATTAACCATTAAGCGCAAACTTCTGAG
CTTGCGTCTACCATTAACATGCTTCGTGAACAACTGCCCAATGA (SEQ ID NO:63)

45 CJUN-II:
ATTGCTCGGATTGAAGAGAAATCAAAACCATTAAGCGCAAACTCTGAG
ATCGCGTCTACCATCAACATGATCCGTGAACAAAGCCAATGA (SEQ ID NO:64)

CJUN-VL:
GTTGCTCGGCTTGAAGAGAAAGTTAAACCTTAAAGCGCAAGTTCTGAGCTTGGCTCTACC
GTTAACATGCTTCGTGAACAACTGCCCAATGA (SEQ ID NO:65)

50 CJUN-LV:
CTTGCTCGGTCGAAGAGAACTTAAACCGTTAAAGCGCAACTGTCTGAGGTCGCGTCTACC
CTGAACATGGTTCGTGAACAACTGCCCAATGA (SEQ ID NO:66)

55 JUND-LI:
CTTAGCCGGATTGAAGAGAACTTAAACCATTAATCTCAACTGACTGAGATCGCGTCTACC
CTGTCTCTTATCCGTGAACAACTGCCCAATGA (SEQ ID NO:67)

- JUND-LL:
CTTAGCCGGCTTGAAGAGAACTTAAGACCCTGAAATCTCAACTGACTGAGCTTGGCTCTACC
CTTTCTTTGCTGCGTGAACAACTTGCCCAATGA (SEQ ID NO:68)
- 5 JUND-IL:
ATCAGCCGGCTTGAAGAGAAAGATTAAGACCCTGAAATCTCAAACTGAGCTTGGCTCTACC
ATTTCTCTTCTGCGTGAACAAATTGCCCAATGA (SEQ ID NO:69)
- JUND-II:
10 ATCAGCCGGATTGAAGAGAAAGATTAAGACCCTCAAACTCTCAAACTGAG
ATTGCGTCTACCAATTTCTTATTGCTGAACAAATTGCCCAATGA (SEQ ID NO:70)
- JUND-VL:
GTTAGCCGGCTTGAAGAGAAAGTTAAGACCCTGAAATCTCAAGTTACTGAG
15 CTTGCGTCTACCGTCTCTTCTGCGTGAACAAAGTTGCCCAATGA (SEQ ID NO:71)
- JUND-LV:
CTTAGCCGGGTTGAAGAGAAAGCTGAAGACCCTCAAACTCTCAACTTACTGAG
GTTGCGTCTACCCCTTCTCTTGTCCGTGAACAACTTGCCCAATGA (SEQ ID NO:72)
- 20 CREB-Pa-LI:
CTTATGTCTATTGAGAAGAACTTGAGGAGATTACCCAGACTCTGATGCAGATCCAGAATGAA
CTGTCTATGATCAAGAATGAGCTGGCCCAATGA (SEQ ID NO:73)
- 25 CREB-Pa-LL:
CTTATGTCTCTGGAGAAGAACTTGAGGAGCTTACCCAGACTCTGATGCAGCTTCAGAATGAA
CTGTCTATGCTGAAGAATGAGCTGGCCCAATGA (SEQ ID NO:74)
- CREB-Pa-IL:
30 ATCATGTCTCTGGAGAAGAAGATCGAGGAGCTTACCCAGACTATCATGCAGCTTCAGAATGA
AATTTCTATGCTGAAGAATGAGATTGCCCAATGA (SEQ ID NO:75)
- CREB-Pa-II:
ATCATGTCTATTGAGAAGAAGATCGAGGAGATCACCCAGACTATCATGCAG
35 ATTCAGAATGAATTCTATGATCAAGAATGAGATTGCCCAATGA (SEQ ID NO:76)
- CREB-Pa-VL:
GTTATGTCTCTTGAAGAAGGTTGAGGAGCTTACCCAGACTGTCTATGCAG
CTTCAGAATGAAGTTTCTATGCTTAAGAATGAGGTTGCCCAATGA (SEQ ID NO:77)
- 40 CREB-Pa-LV:
CTGATGTCTGTGAGAAGAAGCTTGAGGAGGTTACCCAGACTCTCATGCAG
GTTCAGAATGAACCTTCTATGGTTAAGAATGAGCTTGCCCAATGA (SEQ ID NO:78)
- 45 ATF2-LI:
CTTCAGTCTATTGAGAAGAACTTGAGGACATTAGCTCTCTTCTGGGTCAGATCCAATCTGAA
CTGACCCTTATCCGCAATGAGCTGGCCCAATGA (SEQ ID NO:79)
- ATF2-LL:
50 CTTCAGTCTCTTGAAGAAGAACTTGAGGACCTTAGCTCTCTTCTGGGTCAGCTGCAATCTGAG
CTGACCCTTCTGCGCAATGAGCTGGCACAAATGA (SEQ ID NO:80)
- ATF2-IL:
ATTCAGTCTCTGGAGAAGAAAATCGAGGACCTTAGCTCTCTTATTGGTCAGCTTCAATCTGAA
55 ATCACCTTCTTCGCAATGAGATTGCCCAATGA (SEQ ID NO:81)
- ATF2-II:
ATTCAGTCTATTGAGAAGAAAATCGAGGACATTAGCTCTCTTATTGGTCAGATTCAATCTGAA
ATCACCTTATCCGCAATGAGATTGCCCAATGA (SEQ ID NO:82)

Table 5: Designed Hinge Sequences

	D30: KAQASSVPTA QPQAEGSLAK ATTAPATTRN (SEQ ID NO:43)
5	D35: KAQASSVPTA QPQAEGSLAK ATTAPATTRN TGRGG (SEQ ID NO:44)
	ED: KAQASSVPTA QPQAEGSLAK ATTAPATTRN TGRGGEEKKK EKEKEEQEER ETKTPE (SEQ ID NO:45)
10	EDC: KAQASSVPTA QPQAEGSLAK ATTAPATTRN TGRGGEEKKK EKEKEEQEER ETKTPEC (SEQ ID NO:46)
	D63: KAQASSVPTA QPQAEGSLAK ATTAPATTRN TGRGGEEKKK
15	EKEKEEQEER ETKTPECPSH TQP (SEQ ID NO:47)

Example 4:

This example shows that residues at positions other than **a** and **d** can influence the multimeric status of a chimeric protein of the invention. This example also describes how other sequences can be produced in accordance with the invention.

Five candidate leucine zipper domains were selected based on a search of public databases (Table 3B). Multimerization domains were then designed by replacing wild type residues at positions **a** and **d** with leucine or isoleucine (Table 3D); they were synthesised by PCR according to the nucleotide sequences in Table 4B. Each variant was expressed and purified as a component of a chimeric multimeric fusion protein.

With **L** at position **a** and **I** at position **d**, four of the five multimer domains formed tetramer, but the fifth, JUND-LI, formed trimer. With **I** at position **a** and **L** at position **d** ATF-1 formed trimer, but ATF-2, CJUN, and CREB formed dimers. When **L** was placed at both positions **a** and **d**, ATF-2 and CREB formed tetramers, ATF-1 and CJUN formed trimers, and JUND formed dimer. Isoleucine at both positions **a** and **d** formed either trimer or dimer. These results may be contrasted with those obtained with ATF α (Example 1) where the combinations at **a** and **d** **IL** and **LI** formed tetramer while **II** and **LL** formed trimer. The data therefore indicate that sequences outside the **a** and **d** positions can modulate the type of multimer formed.

The residues at positions **b**, **c**, **e**, **f**, and **g** form an interlocked network of inter- and intra- helical interactions that influence the geometry of the helical bundle. This network of intra- and inter-helical interactions can be analyzed from three-dimensional models.

- To do so, a model of the multimerization domain is constructed by homology using a coiled coil of the desired multimerization state as a template. After substituting the sequence of the domain to be analyzed in the proper register relative to the heptad repeat, the structure is energy minimized. Keeping in mind the possibility of alternative side chain rotamers, the surface residues are then analyzed by inspection. If a substitution is contemplated, the propagated effect on the entire network is considered. For example, changing an **e** position to a residue that can make a salt bridge to a **g** position in a neighboring helix may cause a third residue previously interacting with the **g** position to also change its interacting partner(s).
- The effect of a substitution can be predicted with frequent success by this method. In order to confirm the predicted effect, the substituted multimer domain can be screened for its multimerization or other properties (e.g., stability or tightness). For example, the multimeric form produced can be determined by HPLC and/or ultracentrifugation assay methods as described herein (Example 6); the effect on tightness of multimerization can be assayed by determining melting profiles of a polypeptide having the multimerization domain.

Example 5

- This example describes expression and purification of multimeric Fab protein.
- To produce a multimeric Fab, a *SacI*/*EcoRI* DNA fragment encoding the ED hinge (Table 5) derived from IgD and a modified ATF α coiled-coil domain, ATF α (1)-LI+S, was cloned at the 3'-end of the C_H1 coding sequences of Fab19 in the Fab19/Tet plasmid (Figure 1). This plasmid was designated CFY 196.
- To produce the Fab19 and the tetrameric CFY196 proteins, cultures of the *E. coli* strain JM83 expressing the Fab19/Tet or CFY196 plasmids were grown in selective TB medium to an OD₆₀₀ of 2.0. After induction by addition of IPTG to a final concentration of 0.2 mM and incubation for 8 hours at room temperature, cells were harvested by centrifugation at 4,000g for 15 minutes at 4°C. The cell pellet was suspended in 50 ml wash buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 50 μ M PMSF and 5 mM EDTA) per liter culture and repelleted by centrifugation at 4,000 g for 15 minutes at 4°C and frozen. Frozen cell pellets were resuspended in 20 ml/gram wet weight pellet of a lysis buffer (50 mM Tris, pH 8.0, 200 mM NaCl, 5 mM EDTA, 5 mM EGTA, 50 μ M PMSF and

0.1mg/ml lysozyme), and after incubation on ice for 30 minutes the pellets were sonicated, and the lysates clarified by centrifugation at 23,000g for 30 minutes. The supernatant, containing soluble protein, was adjusted to 1M NaCl and loaded on a Protein A column (Amersham/Pharmacia). The column was then washed extensively with 2M NaCl, 25 mM Tris, pH 8.0, 5 mM EDTA and the proteins eluted with 0.1 M glycine, pH 2.5. Eluent pH was neutralized with 1/10 volume of 1 M Tris base pH 9.0.

For the purification of monovalent Fab proteins, protein-containing fractions were pooled and dialyzed against TBS and stored at 4°C. For the purification of multimeric Fab fusion proteins, fractions from the protein A column were dialyzed against 200 mM KCl in 50 mM Hepes pH 7.5, then further purified over a hydroxyapatite column (Macroprep Ceramic Hydroxyapatite Type II, Biorad). After binding the protein in dialysis buffer, the column was washed with binding buffer, then with 200mM KCl/10 mM K phosphate/50 mM Hepes, pH 8.0. The multimeric Fab proteins were eluted with a phosphate gradient in 500mM KCl/ 50 mM Hepes, pH 8.0. Positive fractions were pooled and dialyzed against TBS.

The yield was 1-2 mg/liter of culture in shake flask. The purity of protein preparations were determined by size exclusion chromatography and SDS-PAGE gel (Figure 2).

Example 6

This example describes characterization of the multimeric status of Fab-ATF α domain fusion proteins.

Two assays are described, a sedimentation velocity assay which can determine multimerization state and can distinguish between multimers of different tightness, and an HPLC assay that can distinguish multimerization states after being correlated with the sedimentation velocity data. Sedimentation velocity measures the movement of a solute boundary as the solute moves through solvent under the influence of a centrifugal force. The data are a series of boundary positions recorded over time. The raw data is then processed to give a distribution of sedimentation coefficients (Figure 3). The large peak shows the sedimentation coefficient for the major species in the sample. The number of peaks is an indication of purity, and the broadness of the major peak indicates the homogeneity of the major species.

The oligomeric state of purified multimeric Fab fusion proteins was determined by sedimentation velocity using analytical ultracentrifugation (Modern Analytical Ultracentrifugation, TM Schuster and TM Laue, eds. (1994) Birkhauser, Boston) in combination with light scattering data (Wen, *et al.*, *Analytical Biochem* 240:155 (1996)).

- 5 Trimeric Fab-ATF α domain fusion proteins sediment at about 5.5 S, and tetrameric proteins sediment at 6.5 S. The results of sedimentation studies on Fab-ATF α domain fusion proteins are summarized in Table 6.

- By correlation with sedimentation velocity data, the multimerization state of additional ATF α -based domains was determined by size exclusion HPLC. HPLC size
10 exclusion chromatography was performed on a column selected to resolve species of chimeric multimers ranging from dimers through pentamers. The selected column also resolved species including the Fab moiety of the chimera: Tetramers (6.5S by ultracentrifugation) consistently eluted from the column at 13.7 \pm 0.1 min.; trimers (5.5S) eluted at 14.0 \pm 0.1; and dimers (4.5S) eluted at 15.0 \pm 0.1 min. Data on additional domains
15 is listed in Table 7 along with those determined by sedimentation. The multimeric status of domains derived from ATF1, ATF2, CJUN, JUND and CREB leucine zippers are summarized in Table 8.

Table 6: Multimer Formation

20	Construct	Hinge-ATF α Domains	Sedimentation coeff	Multimer
	CFY192B	ED-ATF α (2)-LI	6.5 S	tetramer
	CFY193B	ED- ATF α (2)-LL	5.5 S	trimer
	CFY195	ED- ATF α (2)-II	5.5 S	trimer
	CFY196	ED- ATF α (1)-LI+S	6.5 S	tetramer
25	CFY197	ED- ATF α (3)-IL	6.5 S	tetramer
	CFY484	D ₃₀ - ATF α (2)-LI	6.5 S	tetramer

Table 7: Multimer Formation by ATF α Variants

ATF α Variant	Hinge	Protein Fused With	Multimeric Status
ATF α -WT	ED-	Fab4.8	Tetramer
ATF α (1)-LI	IgG3	Thioredoxin	Tetramer

ATFa(1)-LI	D-, ED-	Fab4.8, Fab17, Fab19	Tetramer
ATFa(2)-LI	D30-, ED-	Fab4.8, Fab17, Fab19	Tetramer
ATFa(3)-LI	D30-, ED-	Fab17	Tetramer
ATFa-LL	ED-,	Fab4.8, Fab19	Trimer
ATFa-II	D30-, ED-	Fab4.8, Fab19	Trimer
ATFa-IL	ED-,	Fab4.8, Fab19	Tetramer
ATFa-VL	ED-,	Fab4.8	Dimer & Tetramer

Sedimentation data also allows distinguishing between tetramers. For example, CFY 192B, 196 and 484 have the same a and d amino acids, and all form predominantly tetramers. These proteins, however, bear variations in sequence elsewhere. Examination of the c(s) vs. S plots for CFY196 and CFY192B reveals that the former is superior (Figure 5). The distribution of sedimentation coefficient fitted for CFY196 is much narrower than that for CFY192B indicating a more homogeneous tetrameric population for CFY196.

Fab19 ED when fused to ATF(1) α -IL (SEQ ID NO:162) forms a tetramer whose sedimentation velocity analysis showed a broad peak (Figure 7A). Changing a serine to arginine at position e of the third repeat (SEQ ID NO:163), which creates a new potential interhelical salt bridge, gave slight improvement (Figure 7B). Combining these changes with an additional mutation of the isoleucine at position b of the third repeat to glutamine (SEQ ID NO:5) gave a much narrower sedimentation distribution (Figure 7C). Sequences of ATF α -IL mutants:

	1	2	3	4	5
Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c
ATFa(1)-IL:	ISSLEKK	IEELTSQ	IIQLSNE	ITLLRNE	IAQ (SEQ ID NO:162)
ATFa(2)-IL:	ISSLEKK	IEELTSQ	IIQLRNE	ITLLRNE	IAQ (SEQ ID NO:163)
ATFa(3)-IL:	ISSLEKK	IEELTSQ	IQQLRNE	ITLLRNE	IAQ (SEQ ID NO:5)

Table 8: Multimer Formation by Other Multimerization Polypeptides

Domains	Multimeric Status
CREB-Pa-LI	Tetramer
CREB-Pa-LL	Tetramer
CREB-Pa-IL	Dimer

	CREB-Pa-II	degradation products
	JUN-D-LI	Trimer
	JUN-D-LL	Dimer
5	JUN-D-II	degradation products
	JUN-D-IL	degradation products
	CJUN-LI	Tetramer
	CJUN-LL	Trimer
10	CJUN-IL	Dimer
	CJUN-II	degradation products
	ATF-1-LI	Trimer
	ATF-1-LL	Trimer
15	ATF-1-IL	Trimer
	ATF-1-II	Trimer
	ATF2-LI	Tetramer
	ATF2-LL	Tetramer
20	ATF2-IL	Dimer
	ATF2-II	degradation products

25 The sedimentation data correlate well with the results of cell based functional assays (see, *e.g.*, Example 7). These assays indicate that tetrameric proteins exceed trimers in cell protective ability, and trimeric and tetrameric proteins provide greater protection than monovalent Fab. The most homogenous tetramer provides the best protection (Figure 4).

Example 7

30 This example describes biological activities of monovalent Fab19 anti human ICAM-1 protein and multivalent Fab19-EDATF α domain fusion proteins. This example also describes data indicating that trimeric and tetrameric Fab19-EDATF α proteins provide greater protection of cells from HRV infection than monovalent Fab19 and bivalent monoclonal antibody.

35 An HRV protection assay was performed to compare the abilities of the monovalent Fab19 protein and multivalent Fab19-based proteins to protect cells from infection. HeLa cells were plated 1×10^5 per well in a 48-well tissue culture dish 24 hours before the assay. Growth medium was removed and 100 μ l of multivalent or monovalent proteins diluted to the indicated concentrations added to each well. The plates were
40 incubated for one hour in a 37°C incubator, the solution was removed and 200 μ l HRV15

(at MOI of 1) added and then incubated for one hour at 33°C. Cells were washed and 1 ml/well growth medium added. Infected cells were incubated at 33°C for 48 hours, the medium removed and the remaining viable cells stained with crystal violet. Bound crystal violet was extracted with 3 ml methanol per well, then the amount of cell staining was quantified by measuring the A₅₇₀.

The results of the protection assay are illustrated in Figure 4. The percentage protection is calculated for each data point in triplicate using the formula:

$$\% \text{ protection} = \frac{(100)(\text{Absorbance of sample} - \text{Absorbance of virus only})}{(\text{Absorbance of uninfected cells} - \text{Absorbance of virus only})}$$

The protective efficacy is quantitated as EC₅₀, the dose of antibody that gives 50% protection. Based on the data, the EC₅₀ of Fab19 is 76 nM. The tetrameric proteins CFY192B and CFY196 are more effective than the trimeric CFY192B and CFY195. In this group of multimers based on ATFα, CFY196 (Fab19-EDATFα(1)-LI+S) demonstrated the highest protection (EC₅₀ of 0.73 nM), which is 104 times greater protection than monovalent Fab19 protein.

In another study, the protective efficacy of monovalent Fab19, bivalent anti-ICAM-1 monoclonal antibody RR/1 (Chemicon), bivalent CFY202, trimeric CFY193B and tetrameric CFY196 were compared (Figure 5). While monovalent Fab 19 had an EC₅₀ of greater than 200 nM, and bivalent RR/1 has an EC₅₀ greater than 10 nM, the EC₅₀ of CFY 202 was 4.5 nM, of CFY 193B was 1.35 nM and that of CFY 196 was 0.97 nM. These data indicate that tetramer provided the greatest protective efficacy followed by trimer, dimer and monomer.

Example 8

This example describes the construction and multimeric characterization of a non-Fab chimeric polypeptide of the invention, a thioredoxin-ATFαLI fusion protein.

To demonstrate that ATFα leucine zipper domain variants can induce tetrameric complexes when fused with proteins other than Fab, the hinge-ATFα(1)-LI-SG₃H₆ fragment was fused to the C-terminus of thioredoxin in the expression vector pBAD-thio (Invitrogen). The expression construct, pBAD-thio-hinge-ATFα(1)-LI-SG₃H₆, was transferred into the *E. coli* strain TOP-10 and grown in selective TB medium to an OD₆₀₀

of 0.8. After induction by addition of arabinose to 0.02% final concentration, and incubation overnight at room temperature, cells were harvested by centrifugation at 4,000 g for 15 minutes at 4°C. The cell pellets were suspended in lysis buffer (20 mM sodium phosphate, pH 8.0, 1% Triton X-100, 500 mM NaCl, 40 mM imidazole, 1 mM β -mercaptoethanol), 0.2 mM PMSF, 0.5 mg/ml lysozyme and incubated on ice for 20 minutes. The cells were sonicated, and another aliquot of PMSF was added. Cell debris was pelleted by centrifugation at 23,000g and the clarified sonicate was filtered and fractionated by metal affinity chromatography.

Induced histidine-tagged proteins were bound to a Hi Trap metal chelating column (Amersham/Pharmacia) equilibrated with Ni^{2+} according to the manufacturer's directions. The column was then washed with four column volumes of buffer consisting of 100 mM imidazole, 20 mM sodium phosphate, pH 7.4, 500 mM NaCl. Proteins were eluted from the column with 20 mM sodium phosphate, pH 7.4, 1 M Imidazole and collected in fractions. Protein fractions were pooled and dialyzed at 4 °C against phosphate buffered saline (PBS).

The molecular weight of the thioredoxin-ATF α (1)-LI fusion protein was determined by size exclusion chromatography. A Superdex 200 column (7.5 mm x 25 cm) was equilibrated in TBS buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl) at a flow rate of 0.4 ml/min. The column was calibrated with standard proteins (ribonuclease A, 13.7; chymotrypsinogen A, 25 kDa; ovalbumin, 43 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; and β -amylase, 200 kDa). The purified thioredoxin-ATF α LI1 fusion protein was analyzed separately at the same conditions as standard proteins.

The calculated molecular weight of a monomeric thioredoxin-ATF α (1)-LI-+SG₃H₆ fusion protein is 19.6 kDa. However, the calculated molecular mass of purified fusion protein is about 78 kDa. This result demonstrates that the thioredoxin-ATF α (1)LI-+SG₃H₆ fusion protein exists as a tetramer.

Example 9

This example describes inhibiting and treating respiratory syncytial virus (RSV) infection by multivalent ICAM-1 binding proteins of the invention.

RSV appears to bind to ICAM-1 via F protein on the surface of RSV; F protein is also the target of neutralizing antibodies against RSV. A recently published article

purportedly found that human respiratory syncytial virus (RSV) infection of human epithelial cells could be inhibited either by pre-treating RSV with soluble ICAM-1 or pre-treating cells with an anti-ICAM-1 monoclonal antibody (Behera *et al.*, *BBRC* 280:188 (2001)). However, very high antibody concentration (200-400 microgram/ml, >1mM) was required to achieve the apparent inhibition of RSV infection indicating that a monoclonal antibody is unlikely to be an effective therapy against RSV.

Given the high concentration of the monoclonal antibody required for inhibition of RSV infection in vitro, RSV may have relatively high binding affinity, or avidity, for ICAM-1. If so, a multimeric (dimer, trimer, tetramer, pentamer or even higher order oligomer) ICAM-1 binding protein will have better efficacy against RSV infection than a monovalent ICAM-1 antibody. Additionally, although a full-blown RSV infection is a systemic disease, the virus first invades the body at the upper respiratory tract, especially at the nasopharynx area. Blocking RSV from the upper respiratory tract will prevent the entry of RSV, and therefore prevent RSV from invading the lower respiratory tract.

Although not wishing to be bound by theory, multivalent ICAM-1 binding protein appears to block the RSV foothold or entryway into cells of the upper respiratory tract. Consequently, free-floating RSV particles are re-routed by the normal mucocilliary clearing system into the gastrointestinal tract and become harmless.

Example 10

This example describes the construction and expression of polypeptides composed of two different binding specificities.

To produce a molecule with tetrameric binding capacity from a dimerization domain the multimerization domain can be at the center of a molecule linking Fab from two different antibody molecules (see, e.g., Figure 6A). Alternatively, a trimerization or tetramerization domain could be used to produce hexavalent and octavalent molecules, respectively.

In the bispecific multimeric protein, the Fab moieties illustrated with different hatching could have different specificities, such as anti-CD-3 and anti-CD-19. The Fab portion of each protein, consisting of the heavy and light chains, would be linked by a linker sequence to a dimerization domain, illustrated as a hexagon in Figure 6A. This polypeptide is produced from one tricistronic RNA molecule. Alternatively, three promoters could drive the expression of three distinct polypeptides. The coding sequence

for the first polypeptide chain translated from this message is illustrated by the hatched box in Figure 6B, representing the anti-CD-3 light chain (LC-1). The second DNA fragment encodes the central chimeric polypeptide consisting of the anti-CD-3 heavy chain (HC-1) linked to a long hinge derived from IgD, followed by a dimerization domain, a second hinge, followed by the anti-CD19 light chain (LC-2). The third RNA, illustrated by the white box in Figure 6B, would encode the anti-CD-19 heavy chain (HC-2).

An alternative expression construct in which the HC-2 sequences and the LC-2 sequences are switched for sequences encoding an scFv would also be possible, if the affinity of the scFv was as high as desirable.

To express a bispecific tetravalent molecule from a tricistronic message, three DNA fragments would be prepared. The first is an Nde I/ Xba I fragment consisting of the light chain from the anti-CD-3 Fab and the bulk of an intervening sequence (IVS1) which includes a ribosome binding site. Downstream from this fragment the XbaI/SpeI restriction fragment consisting of six pieces fused together using PCR is ligated: the remainder of the IVS1, the heavy chain of Fab, a hinge derived from IgD, the CREB-IL dimerization domain, a second hinge derived from IgD, the light chain of anti-CD-19, and the bulk of the second intervening sequence (IVS2). The third SpeI/ HindIII restriction fragment would contain the remainder of IVS2, the heavy chain of anti-CD-19, and a terminator sequence. Each of the IVS contain a ribosome binding site, and the 3' end of the construction contains the bacteriophage λ t_0 terminator (TER). When translated, each of the three polypeptides will be preceded by an enterotoxin II signal sequence (stII).

For other bispecific molecules, the multimerization domain indicated by the filled box would be replaced by alternative multimerization sequences to form tetravalent, hexavalent, octavalent or higher order binding arms. These three DNA coding sequences could be driven by multiple promoters, or be encoded on distinct vectors. A similar construct could also be used for bispecific antibodies when two Fab molecules of different specificity share a common light chain. In this instance, the sequences encoding HC-2 in Figure 6B would replace those of LC-2, and the TER sequences would replace IVS2. This expression vector would then produce two polypeptide chains in a bicistronic message.

Example 11

This example shows how amino acids adjacent to multimerization domains of the invention can be modified to modulate stability or tightness of the multimers that form.

In chimeric proteins of the invention with the coiled coil domain at the C terminus, the C terminus of the multimerization domain is exposed to solvent. An extra amino acid can be added to the C terminus to make a more stable, hydrophilic ending for the protein. In general, by considering the last two amino acids in the coiled coil domain a terminal amino acid can be selected to add to the C terminus that will not disturb the interactions in which these residues participate. For ATF α (1)-LI, a serine fits these criteria. A serine in this position can stabilize the fold of the multimerization domain by allowing the formation of a series of water-mediated hydrogen bonds that provide a hydrophilic termination for the C terminus of the coiled coil.

ATF α (1)-LI (SEQ ID NO:1) was modified by the addition of a C terminal serine (SEQ ID NO:154). When this multimerization domain was used in an anti-ICAM antibody chimera (CFY196), the purified protein multimer (tetramer) was found to give an exceptionally narrow distribution of sedimentation coefficient (Figure 3C, Example 6), which indicates tighter multimerization, (i.e., the K_D decreases) for the subunits that comprise the multimer. The sequence of ATF(1) α -LI+S:

	1	2	3	4	5
Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c d
ATF α (1)-LI+S:	L S S I E K K	L E E I T S Q	L I Q I S N E	L T L I R N E	L A Q S

(SEQ ID NO:154)

Example 12

This example describes the construction of pentamers with a method of the invention.

To construct a pentamer, the multimerization domain ATF α -II (SEQ ID NO:6), which forms trimers when used in a chimeric anti-ICAM antibody, termed CFY195, was selected. This sequence was chosen because the beta branched isoleucine residues can pack efficiently in the hydrophobic core of a pentamer. Residues at the interface between the hydrophobic core and the solvent exposed exterior of the domain must be changed to be more hydrophobic in order to change the trimer to a pentamer. Accordingly, four residues at e and g positions were changed to leucine. Two additional changes were made at one b and one c position to adjust the network of inter- and intra-helical interactions to

favor pentamer formation. Both of these mutations, which change hydrophobic residues to hydrophilic residues, provide proper orientation of interacting interhelical surfaces. This sequence was designated ATF α -II-g (SEQ ID NO:159).

- Using overlapping oligonucleotides, both ATF α -II-g and several variants (Table 9) containing subsets of the mutations in ATF α -II-g were generated by PCR, subcloned, and sequenced in their entirety. These all have isoleucine in their a and d positions, but differ in the six amino acids that are underlined in Table 9. These domains were each fused to Fab19 and the ED hinge and assessed for their multimerization state by size-exclusion chromatography as described in Example 6.
- Multimeric proteins composed of Fab 19, an ED hinge and the multimerization domains ATF α -II-a through ATF α -II-g (SEQ ID NO:155-159) exhibited a retention time greater than trimeric CFY195, but a, b, and c eluted from the HPLC column as broad peaks, indicating substantial heterogeneity. Only the ATF α -II-f and ATF α -II-g multimers produced the homogeneous species and eluted before tetramer, at a retention time expected for pentamer. The activity of ATF α -II-g multimer was measured in a cell protection assay as described in Example 7. It was found to have similar protective ability to tetrameric CFY196 and superior ability to trimeric CFY195.

Table 9: ATF α -II Leucine Zipper Domain Variants

	1	2	3	4	5
Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c
ATFa-II:	I S S I E K K	I E E I T S Q	I J Q I R N E	I T L I R N E	I A Q (SEQ ID NO:6)
ATFa-II-a:	I S S I E K L	I E E I L S Q	I J Q I R N L	I T L I L N E	I A Q (SEQ ID NO:155)
ATFa-II-b:	I S S I E K K	I E E I L S Q	I Q Q I R N L	I T S I L N E	I A Q (SEQ ID NO:156)
ATFa-II-c:	I S S I E K L	I E E I L S Q	I Q Q I R N L	I T L I R N E	I A Q (SEQ ID NO:157)
ATFa-II-f:	I S S I E K L	I E E I L S Q	I Q Q I R N L	I T S I R N E	I A Q (SEQ ID NO:158)
ATFa-II-g:	I S S I E K L	I E E I L S Q	I Q Q I R N L	I T S I L N E	I A Q (SEQ ID NO:159)

- To further demonstrate that one can change a trimer to a higher order multimer by enlarging the hydrophobic core, a pentamer in the context of another coiled-coil domain, ATF-1, was produced. In Table 10 is the sequence of ATF1-II and a variant, ATF1-IIa, designed to form pentamers. The two sequences differ at the positions indicated by underlined amino acids. Comparison by HPLC of two chimeric proteins prepared from these domains indicate that the amino acid alterations changed the trimeric protein prepared from ATF1-II into a higher order multimer.

Table 10: ATF1-II Leucine Zipper Domain Variants

	1	2	3	4	5
5 Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c
ATF1-II:	I K S I E N R	I A V I E N Q	I K T I I E E	I K T I K D L	I S N (SEQ ID NO:12)
ATF1-II-a:	I K S I E N <u>L</u>	I A <u>Q</u> I L N Q	I K T I I E <u>L</u>	I K T I <u>L</u> D L	I S N (SEQ ID NO:160)

Example 13

10 This example describes a competition ELISA assay for evaluation of binding affinity of monomeric and multimeric Fab antibodies.

A tetrameric Fab19, termed 196TGC, conjugated with horseradish peroxidase (HRP) was developed for use as a tracer. A cysteine was introduced into a tetrameric anti-ICAM antibody by adding the sequence TGC to the C terminus of a chimeric Fab19-
 15 ED protein containing the ATF(1) α -LI multimerization domain (196TGC). HRP was chemically coupled to 196TGC using EZ-Link maleimide activated HRP (Pierce). The sequence of the 196TGC multimerization domain:

	1	2	3	4	5
20 Repeats:	a b c d e f g	a b c d e f g	a b c d e f g	a b c d e f g	a b c d f g
ATF α (1)-TGC:	L S S I E K K	L E E I T S Q	L I Q I S N E	L T L I R N E	L A Q T G C (SEQ ID NO:161)

A 96-well EIA plate (Corning, Inc.) was coated with 100 μ l/well soluble ICAM-1
 25 (Bender MedSystems) at 1 μ g/ml in 0.1 M NaHCO₃. After washing with TBST (50 mM Tris, pH8.0, 150 mM NaCl, 0.05% Tween-20), the plate was blocked with 3% non-fat milk in TBST at room temperature for 1 hour. After washing with TBST, anti-ICAM-1 Fab samples (monomer or multimer) diluted serially in 1% non-fat milk / TBST solution were added and incubated at room temperature for 1 hour. After washing with TBST, the
 30 plate was incubated with the horseradish peroxidase-conjugated anti-ICAM-1 tetrameric antibody (196TGC-HRP) diluted 1:50,000 in 1% non-fat milk/TBST at room temperature for 2 hours. The plate was washed thoroughly with TBST and 100 μ l/well 3,3',5,5'-tetramethylbenzidine substrate solution (Kirkegaard and Perry Laboratories) was added. After 15 min incubation, the color development was stopped by adding
 35 100 ml/well 0.12 N HCl and the absorbance of the wells at 450 nm was measured by a plate reader (ICN).

The percentage of inhibition of tracer antibody (196TGC-HRP) binding was calculated as follows:

$$\% \text{ Inhibition} = 100 \times (A_0 - A_s) / A_0$$

Where A_0 is OD₄₅₀ of the reference well without samples (196TGC-HRP only);

- 5 A_s is the OD₄₅₀ reading from the diluted sample. The relative binding affinity of the anti-ICAM-1 antibodies were represented by the protein concentration that blocks tracer antibody (TGC-HRP) at 50% (IC₅₀).

- Figure 8 shows that monomeric Fab (Fab19) only inhibits 25% tracer antibody (TGC-HRP) binding at the highest concentration tested. However, its trimer (Fab19-EDATFα-II) or its tetramer (Fab19-EDATFα-LI) gave a much higher percentage inhibition. The IC₅₀ for these three molecules are as follows:

	<u>Samples</u>	<u>IC₅₀ (μg/ml)</u>
	Fab19	below IC ₅₀
	Fab19-EDATFα-II	0.54
15	Fab19-EDATF(1)α-LI+S	0.069

Example 14

This example describes additional applications of the invention multimer polypeptides.

- 20 Multimers may be used in any context where greater binding affinity or multivalent binding is desired. The only requirement is the availability of a moiety that can be adapted for use in the construction of a chimeric protein of the invention. The moiety may be a binding protein or any synthetic or natural molecule that can be coupled by a chemical bond to form the chimera. Examples of such moieties include chelators,
- 25 binding peptides, binding proteins, and the like.

- Multimeric molecules of the invention could be used for various environmental applications. For example, invention multimers that bound heavy metals or toxins could be used in bioremediation. Multimers that bound a protein on the surface of a pathogen can be used to neutralize that pathogen in the environment, for example, in a field where
- 30 cattle graze. Multimers of the invention can also be constructed that act as insecticides or herbicides free of the unintended toxicity of many small molecules.

Multimeric molecules of the invention can also have industrial applications. For example, a multimer could be used in industrial chemistry to either remove a product of a reaction, thus speeding the reaction or driving it to completion, or to remove a reactant or catalyst, thus stopping a reaction. In addition, multimeric molecules can be used to
 5 recover substances from dilute solutions. Furthermore, bispecific molecules can be constructed from two successive enzymes in a synthetic pathway in order to increase the reaction rate for the combined steps.

Example 15

10 This example describes additional kinds of multimeric peptide-based drugs that can be made in accordance with the invention.

Binding molecules, such as antibodies against a target can be identified by any means known in the art. For example, CDR regions of an antibody that binds to a target of interest can be transferred to a humanized framework using methods known in the art.
 15 The humanized antibody can optionally be expressed as either ScFv or Fab fragments as part of a chimeric multimeric protein. Particular non-limiting examples of target proteins of interest along with an indication of the medical condition that can be treated by binding of a multimeric protein to the target:

- * Beta tryptase (allergy, inflammation)
- 20 * LFA-1 (transplant rejection)
- * CD105, VEGF (macular degeneration, cancer)
- * IgE (asthma)
- * CD154 (lupus, transplant rejection)
- * CD14 (sepsis)
- 25 * Folate receptor alpha (filovirus infection, e.g., Ebola and Marburg viruses)
- * nectin-1, also known as CD111 – (human herpesviruses)
- * gp120 (HIV-1/AIDS)
- * IL-6 (arthritis)
- * IL-5 (asthma)
- 30 * IL-8 (general inflammation)
- * or any other interleukin (general inflammation)
- * Any growth factor receptor (cancer)

In addition, therapeutic antibodies already developed could be enhanced by application of this invention. Examples include:

- * anti-von Willebrand factor (coronary thrombosis)
- * anti-TNF alpha (Crohn's disease)
- 5 * anti-Her-2 (breast cancer)
- * anti-CD-3 (transplant rejection)
- * anti-CD-20 (non Hodgkin's lymphoma)

10

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:-

1. A multimeric protein comprising an isolated multimerization polypeptide, wherein said isolated multimerization polypeptide comprises SEQ ID NO: 154 or a fragment thereof that is at least 11 amino acids in length.
- 5 2. A multimeric protein comprising an isolated multimerization polypeptide having 70% or greater identity to SEQ ID NO: 154.
3. A multimeric protein of claim 2 wherein said isolated multimerization polypeptide has 75% or greater identity to SEQ ID NO: 154.
4. A multimeric protein of claim 2 wherein said isolated multimerization
- 10 polypeptide has 80% or greater identity to SEQ ID NO: 154.
5. A multimeric protein of claim 2 wherein said isolated multimerization polypeptide has 85% or greater identity to SEQ ID NO: 154.
6. A multimeric protein of claim 2 wherein said isolated multimerization polypeptide has 90% or greater identity to SEQ ID NO: 154.
- 15 7. A multimeric protein of claim 2 wherein said isolated multimerization polypeptide has 95% or greater identity to SEQ ID NO: 154.
8. The multimeric protein of claim 2 wherein said isolated multimerization polypeptide comprises a seven residue zipper repeat sequence (a.b.c.d.e.f.g) having one or more amino acid substitutions, provided that all of positions a or d are either leucine,
- 20 isoleucine or valine.
9. The multimeric protein of claim 2 wherein said isolated multimerization polypeptide comprises a seven residue zipper repeat sequence (a.b.c.d.e.f.g) having one or more amino acid substitutions, provided that one or more of positions a or d are either leucine, isoleucine or valine.
- 25 10. The multimeric protein of claim 9, wherein at least one of positions a or d of said seven residue zipper repeat sequence (a.b.c.d.e.f.g) is an amino acid other than leucine, isoleucine or valine.
11. The multimeric protein of claim 9 comprising 1 to 5 amino acid substitutions.
12. The multimeric protein of claim 2 wherein said isolated multimerization
- 30 polypeptide comprises a seven residue zipper repeat sequence (a.b.c.d.e.f.g) having one or more amino acid substitutions in positions b, c, e, f or g.

13. The multimeric protein of claim 12, said polypeptide having 1 to 5 amino acid substitutions.
14. The multimeric protein of claim 1, wherein said isolated multimerization polypeptide is at least 15 amino acids in length.
- 5 15. The multimeric protein of claim 1, wherein said isolated multimerization polypeptide is at least 18 amino acids in length.
16. The multimeric protein of claim 1, wherein said isolated multimerization polypeptide is at least 22 amino acids in length.
17. The multimeric protein of claim 1, wherein said isolated multimerization
10 polypeptide is at least 27 amino acids in length.
18. The multimeric protein of claim 1, wherein said isolated multimerization polypeptide is at least 31 amino acids in length.
19. The multimeric protein containing the isolated multimerization polypeptide of claim 1, wherein said isolated multimerization polypeptide is a sequence less than about
15 125 amino acids in length and comprises a hinge domain or region.
20. The multimeric protein containing the isolated multimerization polypeptide of claim 1, wherein said isolated multimerization polypeptide is a sequence less than about 100 amino acids in length and comprises a hinge domain or region.
21. The multimeric protein containing the isolated multimerization polypeptide of
20 claim 1, wherein said isolated multimerization polypeptide is a sequence less than about 75 amino acids in length and comprises a hinge domain or region.
22. The multimeric protein containing the isolated multimerization polypeptide of claim 1, wherein said isolated multimerization polypeptide is a sequence less than about 50 amino acids in length.
- 25 23. A multimeric protein of claim 1 wherein said multimerization polypeptide is fused to a heterologous polypeptide to form a chimeric polypeptide.
24. A multimeric protein of claim 23, wherein the multimerization polypeptide is fused to the amino terminus of the heterologous polypeptide.
25. A multimeric protein of claim 23, wherein the multimerization polypeptide is
30 fused to the carboxy terminus of the heterologous polypeptide.
26. The multimeric protein of claim 23, wherein the multimerization polypeptide has 65% or greater identity to an isolated multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.

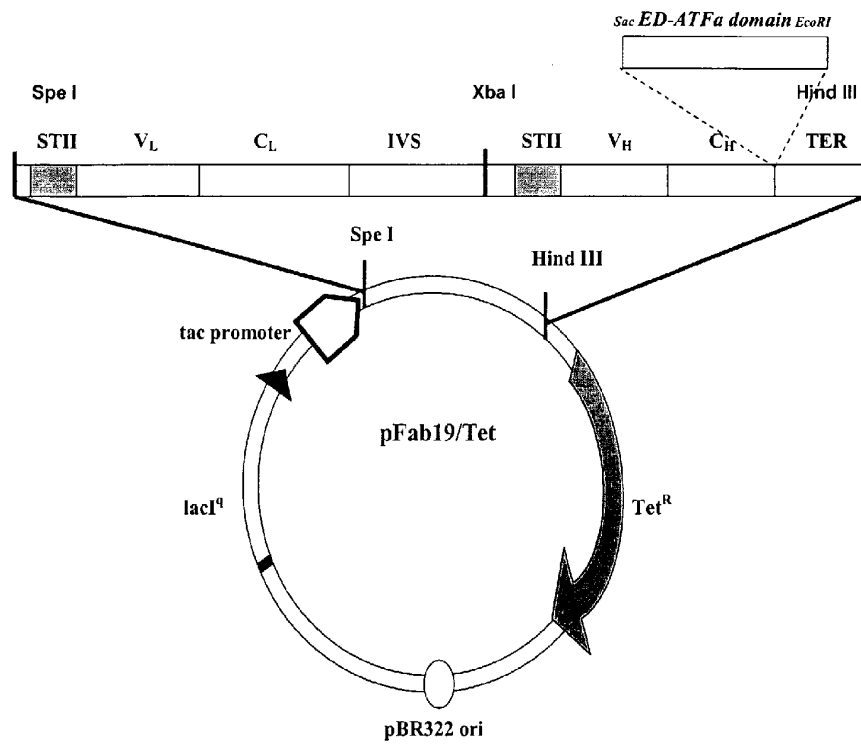
27. The multimeric protein of claim 23, wherein the multimerization polypeptide is a subsequence of the multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.
28. The multimeric protein of claim 23, wherein the multimerization polypeptide has
5 one or more amino acid substitutions, provided that one or more of positions **a** and **d** of a seven residue zipper repeat sequence (**a.b.c.d.e.f.g**) are either leucine, isoleucine or valine, and said chimeric polypeptide being capable of multimerization.
29. The multimeric protein of claim 23, wherein the chimeric polypeptide has a sequence length from about 18-30, 30-50, 50-75, 75-100, 100-150, 150-200, 200-250,
10 250-500 or 500-1000 amino acids.
30. A multimeric protein of claim 23, wherein the heterologous polypeptide is selected from: a binding protein, enzyme, receptor, ligand, nucleic acid binding protein, growth regulatory factor, differentiative factor, or chemotactic factor.
31. A multimeric protein of claim 30, wherein the binding protein comprises an
15 antigen binding polypeptide.
32. A multimeric protein of claim 31, wherein the antigen binding polypeptide comprises at least one antibody variable domain.
33. A multimeric protein of claim 32, wherein the antibody variable domain is human or humanized.
- 20 34. A multimeric protein of claim 31, wherein the antigen binding polypeptide comprises a single chain antibody, Fab, Fab', (Fab')₂ or Fv antibody subsequence.
35. A multimeric protein of claim 31, wherein the antigen binding polypeptide comprises a multispecific or multifunctional antibody.
36. A multimeric protein of claim 31, wherein the antigen binding polypeptide binds
25 to ICAM-I or an epitope thereof.
37. A multimeric protein of claim 31, wherein the antigen binding polypeptide inhibits human rhinovirus infection of a cell that expresses ICAM-I.
38. A multimeric protein of claim 31, wherein the antigen binding polypeptide forms a hetero- or homo-dimer, -trimer, -tetramer or higher order oligomer.
- 30 39. A multimeric protein of claim 38, wherein the K_D of the monomers comprising the homo-dimer, -trimer, tetramer or higher order oligomer is 1×10^{-7} or less.
40. A multimeric protein of claim 38, wherein the K_D of the monomers comprising the homo-dimer, trimer, -tetramer or higher order oligomer is 1×10^{-8} or less.

41. A homo- or hetero-dimer, trimer, tetramer or higher order polypeptide oligomer comprising at least one multimeric protein of claim 23.
42. A multimeric protein of claim 23, wherein the polypeptide contains a linker between the multimerization polypeptide and the heterologous polypeptide.
- 5 43. A multimeric protein of claim 42, wherein the linker comprises a human or humanized amino acid sequence.
44. A multimeric protein of claim 42, wherein the linker comprises an amino acid sequence from about 5 to 20 amino acids.
45. A multimeric protein of claim 42, wherein the linker comprises an amino acid
10 sequence from about 10 to 30 amino acids.
46. A multimeric protein of claim 42, wherein the linker comprises an amino acid sequence from about 25 to 50 amino acids.
47. A multimeric protein of claim 42, wherein the linker comprises an amino acid sequence from about 30 to 60 amino acids.
- 15 48. A multimeric protein of claim 42, wherein the linker comprises an amino acid sequence from about 50 to 75 amino acids.
49. A multimeric protein of claim 42, wherein the linker comprises an amino acid sequence set forth in any one of SEQ ID NO:43, SEQ ID NO:44, SEQ ID NO:45, SEQ ID NO:46 or SEQ ID NO:47.
- 20 50. A multimeric protein comprising an isolated multimerization polypeptide, wherein said isolated multimerization polypeptide comprises at least 28 contiguous amino acids of SEQ ID NO: 154.
51. A multimeric protein of claim 1, wherein said isolated multimerization polypeptide comprises an amino acid sequence according to the formula: X_1 -Ser-Ser- X_2 -
25 Glu-Lys-Lys- X_3 -Glu-Glu- X_4 -Thr-Ser-Gln- X_5 - X_6 -Gln- X_7 - X_8 -Asn-Glu- X_9 -Thr-Leu- X_{10} -Arg-Asn-Glu- X_{11} -Ala-Gln- X_{12} , wherein X_1 , X_2 , X_4 , X_7 , X_9 , X_{10} , X_{11} are selected from Val, Leu or Ile, X_3 is selected from Ala, Val, Leu, or Ile, X_5 is selected from Asn, Val, Leu, or Ile, X_6 is selected from Ile or Gln, X_8 is selected from Arg or Ser, and X_{12} is Ser or is entirely missing.
- 30 52. A pharmaceutical formulation comprising a multimeric protein of any one of claims 1 to 40, 42 to 50 or 67 to 70.

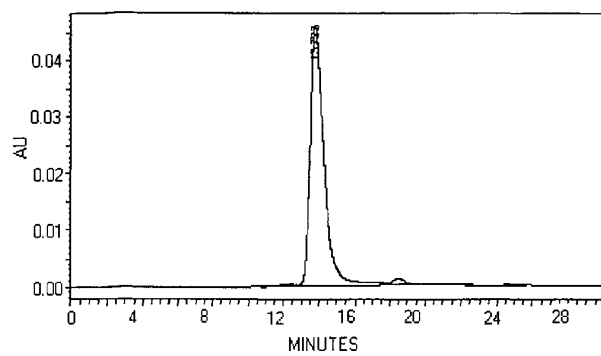
53. A pharmaceutical formulation of claim 52 wherein said multimeric protein is selected from a binding protein, enzyme, receptor, ligand, nucleic acid binding protein, growth regulatory factor, differentiative factor, and chemotactic factor.
54. A pharmaceutical formulation of claim 53 wherein said multimeric protein is a
5 binding protein.
55. A pharmaceutical formulation of claim 54 wherein the binding protein comprises an antigen binding polypeptide selected from an antibody, a single chain antibody, Fab, Fab', (Fab')₂, or Fv antibody subsequence.
56. A pharmaceutical formulation of claim 53 wherein said multimeric protein
10 comprises an enzyme.
57. A pharmaceutical formulation of claim 55 wherein said binding protein is humanized.
58. A pharmaceutical formulation of claim 55 wherein the multimeric protein forms a trimer, tetramer or pentamer, wherein said multimeric protein comprises homo- or hetero-
15 multimers.
59. A pharmaceutical formulation of claim 58 wherein the multimeric protein forms a tetramer.
60. A pharmaceutical formulation of claim 58 wherein the multimeric protein is a homomeric tetramer.
- 20 61. A pharmaceutical formulation of claim 55 in an amount effective to inhibit respiratory syncytial virus infection in a subject.
62. A pharmaceutical formulation of claim 55 in an amount effective to inhibit human rhinovirus infection in a subject.
63. A pharmaceutical formulation of claim 55 for administration to a subject at risk of
25 having asthma.
64. A pharmaceutical formulation of claim 55 that is formulated for localized administration to a subject.
65. A pharmaceutical formulation of claim 55 that is formulated for inhalation or intranasal administration.
- 30 66. A pharmaceutical formulation of claim 55 in the form of a kit comprising instructions for administration.

67. The multimeric protein of claim 23, wherein the multimerization polypeptide has 75% or greater identity to an isolated multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.
68. The multimeric protein of claim 25, wherein the multimerization polypeptide has 85% or greater identity to an isolated multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.
69. The multimeric protein of claim 25, wherein the multimerization polypeptide has 95% or greater identity to an isolated multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.
70. The multimeric protein of claim 25, wherein the multimerization polypeptide has 99% or greater identity to an isolated multimerization polypeptide of claim 1, said chimeric polypeptide being capable of conferring multimerization.
71. A method of treating respiratory syncytial virus infection in a subject said method comprising the step of administering to a subject in need of treatment a multimeric protein of any one of claims 1 to 40, 42 to 51 or 67 to 70 or a pharmaceutical formulation of any one of claims 52 to 66.
72. A method of treating human rhinovirus infection in a subject said method comprising the step of administering to a subject in need of treatment a multimeric protein of any one of claims 1 to 40, 42 to 51 or 67 to 70 or a pharmaceutical formulation of any one of claims 52 to 66.
73. A method of treating asthma in a subject said method comprising the step of administering to a subject need of treatment a multimeric protein of any one of claims 1 to 40, 42 to 51 or 67 to 70 or a pharmaceutical formulation of any one of claims 52 to 66.
74. Use of a multimeric protein of any one of claims 1 to 40, 42 to 51 or 67 to 70 in the manufacture of a medicament for the treatment of respiratory syncytial virus, human rhinovirus, or asthma.
75. A multimeric protein; a homo- or hetero-dimer, trimer, tetramer or higher order polypeptide oligomer; a pharmaceutical formulation; a method of treatment; or use of a multimeric protein, substantially as herein described with reference to any one or more of the examples but excluding comparative examples.

Figure 1



A



B

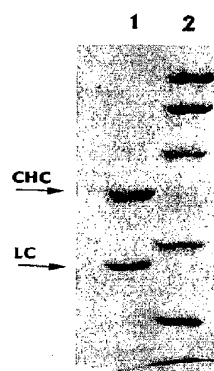
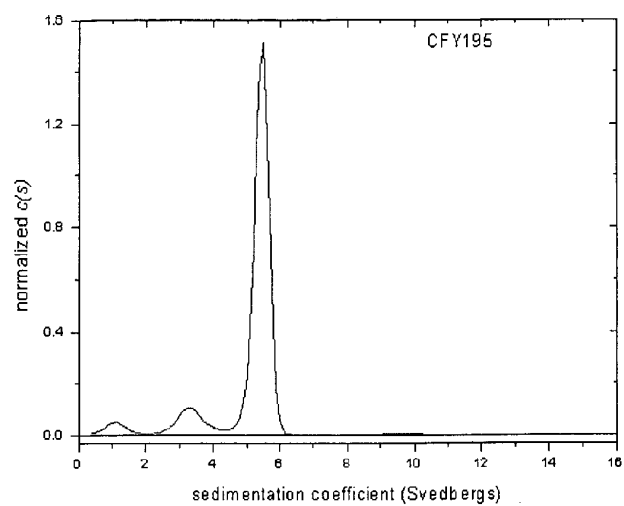
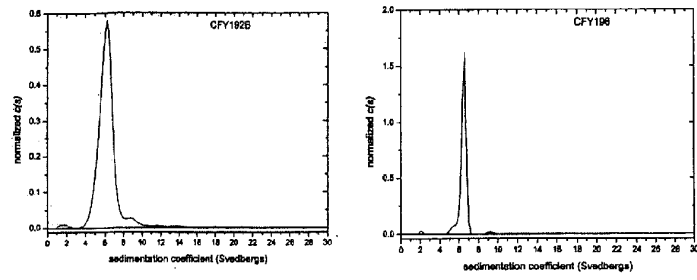
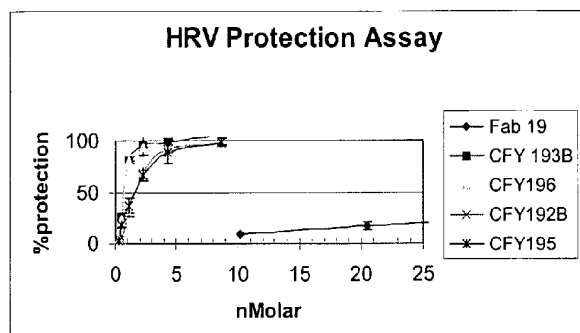


Figure 2

**Figure 3A**



Figures 3B and 3C

**Figure 4**

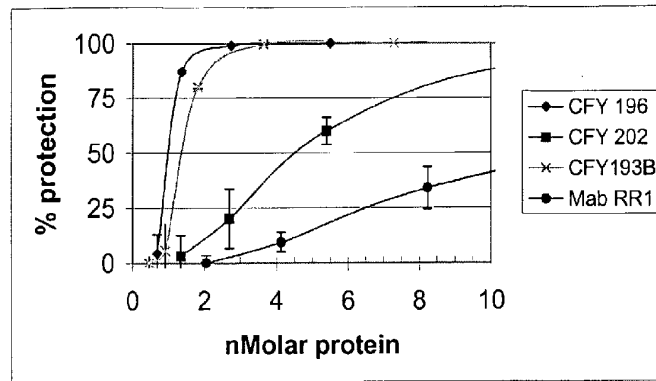
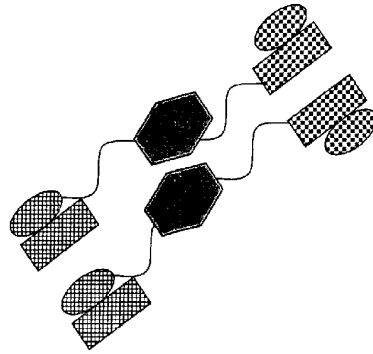


Figure 5

A



B

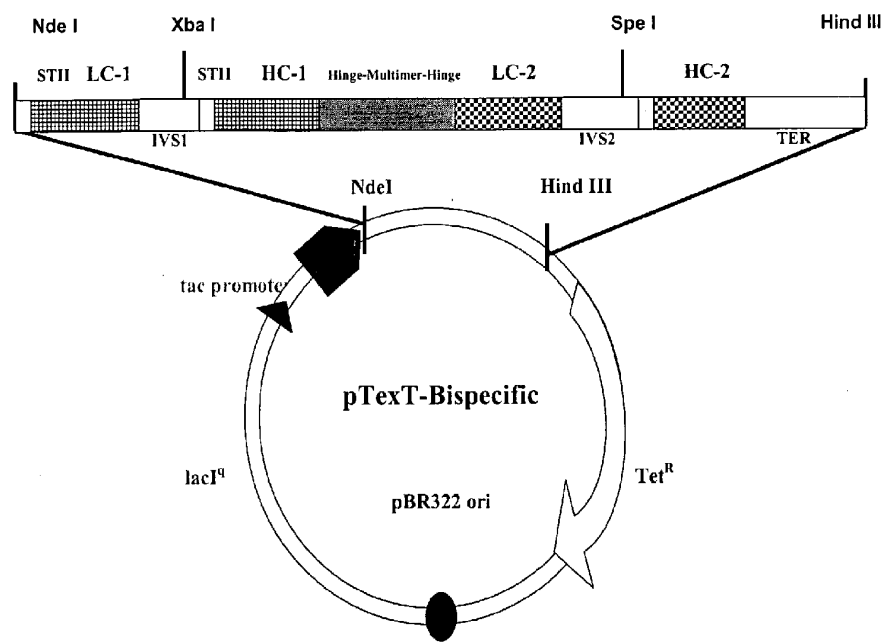


Figure 6

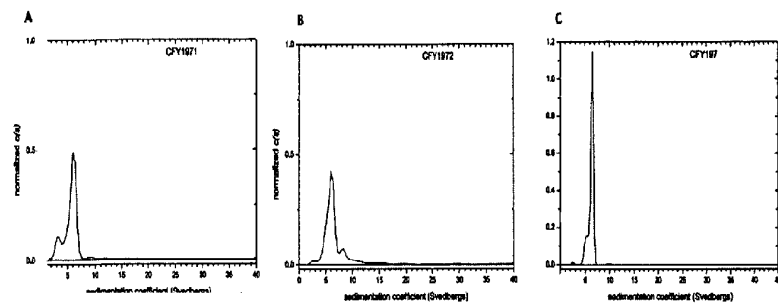
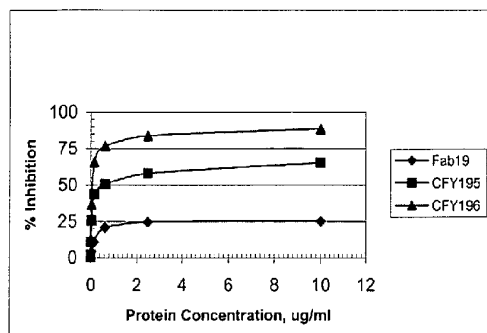


Figure 7

**Figure 8**