PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : C07K 14/00	A2	(11) International Publication Number: WO 00/18796
		(43) International Publication Date: 6 April 2000 (06.04.00)

(21) International Application Number: PCT/US99/22970

(22) International Filing Date: 1 October 1999 (01.10.99)

(30) Priority Data:
09/164,740 1 October 1998 (01.10.98) US

(71) Applicant: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(72) Inventors: LOK, Si; 806 NW 52nd Street, Seattle, WA 98107 (US). SHEPPARD, Paul, O.; 20717 NE 2nd Street, Redmond, WA 98053 (US).

(74) Agent: LUNN, Paul, G.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: SECRETORY PROTEIN – 48

(57) Abstract

Secretory Protein – 48 (Zsig48), polypeptides, polynucleotides encoding said polypeptides, antibodies which specifically bind to Zsig48 and anti–idiotypic antibodies. Zsig48 polypeptides cause leukocytes to proliferate and thus can be used to elevate leukocytes in cancer treatments and in immunosuppressed patients.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

SECRETORY PROTEIN - 48

TECHNICAL FIELD

The present invention relates generally to a new cytokine having diagnostic and therapeutic uses. In particular, the present invention relates to a novel secreted protein designated `Secretory Protein - 48' or , Zsig48 for short, and to nucleic acid molecules encoding Zsig48.

BACKGROUND OF THE INVENTION

Proliferation, maintenance, survival and differentiation of cells of multicellular organisms are controlled by hormones and polypeptide growth factors. These diffusable molecules allow cells to communicate with each other and act in concert to form cells and organs, and to repair and regenerate damaged tissue. Examples of hormones and growth factors include the steroid hormones (e.g. estrogen, testosterone), parathyroid hormone, follicle stimulating hormone, the interleukins, platelet derived growth factor (PDGF), epidermal growth factor (EGF), granulocyte-macrophage colony stimulating factor (GM-CSF), erythropoietin (EPO) and calcitonin.

Hormones and growth factors influence cellular metabolism by binding to proteins. Proteins may be integral membrane proteins that are linked to signaling pathways within the cell, such as second messenger systems. Other classes of proteins are soluble molecules, such as the transcription factors.

Of particular interest are cytokines, molecules that promote the proliferation, maintenance, survival or differentiation of cells. Examples of cytokines include

erythropoietin (EPO), which stimulates the development of red blood cells; thrombopoietin (TPO), which stimulates development of cells of the megakaryocyte lineage; and granulocyte-colony stimulating factor (G-CSF), which stimulates development of neutrophils. These cytokines are useful in restoring normal blood cell levels in patients suffering from anemia or receiving chemotherapy for cancer. The demonstrated *in vivo* activities of these cytokines illustrates the enormous clinical potential of, and need for, other cytokines, cytokine agonists, and cytokine antagonists.

SUMMARY OF THE INVENTION

The present invention fills this need by providing for a secretory protein, designated `Zsig48' which can stimulate the proliferation of peripheral blood mononuclear cells, *i.e.*, T-cells, B-cells and monocytes, (PBMNCs). The present invention also provides Zsig48 polypeptides and Zsig48 fusion proteins, as well as nucleic acid molecules encoding such polypeptides and proteins.

The human Zsig48 polypeptide with signal sequence is comprised of a sequence which is 105 amino acid residues as shown in SEQ ID NOs: 1 and 2. The signal sequence is comprised of amino acid residues 1-26 of SEQ ID NO:2. A mature Zsig48 polypeptide thus is comprised of amino acid residues 27, a leucine, to and including amino acid residue 105, a histidine, of SEQ ID NO:2 also defined by SEQ ID NO:3. An alternative mature Zsig48 polypeptide is comprised of amino acid residues 29, a leucine, to and including amino acid residue 105 of SEQ ID NO: 2, also defined by SEQ ID NO:4. In yet a third alternative signal peptidase cleavage site, the signal sequence is comprised

of amino acid residues 1-40, the mature sequence being comprised of amino acid residue 41, a proline, to and including amino acid residue 105 of SEQ ID NO:2. This mature sequence is also defined by SEQ ID NO:5. Another mature sequence of Zsig48 extends from amino acid residue 26 to and including amino acid residue 105 of SEQ ID NO:2, also represented by SEQ ID NO:16.

The present invention further provides pharmaceutical compositions that comprise such polypeptides, and a pharmaceutically acceptable carrier.

The present invention also includes variant human Zsig48 polypeptides, wherein the variant polypeptide shares an identity with the amino acid sequence of SEQ ID NOs:2, 3, 4, 5 or 16 selected from the group consisting of at least 70% identity, at least 80% identity, at least 90% identity, at least 95% identity, or greater than 95% identity, and wherein any difference between the amino acid sequence of the variant polypeptide and the amino acid sequence of SEQ ID NOs:2, 3, 4 and 5 is due to one or more conservative amino acid substitutions.

An additional embodiment of the present invention relates to a peptide or polypeptide which has the amino acid sequence of an epitope-bearing portion of a Zsig48 polypeptide having an amino acid sequence described above. Peptides or polypeptides having the amino acid sequence of an epitope-bearing portion of a Zsig48 polypeptide of the present invention include portions of such polypeptides with at least nine, preferably at least 15 and more preferably at least 30 to 50 amino acids, although epitope-bearing polypeptides of any length up to and including the entire amino acid sequence of a polypeptide of the present invention described above are also included in the present invention. Also claimed are any of these polypeptides that are fused to another

polypeptide or carrier molecule. Examples of such polypeptides are those polypeptides comprised of one or more of SEQ ID NOs: 8, 9, 10, 11, or 12.

The present invention also provides for isolated polynucleotides which encode the above-described polypeptides.

The present invention also provides isolated nucleic acid molecules that encode a Zsig48 polypeptide, wherein the nucleic acid molecule is selected from the group consisting of (a) a nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, (b) a nucleic acid molecule which encodes isolated polypeptides having an amino acid sequence that is at least 70%, 80%, 90%, 95% or 99% identical to an amino acid sequence selected from the group consisting of the polypeptide defined by SEQ ID NOs:2, 3, 4 or 5; (c) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule consisting of the nucleotide sequence of SEQ ID NO:1, or the complement of SEQ ID NO:1, and (d) a nucleic acid molecule that remains hybridized following stringent wash conditions to a nucleic acid molecule which encodes an isolated polypeptides having an amino acid sequence that is at least 70%, 80%, 90%, 95% or 99% identical to an amino acid sequence selected from the group consisting of the polypeptide defined by SEQ ID NOs:2, 3, 4 and 5. SEQ ID NO:14 shows a genomic sequence of *Zsig48*.

The present invention also provides vectors and expression vectors comprising such nucleic acid molecules, recombinant host cells comprising such vectors and expression vectors, and recombinant viruses comprising such expression vectors. These expression vectors and recombinant host cells can be used to prepare Zsig48 polypeptides. In addition, the present invention provides

pharmaceutical compositions, comprising a pharmaceutically acceptable carrier and at least one of such an expression vector or recombinant virus. Preferably, such pharmaceutical compositions comprise a human *Zsig48* gene, or a variant thereof.

The present invention further contemplates antibodies and antibody fragments that specifically bind with Zsig48 polypeptides. Such antibodies include polyclonal antibodies, murine monoclonal antibodies, humanized antibodies derived from murine monoclonal antibodies, and human monoclonal antibodies. Examples of antibody fragments include $F(ab')_2$, $F(ab)_2$, Fab', Fab, Fv, scFv, and minimal recognition units.

In addition, the presence of Zsig48 polypeptide in a biological sample can be detected by methods that comprise the steps of (a) contacting the biological sample with an antibody, or an antibody fragment, that specifically binds with a polypeptide having the amino acid sequence of either SEQ ID NOs:2 3, 4 or 5, wherein the contacting is performed under conditions that allow the binding of the antibody or antibody fragment to the biological sample, and (b) detecting any of the bound antibody or bound antibody fragment.

The present invention also contemplates isolated nucleic acid molecules comprising a nucleotide sequence that encodes an Zsig48 secretion signal sequence and a nucleotide sequence that encodes a biologically active polypeptide, wherein the Zsig48 secretion signal sequence comprises an amino acid sequence of residues 1 to 26, 1 to 28 or 1 to 40 of SEQ ID NO:2. Illustrative biologically active polypeptides include Factor VIIa, proinsulin, insulin, follicle stimulating hormone, tissue type plasminogen activator, tumor necrosis factor, interleukin, colony stimulating factor, interferon, erythropoietin, and

thrombopoietin. Moreover, the present invention provides fusion proteins comprising a Zsig48 secretion signal sequence and a polypeptide, wherein the Zsig48 secretion signal sequence comprises an amino acid sequence of residues 1 to 26, 1 to 28 or 1 to 40 of SEQ ID NO:2.

The present invention also contemplates anti-idiotype antibodies, or anti-idiotype antibody fragments, that specifically bind with an anti-Zsig48 antibody or antibody fragment, wherein the anti-idiotype antibody, or anti-idiotype antibody fragment, possesses the ability to cause proliferation of T-cells, B-cells or monocytes.

The present invention further includes methods for detecting an alteration in a chromosome containing *Zsig48*. Illustrative chromosomal aberrations at the *Zsig48* gene locus include aneuploidy, gene copy number changes, insertions, deletions, restriction site changes and rearrangements. These aberrations can occur within flanking sequences, including upstream promoter and regulatory regions, and can be manifested as physical alterations within a coding sequence or changes in gene expression level. Such methods are effected by examining the *Zsig48* gene and its gene products. In general, suitable assay methods include molecular genetic techniques known to those in the art, such as restriction fragment length polymorphism analysis, short tandem repeat analysis employing polymerase chain reaction techniques, ligation chain reaction, ribonuclease protection assays, use of single-nucleotide polymorphisms, protein truncation assays, and other genetic linkage techniques known in the art.

In particular, the present invention provides methods for diagnosing an alteration in the *Zsig48* gene of an individual, comprising: (a) amplifying nucleic acid molecules that encode Zsig48 from RNA isolated from a

biological sample of the individual, and (b) detecting a mutation in the amplified nucleic acid molecules, wherein the presence of a mutation indicates an alteration in the Zsig48 gene. Similarly, methods of detecting a chromosome abnormality in a subject comprise: (a) amplifying nucleic acid molecules that encode Zsig48 from RNA isolated from a biological sample of the subject, and (b) detecting a mutation in the amplified nucleic acid molecules, wherein the presence of a mutation indicates a chromosome abnormality. In variations of these methods, the detecting step is performed by comparing the nucleotide sequence of the amplified nucleic acid molecules to the nucleotide sequence of SEQ ID NOs:1. Alternatively, the detecting step can be performed by fractionating the amplified nucleic acid molecules and control nucleic acid molecules that encode the amino acid sequence of SEQ ID NOs:2, and comparing the lengths of the fractionated amplified and control nucleic acid molecules. Exemplary methods for amplification include polymerase chain reaction or reverse transcriptase-polymerase chain reaction.

The present invention further provides for a method for promoting the proliferation of leukocytes comprising bringing the leukocytes into contact with Zsig48.

These and other aspects of the invention will become evident upon reference to the following detailed description and the attached drawings. In addition, various references are identified below and are incorporated by reference in their entirety.

DETAILED DESCRIPTION OF THE INVENTION

The teachings of all the references cited herein are incorporated in their entirety herein by reference.

Definitions

In the description that follows, a number of terms are used extensively. The following definitions are provided to facilitate understanding of the invention.

As used herein, "nucleic acid" or "nucleic acid molecule" refers to polynucleotides, such as deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), oligonucleotides, fragments generated by the polymerase chain reaction (PCR), and fragments generated by any of ligation, scission, endonuclease action, and exonuclease action. Nucleic acid molecules can be composed of monomers that are naturally-occurring nucleotides (such as DNA and RNA), or analogs of naturally-occurring nucleotides (*e.g.*, α-enantiomeric forms of naturally-occurring nucleotides), or a combination of both. Modified nucleotides can have alterations in sugar moieties and/or in pyrimidine or purine base moieties. Sugar modifications include, for example, replacement of one or more hydroxyl groups with halogens, alkyl groups, amines, and azido groups, or sugars can be functionalized as ethers or esters. Moreover, the entire sugar moiety can be replaced with sterically and electronically similar structures, such as aza-sugars and carbocyclic sugar analogs. Examples of modifications in a base moiety include alkylated purines and pyrimidines, acylated purines or pyrimidines, or other well-known heterocyclic substitutes. Nucleic acid monomers can be linked by phosphodiester bonds or analogs of such linkages. Analogs of phosphodiester linkages include phosphorothioate, phosphorodithioate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoranilidate, phosphoramidate, and the like. The

term "nucleic acid molecule" also includes so-called "peptide nucleic acids," which comprise naturally-occurring or modified nucleic acid bases attached to a polyamide backbone. Nucleic acids can be either single stranded or double stranded.

The term "complement of a nucleic acid molecule" refers to a nucleic acid molecule having a complementary nucleotide sequence and reverse orientation as compared to a reference nucleotide sequence. For example, the sequence 5' ATGCACGGG 3' is complementary to 5' CCCGTGCAT 3'.

The term "contig" denotes a nucleic acid molecule that has a contiguous stretch of identical or complementary sequence to another nucleic acid molecule. Contiguous sequences are said to "overlap" a given stretch of a nucleic acid molecule either in their entirety or along a partial stretch of the nucleic acid molecule.

The term "degenerate nucleotide sequence" denotes a sequence of nucleotides that includes one or more degenerate codons as compared to a reference nucleic acid molecule that encodes a polypeptide. Degenerate codons contain different triplets of nucleotides, but encode the same amino acid residue (*i.e.*, GAU and GAC triplets each encode Asp).

The term "structural gene" refers to a nucleic acid molecule that is transcribed into messenger RNA (mRNA), which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

An "isolated nucleic acid molecule" is a nucleic acid molecule that is not integrated in the genomic DNA of an organism. For example, a DNA molecule that encodes a growth factor that has been separated from the genomic DNA

of a cell is an isolated DNA molecule. Another example of an isolated nucleic acid molecule is a chemically-synthesized nucleic acid molecule that is not integrated in the genome of an organism. A nucleic acid molecule that has been isolated from a particular species is smaller than the complete DNA molecule of a chromosome from that species.

A "nucleic acid molecule construct" is a nucleic acid molecule, either single- or double-stranded, that has been modified through human intervention to contain segments of nucleic acid combined and juxtaposed in an arrangement not existing in nature.

"Linear DNA" denotes non-circular DNA molecules having free 5' and 3' ends. Linear DNA can be prepared from closed circular DNA molecules, such as plasmids, by enzymatic digestion or physical disruption.

"Complementary DNA (cDNA)" is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complementary DNA strand. The term "cDNA" also refers to a clone of a cDNA molecule synthesized from an RNA template.

A "promoter" is a nucleotide sequence that directs the transcription of a structural gene. Typically, a promoter is located in the 5' non-coding region of a gene, proximal to the transcriptional start site of a structural gene. Sequence elements within promoters that function in the initiation of transcription are often characterized by consensus nucleotide sequences. These

promoter elements include RNA polymerase binding sites, TATA sequences, CAAT sequences, differentiation-specific elements (DSEs; McGehee *et al.*, *Mol. Endocrinol.* 7:551 (1993)), cyclic AMP response elements (CREs), serum response elements (SREs; Treisman, *Seminars in Cancer Biol. 1*:47 (1990)), glucocorticoid response elements (GREs), and binding sites for other transcription factors, such as CRE/ATF (O'Reilly *et al.*, *J. Biol. Chem. 267*:19938 (1992)), AP2 (Ye *et al.*, *J. Biol. Chem. 269*:25728 (1994)), SP1, cAMP response element binding protein (CREB; Loeken, *Gene Expr. 3*:253 (1993)) and octamer factors (see, in general, Watson *et al.*, eds., *Molecular Biology of the Gene*, 4th ed. (The Benjamin/Cummings Publishing Company, Inc. 1987), and Lemaigre and Rousseau, *Biochem. J. 303*:1 (1994)). If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter. Repressible promoters are also known.

A "core promoter" contains essential nucleotide sequences for promoter function, including the TATA box and start of transcription. By this definition, a core promoter may or may not have detectable activity in the absence of specific sequences that may enhance the activity or confer tissue specific activity.

A "regulatory element" is a nucleotide sequence that modulates the activity of a core promoter. For example, a regulatory element may contain a nucleotide sequence that binds with cellular factors enabling transcription exclusively or preferentially in particular cells, tissues, or organelles. These types of regulatory elements are normally associated with genes that are expressed in a "cell-specific," "tissue-specific," or "organelle-specific" manner. For example, the *Zsig48*

regulatory element preferentially induces gene expression in placenta, kidney, heart or leukocytes.

An "enhancer" is a type of regulatory element that can increase the efficiency of transcription, regardless of the distance or orientation of the enhancer relative to the start site of transcription.

"Heterologous DNA" refers to a DNA molecule, or a population of DNA molecules, that does not exist naturally within a given host cell. DNA molecules heterologous to a particular host cell may contain DNA derived from the host cell species (*i.e.*, endogenous DNA) so long as that host DNA is combined with non-host DNA (*i.e.*, exogenous DNA). For example, a DNA molecule containing a non-host DNA segment encoding a polypeptide operably linked to a host DNA segment comprising a transcription promoter is considered to be a heterologous DNA molecule. Conversely, a heterologous DNA molecule can comprise an endogenous gene operably linked with an exogenous promoter. As another illustration, a DNA molecule comprising a gene derived from a wild-type cell is considered to be heterologous DNA if that DNA molecule is introduced into a mutant cell that lacks the wild-type gene.

A "polypeptide" is a polymer of amino acid residues joined by peptide bonds, whether produced naturally or synthetically. Polypeptides of less than about 10 amino acid residues are commonly referred to as "peptides."

A "protein" is a macromolecule comprising one or more polypeptide chains. A protein may also comprise non-peptidic components, such as carbohydrate groups. Carbohydrates and other non-peptidic substituents may be added to a protein by the cell in which the protein is

produced, and will vary with the type of cell. Proteins are defined herein in terms of their amino acid backbone structures; substituents such as carbohydrate groups are generally not specified, but may be present nonetheless.

A peptide or polypeptide encoded by a non-host DNA molecule is a "heterologous" peptide or polypeptide.

An "integrated genetic element" is a segment of DNA that has been incorporated into a chromosome of a host cell after that element is introduced into the cell through human manipulation. Within the present invention, integrated genetic elements are most commonly derived from linearized plasmids that are introduced into the cells by electroporation or other techniques. Integrated genetic elements are passed from the original host cell to its progeny.

A "cloning vector" is a nucleic acid molecule, such as a plasmid, cosmid, or bacteriophage, that has the capability of replicating autonomously in a host cell. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites that allow insertion of a nucleic acid molecule in a determinable fashion without loss of an essential biological function of the vector, as well as nucleotide sequences encoding a marker gene that is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

An "expression vector" is a nucleic acid molecule encoding a gene that is expressed in a host cell. Typically, an expression vector comprises a transcription promoter, a gene, and a transcription terminator. Gene expression is usually placed under the control of a promoter, and such a gene is said to be "operably linked

to" the promoter. Similarly, a regulatory element and a core promoter are operably linked if the regulatory element modulates the activity of the core promoter.

A "recombinant host" is a cell that contains a heterologous nucleic acid molecule, such as a cloning vector or expression vector. In the present context, an example of a recombinant host is a cell that produces Zsig48 from an expression vector. In contrast, Zsig48 can be produced by a cell that is a "natural source" of Zsig48, and that lacks an expression vector.

"Integrative transformants" are recombinant host cells, in which heterologous DNA has become integrated into the genomic DNA of the cells.

A "fusion protein" is a hybrid protein expressed by a nucleic acid molecule comprising nucleotide sequences of at least two genes. For example, a fusion protein can comprise at least part of an Zsig48 polypeptide fused with a polypeptide that binds an affinity matrix. Such a fusion protein provides a means to isolate large quantities of Zsig48 using affinity chromatography.

The term "receptor" denotes a cell-associated protein that binds to a bioactive molecule termed a "ligand." This interaction mediates the effect of the ligand on the cell. Receptors can be membrane bound, cytosolic or nuclear; monomeric (*e.g.*, thyroid stimulating hormone receptor, beta-adrenergic receptor) or multimeric (*e.g.*, PDGF receptor, growth hormone receptor, IL-3 receptor, GM-CSF receptor, G-CSF receptor, erythropoietin receptor and IL-6 receptor). Membrane-bound receptors are characterized by a multi-domain structure comprising an extracellular ligand-binding domain and an intracellular effector domain that is typically involved in signal transduction. In certain membrane-bound receptors, the

extracellular ligand-binding domain and the intracellular effector domain are located in separate polypeptides that comprise the complete functional receptor.

In general, the binding of ligand to receptor results in a conformational change in the receptor that causes an interaction between the effector domain and other molecule(s) in the cell, which in turn leads to an alteration in the metabolism of the cell. Metabolic events that are often linked to receptor-ligand interactions include gene transcription, phosphorylation, dephosphorylation, increases in cyclic AMP production, mobilization of cellular calcium, mobilization of membrane lipids, cell adhesion, hydrolysis of inositol lipids and hydrolysis of phospholipids.

The term "secretory signal sequence" denotes a DNA sequence that encodes a peptide (a "secretory peptide") that, as a component of a larger polypeptide, directs the larger polypeptide through a secretory pathway of a cell in which it is synthesized. The larger polypeptide is commonly cleaved to remove the secretory peptide during transit through the secretory pathway.

An "isolated polypeptide" is a polypeptide that is essentially free from contaminating cellular components, such as carbohydrate, lipid, or other proteinaceous impurities associated with the polypeptide in nature. Typically, a preparation of isolated polypeptide contains the polypeptide in a highly purified form, *i.e.*, at least about 80% pure, at least about 90% pure, at least about 95% pure, greater than 95% pure, or greater than 99% pure. One way to show that a particular protein preparation contains an isolated polypeptide is by the appearance of a single band following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis of the protein preparation and Coomassie Brilliant Blue staining

of the gel. However, the term "isolated" does not exclude the presence of the same polypeptide in alternative physical forms, such as dimers or alternatively glycosylated or derivatized forms.

The terms "amino-terminal or N-terminal" and "carboxyl-terminal or C-terminal" are used herein to denote positions within polypeptides. Where the context allows, these terms are used with reference to a particular sequence or portion of a polypeptide to denote proximity or relative position. For example, a certain sequence positioned carboxyl-terminal to a reference sequence within a polypeptide is located proximal to the carboxyl terminus of the reference sequence, but is not necessarily at the carboxyl terminus of the complete polypeptide.

The term "expression" refers to the biosynthesis of a gene product. For example, in the case of a structural gene, expression involves transcription of the structural gene into mRNA and the translation of mRNA into one or more polypeptides.

The term "splice variant" is used herein to denote alternative forms of RNA transcribed from a gene. Splice variation arises naturally through use of alternative splicing sites within a transcribed RNA molecule, or less commonly between separately transcribed RNA molecules, and may result in several mRNAs transcribed from the same gene. Splice variants may encode polypeptides having altered amino acid sequence. The term splice variant is also used herein to denote a polypeptide encoded by a splice variant of an mRNA transcribed from a gene.

As used herein, the term "immunomodulator" includes cytokines, stem cell growth factors,

lymphotoxins, co-stimulatory molecules, hematopoietic factors, and synthetic analogs of these molecules.

The term "complement/anti-complement pair" denotes non-identical moieties that form a non-covalently associated, stable pair under appropriate conditions. For instance, biotin and avidin (or streptavidin) are prototypical members of a complement/anti-complement pair. Other exemplary complement/anti-complement pairs include receptor/ligand pairs, antibody/antigen (or hapten or epitope) pairs, sense/antisense polynucleotide pairs, and the like. Where subsequent dissociation of the complement/anti-complement pair is desirable, the complement/anti-complement pair preferably has a binding affinity of less than 10^9 M^{-1}.

An "anti-idiotype antibody" is an antibody that binds with the variable region domain of an immunoglobulin. In the present context, an anti-idiotype antibody binds with the variable region of an anti-Zsig48-antibody, and thus, an anti-idiotype antibody mimics an epitope of Zsig48.

An "antibody fragment" is a portion of an antibody such as $F(ab')_2$, $F(ab)_2$, Fab', Fab, and the like. Regardless of structure, an antibody fragment binds with the same antigen that is recognized by the intact antibody. For example, a Zsig48 monoclonal antibody fragment binds with an epitope of Zsig48.

The term "antibody fragment" also includes a synthetic or a genetically engineered polypeptide that binds to a specific antigen, such as polypeptides consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, recombinant single chain polypeptide molecules in which light and heavy variable regions are

connected by a peptide linker ("scFv proteins"), and minimal recognition units consisting of the amino acid residues that mimic the hypervariable region.

A "chimeric antibody" is a recombinant protein that contains the variable domains and complementary determining regions derived from a rodent antibody, while the remainder of the antibody molecule is derived from a human antibody.

"Humanized antibodies" are recombinant proteins in which murine complementarity determining regions of a monoclonal antibody have been transferred from heavy and light variable chains of the murine immunoglobulin into a human variable domain.

As used herein, a "therapeutic agent" is a molecule or atom which is conjugated to an antibody moiety to produce a conjugate which is useful for therapy. Examples of therapeutic agents include drugs, toxins, immunomodulators, chelators, boron compounds, photoactive agents or dyes, and radioisotopes.

A "detectable label" is a molecule or atom which can be conjugated to an antibody moiety to produce a molecule useful for diagnosis. Examples of detectable labels include chelators, photoactive agents, radioisotopes, fluorescent agents, paramagnetic ions, or other marker moieties.

The term "affinity tag" is used herein to denote a polypeptide segment that can be attached to a second polypeptide to provide for purification or detection of the second polypeptide or provide sites for attachment of the second polypeptide to a substrate. In principal, any peptide or protein for which an antibody or other specific binding agent is available can be used as an affinity tag.

Affinity tags include a poly-histidine tract, protein A (Nilsson *et al.*, *EMBO J.* 4:1075 (1985); Nilsson *et al.*, *Methods Enzymol.* 198:3 (1991)), glutathione S transferase (Smith and Johnson, *Gene* 67:31 (1988)), Glu-Glu affinity tag (Grussenmeyer *et al.*, *Proc. Natl. Acad. Sci. USA* 82:7952 (1985)), substance P, FLAG peptide (Hopp *et al.*, *Biotechnology* 6:1204 (1988)), streptavidin binding peptide, or other antigenic epitope or binding domain. See, in general, Ford *et al.*, *Protein Expression and Purification* 2:95 (1991). DNAs encoding affinity tags are available from commercial suppliers (*e.g.*, Pharmacia Biotech, Piscataway, NJ).

A "naked antibody" is an entire antibody, as opposed to an antibody fragment, which is not conjugated with a therapeutic agent. Naked antibodies include both polyclonal and monoclonal antibodies, as well as certain recombinant antibodies, such as chimeric and humanized antibodies.

As used herein, the term "antibody component" includes both an entire antibody and an antibody fragment.

An "immunoconjugate" is a conjugate of an antibody component with a therapeutic agent or a detectable label.

As used herein, the term "antibody fusion protein" refers to a recombinant molecule that comprises an antibody component and a therapeutic agent. Examples of therapeutic agents suitable for such fusion proteins include immunomodulators ("antibody-immunomodulator fusion protein") and toxins ("antibody-toxin fusion protein").

A "tumor associated antigen" is a protein normally not expressed, or expressed at lower levels, by a normal counterpart cell. Examples of tumor associated

antigens include alpha-fetoprotein, carcinoembryonic antigen, and Her-2/neu. Many other illustrations of tumor associated antigens are known to those of skill in the art. See, for example, Urban *et al.*, *Ann. Rev. Immunol.* *10*:617 (1992).

As used herein, an "infectious agent" denotes both microbes and parasites. A "microbe" includes viruses, bacteria, rickettsia, mycoplasma, protozoa, fungi and like microorganisms. A "parasite" denotes infectious, generally microscopic or very small multicellular invertebrates, or ova or juvenile forms thereof, which are susceptible to immune-mediated clearance or lytic or phagocytic destruction, such as malarial parasites, spirochetes, and the like.

An "infectious agent antigen" is an antigen associated with an infectious agent.

A "target polypeptide" or a "target peptide" is an amino acid sequence that comprises at least one epitope, and that is expressed on a target cell, such as a tumor cell, or a cell that carries an infectious agent antigen. T cells recognize peptide epitopes presented by a major histocompatibility complex molecule to a target polypeptide or target peptide and typically lyse the target cell or recruit other immune cells to the site of the target cell, thereby killing the target cell.

An "antigenic peptide" is a peptide which will bind a major histocompatibility complex molecule to form an MHC-peptide complex which is recognized by a T cell, thereby inducing a cytotoxic lymphocyte response upon presentation to the T cell. Thus, antigenic peptides are capable of binding to an appropriate major histocompatibility complex molecule and inducing a cytotoxic T cells response, such as cell lysis or specific

cytokine release against the target cell which binds or expresses the antigen. The antigenic peptide can be bound in the context of a class I or class II major histocompatibility complex molecule, on an antigen presenting cell or on a target cell.

In eukaryotes, RNA polymerase II catalyzes the transcription of a structural gene to produce mRNA. A nucleic acid molecule can be designed to contain an RNA polymerase II template in which the RNA transcript has a sequence that is complementary to that of a specific mRNA. The RNA transcript is termed an "anti-sense RNA" and a nucleic acid molecule that encodes the anti-sense RNA is termed an "anti-sense gene." Anti-sense RNA molecules are capable of binding to mRNA molecules, resulting in an inhibition of mRNA translation.

An "anti-sense oligonucleotide specific for Zsig48" or an "*Zsig48* anti-sense oligonucleotide" is an oligonucleotide having a sequence (a) capable of forming a stable triplex with a portion of a *Zsig48* gene, or (b) capable of forming a stable duplex with a portion of an mRNA transcript of the *Zsig48* gene.

A "ribozyme" is a nucleic acid molecule that contains a catalytic center. The term includes RNA enzymes, self-splicing RNAs, self-cleaving RNAs, and nucleic acid molecules that perform these catalytic functions. A nucleic acid molecule that encodes a ribozyme is termed a "ribozyme gene."

An "external guide sequence" is a nucleic acid molecule that directs the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, resulting in the cleavage of the mRNA by RNase P. A nucleic acid molecule that encodes an external guide sequence is termed an "external guide sequence gene."

The term "variant human *Zsig48* gene" refers to nucleic acid molecules that encode a polypeptide having an amino acid sequence that is a modification of SEQ ID NO:2. Such variants include naturally-occurring polymorphisms of *Zsig48* genes, as well as synthetic genes that contain conservative amino acid substitutions of the amino acid sequence of SEQ ID NOs:2, 3, 4 or 5. Additional variant forms of *Zsig48* genes are nucleic acid molecules that contain insertions or deletions of the nucleotide sequences described herein. A variant *Zsig48* gene can be identified by determining whether the gene hybridizes with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, or its complement, under stringent conditions.

Alternatively, variant *Zsig48*genes can be identified by sequence comparison. Two amino acid sequences have "100% amino acid sequence identity" if the amino acid residues of the two amino acid sequences are the same when aligned for maximal correspondence. Similarly, two nucleotide sequences have "100% nucleotide sequence identity" if the nucleotide residues of the two nucleotide sequences are the same when aligned for maximal correspondence. Sequence comparisons can be performed using standard software programs such as those included in the LASERGENE bioinformatics computing suite, which is produced by DNASTAR (Madison, Wisconsin). Other methods for comparing two nucleotide or amino acid sequences by determining optimal alignment are well-known to those of skill in the art (see, for example, Peruski and Peruski, *The Internet and the New Biology: Tools for Genomic and Molecular Research* (ASM Press, Inc. 1997), Wu et al. (eds.), "Information Superhighway and Computer Databases of Nucleic Acids and Proteins," in *Methods in Gene Biotechnology*, pages 123-151 (CRC Press, Inc. 1997), and Bishop (ed.), *Guide to Human Genome Computing*, 2nd Edition

(Academic Press, Inc. 1998)). Particular methods for determining sequence identity are described below.

Regardless of the particular method used to identify a variant *Zsig48* gene or variant Zsig48 polypeptide, a variant gene or polypeptide encoded by a variant gene is functionally characterized by its ability to bind specifically to an anti-Zsig48 antibody.

The term "allelic variant" is used herein to denote any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in phenotypic polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequence.

The term "ortholog" denotes a polypeptide or protein obtained from one species that is the functional counterpart of a polypeptide or protein from a different species. Sequence differences among orthologs are the result of speciation.

"Paralogs" are distinct but structurally related proteins made by an organism. Paralogs are believed to arise through gene duplication. For example, α-globin, β-globin, and myoglobin are paralogs of each other.

Due to the imprecision of standard analytical methods, molecular weights and lengths of polymers are understood to be approximate values. When such a value is expressed as "about" X or "approximately" X, the stated value of X will be understood to be accurate to ±10%.

Introduction

Zsig48 as shown in SEQ ID NO:2 has a single disulfide bond between the cysteine residue 48 and cysteine residue 81, and an amphiphilic helix, *i.e.* a helix which contains both a hydrophilic and a hydrophobic face, from amino acid residue 88, a proline, to amino acid residue 105, a histidine of SEQ ID NO:2. Thus, the structure of Zsig48, especially the C-terminal helix suggests that Zsig48 may be a peptide ligand for the G-protein coupled 7-transmembrane domain (TMD) class of receptors. G-protein -linked receptors act indirectly to regulate the activity of a separate plasma membrane-bound target protein, which can be an enzyme or an ion channel. The interaction between the receptor and the target protein is mediated by a third protein, called a trimeric GTP-binding regulatory protein, *i.e.* a G protein. Thus, Zsig48 binds to its G-protein coupled 7-TMD receptor. The receptor then converts this extracellular event into one or intracellular signals that alter the behavior of the target cell. Accordingly, general utility claims for ligands of this receptor class would be applicable for Zsig48. These would include: alteration of cellular metabolism and secretion, ion transport, cell proliferation, differentiation.

It has been determined that Zsig48 is highly expressed in the heart, placenta, and kidney. That Zsig48 is highly expressed in the placenta would suggests that the polypeptide may function in fetal development directly or indirectly by providing support functions to the placenta such as blood vessel development or the transport of metabolites. Transcript appearance in the heart further suggests that Zsig48 may be important as a autocrine or paracrine regulator of myocardial function. It has been

further determined by RT-PCR that Zsig48 is expressed in leukocytes.

Example 3 below shows that Zsig48 can be used to promote proliferation of leukocytes in an individual.

Production of the Human Zsig48 Gene

Polynucleotides, generally a cDNA sequence, of the present invention encode the described polypeptides herein. A cDNA sequence which encodes a polypeptide of the present invention is comprised of a series of codons, each amino acid residue of the polypeptide being encoded by a codon and each codon being comprised of three nucleotides. The amino acid residues are encoded by their respective codons as follows.

Alanine (Ala) is encoded by GCA, GCC, GCG or GCT;

Cysteine (Cys) is encoded by TGC or TGT;

Aspartic acid (Asp) is encoded by GAC or GAT;

Glutamic acid (Glu) is encoded by GAA or GAG;

Phenylalanine (Phe) is encoded by TTC or TTT;

Glycine (Gly) is encoded by GGA, GGC, GGG or GGT;

Histidine (His) is encoded by CAC or CAT;

Isoleucine (Ile) is encoded by ATA, ATC or ATT;

Lysine (Lys) is encoded by AAA, or AAG;

Leucine (Leu) is encoded by TTA, TTG, CTA, CTC, CTG or CTT;

Methionine (Met) is encoded by ATG;

Asparagine (Asn) is encoded by AAC or AAT;

Proline (Pro) is encoded by CCA, CCC, CCG or CCT;

Glutamine (Gln) is encoded by CAA or CAG;

Arginine (Arg) is encoded by AGA, AGG, CGA, CGC, CGG or CGT;

Serine (Ser) is encoded by AGC, AGT, TCA, TCC, TCG or TCT;

Threonine (Thr) is encoded by ACA, ACC, ACG or ACT;

Valine (Val) is encoded by GTA, GTC, GTG or GTT;

Tryptophan (Trp) is encoded by TGG; and

Tyrosine (Tyr) is encoded by TAC or TAT.

It is to be recognized that according to the present invention, when a polynucleotide is claimed as described herein, it is understood that what is claimed are both the sense strand, the anti-sense strand, and the DNA as double-stranded having both the sense and anti-sense strand annealed together by their respective hydrogen bonds. Also claimed is the messenger RNA (mRNA) which encodes the polypeptides of the president invention, and which mRNA is encoded by the cDNA described herein. Messenger RNA (mRNA) will encode a polypeptide using the same codons as those defined herein, with the exception that each thymine nucleotide (T) is replaced by a uracil nucleotide (U).

Nucleic acid molecules encoding a human *Zsig48* gene can be obtained by screening a human cDNA or genomic library using polynucleotide probes based upon SEQ ID NO:1. These techniques are standard and well-established.

As an illustration, a nucleic acid molecule that encodes a human *Zsig48* gene can be isolated from a human cDNA library. In this case, the first step would be to prepare the cDNA library by isolating RNA from placenta, kidney, leukocytes or heart tissue using methods well-known to those of skill in the art. In general, RNA isolation techniques must provide a method for breaking cells, a means of inhibiting RNase-directed degradation of RNA, and a method of separating RNA from DNA, protein, and polysaccharide contaminants. For example, total RNA can be

isolated by freezing tissue in liquid nitrogen, grinding the frozen tissue with a mortar and pestle to lyse the cells, extracting the ground tissue with a solution of phenol/chloroform to remove proteins, and separating RNA from the remaining impurities by selective precipitation with lithium chloride (see, for example, Ausubel *et al.* (eds.), *Short Protocols in Molecular Biology, 3rd Edition*, pages 4-1 to 4-6 (John Wiley & Sons 1995) ["Ausubel (1995)"]; Wu *et al.*, *Methods in Gene Biotechnology*, pages 33-41 (CRC Press, Inc. 1997) ["Wu (1997)"]).

Alternatively, total RNA can be isolated from placental, leukocyte, kidney or heart tissue by extracting ground tissue with guanidinium isothiocyanate, extracting with organic solvents, and separating RNA from contaminants using differential centrifugation (see, for example, Chirgwin *et al.*, *Biochemistry 18*:52 (1979); Ausubel (1995) at pages 4-1 to 4-6; Wu (1997) at pages 33-41).

In order to construct a cDNA library, poly(A)$^+$ RNA must be isolated from a total RNA preparation. Poly(A)$^+$ RNA can be isolated from total RNA using the standard technique of oligo(dT)-cellulose chromatography (see, for example, Aviv and Leder, *Proc. Nat'l Acad. Sci. USA 69*:1408 (1972); Ausubel (1995) at pages 4-11 to 4-12).

Double-stranded cDNA molecules are synthesized from poly(A)$^+$ RNA using techniques well-known to those in the art. (see, for example, Wu (1997) at pages 41-46). Moreover, commercially available kits can be used to synthesize double-stranded cDNA molecules. For example, such kits are available from Life Technologies, Inc. (Gaithersburg, MD), CLONTECH Laboratories, Inc. (Palo Alto, CA), Promega Corporation (Madison, WI) and STRATAGENE (La Jolla, CA).

Various cloning vectors are appropriate for the construction of a cDNA library. For example, a cDNA library can be prepared in a vector derived from bacteriophage, such as a λgt10 vector. See, for example, Huynh *et al.*, "Constructing and Screening cDNA Libraries in λgt10 and λgt11," in *DNA Cloning: A Practical Approach Vol. I*, Glover (ed.), page 49 (IRL Press, 1985); Wu (1997) at pages 47-52.

Alternatively, double-stranded cDNA molecules can be inserted into a plasmid vector, such as a pBLUESCRIPT vector (STRATAGENE; La Jolla, CA), a LAMDAGEM-4 (Promega Corp.) or other commercially available vectors. Suitable cloning vectors also can be obtained from the American Type Culture Collection (Manassas, VA).

To amplify the cloned cDNA molecules, the cDNA library is inserted into a prokaryotic host, using standard techniques. For example, a cDNA library can be introduced into competent *E. coli* DH5 cells, which can be obtained, for example, from Life Technologies, Inc. (Gaithersburg, MD).

A human genomic library can be prepared by means well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327). Genomic DNA can be isolated by lysing tissue with the detergent Sarkosyl, digesting the lysate with proteinase K, clearing insoluble debris from the lysate by centrifugation, precipitating nucleic acid from the lysate using isopropanol, and purifying resuspended DNA on a cesium chloride density gradient.

DNA fragments that are suitable for the production of a genomic library can be obtained by the random shearing of genomic DNA or by the partial digestion of genomic DNA with restriction endonucleases. Genomic DNA

fragments can be inserted into a vector, such as a bacteriophage or cosmid vector, in accordance with conventional techniques, such as the use of restriction enzyme digestion to provide appropriate termini, the use of alkaline phosphatase treatment to avoid undesirable joining of DNA molecules, and ligation with appropriate ligases. Techniques for such manipulation are well-known in the art (see, for example, Ausubel (1995) at pages 5-1 to 5-6; Wu (1997) at pages 307-327).

Nucleic acid molecules that encode a human *Zsig48* gene can also be obtained using the polymerase chain reaction (PCR) with oligonucleotide primers having nucleotide sequences that are based upon the nucleotide sequences of the human *Zsig48* gene, as described herein. General methods for screening libraries with PCR are provided by, for example, Yu *et al.*, "Use of the Polymerase Chain Reaction to Screen Phage Libraries," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 211-215 (Humana Press, Inc. 1993). Moreover, techniques for using PCR to isolate related genes are described by, for example, Preston, "Use of Degenerate Oligonucleotide Primers and the Polymerase Chain Reaction to Clone Gene Family Members," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 317-337 (Humana Press, Inc. 1993). Alternatively, human genomic libraries can be obtained from commercial sources such as Research Genetics (Huntsville, AL) and the American Type Culture Collection (Manassas, VA). A library containing cDNA or genomic clones can be screened with one or more polynucleotide probes based upon SEQ ID NO:1, using standard methods (see, for example, Ausubel (1995) at pages 6-1 to 6-11).

Anti-Zsig48 antibodies, produced as described below, can also be used to isolate DNA sequences that

encode human *Zsig48* genes from cDNA libraries. For example, the antibodies can be used to screen λgt11 expression libraries, or the antibodies can be used for immunoscreening following hybrid selection and translation (see, for example, Ausubel (1995) at pages 6-12 to 6-16; Margolis *et al.*, "Screening λ expression libraries with antibody and protein probes," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 1-14 (Oxford University Press 1995)).

As an alternative, a *Zsig48* gene can be obtained by synthesizing nucleic acid molecules using mutually priming long oligonucleotides and the nucleotide sequences described herein (see, for example, Ausubel (1995) at pages 8-8 to 8-9). Established techniques using the polymerase chain reaction provide the ability to synthesize DNA molecules at least two kilobases in length (Adang *et al.*, *Plant Molec. Biol. 21*:1131 (1993), Bambot *et al.*, *PCR Methods and Applications 2*:266 (1993), Dillon *et al.*, "Use of the Polymerase Chain Reaction for the Rapid Construction of Synthetic Genes," in *Methods in Molecular Biology, Vol. 15: PCR Protocols: Current Methods and Applications*, White (ed.), pages 263-268, (Humana Press, Inc. 1993), and Holowachuk *et al.*, *PCR Methods Appl.* 4:299 (1995)).

The nucleic acid molecules of the present invention can also be synthesized with "DNA synthesizers" using protocols such as the phosphoramidite method. If chemically-synthesized double stranded DNA is required for an application such as the synthesis of a gene or a gene fragment, then each complementary strand is made separately. The production of short genes (60 to 80 base pairs) is technically straightforward and can be accomplished by synthesizing the complementary strands and then annealing them. For the production of longer genes (>300 base pairs), however, special strategies may be

required, because the coupling efficiency of each cycle during chemical DNA synthesis is seldom 100%. To overcome this problem, synthetic genes (double-stranded) are assembled in modular form from single-stranded fragments that are from 20 to 100 nucleotides in length. For reviews on polynucleotide synthesis, see, for example, Glick and Pasternak, *Molecular Biotechnology, Principles and Applications of Recombinant DNA* (ASM Press 1994), Itakura *et al.*, *Annu. Rev. Biochem.* 53:323 (1984), and Climie *et al.*, *Proc. Nat'l Acad. Sci. USA* 87:633 (1990).

The sequence of a *Zsig48* cDNA or *Zsig48* genomic fragment can be determined using standard methods. Moreover, the identification of genomic fragments containing a *Zsig48* promoter or regulatory element can be achieved using well-established techniques, such as deletion analysis (see, generally, Ausubel (1995)).

Cloning of 5' flanking sequences also facilitates production of Zsig48 proteins by "gene activation," following the methods disclosed in U.S. Patent No. 5,641,670. Briefly, expression of an endogenous *Zsig48* gene in a cell is altered by introducing into the *Zsig48* locus a DNA construct comprising at least a targeting sequence, a regulatory sequence, an exon, and an unpaired splice donor site. The targeting sequence is a *Zsig48* 5' non-coding sequence that permits homologous recombination of the construct with the endogenous *Zsig48* locus, wherein the sequences within the construct become operably linked with the endogenous *Zsig48* coding sequence. In this way, an endogenous *Zsig48* promoter can be replaced or supplemented with other regulatory sequences to provide enhanced, tissue-specific, or otherwise regulated expression.

Production of Zsig48 Gene Variants

The present invention provides a variety of nucleic acid molecules, including DNA and RNA molecules, that encode the Zsig48 polypeptides disclosed herein. Those skilled in the art will readily recognize that, in view of the degeneracy of the genetic code, considerable sequence variation is possible among these polynucleotide molecules.

Different species can exhibit "preferential codon usage." In general, see, Grantham *et al.*, *Nuc. Acids Res. 8*:1893 (1980), Haas *et al. Curr. Biol. 6*:315 (1996), Wain-Hobson *et al.*, *Gene 13*:355 (1981), Grosjean and Fiers, *Gene 18*:199 (1982), Holm, *Nuc. Acids Res. 14*:3075 (1986), Ikemura, *J. Mol. Biol. 158*:573 (1982), Sharp and Matassi, *Curr. Opin. Genet. Dev. 4*:851 (1994), Kane, *Curr. Opin. Biotechnol. 6*:494 (1995), and Makrides, *Microbiol. Rev. 60*:512 (1996). As used herein, the term "preferential codon usage" or "preferential codons" is a term of art referring to protein translation codons that are most frequently used in cells of a certain species, thus favoring one or a few representatives of the possible codons encoding each amino acid. For example, the amino acid Threonine (Thr) may be encoded by ACA, ACC, ACG, or ACT, but in mammalian cells ACC is the most commonly used codon; in other species, for example, insect cells, yeast, viruses or bacteria, different Thr codons may be preferential. Preferential codons for a particular species can be introduced into the polynucleotides of the present invention by a variety of methods known in the art. Introduction of preferential codon sequences into recombinant DNA can, for example, enhance production of the protein by making protein translation more efficient within a particular cell type or species.

The present invention further provides variant polypeptides and nucleic acid molecules that represent

counterparts from other species (orthologs). These species include, but are not limited to mammalian, avian, amphibian, reptile, fish, insect and other vertebrate and invertebrate species. Of particular interest are Zsig48 polypeptides from other mammalian species, including murine, porcine, ovine, bovine, canine, feline, equine, and other primate polypeptides. Orthologs of human Zsig48 can be cloned using information and compositions provided by the present invention in combination with conventional cloning techniques. For example, a cDNA can be cloned using mRNA obtained from a tissue or cell type that expresses Zsig48 as disclosed herein. Suitable sources of mRNA can be identified by probing northern blots with probes designed from the sequences disclosed herein. A library is then prepared from mRNA of a positive tissue or cell line.

A Zsig48-encoding cDNA can be isolated by a variety of methods, such as by probing with a complete or partial human cDNA or with one or more sets of degenerate probes based on the disclosed sequences. A cDNA can also be cloned using the polymerase chain reaction with primers designed from the representative human Zsig48 sequences disclosed herein. Within an additional method, the cDNA library can be used to transform or transfect host cells, and expression of the cDNA of interest can be detected with an antibody to Zsig48 polypeptide. Similar techniques can also be applied to the isolation of genomic clones, and to the isolation of nucleic molecules that encode murine Zsig48.

Those skilled in the art will recognize that the sequence disclosed in SEQ ID NO:1 represents a single allele of human Zsig48, and that allelic variation and alternative splicing are expected to occur. Allelic variants of this sequence can be cloned by probing cDNA or genomic libraries from different individuals according to

standard procedures. Allelic variants of the nucleotide sequence shown in SEQ ID NO:1, including those containing silent mutations and those in which mutations result in amino acid sequence changes, are within the scope of the present invention, as are proteins which are allelic variants of SEQ ID NOs:2, 3, 4 and 5. cDNA molecules generated from alternatively spliced mRNAs, which retain the properties of the Zsig48 polypeptide are included within the scope of the present invention, as are polypeptides encoded by such cDNAs and mRNAs. Allelic variants and splice variants of these sequences can be cloned by probing cDNA or genomic libraries from different individuals or tissues according to standard procedures known in the art.

Within preferred embodiments of the invention, isolated nucleic acid molecules that encode human Zsig48 can hybridize to nucleic acid molecules having the nucleotide sequence of SEQ ID NO:1, or a sequence complementary thereto, under "stringent conditions." In general, stringent conditions are selected to be about 5°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength and pH. The T_m is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe.

As an illustration, a nucleic acid molecule encoding a variant Zsig48 polypeptide can be hybridized with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) at 42°C overnight in a solution comprising 50% formamide, 5xSSC (1xSSC: 0.15 M sodium chloride and 15 mM sodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution (100x Denhardt's solution: 2% (w/v) Ficoll 400, 2% (w/v) polyvinylpyrrolidone, and 2% (w/v) bovine serum albumin), 10% dextran sulfate, and 20 µg/ml denatured, sheared

salmon sperm DNA. One of skill in the art can devise variations of these hybridization conditions. For example, the hybridization mixture can be incubated at a higher temperature, such as about 65°C, in a solution that does not contain formamide. Moreover, premixed hybridization solutions are available (*e.g.*, EXPRESSHYB Hybridization Solution from CLONTECH Laboratories, Inc.), and hybridization can be performed according to the manufacturer's instructions.

Following hybridization, the nucleic acid molecules can be washed to remove non-hybridized nucleic acid molecules under stringent conditions, or under highly stringent conditions. Typical stringent washing conditions include washing in a solution of 0.5x - 2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 55 - 65°C. That is, nucleic acid molecules encoding a variant Zsig48 polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, including 0.5x SSC with 0.1% SDS at 55°C, or 2xSSC with 0.1% SDS at 65°C. One of skill in the art can readily devise equivalent conditions, for example, by substituting SSPE for SSC in the wash solution.

Typical highly stringent washing conditions include washing in a solution of 0.1x - 0.2x SSC with 0.1% sodium dodecyl sulfate (SDS) at 50 - 65°C. In other words, nucleic acid molecules encoding a variant Zsig48 polypeptide hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, including 0.1x SSC with 0.1% SDS at 50°C, or 0.2xSSC with 0.1% SDS at 65°C.

The present invention also provides isolated Zsig48 polypeptides that have a substantially similar sequence identity to the polypeptides of SEQ ID NO:2, SEQ ID NO:5, or their orthologs. The term "substantially similar sequence identity" is used herein to denote polypeptides having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the sequences shown in SEQ ID NO2:2, 3, 4 and 5, or their orthologs.

The present invention also contemplates *Zsig48* variant nucleic acid molecules that can be identified using two criteria: a determination of the similarity between the encoded polypeptide with the amino acid sequence of SEQ ID NO:2, and a hybridization assay, as described above. Such *Zsig48* variants include nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under stringent washing conditions, in which the wash stringency is equivalent to 0.5x - 2x SSC with 0.1% SDS at 55 - 65°C, and (2) that encode a polypeptide having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NOs:2, 3, 4 or 5. Alternatively, *Zsig48* variants can be characterized as nucleic acid molecules (1) that hybridize with a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1 (or its complement) under highly stringent washing conditions, in which the wash stringency is equivalent to 0.1x - 0.2x SSC with 0.1% SDS at 50 - 65°C, and (2) that encode a polypeptide having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the amino acid sequence of SEQ ID NOs:2, 3, 4 or 5.

The present invention also contemplates human *Zsig48* variant nucleic acid molecules identified by at

least one of hybridization analysis and sequence identity determination, with reference to SEQ ID NOs:2, 3, 4 or 5.

Percent sequence identity is determined by conventional methods. See, for example, Altschul *et al.*, *Bull. Math. Bio.* *48*:603 (1986), and Henikoff and Henikoff, *Proc. Natl. Acad. Sci. USA* *89*:10915 (1992). Briefly, two amino acid sequences are aligned to optimize the alignment scores using a gap opening penalty of 10, a gap extension penalty of 1, and the "BLOSUM 62" scoring matrix of Henikoff and Henikoff (*ibid.*) as shown in Table 1(amino acids are indicated by the standard one-letter codes). The percent identity is then calculated as: ([Total number of identical matches]/ [length of the longer sequence plus the number of gaps introduced into the longer sequence in order to align the two sequences])(100).

Table 1

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	4																			
R	-1	5																		
N	-2	0	6																	
D	-2	-2	1	6																
C	0	-3	-3	-3	9															
Q	-1	1	0	0	-3	5														
E	-1	0	0	2	-4	2	5													
G	0	-2	0	-1	-3	-2	-2	6												
H	-2	0	1	-1	-3	0	0	-2	8											
I	-1	-3	-3	-3	-1	-3	-3	-4	-3	4										
L	-1	-2	-3	-4	-1	-2	-3	-4	-3	2	4									
K	-1	2	0	-1	-3	1	1	-2	-1	-3	-2	5								
M	-1	-1	-2	-3	-1	0	-2	-3	-2	1	2	-1	5							
F	-2	-3	-3	-3	-2	-3	-3	-3	-1	0	0	-3	0	6						
P	-1	-2	-2	-1	-3	-1	-1	-2	-2	-3	-3	-1	-2	-4	7					
S	1	-1	1	0	-1	0	0	0	-1	-2	-2	0	-1	-2	-1	4				
T	0	-1	0	-1	-1	-1	-1	-2	-2	-1	-1	-1	-1	-2	-1	1	5			
W	-3	-3	-4	-4	-2	-2	-3	-2	-2	-3	-2	-3	-1	1	-4	-3	-2	11		
Y	-2	-2	-2	-3	-2	-1	-2	-3	2	-1	-1	-2	-1	3	-3	-2	-2	2	7	
V	0	-3	-3	-3	-1	-2	-2	-3	-3	3	1	-2	1	-1	-2	-2	0	-3	-1	4

Those skilled in the art appreciate that there are many established algorithms available to align two amino acid sequences. The "FASTA" similarity search algorithm of Pearson and Lipman is a suitable protein alignment method for examining the level of identity shared by an amino acid sequence disclosed herein and the amino acid sequence of a putative Zsig48 variant. The FASTA algorithm is described by Pearson and Lipman, *Proc. Nat'l Acad. Sci. USA 85*:2444 (1988), and by Pearson, *Meth. Enzymol. 183*:63 (1990). Briefly, FASTA first characterizes sequence similarity by identifying regions shared by the query sequence (*e.g.*, SEQ ID NO:2) and a test sequence that have either the highest density of identities (if the ktup variable is 1) or pairs of identities (if ktup=2), without considering conservative amino acid substitutions, insertions, or deletions. The ten regions with the highest density of identities are then re-scored by comparing the similarity of all paired amino acids using an amino acid substitution matrix, and the ends of the regions are "trimmed" to include only those residues that contribute to the highest score. If there are several regions with scores greater than the "cutoff" value (calculated by a predetermined formula based upon the length of the sequence and the ktup value), then the trimmed initial regions are examined to determine whether the regions can be joined to form an approximate alignment with gaps. Finally, the highest scoring regions of the two amino acid sequences are aligned using a modification of the Needleman-Wunsch-Sellers algorithm (Needleman and Wunsch, *J. Mol. Biol. 48*:444 (1970); Sellers, *SIAM J. Appl. Math. 26*:787 (1974)), which allows for amino acid insertions and deletions. Illustrative parameters for FASTA analysis are: ktup=1, gap opening penalty=10, gap extension penalty=1, and substitution matrix=BLOSUM62. These parameters can be introduced into a FASTA program by modifying the scoring matrix file

("SMATRIX"), as explained in Appendix 2 of Pearson, *Meth. Enzymol. 183*:63 (1990).

FASTA can also be used to determine the sequence identity of nucleic acid molecules using a ratio as disclosed above. For nucleotide sequence comparisons, the ktup value can range between one to six, preferably from three to six, most preferably three, with other parameters set as described above.

The present invention includes nucleic acid molecules that encode a polypeptide having a conservative amino acid change, compared with the amino acid sequence of SEQ ID NOs:2, 3, 4 or 5. That is, variants can be obtained that contain one or more amino acid substitutions of SEQ ID NOs:2, 3, 4 or 5, in which an alkyl amino acid is substituted for an alkyl amino acid in an Zsig48 amino acid sequence, an aromatic amino acid is substituted for an aromatic amino acid in an Zsig48 amino acid sequence, a sulfur-containing amino acid is substituted for a sulfur-containing amino acid in an Zsig48 amino acid sequence, a hydroxy-containing amino acid is substituted for a hydroxy-containing amino acid in an Zsig48 amino acid sequence, an acidic amino acid is substituted for an acidic amino acid in a Zsig48 amino acid sequence, a basic amino acid is substituted for a basic amino acid in a Zsig48 amino acid sequence, or a dibasic monocarboxylic amino acid is substituted for a dibasic monocarboxylic amino acid in an Zsig48 amino acid sequence.

Among the common amino acids, for example, a "conservative amino acid substitution" is illustrated by a substitution among amino acids within each of the following groups: (1) glycine, alanine, valine, leucine, and isoleucine, (2) phenylalanine, tyrosine, and tryptophan, (3) serine and threonine, (4) aspartate and

glutamate, (5) glutamine and asparagine, and (6) lysine, arginine and histidine.

Table 2

Conservative amino acid substitutions

Basic:	arginine
	lysine
	histidine
Acidic:	glutamic acid
	aspartic acid
Polar:	glutamine
	asparagine
Hydrophobic:	leucine
	isoleucine
	valine
Aromatic:	phenylalanine
	tryptophan
	tyrosine
Small:	glycine
	alanine
	serine
	threonine
	methionine

The BLOSUM62 table is an amino acid substitution matrix derived from about 2,000 local multiple alignments of protein sequence segments, representing highly conserved regions of more than 500 groups of related proteins (Henikoff and Henikoff, *Proc. Nat'l Acad. Sci. USA 89*:10915 (1992)). Accordingly, the BLOSUM62 substitution frequencies can be used to define conservative amino acid substitutions that may be introduced into the amino acid sequences of the present

invention. Although it is possible to design amino acid substitutions based solely upon chemical properties (as discussed above), the language "conservative amino acid substitution" preferably refers to a substitution represented by a BLOSUM62 value of greater than -1. For example, an amino acid substitution is conservative if the substitution is characterized by a BLOSUM62 value of 0, 1, 2, or 3. According to this system, preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 1 (*e.g.*, 1, 2 or 3), while more preferred conservative amino acid substitutions are characterized by a BLOSUM62 value of at least 2 (*e.g.*, 2 or 3).

Particular variants of human Zsig48 are characterized by having at least 70%, at least 80%, at least 90%, at least 95% or greater than 95% sequence identity to the corresponding human (*i.e.*, SEQ ID NOs: 2, 3, 4 or 5) amino acid sequences, wherein the variation in amino acid sequence is due to one or more conservative amino acid substitutions.

Conservative amino acid changes in an *Zsig48* gene can be introduced by substituting nucleotides for the nucleotides recited in SEQ ID NO:1. Such "conservative amino acid" variants can be obtained, for example, by oligonucleotide-directed mutagenesis, linker-scanning mutagenesis, mutagenesis using the polymerase chain reaction, and the like (see Ausubel (1995) at pages 8-10 to 8-22; and McPherson (ed.), *Directed Mutagenesis: A Practical Approach* (IRL Press 1991)). The ability of such variants to promote proliferation of T-cells, B-cells or monocytes using a standard method, such as the assay described herein. Alternatively, a variant Zsig48 polypeptide can be identified by the ability to specifically bind anti- Zsig48 antibodies.

The proteins of the present invention can also comprise non-naturally occurring amino acid residues. Non-naturally occurring amino acids include, without limitation, *trans*-3-methylproline, 2,4-methanoproline, *cis*-4-hydroxyproline, *trans*-4-hydroxyproline, *N*-methylglycine, *allo*-threonine, methylthreonine, hydroxyethylcysteine, hydroxyethylhomocysteine, nitroglutamine, homoglutamine, pipecolic acid, thiazolidine carboxylic acid, dehydroproline, 3- and 4-methylproline, 3,3-dimethylproline, *tert*-leucine, norvaline, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine, and 4-fluorophenylalanine. Several methods are known in the art for incorporating non-naturally occurring amino acid residues into proteins. For example, an *in vitro* system can be employed wherein nonsense mutations are suppressed using chemically aminoacylated suppressor tRNAs. Methods for synthesizing amino acids and aminoacylating tRNA are known in the art. Transcription and translation of plasmids containing nonsense mutations is typically carried out in a cell-free system comprising an *E. coli* S30 extract and commercially available enzymes and other reagents. Proteins are purified by chromatography. See, for example, Robertson *et al.*, *J. Am. Chem. Soc.* *113*:2722 (1991), Ellman *et al.*, *Methods Enzymol.* *202*:301 (1991), Chung *et al.*, *Science* *259*:806 (1993), and Chung *et al.*, *Proc. Nat'l Acad. Sci. USA* *90*:10145 (1993).

In a second method, translation is carried out in *Xenopus* oocytes by microinjection of mutated mRNA and chemically aminoacylated suppressor tRNAs (Turcatti *et al.*, *J. Biol. Chem.* *271*:19991 (1996)). Within a third method, *E. coli* cells are cultured in the absence of a natural amino acid that is to be replaced (*e.g.*, phenylalanine) and in the presence of the desired non-naturally occurring amino acid(s) (*e.g.*, 2-azaphenylalanine, 3-azaphenylalanine, 4-azaphenylalanine,

or 4-fluorophenylalanine). The non-naturally occurring amino acid is incorporated into the protein in place of its natural counterpart. See, Koide *et al.*, *Biochem.* *33*:7470 (1994). Naturally occurring amino acid residues can be converted to non-naturally occurring species by *in vitro* chemical modification. Chemical modification can be combined with site-directed mutagenesis to further expand the range of substitutions (Wynn and Richards, *Protein Sci.* *2*:395 (1993)).

A limited number of non-conservative amino acids, amino acids that are not encoded by the genetic code, non-naturally occurring amino acids, and unnatural amino acids may be substituted for Zsig48 amino acid residues.

Essential amino acids in the polypeptides of the present invention can be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis [Cunningham and Wells, *Science* *244*:1081 (1989), Bass *et al.*, *Proc. Nat'l Acad. Sci. USA* *88*:4498 (1991), Coombs and Corey, "Site-Directed Mutagenesis and Protein Engineering," in *Proteins: Analysis and Design*, Angeletti (ed.), pages 259-311 (Academic Press, Inc. 1998)]. In the latter technique, single alanine mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity as disclosed below to identify amino acid residues that are critical to the activity of the molecule. See also, Hilton *et al.*, *J. Biol. Chem.* *271*:4699 (1996). Sequence analysis can also identify motifs that reside within human Zsig48 polypeptides.

Although sequence analysis can be used to identify Zsig48 ligand binding sites, the location of Zsig48 ligand binding domains can also be determined by

physical analysis of structure, as determined by such techniques as nuclear magnetic resonance, crystallography, electron diffraction or photoaffinity labeling, in conjunction with mutation of putative contact site amino acids. See, for example, de Vos *et al.*, *Science 255*:306 (1992), Smith *et al.*, *J. Mol. Biol. 224*:899 (1992), and Wlodaver *et al.*, *FEBS Lett. 309*:59 (1992). Moreover, Zsig48 labeled with biotin or FITC can be used for expression cloning of Zsig48 ligand.

Multiple amino acid substitutions can be made and tested using known methods of mutagenesis and screening, such as those disclosed by Reidhaar-Olson and Sauer (*Science 241*:53 (1988)) or Bowie and Sauer (*Proc. Nat'l Acad. Sci. USA 86*:2152 (1989)). Briefly, these authors disclose methods for simultaneously randomizing two or more positions in a polypeptide, selecting for functional polypeptide, and then sequencing the mutagenized polypeptides to determine the spectrum of allowable substitutions at each position. Other methods that can be used include phage display (*e.g.*, Lowman *et al.*, *Biochem. 30*:10832 (1991), Ladner *et al.*, U.S. Patent No. 5,223,409, Huse, international publication No. WO 92/06204, and region-directed mutagenesis (Derbyshire *et al.*, *Gene 46*:145 (1986), and Ner *et al.*, *DNA 7*:127, (1988)).

Variants of the disclosed Zsig48 nucleotide and polypeptide sequences can also be generated through DNA shuffling as disclosed by Stemmer, *Nature 370*:389 (1994), Stemmer, *Proc. Nat'l Acad. Sci. USA 91*:10747 (1994), and international publication No. WO 97/20078. Briefly, variant DNAs are generated by *in vitro* homologous recombination by random fragmentation of a parent DNA followed by reassembly using PCR, resulting in randomly introduced point mutations. This technique can be modified by using a family of parent DNAs, such as allelic

variants or DNAs from different species, to introduce additional variability into the process. Selection or screening for the desired activity, followed by additional iterations of mutagenesis and assay provides for rapid "evolution" of sequences by selecting for desirable mutations while simultaneously selecting against detrimental changes.

Mutagenesis methods as disclosed herein can be combined with high-throughput, automated screening methods to detect activity of cloned, mutagenized polypeptides in host cells. Mutagenized DNA molecules that encode biologically active polypeptides, or polypeptides that bind with anti-Zsig48 antibodies, can be recovered from the host cells and rapidly sequenced using modern equipment. These methods allow the rapid determination of the importance of individual amino acid residues in a polypeptide of interest, and can be applied to polypeptides of unknown structure.

The present invention also includes "functional fragments" of Zsig48 polypeptides and nucleic acid molecules encoding such functional fragments. Routine deletion analyses of nucleic acid molecules can be performed to obtain functional fragments of a nucleic acid molecule that encodes a Zsig48 polypeptide. As an illustration, DNA molecules having the nucleotide sequence of SEQ ID NO:1 can be digested with *Bal*31 nuclease to obtain a series of nested deletions. One alternative to exonuclease digestion is to use oligonucleotide-directed mutagenesis to introduce deletions or stop codons to specify production of a desired fragment. Alternatively, particular fragments of a *Zsig48* gene can be synthesized using the polymerase chain reaction.

The present invention also contemplates functional fragments of a *Zsig48* gene that has amino acid

changes, compared with the amino acid sequence of SEQ ID NO:2. A variant *Zsig48* gene can be identified on the basis of structure by determining the level of identity with nucleotide and amino acid sequences of SEQ ID NOs: 2,3, 4 or 5 as discussed above. An alternative approach to identifying a variant gene on the basis of structure is to determine whether a nucleic acid molecule encoding a potential variant *Zsig48* gene can hybridize to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, as discussed above.

The present invention also provides polypeptide fragments or peptides comprising an [-bearing portion of a Zsig48 polypeptide described herein. Such fragments or peptides may comprise an "immunogenic epitope," which is a part of a protein that elicits an antibody response when the entire protein is used as an immunogen. Immunogenic epitope-bearing peptides can be identified using standard methods (see, for example, Geysen *et al.*, *Proc. Nat'l Acad. Sci. USA 81*:3998 (1983)] Examples of such epitopes (determined by Jameson-Wolf method, DNAstar described below) are a polypeptide comprised of amino acid residue 38, a serine to and including amino acid residue 57, a threonine, of SEQ ID NO:2 also defined by SEQ ID NO:8; a polypeptide comprised of amino acid residue 38, a serine, to and including amino acid residue 79, a threonine, of SEQ ID NO:2, also defined by SEQ ID NO:9; a polypeptide comprised of amino acid residue 38, a serine, to and including amino acid residue 102, a serine of SEQ ID NO:2 also defined by SEQ ID NO:10; a polypeptide comprised of amino acid residue 60, a threonine, to and including amino acid residue 79, a threonine, of SEQ ID NO:2 also defined by SEQ ID NO:11; and a polypeptide comprised of amino acid residue 60, a threonine, to and including amino acid residue 102, a serine of SEQ ID NO:2, also defined by SEQ ID NO:12.

In contrast, polypeptide fragments or peptides may comprise an "antigenic epitope," which is a region of a protein molecule to which an antibody can specifically bind. Certain epitopes consist of a linear or contiguous stretch of amino acids, and the antigenicity of such an epitope is not disrupted by denaturing agents. It is known in the art that relatively short synthetic peptides that can mimic epitopes of a protein can be used to stimulate the production of antibodies against the protein (see, for example, Sutcliffe *et al.*, *Science 219*:660 (1983)). Accordingly, antigenic epitope-bearing peptides and polypeptides of the present invention are useful to raise antibodies that bind with the polypeptides described herein.

Antigenic epitope-bearing peptides and polypeptides preferably contain at least four to ten amino acids, at least ten to fifteen amino acids, or about 15 to about 30 amino acids of SEQ ID NO:2. Such epitope-bearing peptides and polypeptides can be produced by fragmenting a Zsig48 polypeptide, or by chemical peptide synthesis, as described herein. Moreover, epitopes can be selected by phage display of random peptide libraries (see, for example, Lane and Stephen, *Curr. Opin. Immunol.* 5:268 (1993), and Cortese *et al.*, *Curr. Opin. Biotechnol.* 7:616 (1996)). Standard methods for identifying epitopes and producing antibodies from small peptides that comprise an epitope are described, for example, by Mole, "Epitope Mapping," in *Methods in Molecular Biology, Vol. 10*, Manson (ed.), pages 105-116 (The Humana Press, Inc. 1992), Price, "Production and Characterization of Synthetic Peptide-Derived Antibodies," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 60-84 (Cambridge University Press 1995), and Coligan *et al.* (eds.), *Current Protocols in Immunology*, pages 9.3.1 - 9.3.5 and pages 9.4.1 - 9.4.11 (John Wiley & Sons 1997).

Regardless of the particular nucleotide sequence of a variant *Zsig48* gene, the gene encodes a polypeptide that is characterized by its ability to bind specifically to an anti-Zsig48 antibody.

For any Zsig48 polypeptide, including variants and fusion proteins, one of ordinary skill in the art can readily generate a fully degenerate polynucleotide sequence encoding that variant using the information set forth in Tables 1 and 2 above. Moreover, those of skill in the art can use standard software to devise Zsig48 variants based upon the nucleotide and amino acid sequences described herein. Accordingly, the present invention includes a computer-readable medium encoded with a data st7ructure that provides at least one of the following sequences: SEQ ID NO:1, 2, 3, 4, 5, 8, 9, 10, 11, and 12. For example, a computer-readable medium can be encoded with a data structure that provides at least one of the following sequences: SEQ ID NO:1, SEQ ID NO:2. Suitable forms of computer-readable media include magnetic media and optically-readable media. Examples of magnetic media include a hard or fixed drive, a random access memory (RAM) chip, a floppy disk, digital linear tape (DLT), a disk cache, and a ZIP disk. Optically readable media are exemplified by compact discs (*e.g.*, CD-read only memory (ROM), CD-re-writable (RW), and CD-recordable), and digital versatile/video discs (DVD) (*e.g.*, DVD-ROM, DVD-RAM, and DVD+RW).

Production of Zsig48 Fusion Proteins and Conjugates

Fusion proteins of Zsig48 can be used to express Zsig48 in a recombinant host, and to isolate expressed Zsig48. As described below, particular Zsig48 fusion proteins also have uses in diagnosis and therapy.

One type of fusion protein comprises a peptide that guides a Zsig48 polypeptide from a recombinant host cell. To direct a Zsig48 into the secretory pathway of a eukaryotic host cell, a secretory signal sequence (also known as a signal peptide, a leader sequence, prepro sequence or pre sequence) is provided in the Zsig48 expression vector. While the secretory signal sequence may be derived from Zsig48, a suitable signal sequence may also be derived from another secreted protein or synthesized *de novo*. The secretory signal sequence is operably linked to an Zsig48-encoding sequence such that the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleotide sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleotide sequence of interest (see, *e.g.*, Welch *et al.*, U.S. Patent No. 5,037,743; Holland *et al.*, U.S. Patent No. 5,143,830).

Although the secretory signal sequence of Zsig48 or another protein produced by mammalian cells (*e.g.*, tissue-type plasminogen activator signal sequence, as described, for example, in U.S. Patent No. 5,641,655) is useful for expression of Zsig48 in recombinant mammalian hosts, a yeast signal sequence is preferred for expression in yeast cells. Examples of suitable yeast signal sequences are those derived from yeast mating pheromone α-factor (encoded by the *MFα1* gene), invertase (encoded by the *SUC2* gene), or acid phosphatase (encoded by the *PHO5* gene). See, for example, Romanos *et al.*, "Expression of Cloned Genes in Yeast," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (eds.), pages 123-167 (Oxford University Press 1995).

In bacterial cells, it is often desirable to express a heterologous protein as a fusion protein to decrease toxicity, increase stability, and to enhance recovery of the expressed protein. For example, Zsig48 can be expressed as a fusion protein comprising a glutathione S-transferase polypeptide. Glutathione S-transferase fusion proteins are typically soluble, and easily purifiable from *E. coli* lysates on immobilized glutathione columns. In similar approaches, a Zsig48 fusion protein comprising a maltose binding protein polypeptide can be isolated with an amylose resin column, while a fusion protein comprising the C-terminal end of a truncated Protein A gene can be purified using IgG-Sepharose. Established techniques for expressing a heterologous polypeptide as a fusion protein in a bacterial cell are described, for example, by Williams *et al.*, "Expression of Foreign Proteins in *E. coli* Using Plasmid Vectors and Purification of Specific Polyclonal Antibodies," in *DNA Cloning 2: A Practical Approach*, 2nd Edition, Glover and Hames (Eds.), pages 15-58 (Oxford University Press 1995). In addition, commercially available expression systems are available. For example, the PINPOINT Xa protein purification system (Promega Corporation; Madison, WI) provides a method for isolating a fusion protein comprising a polypeptide that becomes biotinylated during expression with a resin that comprises avidin.

Peptide tags that are useful for isolating heterologous polypeptides expressed by either prokaryotic or eukaryotic cells include polyHistidine tags (which have an affinity for nickel-chelating resin), *c-myc* tags, calmodulin binding protein (isolated with calmodulin affinity chromatography), substance P, the RYIRS tag (which binds with anti-RYIRS antibodies), the Glu-Glu tag, and the FLAG tag (which binds with anti-FLAG antibodies). See, for example, Luo *et al.*, *Arch. Biochem. Biophys.*

329:215 (1996), Morganti *et al.*, *Biotechnol. Appl. Biochem. 23*:67 (1996), and Zheng *et al.*, *Gene 186*:55 (1997). Nucleic acid molecules encoding such peptide tags are available, for example, from Sigma-Aldrich Corporation (St. Louis, MO).

The present invention also contemplates that the use of the secretory signal sequence contained in the Zsig48 polypeptides of the present invention to direct other polypeptides into the secretory pathway. A signal fusion polypeptide can be made wherein a secretory signal sequence derived from amino acid residues 1 to 26 or 1-28 or 1-40 of SEQ ID NO:2 is operably linked to another polypeptide using methods known in the art and disclosed herein. The secretory signal sequence contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Such constructs have numerous applications known in the art. For example, these novel secretory signal sequence fusion constructs can direct the secretion of an active component of a normally non-secreted protein, such as a receptor. Such fusions may be used in a transgenic animal or in a cultured recombinant host to direct peptides through the secretory pathway. With regard to the latter, exemplary polypeptides include pharmaceutically active molecules such as Factor VIIa, proinsulin, insulin, follicle stimulating hormone, tissue type plasminogen activator, tumor necrosis factor, interleukins (*e.g.*, interleukin-1 (IL-1), IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-14, and IL-15), colony stimulating factors (*e.g.*, granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF)), interferons (*e.g.*, interferons-α, -β, -γ, -ω, -δ, and -τ), the stem cell growth factor designated "S1 factor," erythropoietin, and thrombopoietin. The Zsig48 secretory signal sequence

contained in the fusion polypeptides of the present invention is preferably fused amino-terminally to an additional peptide to direct the additional peptide into the secretory pathway. Fusion proteins comprising a Zsig48 secretory signal sequence can be constructed using standard techniques.

Another form of fusion protein comprises an Zsig48 polypeptide and an immunoglobulin heavy chain constant region, typically an F_c fragment, which contains two or three constant region domains and a hinge region but lacks the variable region. As an illustration, Chang *et al.*, U.S. Patent No. 5,723,125, describe a fusion protein comprising a human interferon and a human immunoglobulin Fc fragment. The C-terminal of the interferon is linked to the N-terminal of the Fc fragment by a peptide linker moiety. An example of a peptide linker is a peptide comprising primarily a T cell inert sequence, which is immunologically inert. An exemplary peptide linker has the amino acid sequence: GGSGG SGGGG SGGGG S (SEQ ID NO:17). In this fusion protein, a preferred Fc moiety is a human γ4 chain, which is stable in solution and has little or no complement activating activity. Accordingly, the present invention contemplates a Zsig48 fusion protein that comprises a Zsig48 moiety and a human Fc fragment, wherein the C-terminus of the Zsig48 moiety is attached to the N-terminus of the Fc fragment via a peptide linker, such as a peptide consisting of the amino acid sequence of SEQ ID NO:20. The Zsig48 moiety can be a Zsig48 molecule or a fragment thereof.

In another variation, a Zsig48 fusion protein comprises an IgG sequence, a Zsig48 moiety covalently joined to the aminoterminal end of the IgG sequence, and a signal peptide that is covalently joined to the aminoterminal of the Zsig48 moiety, wherein the IgG sequence consists of the following elements in the

following order: a hinge region, a CH_2 domain, and a CH_3 domain. Accordingly, the IgG sequence lacks a CH_1 domain. This general approach to producing fusion proteins that comprise both antibody and nonantibody portions has been described by LaRochelle *et al.*, EP 742830 (WO 95/21258).

Fusion proteins comprising a Zsig48 moiety and an Fc moiety can be used, for example, as an *in vitro* assay tool. For example, the presence of a Zsig48 ligand in a biological sample can be detected using a Zsig48-immunoglobulin fusion protein, in which the Zsig48 moiety is used to target the cognate ligand, and a macromolecule, such as Protein A or anti-Fc antibody, is used to detect the bound fusion protein-receptor complex. Moreover, such fusion proteins can be used to identify agonists and antagonists that interfere with the binding of Zsig48 to its ligand.

In addition, antibody-Zsig48 fusion proteins, comprising antibody variable domains, are useful as therapeutic proteins, in which the antibody moiety binds with a target antigen, such as a tumor associated antigen. Methods of making antibody-cytokine fusion proteins are known to those of skill in the art. For example, antibody fusion proteins comprising an interleukin-2 moiety are described by Boleti *et al.*, *Ann. Oncol.* *6*:945 (1995), Nicolet *et al.*, *Cancer Gene Ther.* *2*:161 (1995), Becker *et al.*, *Proc. Nat'l Acad. Sci. USA* *93*:7826 (1996), Hank *et al.*, *Clin. Cancer Res.* *2*:1951 (1996), and Hu *et al.*, *Cancer Res.* *56*:4998 (1996). Moreover, Yang *et al.*, *Hum. Antibodies Hybridomas* *6*:129 (1995), and Xiang *et al.*, *J. Biotechnol.* *53*:3 (1997), describe fusion proteins that include an $F(ab')_2$ fragment and a tumor necrosis factor alpha moiety. Additional cytokine-antibody fusion proteins include IL-8, IL-12, or interferon-τ as the cytokine moiety (Holzer *et al.*, *Cytokine* *8*:214 (1996); Gillies *et al.*, *J. Immunol.* *160*:6195 (1998); Xiang *et al.*,

Hum. Antibodies Hybridomas 7:2 (1996)). Also see, Gillies, U.S. Patent No. 5,650,150.

Fusion proteins can be prepared by methods known to those skilled in the art by preparing each component of the fusion protein and chemically conjugating them. Alternatively, a polynucleotide encoding both components of the fusion protein in the proper reading frame can be generated using known techniques and expressed by the methods described herein. General methods for enzymatic and chemical cleavage of fusion proteins are described, for example, by Ausubel (1995) at pages 16-19 to 16-25.

The present invention also contemplates chemically modified Zsig48 compositions, in which an Zsig48 polypeptide is linked with a polymer. Typically, the polymer is water soluble so that the Zsig48 conjugate does not precipitate in an aqueous environment, such as a physiological environment. An example of a suitable polymer is one that has been modified to have a single reactive group, such as an active ester for acylation, or an aldehyde for alkylation, In this way, the degree of polymerization can be controlled. An example of a reactive aldehyde is polyethylene glycol propionaldehyde, or mono-(C1-C10) alkoxy, or aryloxy derivatives thereof (see, for example, Harris, *et al.*, U.S. Patent No. 5,252,714). The polymer may be branched or unbranched. Moreover, a mixture of polymers can be used to produce Zsig48 conjugates.

Zsig48 conjugates used for therapy should preferably comprise pharmaceutically acceptable water-soluble polymer moieties. Suitable water-soluble polymers include polyethylene glycol (PEG), monomethoxy-PEG, mono-(C1-C10)alkoxy-PEG, aryloxy-PEG, poly-(N-vinyl pyrrolidone)PEG, tresyl monomethoxy PEG, PEG propionaldehyde, *bis*-succinimidyl carbonate PEG, propylene

glycol homopolymers, a polypropylene oxide/ethylene oxide co-polymer, polyoxyethylated polyols (*e.g.*, glycerol), polyvinyl alcohol, dextran, cellulose, or other carbohydrate-based polymers. Suitable PEG may have a molecular weight from about 600 to about 60,000, including, for example, 5,000, 12,000, 20,000 and 25,000. A Zsig48 conjugate can also comprise a mixture of such water-soluble polymers.

PEGylation by acylation typically requires reacting an active ester derivative of PEG with a Zsig48 polypeptide. An example of an activated PEG ester is PEG esterified to *N*-hydroxysuccinimide. As used herein, the term "acylation" includes the following types of linkages between Zsig48 and a water soluble polymer: amide, carbamate, urethane, and the like. Methods for preparing PEGylated Zsig48 by acylation will typically comprise the steps of (a) reacting a Zsig48 polypeptide with PEG (such as a reactive ester of an aldehyde derivative of PEG) under conditions whereby one or more PEG groups attach to Zsig48, and (b) obtaining the reaction product(s). Generally, the optimal reaction conditions for acylation reactions will be determined based upon known parameters and desired results. For example, the larger the ratio of PEG:Zsig48, the greater the percentage of polyPEGylated Zsig48 product.

The product of PEGylation by acylation is typically a polyPEGylated Zsig48 product, wherein the lysine ε-amino groups are PEGylated via an acyl linking group. An example of a connecting linkage is an amide. Typically, the resulting Zsig48 will be at least 95% mono-, di-, or tri-pegylated, although some species with higher degrees of PEGylation may be formed depending upon the reaction conditions. PEGylated species can be separated from unconjugated Zsig48 polypeptides using standard purification methods, such as dialysis, ultrafiltration,

ion exchange chromatography, affinity chromatography, and the like.

PEGylation by alkylation generally involves reacting a terminal aldehyde derivative of PEG with Zsig48 in the presence of a reducing agent. PEG groups are preferably attached to the polypeptide via a $-CH_2-NH$ group.

Derivatization via reductive alkylation to produce a monoPEGylated product takes advantage of the differential reactivity of different types of primary amino groups available for derivatization. Typically, the reaction is performed at a pH that allows one to take advantage of the pKa differences between the ε-amino groups of the lysine residues and the α-amino group of the *N*-terminal residue of the protein. By such selective derivatization, attachment of a water-soluble polymer that contains a reactive group such as an aldehyde, to a protein is controlled. The conjugation with the polymer occurs predominantly at the *N*-terminus of the protein without significant modification of other reactive groups such as the lysine side chain amino groups. The present invention provides a substantially homogenous preparation of Zsig48 monopolymer conjugates.

Reductive alkylation to produce a substantially homogenous population of monopolymer Zsig48 conjugate molecule can comprise the steps of: (a) reacting a Zsig48 polypeptide with a reactive PEG under reductive alkylation conditions at a pH suitable to permit selective modification of the α-amino group at the amino terminus of the Zsig48, and (b) obtaining the reaction product(s). The reducing agent used for reductive alkylation should be stable in aqueous solution and preferably be able to reduce only the Schiff base formed in the initial process of reductive alkylation. Preferred reducing agents include sodium borohydride, sodium cyanoborohydride,

dimethylamine borane, trimethylamine borane, and pyridine borane.

For a substantially homogenous population of monopolymer Zsig48 conjugates, the reductive alkylation reaction conditions are those which permit the selective attachment of the water soluble polymer moiety to the *N*-terminus of Zsig48. Such reaction conditions generally provide for pKa differences between the lysine amino groups and the α-amino group at the *N*-terminus. The pH also affects the ratio of polymer to protein to be used. In general, if the pH is lower, a larger excess of polymer to protein will be desired because the less reactive the *N*-terminal α-group, the more polymer is needed to achieve optimal conditions. If the pH is higher, the polymer: Zsig48 need not be as large because more reactive groups are available. Typically, the pH will fall within the range of 3 - 9, or 3 - 6.

Another factor to consider is the molecular weight of the water-soluble polymer. Generally, the higher the molecular weight of the polymer, the fewer number of polymer molecules which may be attached to the protein. For PEGylation reactions, the typical molecular weight is about 2 kDa to about 100 kDa, about 5 kDa to about 50 kDa, or about 12 kDa to about 25 kDa. The molar ratio of water-soluble polymer to Zsig48 will generally be in the range of 1:1 to 100:1. Typically, the molar ratio of water-soluble polymer to Zsig48 will be 1:1 to 20:1 for polyPEGylation, and 1:1 to 5:1 for monoPEGylation.

General methods for producing conjugates comprising Zsig48 and water-soluble polymer moieties are known in the art. See, for example, Karasiewicz *et al.*, U.S. Patent No. 5,382,657, Greenwald *et al.*, U.S. Patent No. 5,738, 846, Nieforth *et al.*, *Clin. Pharmacol. Ther.*

59:636 (1996), Monkarsh *et al.*, *Anal. Biochem.* *247*:434 (1997)).

Production of Zsig48 Polypeptides in Cultured Cells

The polypeptides of the present invention, including full-length polypeptides, functional fragments, and fusion proteins, can be produced in recombinant host cells following conventional techniques. To express a *Zsig48* gene, a nucleic acid molecule encoding the polypeptide must be operably linked to regulatory sequences that control transcriptional expression in an expression vector and then, introduced into a host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors can include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Expression vectors that are suitable for production of a foreign protein in eukaryotic cells typically contain (1) prokaryotic DNA elements coding for a bacterial replication origin and an antibiotic resistance marker to provide for the growth and selection of the expression vector in a bacterial host; (2) eukaryotic DNA elements that control initiation of transcription, such as a promoter; and (3) DNA elements that control the processing of transcripts, such as a transcription termination/polyadenylation sequence. As discussed above, expression vectors can also include nucleotide sequences encoding a secretory sequence that directs the heterologous polypeptide into the secretory pathway of a host cell. For example, a *Zsig48* expression vector may comprise a *Zsig48* gene and a secretory sequence derived from a *Zsig48* gene or another secreted gene.

Zsig48 proteins of the present invention may be expressed in mammalian cells. Examples of suitable

mammalian host cells include African green monkey kidney cells (Vero; ATCC CRL 1587), human embryonic kidney cells (293-HEK; ATCC CRL 1573), baby hamster kidney cells (BHK-21, BHK-570; ATCC CRL 8544, ATCC CRL 10314), canine kidney cells (MDCK; ATCC CCL 34), Chinese hamster ovary cells (CHO-K1; ATCC CCL61; CHO DG44 [Chasin *et al.*, *Som. Cell. Molec. Genet.* *12*:555 (1986)]), rat pituitary cells (GH1; ATCC CCL82), HeLa S3 cells (ATCC CCL2.2), rat hepatoma cells (H-4-II-E; ATCC CRL 1548) SV40-transformed monkey kidney cells (COS-1; ATCC CRL 1650) and murine embryonic cells (NIH-3T3; ATCC CRL 1658).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, simian virus, or the like, in which the regulatory signals are associated with a particular gene which has a high level of expression. Suitable transcriptional and translational regulatory sequences also can be obtained from mammalian genes, such as *actin*, *collagen*, *myosin*, and *metallothionein* genes.

Transcriptional regulatory sequences include a promoter region sufficient to direct the initiation of RNA synthesis. Suitable eukaryotic promoters include the promoter of the mouse *metallothionein I* gene (Hamer *et al.*, *J. Molec. Appl. Genet.* *1*:273 (1982)), the *TK* promoter of *Herpes* virus (McKnight, *Cell* *31*:355 (1982)), the *SV40* early promoter (Benoist *et al.*, *Nature* *290*:304 (1981)), the *Rous* sarcoma virus promoter (Gorman *et al.*, *Proc. Nat'l Acad. Sci. USA* *79*:6777 (1982)), the cytomegalovirus promoter (Foecking *et al.*, *Gene* *45*:101 (1980)), and the mouse mammary tumor virus promoter (see, generally, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163-181 (John Wiley & Sons, Inc. 1996)).

Alternatively, a prokaryotic promoter, such as the bacteriophage T3 RNA polymerase promoter, can be used to control *Zsig48* gene expression in mammalian cells if the prokaryotic promoter is regulated by a eukaryotic promoter (Zhou *et al.*, *Mol. Cell. Biol. 10*:4529 (1990), and Kaufman *et al.*, *Nucl. Acids Res. 19*:4485 (1991)).

An expression vector can be introduced into host cells using a variety of standard techniques including calcium phosphate transfection, liposome-mediated transfection, microprojectile-mediated delivery, electroporation, and the like. Preferably, the transfected cells are selected and propagated to provide recombinant host cells that comprise the expression vector stably integrated in the host cell genome. Techniques for introducing vectors into eukaryotic cells and techniques for selecting such stable transformants using a dominant selectable marker are described, for example, by Ausubel (1995) and by Murray (ed.), *Gene Transfer and Expression Protocols* (Humana Press 1991).

For example, one suitable selectable marker is a gene that provides resistance to the antibiotic neomycin. In this case, selection is carried out in the presence of a neomycin-type drug, such as G-418 or the like. Selection systems can also be used to increase the expression level of the gene of interest, a process referred to as "amplification." Amplification is carried out by culturing transfectants in the presence of a low level of the selective agent and then increasing the amount of selective agent to select for cells that produce high levels of the products of the introduced genes. A preferred amplifiable selectable marker is dihydrofolate reductase, which confers resistance to methotrexate. Other drug resistance genes (*e.g.*, hygromycin resistance, multi-drug resistance, puromycin acetyltransferase) can

also be used. Alternatively, markers that introduce an altered phenotype, such as green fluorescent protein, or cell surface proteins such as CD4, CD8, Class I MHC, placental alkaline phosphatase may be used to sort transfected cells from untransfected cells by such means as FACS sorting or magnetic bead separation technology.

Zsig48 polypeptides can also be produced by cultured mammalian cells using a viral delivery system. Exemplary viruses for this purpose include adenovirus, herpesvirus, vaccinia virus and adeno-associated virus (AAV). Adenovirus, a double-stranded DNA virus, is currently the best studied gene transfer vector for delivery of heterologous nucleic acid (for a review, see Becker *et al.*, *Meth. Cell Biol.* *43*:161 (1994), and Douglas and Curiel, *Science & Medicine* *4*:44 (1997)). Advantages of the adenovirus system include the accommodation of relatively large DNA inserts, the ability to grow to high-titer, the ability to infect a broad range of mammalian cell types, and flexibility that allows use with a large number of available vectors containing different promoters.

By deleting portions of the adenovirus genome, larger inserts (up to 7 kb) of heterologous DNA can be accommodated. These inserts can be incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. An option is to delete the essential *E1* gene from the viral vector, which results in the inability to replicate unless the *E1* gene is provided by the host cell. Adenovirus vector-infected human 293 cells (ATCC Nos. CRL-1573, 45504, 45505), for example, can be grown as adherent cells or in suspension culture at relatively high cell density to produce significant amounts of protein (see Garnier *et al.*, *Cytotechnol.* *15*:145 (1994)).

Zsig48 genes may also be expressed in other higher eukaryotic cells, such as avian, fungal, insect, yeast, or plant cells. The baculovirus system provides an efficient means to introduce cloned Zsig48 genes into insect cells. Suitable expression vectors are based upon the *Autographa californica* multiple nuclear polyhedrosis virus (AcMNPV), and contain well-known promoters such as *Drosophila heat shock protein* (*hsp*) 70 promoter, *Autographa californica nuclear polyhedrosis virus immediate-early* gene promoter (*ie-1*) and the *delayed early 39K* promoter, baculovirus *p10* promoter, and the *Drosophila metallothionein* promoter. A second method of making recombinant baculovirus utilizes a transposon-based system described by Luckow (Luckow, *et al., J. Virol. 67*:4566 (1993)). This system, which utilizes transfer vectors, is sold in the BAC-to-BAC kit (Life Technologies, Rockville, MD). This system utilizes a transfer vector, PFASTBAC (Life Technologies) containing a Tn7 transposon to move the DNA encoding the Zsig48 polypeptide into a baculovirus genome maintained in *E. coli* as a large plasmid called a "bacmid." See, Hill-Perkins and Possee, *J. Gen. Virol. 71*:971 (1990), Bonning, *et al., J. Gen. Virol. 75*:1551 (1994), and Chazenbalk, and Rapoport, *J. Biol. Chem. 270*:1543 (1995). In addition, transfer vectors can include an in-frame fusion with DNA encoding an epitope tag at the C- or N-terminus of the expressed Zsig48 polypeptide, for example, a Glu-Glu epitope tag (Grussenmeyer *et al., Proc. Nat'l Acad. Sci. 82*:7952 (1985)). Using a technique known in the art, a transfer vector containing a *Zsig48* gene is transformed into *E. coli*, and screened for bacmids which contain an interrupted *lacZ* gene indicative of recombinant baculovirus. The bacmid DNA containing the recombinant baculovirus genome is then isolated using common techniques.

The illustrative pFASTBAC vector can be modified to a considerable degree. For example, the polyhedrin promoter can be removed and substituted with the baculovirus basic protein promoter (also known as P*cor*, p6.9 or MP promoter) which is expressed earlier in the baculovirus infection, and has been shown to be advantageous for expressing secreted proteins (see, for example, Hill-Perkins and Possee, *J. Gen. Virol.* 71:971 (1990), Bonning, *et al.*, *J. Gen. Virol.* 75:1551 (1994), and Chazenbalk and Rapoport, *J. Biol. Chem.* 270:1543 (1995). In such transfer vector constructs, a short or long version of the basic protein promoter can be used. Moreover, transfer vectors can be constructed which replace the native Zsig48 secretory signal sequences with secretory signal sequences derived from insect proteins. For example, a secretory signal sequence from Ecdysteroid Glucosyltransferase (EGT), honey bee Melittin (Invitrogen Corporation; Carlsbad, CA), or baculovirus gp67 (PharMingen: San Diego, CA) can be used in constructs to replace the native Zsig48 secretory signal sequence.

The recombinant virus or bacmid is used to transfect host cells. Suitable insect host cells include cell lines derived from IPLB-*Sf*-21, a *Spodoptera frugiperda* pupal ovarian cell line, such as *Sf*9 (ATCC CRL 1711), *Sf*21AE, and *Sf*21 (Invitrogen Corporation; San Diego, CA), as well as *Drosophila* Schneider-2 cells, and the HIGH FIVEO cell line (Invitrogen) derived from *Trichoplusia ni* (U.S. Patent No. 5,300,435). Commercially available serum-free media can be used to grow and to maintain the cells. Suitable media are Sf900 II™ (Life Technologies) or ESF 921™ (Expression Systems) for the Sf9 cells; and Ex-cell0405™ (JRH Biosciences, Lenexa, KS) or Express FiveO™ (Life Technologies) for the *T. ni* cells. When recombinant virus is used, the cells are typically grown up from an inoculation density of approximately 2-5 x 10^5 cells to a density of 1-2 x 10^6 cells at which time a

recombinant viral stock is added at a multiplicity of infection (MOI) of 0.1 to 10, more typically near 3.

Established techniques for producing recombinant proteins in baculovirus systems are provided by Bailey *et al.*, "Manipulation of Baculovirus Vectors," in *Methods in Molecular Biology, Volume 7: Gene Transfer and Expression Protocols*, Murray (ed.), pages 147-168 (The Humana Press, Inc. 1991), by Patel *et al.*, "The baculovirus expression system," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 205-244 (Oxford University Press 1995), by Ausubel (1995) at pages 16-37 to 16-57, by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995), and by Lucknow, "Insect Cell Expression Technology," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 183-218 (John Wiley & Sons, Inc. 1996).

Fungal cells, including yeast cells, can also be used to express the genes described herein. Yeast species of particular interest in this regard include *Saccharomyces cerevisiae*, *Pichia pastoris*, and *Pichia methanolica*. Suitable promoters for expression in yeast include promoters from *GAL1* (galactose), *PGK* (phosphoglycerate kinase), *ADH* (alcohol dehydrogenase), *AOX1* (alcohol oxidase), HIS4 (histidinol dehydrogenase), and the like. Many yeast cloning vectors have been designed and are readily available. These vectors include YIp-based vectors, such as YIp5, YRp vectors, such as YRp17, YEp vectors such as YEp13 and YCp vectors, such as YCp19. Methods for transforming *S. cerevisiae* cells with exogenous DNA and producing recombinant polypeptides therefrom are disclosed by, for example, Kawasaki, U.S. Patent No. 4,599,311, Kawasaki *et al.*, U.S. Patent No. 4,931,373, Brake, U.S. Patent No. 4,870,008, Welch *et al.*, U.S. Patent No. 5,037,743, and Murray *et al.*, U.S. Patent No. 4,845,075. Transformed cells are selected by

phenotype determined by the selectable marker, commonly drug resistance or the ability to grow in the absence of a particular nutrient (*e.g.*, leucine). A preferred vector system for use in *Saccharomyces cerevisiae* is the *POT1* vector system disclosed by Kawasaki *et al.* (U.S. Patent No. 4,931,373), which allows transformed cells to be selected by growth in glucose-containing media. Additional suitable promoters and terminators for use in yeast include those from glycolytic enzyme genes (see, *e.g.*, Kawasaki, U.S. Patent No. 4,599,311, Kingsman *et al.*, U.S. Patent No. 4,615,974, and Bitter, U.S. Patent No. 4,977,092) and alcohol dehydrogenase genes. See also U.S. Patents Nos. 4,990,446, 5,063,154, 5,139,936, and 4,661,454.

Transformation systems for other yeasts, including *Hansenula polymorpha, Schizosaccharomyces pombe, Kluyveromyces lactis, Kluyveromyces fragilis, Ustilago maydis, Pichia pastoris, Pichia methanolica, Pichia guillermondii* and *Candida maltosa* are known in the art. See, for example, Gleeson *et al., J. Gen. Microbiol.* *132*:3459 (1986), and Cregg, U.S. Patent No. 4,882,279. *Aspergillus* cells may be utilized according to the methods of McKnight *et al.*, U.S. Patent No. 4,935,349. Methods for transforming *Acremonium chrysogenum* are disclosed by Sumino *et al.*, U.S. Patent No. 5,162,228. Methods for transforming *Neurospora* are disclosed by Lambowitz, U.S. Patent No. 4,486,533.

For example, the use of *Pichia methanolica* as host for the production of recombinant proteins is disclosed by Raymond, U.S. Patent No. 5,716,808, Raymond, U.S. Patent No. 5,736,383, Raymond *et al., Yeast* *14*:11-23 (1998), and in international publication Nos. WO 97/17450, WO 97/17451, WO 98/02536, and WO 98/02565. DNA molecules for use in transforming *P. methanolica* will commonly be prepared as double-stranded, circular plasmids, which are

preferably linearized prior to transformation. For polypeptide production in *P. methanolica*, it is preferred that the promoter and terminator in the plasmid be that of a *P. methanolica* gene, such as a *P. methanolica* alcohol utilization gene (*AUG1* or *AUG2*). Other useful promoters include those of the dihydroxyacetone synthase (DHAS), formate dehydrogenase (FMD), and catalase (CAT) genes. To facilitate integration of the DNA into the host chromosome, it is preferred to have the entire expression segment of the plasmid flanked at both ends by host DNA sequences. A preferred selectable marker for use in *Pichia methanolica* is a *P. methanolica ADE2* gene, which encodes phosphoribosyl-5-aminoimidazole carboxylase (AIRC; EC 4.1.1.21), and which allows *ade2* host cells to grow in the absence of adenine. For large-scale, industrial processes where it is desirable to minimize the use of methanol, it is preferred to use host cells in which both methanol utilization genes (*AUG1* and *AUG2*) are deleted. For production of secreted proteins, host cells deficient in vacuolar protease genes (*PEP4* and *PRB1*) are preferred. Electroporation is used to facilitate the introduction of a plasmid containing DNA encoding a polypeptide of interest into *P. methanolica* cells. *P. methanolica* cells can be transformed by electroporation using an exponentially decaying, pulsed electric field having a field strength of from 2.5 to 4.5 kV/cm, preferably about 3.75 kV/cm, and a time constant (t) of from 1 to 40 milliseconds, most preferably about 20 milliseconds.

Expression vectors can also be introduced into plant protoplasts, intact plant tissues, or isolated plant cells. Methods for introducing expression vectors into plant tissue include the direct infection or co-cultivation of plant tissue with *Agrobacterium tumefaciens*, microprojectile-mediated delivery, DNA injection, electroporation, and the like. See, for example, Horsch *et al.*, *Science* 227:1229 (1985), Klein *et al.*, *Biotechnology*

10:268 (1992), and Miki *et al.*, "Procedures for Introducing Foreign DNA into Plants," in *Methods in Plant Molecular Biology and Biotechnology*, Glick *et al.* (eds.), pages 67-88 (CRC Press, 1993).

Alternatively, *Zsig48* genes can be expressed in prokaryotic host cells. Suitable promoters that can be used to express Zsig48 polypeptides in a prokaryotic host are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the *trp*, *recA*, heat shock, *lacUV5*, *tac*, *lpp-lacSpr*, *phoA*, and *lacZ* promoters of *E. coli*, promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters have been reviewed by Glick, *J. Ind. Microbiol. 1*:277 (1987), Watson *et al.*, *Molecular Biology of the Gene, 4th Ed.* (Benjamin Cummins 1987), and by Ausubel *et al.* (1995).

Preferred prokaryotic hosts include *E. coli* and *Bacillus subtilus*. Suitable strains of *E. coli* include BL21(DE3), BL21(DE3)pLysS, BL21(DE3)pLysE, DH1, DH4I, DH5, DH5I, DH5IF', DH5IMCR, DH10B, DH10B/p3, DH11S, C600, HB101, JM101, JM105, JM109, JM110, K38, RR1, Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (ed.), *Molecular Biology Labfax* (Academic Press 1991)). Suitable strains of *Bacillus subtilus* include BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in *DNA Cloning: A Practical Approach*, Glover (ed.) (IRL Press 1985)).

When expressing a Zsig48 polypeptide in bacteria such as *E. coli*, the polypeptide may be retained in the cytoplasm, typically as insoluble granules, or may be

directed to the periplasmic space by a bacterial secretion sequence. In the former case, the cells are lysed, and the granules are recovered and denatured using, for example, guanidine isothiocyanate or urea. The denatured polypeptide can then be refolded and dimerized by diluting the denaturant, such as by dialysis against a solution of urea and a combination of reduced and oxidized glutathione, followed by dialysis against a buffered saline solution. In the latter case, the polypeptide can be recovered from the periplasmic space in a soluble and functional form by disrupting the cells (by, for example, sonication or osmotic shock) to release the contents of the periplasmic space and recovering the protein, thereby obviating the need for denaturation and refolding.

Methods for expressing proteins in prokaryotic hosts are well-known to those of skill in the art (see, for example, Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995), Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, page 137 (Wiley-Liss, Inc. 1995), and Georgiou, "Expression of Proteins in Bacteria," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), page 101 (John Wiley & Sons, Inc. 1996)). Standard methods for introducing expression vectors into bacterial, yeast, insect, and plant cells are provided, for example, by Ausubel (1995).

General methods for expressing and recovering foreign protein produced by a mammalian cell system are provided by, for example, Etcheverry, "Expression of Engineered Proteins in Mammalian Cell Culture," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 163 (Wiley-Liss, Inc. 1996). Standard

techniques for recovering protein produced by a bacterial system is provided by, for example, Grisshammer *et al.*, "Purification of over-produced proteins from *E. coli* cells," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), pages 59-92 (Oxford University Press 1995). Established methods for isolating recombinant proteins from a baculovirus system are described by Richardson (ed.), *Baculovirus Expression Protocols* (The Humana Press, Inc. 1995).

Isolation of Zsig48 Polypeptides

It is preferred to purify the polypeptides of the present invention to at least about 80% purity, more preferably to at least about 90% purity, even more preferably to at least about 95% purity, or even greater than 95% purity with respect to contaminating macromolecules, particularly other proteins and nucleic acids, and free of infectious and pyrogenic agents. The polypeptides of the present invention may also be purified to a pharmaceutically pure state, which is greater than 99.9% pure. Preferably, a purified polypeptide is substantially free of other polypeptides, particularly other polypeptides of animal origin.

Fractionation and/or conventional purification methods can be used to obtain preparations of Zsig48 purified from natural sources (*e.g.*, placenta or leukocytes), and recombinant Zsig48 polypeptides and Zsig48 polypeptides purified from recombinant host cells. In general, ammonium sulfate precipitation and acid or chaotrope extraction may be used for fractionation of samples. Exemplary purification steps may include hydroxyapatite, size exclusion, FPLC and reverse-phase high performance liquid chromatography. Suitable chromatographic media include derivatized dextrans, agarose, cellulose, polyacrylamide, specialty silicas, and

the like. PEI, DEAE, QAE and Q derivatives are preferred. Exemplary chromatographic media include those media derivatized with phenyl, butyl, or octyl groups, such as Phenyl-Sepharose FF (Pharmacia), Toyopearl butyl 650 (Toso Haas, Montgomeryville, PA), Octyl-Sepharose (Pharmacia) and the like; or polyacrylic resins, such as Amberchrom CG 71 (Toso Haas) and the like. Suitable solid supports include glass beads, silica-based resins, cellulosic resins, agarose beads, cross-linked agarose beads, polystyrene beads, cross-linked polyacrylamide resins and the like that are insoluble under the conditions in which they are to be used. These supports may be modified with reactive groups that allow attachment of proteins by amino groups, carboxyl groups, sulfhydryl groups, hydroxyl groups and/or carbohydrate moieties.

Examples of coupling chemistries include cyanogen bromide activation, N-hydroxysuccinimide activation, epoxide activation, sulfhydryl activation, hydrazide activation, and carboxyl and amino derivatives for carbodiimide coupling chemistries. These and other solid media are well known and widely used in the art, and are available from commercial suppliers. Selection of a particular method for polypeptide isolation and purification is a matter of routine design and is determined in part by the properties of the chosen support. See, for example, *Affinity Chromatography: Principles & Methods* (Pharmacia LKB Biotechnology 1988), and Doonan, *Protein Purification Protocols* (The Humana Press 1996).

Additional variations in Zsig48 isolation and purification can be devised by those of skill in the art. For example, anti-Zsig48 antibodies, obtained as described below, can be used to isolate large quantities of protein by immunoaffinity purification. The use of monoclonal antibody columns to purify interferons from recombinant

cells and from natural sources has been described, for example, by Staehelin *et al.*, *J. Biol. Chem.* *256*:9750 (1981), and by Adolf *et al.*, *J. Biol. Chem.* *265*:9290 (1990). Moreover, methods for binding receptors, such as Zsig48, to ligand polypeptides bound to support media are well known in the art.

The polypeptides of the present invention can also be isolated by exploitation of particular properties. For example, immobilized metal ion adsorption (IMAC) chromatography can be used to purify histidine-rich proteins, including those comprising polyhistidine tags. Briefly, a gel is first charged with divalent metal ions to form a chelate (Sulkowski, *Trends in Biochem.* *3*:1 (1985)). Histidine-rich proteins will be adsorbed to this matrix with differing affinities, depending upon the metal ion used, and will be eluted by competitive elution, lowering the pH, or use of strong chelating agents. Other methods of purification include purification of glycosylated proteins by lectin affinity chromatography and ion exchange chromatography (M. Deutscher, (ed.), *Meth. Enzymol.* *182*:529 (1990)). For example, the interferon-γ isolation method of Rinderknecht *et al.*, *J. Biol. Chem.* *259*:6790 (1984), requires the binding of the interferon with concanavalin A-sepharose in one step. Within additional embodiments of the invention, a fusion of the polypeptide of interest and an affinity tag (*e.g.*, maltose-binding protein, an immunoglobulin domain) may be constructed to facilitate purification.

Zsig48 polypeptides or fragments thereof may also be prepared through chemical synthesis, as described below. Zsig48 polypeptides may be monomers or multimers; glycosylated or non-glycosylated; PEGylated or non-PEGylated; and may or may not include an initial methionine amino acid residue.

Peptides and polypeptides of the present invention comprise at least six, preferably at least nine, and more preferably at least 15 contiguous amino acid residues of SEQ ID NOs:2, 3, 4 or 5. Within certain embodiments of the invention, the polypeptides comprise 20, 30, 40, 50, 100, or more contiguous residues of these amino acid sequences, for example, SEQ ID NOs: 8-12. Nucleic acid molecules encoding such peptides and polypeptides are useful as polymerase chain reaction primers and probes.

Chemical Synthesis of Zsig48 Polypeptides

Zsig48 polypeptides of the present invention can also be synthesized by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. The polypeptides are preferably prepared by solid phase peptide synthesis, for example as described by Merrifield, *J. Am. Chem. Soc.* *85*:2149 (1963).

Solid phase synthesis is usually carried out from the carboxyl-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is formed when the attachment is made to a chloromethyl, chlortrityl or hydroxymethyl resin, and the resulting polypeptide will have a free carboxyl group at the C-terminus. Alternatively, when an amide resin such as benzhydrylamine or *p*-methylbenzhydrylamine resin (for tBoc chemistry) and Rink amide or PAL resin (for Fmoc chemistry) are used, an amide bond is formed and the resulting polypeptide will have a carboxamide group at the C-terminus. These resins, whether polystyrene- or polyamide-based or polyethyleneglycol-grafted, with or without a handle or linker, with or without the first amino acid attached, are commercially available, and their preparations have been

described by Stewart *et al.*, "Solid Phase Peptide Synthesis" (2nd Edition), (Pierce Chemical Co. 1984), Bayer and Rapp, *Chem. Pept. Prot.* 3:3 (1986), Atherton *et al.*, *Solid Phase Peptide Synthesis: A Practical Approach* (IRL Press 1989), and by Lloyd-Williams *et al.*, *Chemical Approaches to the Synthesis of Peptides and Proteins* (CRC Press, Inc. 1997).

The "native chemical ligation" approach to producing polypeptides is one variation of total chemical synthesis strategy (see, for example, Dawson *et al.*, *Science 266*:776 (1994), Hackeng *et al.*, *Proc. Nat'l Acad. Sci. USA 94*:7845 (1997), and Dawson, *Methods Enzymol. 287*: 34 (1997)). According to this method, an N-terminal cysteine-containing peptide is chemically ligated to a peptide having a C-terminal thioester group to form a normal peptide bond at the ligation site.

The "expressed protein ligation" method is a semi-synthesis variation of the ligation approach (see, for example, Muir *et al*, *Proc. Nat'l Acad. Sci. USA 95*:6705 (1998); Severinov and Muir, *J. Biol. Chem. 273*:16205 (1998)). Here, synthetic peptides and protein cleavage fragments are linked to form the desired protein product. This method is particularly useful for the site-specific incorporation of unnatural amino acids (*e.g.*, amino acids comprising biophysical or biochemical probes) into proteins.

In an approach illustrated by Muir *et al*, *Proc. Nat'l Acad. Sci. USA 95*:6705 (1998), a gene or gene fragment is cloned into the PCYB2-IMPACT vector (New England Biolabs, Inc.; Beverly, MA) using the *Nde*I and *Sma*I restriction sites. As a result, the gene or gene fragment is expressed in frame fused with a chitin binding domain sequence, and a Pro-Gly is appended to the native C terminus of the protein of interest. The presence of a C-

terminal glycine reduces the chance of side reactions, because the glycine residue accelerates native chemical ligation. Affinity chromatography with a chitin resin is used to purify the expressed fusion protein, and the chemical ligation step is initiated by incubating the resin-bound protein with thiophenol and synthetic peptide in buffer. This mixture produces the *in situ* generation of a highly reactive phenyl $^{\alpha}$thioester derivative of the protein that rapidly ligates with the synthetic peptide to produce the desired semi-synthetic protein.

A general class of Zsig48 analogs is provided by anti-idiotype antibodies, and fragments thereof, as described below. Moreover, recombinant antibodies comprising anti-idiotype variable domains can be used as analogs (see, for example, Monfardini *et al., Proc. Assoc. Am. Physicians 108*:420 (1996)). Since the variable domains of anti-idiotype Zsig48 antibodies mimic Zsig48, these domains can provide either Zsig48 agonist or antagonist activity.

A third approach to identifying Zsig48 analogs is provided by the use of combinatorial libraries. Methods for constructing and screening phage display and other combinatorial libraries are provided, for example, by Kay *et al., Phage Display of Peptides and Proteins* (Academic Press 1996), Verdine, U.S. Patent No. 5,783,384, Kay, *et. al.*, U.S. Patent No. 5,747,334, and Kauffman *et al.*, U.S. Patent No. 5,723,323.

As a receptor, the activity of Zsig48 can be measured by a silicon-based biosensor microphysiometer which measures the extracellular acidification rate or proton excretion associated with receptor binding and subsequent cellular responses. An exemplary device is the CYTOSENSOR Microphysiometer manufactured by Molecular Devices Corp. (Sunnyvale, CA). A variety of cellular

responses, such as cell proliferation, ion transport, energy production, inflammatory response, regulatory and receptor activation, and the like, can be measured by this method (see, for example, McConnell *et al.*, *Science* *257*:1906 (1992), Pitchford *et al.*, *Meth. Enzymol.* *228*:84 (1997), Arimilli *et al.*, *J. Immunol. Meth.* *212*:49 (1998), and Van Liefde *et al.*, *Eur. J. Pharmacol.* *346*:87 (1998)). Moreover, the microphysiometer can be used for assaying adherent or non-adherent eukaryotic or prokaryotic cells.

Zsig48, its analogs, and anti-iodiotype Zsig48 antibodies can be used to identify and to isolate Zsig48 ligands. For example, proteins and peptides of the present invention can be immobilized on a column and used to bind ligand proteins from tissue and serum preparations that are run over the column (Hermanson *et al.* (eds.), *Immobilized Affinity Ligand Techniques*, pages 195-202 (Academic Press 1992)). Radiolabeled or affinity labeled Zsig48 polypeptides can also be used to identify or to localize Zsig48 ligands in a biological sample (see, for example, Deutscher (ed.), *Methods in Enzymol.*, vol. 182, pages 721-37 (Academic Press 1990); Brunner *et al.*, *Ann. Rev. Biochem.* *62*:483 (1993); Fedan *et al.*, *Biochem. Pharmacol.* *33*:1167 (1984)). Also see, Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996), who describe the use of anti-idiotype antibodies for receptor identification.

In addition, a solid phase system can be used to identify a Zsig48 ligand, or an agonist or antagonist of a Zsig48 ligand. For example, a Zsig48 polypeptide or Zsig48 fusion protein can be immobilized onto the surface of a receptor chip of a commercially available biosensor instrument (BIACORE, Biacore AB; Uppsala, Sweden). The use of this instrument is disclosed, for example, by Karlsson, *Immunol. Methods* *145*:229 (1991), and Cunningham and Wells, *J. Mol. Biol.* *234*:554 (1993).

As an illustration, a Zsig48 polypeptide or fusion protein is covalently attached, using amine or sulfhydryl chemistry, to dextran fibers that are attached to gold film within a flow cell. A test sample is then passed through the cell. If a receptor is present in the sample, it will bind to the immobilized polypeptide or fusion protein, causing a change in the refractive index of the medium, which is detected as a change in surface plasmon resonance of the gold film. This system allows the determination of on- and off-rates, from which binding affinity can be calculated, and assessment of stoichiometry of binding. This system can also be used to examine antibody-antigen interactions, and the interactions of other complement/anti-complement pairs.

Production of Antibodies to Zsig48 Proteins

Antibodies to Zsig48 can be obtained, for example, using the product of a *Zsig48* expression vector or Zsig48 isolated from a natural source as an antigen. Particularly useful anti-Zsig48 antibodies "bind specifically" to Zsig48. Antibodies are considered to be specifically binding if the antibodies exhibit at least one of the following two properties: (1) antibodies bind to Zsig48 with a threshold level of binding activity, and (2) antibodies do not significantly cross-react with polypeptides related to Zsig48.

With regard to the first characteristic, antibodies specifically bind if they bind to a Zsig48 polypeptide, peptide or epitope with a binding affinity (K_a) of $10^6\ M^{-1}$ or greater, preferably $10^7\ M^{-1}$ or greater, more preferably $10^8\ M^{-1}$ or greater, and most preferably $10^9\ M^{-1}$ or greater. The binding affinity of an antibody can be readily determined by one of ordinary skill in the art, for example, by Scatchard analysis [Scatchard, *Ann. NY*

Acad. Sci. 51:660 (1949)]. With regard to the second characteristic, antibodies do not significantly cross-react with related polypeptide molecules, for example, if they detect Zsig48, but not known related polypeptides using a standard Western blot analysis.

Anti- Zsig48 antibodies can be produced using antigenic Zsig48 epitope-bearing peptides and polypeptides. Antigenic epitope-bearing peptides and polypeptides of the present invention contain a sequence of at least nine, preferably between 15 to about 30 amino acids contained within SEQ ID NO:2, for example, SEQ ID NOs: 8-12. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of the invention, containing from 30 to 50 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are useful for inducing antibodies that bind with Zsig48. It is desirable that the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues, while hydrophobic residues are preferably avoided). Moreover, amino acid sequences containing proline residues may be also be desirable for antibody production.

As an illustration, potential antigenic sites in human Zsig48 were identified using the Jameson-Wolf method, Jameson and Wolf, *CABIOS* 4:181, (1988), as implemented by the PROTEAN program (version 3.14) of LASERGENE (DNASTAR; Madison, WI). Default parameters were used in this analysis. These resulted in the polypeptide of SEQ ID NOs:8, 9, 10, 11 and 12 described above.

The Jameson-Wolf method predicts potential antigenic determinants by combining six major subroutines for protein structural prediction. Briefly, the Hopp-

Woods method, Hopp *et al.*, *Proc. Nat'l Acad. Sci. USA* *78*:3824 (1981), was first used to identify amino acid sequences representing areas of greatest local hydrophilicity (parameter: seven residues averaged). In the second step, Emini's method, Emini *et al.*, *J. Virology* *55*:836 (1985), was used to calculate surface probabilities (parameter: surface decision threshold (0.6) = 1). Third, the Karplus-Schultz method, Karplus and Schultz, *Naturwissenschaften* *72*:212 (1985), was used to predict backbone chain flexibility (parameter: flexibility threshold (0.2) = 1). In the fourth and fifth steps of the analysis, secondary structure predictions were applied to the data using the methods of Chou-Fasman, Chou, "Prediction of Protein Structural Classes from Amino Acid Composition," in *Prediction of Protein Structure and the Principles of Protein Conformation*, Fasman (ed.), pages 549-586 (Plenum Press 1990), and Garnier-Robson, Garnier *et al.*, *J. Mol. Biol.* *120*:97 (1978) (Chou-Fasman parameters: conformation table = 64 proteins; α region threshold = 103; β region threshold = 105; Garnier-Robson parameters: α and β decision constants = 0). In the sixth subroutine, flexibility parameters and hydropathy/solvent accessibility factors were combined to determine a surface contour value, designated as the "antigenic index." Finally, a peak broadening function was applied to the antigenic index, which broadens major surface peaks by adding 20, 40, 60, or 80% of the respective peak value to account for additional free energy derived from the mobility of surface regions relative to interior regions. This calculation was not applied, however, to any major peak that resides in a helical region, since helical regions tend to be less flexible.

The results of this analysis indicated that the following amino acid sequences of SEQ ID NO:2 would provide suitable antigenic peptides: SEQ ID NOs: 8, 9, 10, 11 and 12 as described above.

Polyclonal antibodies to recombinant Zsig48 protein or to Zsig48 isolated from natural sources can be prepared using methods well-known to those of skill in the art. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Immunochemical Protocols* (Manson, ed.), pages 1-5 (Humana Press 1992), and Williams *et al.*, "Expression of foreign proteins in *E. coli* using plasmid vectors and purification of specific polyclonal antibodies," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 15 (Oxford University Press 1995). The immunogenicity of a Zsig48 polypeptide can be increased through the use of an adjuvant, such as alum (aluminum hydroxide) or Freund's complete or incomplete adjuvant. Polypeptides useful for immunization also include fusion polypeptides, such as fusions of Zsig48 or a portion thereof with an immunoglobulin polypeptide or with maltose binding protein. The polypeptide immunogen may be a full-length molecule or a portion thereof. If the polypeptide portion is "hapten-like," such portion may be advantageously joined or linked to a macromolecular carrier (such as keyhole limpet hemocyanin (KLH), bovine serum albumin (BSA) or tetanus toxoid) for immunization.

Although polyclonal antibodies are typically raised in animals such as horses, cows, dogs, chicken, rats, mice, rabbits, guinea pigs, goats, or sheep, an anti- Zsig48 antibody of the present invention may also be derived from a subhuman primate antibody. General techniques for raising diagnostically and therapeutically useful antibodies in baboons may be found, for example, in Goldenberg *et al.*, international patent publication No. WO 91/11465, and in Losman *et al.*, *Int. J. Cancer 46*:310 (1990).

Alternatively, monoclonal anti-Zsig48 antibodies can be generated. Rodent monoclonal antibodies to specific antigens may be obtained by methods known to those skilled in the art (see, for example, Kohler *et al.*, *Nature 256*:495 (1975), Coligan *et al.* (eds.), *Current Protocols in Immunology, Vol. 1*, pages 2.5.1-2.6.7 (John Wiley & Sons 1991) ["Coligan"], Picksley *et al.*, "Production of monoclonal antibodies against proteins expressed in *E. coli*," in *DNA Cloning 2: Expression Systems, 2nd Edition*, Glover *et al.* (eds.), page 93 (Oxford University Press 1995)).

Briefly, monoclonal antibodies can be obtained by injecting mice with a composition comprising an Zsig48 gene product, verifying the presence of antibody production by removing a serum sample, removing the spleen to obtain B-lymphocytes, fusing the B-lymphocytes with myeloma cells to produce hybridomas, cloning the hybridomas, selecting positive clones which produce antibodies to the antigen, culturing the clones that produce antibodies to the antigen, and isolating the antibodies from the hybridoma cultures.

In addition, an anti-Zsig48 antibody of the present invention may be derived from a human monoclonal antibody. Human monoclonal antibodies are obtained from transgenic mice that have been engineered to produce specific human antibodies in response to antigenic challenge. In this technique, elements of the human heavy and light chain locus are introduced into strains of mice derived from embryonic stem cell lines that contain targeted disruptions of the endogenous heavy chain and light chain loci. The transgenic mice can synthesize human antibodies specific for human antigens, and the mice can be used to produce human antibody-secreting hybridomas. Methods for obtaining human antibodies from transgenic mice are described, for example, by Green *et al.*, *Nature Genet.*

7:13 (1994), Lonberg *et al.*, *Nature 368*:856 (1994), and Taylor *et al.*, *Int. Immun. 6*:579 (1994).

Monoclonal antibodies can be isolated and purified from hybridoma cultures by a variety of well-established techniques. Such isolation techniques include affinity chromatography with Protein-A Sepharose, size-exclusion chromatography, and ion-exchange chromatography (see, for example, Coligan at pages 2.7.1-2.7.12 and pages 2.9.1-2.9.3; Baines *et al.*, "Purification of Immunoglobulin G (IgG)," in *Methods in Molecular Biology, Vol. 10*, pages 79-104 (The Humana Press, Inc. 1992)).

For particular uses, it may be desirable to prepare fragments of anti-Zsig48 antibodies. Such antibody fragments can be obtained, for example, by proteolytic hydrolysis of the antibody. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. As an illustration, antibody fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted $F(ab')_2$. This fragment can be further cleaved using a thiol reducing agent to produce 3.5S Fab' monovalent fragments. Optionally, the cleavage reaction can be performed using a blocking group for the sulfhydryl groups that result from cleavage of disulfide linkages. As an alternative, an enzymatic cleavage using pepsin produces two monovalent Fab fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. patent No. 4,331,647, Nisonoff *et al.*, *Arch Biochem. Biophys. 89*:230 (1960), Porter, *Biochem. J. 73*:119 (1959), Edelman *et al.*, in *Methods in Enzymology Vol. 1*, page 422 (Academic Press 1967), and by Coligan at pages 2.8.1-2.8.10 and 2.10.-2.10.4.

Other methods of cleaving antibodies, 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, so long as the fragments bind to the antigen that is recognized by the intact antibody.

For example, Fv fragments comprise an association of V_H and V_L chains. This association can be noncovalent, as described by Inbar *et al.*, *Proc. Nat'l Acad. Sci. USA 69*:2659 (1972). Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde (see, for example, Sandhu, *Crit. Rev. Biotech. 12*:437 (1992)).

The Fv fragments may comprise V_H and V_L chains which are connected by a peptide linker. These single-chain antigen binding proteins (scFv) are prepared by constructing a structural gene comprising DNA sequences encoding the V_H and V_L domains which are connected by an oligonucleotide. The structural gene is inserted into an expression vector which is subsequently introduced into a host cell, such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing scFvs are described, for example, by Whitlow *et al.*, *Methods: A Companion to Methods in Enzymology 2*:97 (1991) (also see, Bird *et al.*, *Science 242*:423 (1988), Ladner *et al.*, U.S. Patent No. 4,946,778, Pack *et al.*, *Bio/Technology 11*:1271 (1993), and Sandhu, *supra*).

As an illustration, a scFV can be obtained by exposing lymphocytes to Zsig48 polypeptide *in vitro*, and selecting antibody display libraries in phage or similar vectors (for instance, through use of immobilized or labeled Zsig48 protein or peptide). Genes encoding polypeptides having potential Zsig48 polypeptide binding domains can be obtained by screening random peptide

libraries displayed on phage (phage display) or on bacteria, such as *E. coli*. Nucleotide sequences encoding the polypeptides can be obtained in a number of ways, such as through random mutagenesis and random polynucleotide synthesis. These random peptide display libraries can be used to screen for peptides which interact with a known target which can be a protein or polypeptide, such as a ligand or receptor, a biological or synthetic macromolecule, or organic or inorganic substances. Techniques for creating and screening such random peptide display libraries are known in the art (Ladner *et al.*, U.S. Patent No. 5,223,409, Ladner *et al.*, U.S. Patent No. 4,946,778, Ladner *et al.*, U.S. Patent No. 5,403,484, Ladner *et al.*, U.S. Patent No. 5,571,698, and Kay *et al.*, *Phage Display of Peptides and Proteins* (Academic Press, Inc. 1996)) and random peptide display libraries and kits for screening such libraries are available commercially, for instance from CLONTECH Laboratories, Inc. (Palo Alto, CA), Invitrogen Inc. (San Diego, CA), New England Biolabs, Inc. (Beverly, MA), and Pharmacia LKB Biotechnology Inc. (Piscataway, NJ). Random peptide display libraries can be screened using the Zsig48 sequences disclosed herein to identify proteins which bind to Zsig48.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells (see, for example, Larrick *et al.*, *Methods: A Companion to Methods in Enzymology* 2:106 (1991), Courtenay-Luck, "Genetic Manipulation of Monoclonal Antibodies," in *Monoclonal Antibodies: Production, Engineering and Clinical Application*, Ritter *et al.* (eds.), page 166 (Cambridge University Press 1995),

and Ward *et al.*, "Genetic Manipulation and Expression of Antibodies," in *Monoclonal Antibodies: Principles and Applications*, Birch *et al.*, (eds.), page 137 (Wiley-Liss, Inc. 1995)).

Alternatively, an anti-Zsig48 antibody may be derived from a "humanized" monoclonal antibody. Humanized monoclonal antibodies are produced by transferring mouse complementary determining regions from heavy and light variable chains of the mouse immunoglobulin into a human variable domain. Typical residues of human antibodies are then substituted in the framework regions of the murine counterparts. The use of antibody components derived from humanized monoclonal antibodies obviates potential problems associated with the immunogenicity of murine constant regions. General techniques for cloning murine immunoglobulin variable domains are described, for example, by Orlandi *et al.*, *Proc. Nat'l Acad. Sci. USA 86*:3833 (1989). Techniques for producing humanized monoclonal antibodies are described, for example, by Jones *et al.*, *Nature 321*:522 (1986), Carter *et al.*, *Proc. Nat'l Acad. Sci. USA 89*:4285 (1992), Sandhu, *Crit. Rev. Biotech. 12*:437 (1992), Singer *et al.*, *J. Immun. 150*:2844 (1993), Sudhir (ed.), *Antibody Engineering Protocols* (Humana Press, Inc. 1995), Kelley, "Engineering Therapeutic Antibodies," in *Protein Engineering: Principles and Practice*, Cleland *et al.* (eds.), pages 399-434 (John Wiley & Sons, Inc. 1996), and by Queen *et al.*, U.S. Patent No. 5,693,762 (1997).

Polyclonal anti-idiotype antibodies can be prepared by immunizing animals with anti-Zsig48 antibodies or antibody fragments, using standard techniques. See, for example, Green *et al.*, "Production of Polyclonal Antisera," in *Methods In Molecular Biology: Immunochemical Protocols*, Manson (ed.), pages 1-12 (Humana Press 1992). Also, see Coligan at pages 2.4.1-2.4.7. Alternatively,

monoclonal anti-idiotype antibodies can be prepared using anti-Zsig48 antibodies or antibody fragments as immunogens with the techniques, described above. As another alternative, humanized anti-idiotype antibodies or subhuman primate anti-idiotype antibodies can be prepared using the above-described techniques. Methods for producing anti-idiotype antibodies are described, for example, by Irie, U.S. Patent No. 5,208,146, Greene, *et. al.*, U.S. Patent No. 5,637,677, and Varthakavi and Minocha, *J. Gen. Virol.* 77:1875 (1996).

Diagnostic Application of Zsig48 Nucleotide Sequences

Nucleic acid molecules can be used to detect the expression of a Zsig48 gene in a biological sample. Probe molecules include double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1 or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the like.

In a basic assay, a single-stranded probe molecule is incubated with RNA, isolated from a biological sample, under conditions of temperature and ionic strength that promote base pairing between the probe and target *Zsig48* RNA species. After separating unbound probe from hybridized molecules, the amount of hybrids is detected. Illustrative biological samples include blood, urine, saliva, tissue biopsy, and autopsy material.

Well-established hybridization methods of RNA detection include northern analysis and dot/slot blot hybridization (see, for example, Ausubel (1995) at pages 4-1 to 4-27, and Wu *et al.* (eds.), "Analysis of Gene Expression at the RNA Level," in *Methods in Gene Biotechnology*, pages 225-239 (CRC Press, Inc. 1997)).

Nucleic acid probes can be detectably labeled with radioisotopes such as ^{32}P or ^{35}S. Alternatively, *Zsig48* RNA can be detected with a nonradioactive hybridization method (see, for example, Isaac (ed.), *Protocols for Nucleic Acid Analysis by Nonradioactive Probes* (Humana Press, Inc. 1993)). Typically, nonradioactive detection is achieved by enzymatic conversion of chromogenic or chemiluminescent substrates. Illustrative nonradioactive moieties include biotin, fluorescein, and digoxigenin.

Zsig48 oligonucleotide probes are also useful for *in vivo* diagnosis. As an illustration, ^{18}F-labeled oligonucleotides can be administered to a subject and visualized by positron emission tomography (Tavitian *et al.*, *Nature Medicine* 4:467 (1998)).

Numerous diagnostic procedures take advantage of the polymerase chain reaction (PCR) to increase sensitivity of detection methods. Standard techniques for performing PCR are well-known (see, generally, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), White (ed.), *PCR Protocols: Current Methods and Applications* (Humana Press, Inc. 1993), Cotter (ed.), *Molecular Diagnosis of Cancer* (Humana Press, Inc. 1996), Hanausek and Walaszek (eds.), *Tumor Marker Protocols* (Humana Press, Inc. 1998), Lo (ed.), *Clinical Applications of PCR* (Humana Press, Inc. 1998), and Meltzer (ed.), *PCR in Bioanalysis* (Humana Press, Inc. 1998)).

One variation of PCR for diagnostic assays is reverse transcriptase-PCR (RT-PCR). In the RT-PCR technique, RNA is isolated from a biological sample, reverse transcribed to cDNA, and the cDNA is incubated with *Zsig48* primers (see, for example, Wu *et al.* (eds.), "Rapid Isolation of Specific cDNAs or Genes by PCR," in *Methods in Gene Biotechnology*, pages 15-28 (CRC Press,

Inc. 1997)). PCR is then performed and the products are analyzed using standard techniques.

As an illustration, RNA is isolated from biological sample using, for example, the gunadinium-thiocyanate cell lysis procedure described above. Alternatively, a solid-phase technique can be used to isolate mRNA from a cell lysate. A reverse transcription reaction can be primed with the isolated RNA using random oligonucleotides, short homopolymers of dT, or *Zsig48* anti-sense oligomers. Oligo-dT primers offer the advantage that various mRNA nucleotide sequences are amplified that can provide control target sequences. *Zsig48* sequences are amplified by the polymerase chain reaction using two flanking oligonucleotide primers that are typically 20 bases in length.

PCR amplification products can be detected using a variety of approaches. For example, PCR products can be fractionated by gel electrophoresis, and visualized by ethidium bromide staining. Alternatively, fractionated PCR products can be transferred to a membrane, hybridized with a detectably-labeled *Zsig48* probe, and examined by autoradiography. Additional alternative approaches include the use of digoxigenin-labeled deoxyribonucleic acid triphosphates to provide chemiluminescence detection, and the C-TRAK colorimetric assay.

Another approach for detection of Zsig48 expression is cycling probe technology (CPT), in which a single-stranded DNA target binds with an excess of DNA-RNA-DNA chimeric probe to form a complex, the RNA portion is cleaved with RNAase H, and the presence of cleaved chimeric probe is detected (see, for example, Beggs *et al.*, *J. Clin. Microbiol.* *34*:2985 (1996), Bekkaoui *et al.*, *Biotechniques* *20*:240 (1996)). Alternative methods for detection of *Zsig48* sequences can utilize approaches such

as nucleic acid sequence-based amplification (NASBA), cooperative amplification of templates by cross-hybridization (CATCH), and the ligase chain reaction (LCR) (see, for example, Marshall *et al.*, U.S. Patent No. 5,686,272 (1997), Dyer *et al.*, *J. Virol. Methods 60*:161 (1996), Ehricht *et al.*, *Eur. J. Biochem. 243*:358 (1997), and Chadwick *et al.*, *J. Virol. Methods 70*:59 (1998)). Other standard methods are known to those of skill in the art.

Zsig48 probes and primers can also be used to detect and to localize *Zsig48* gene expression in tissue samples. Methods for such *in situ* hybridization are well-known to those of skill in the art (see, for example, Choo (ed.), *In Situ Hybridization Protocols* (Humana Press, Inc. 1994), Wu *et al.* (eds.), "Analysis of Cellular DNA or Abundance of mRNA by Radioactive *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 259-278 (CRC Press, Inc. 1997), and Wu *et al.* (eds.), "Localization of DNA or Abundance of mRNA by Fluorescence *In Situ* Hybridization (RISH)," in *Methods in Gene Biotechnology*, pages 279-289 (CRC Press, Inc. 1997)). Various additional diagnostic approaches are well-known to those of skill in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Coleman and Tsongalis, *Molecular Diagnostics* (Humana Press, Inc. 1996), and Elles, *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc., 1996)).

Nucleic acid molecules comprising *Zsig48* nucleotide sequences can also be used to determine whether a subject's chromosomes contain a mutation in the *Zsig48* gene which has been mapped to chromosome 7q36.3. Detectable chromosomal aberrations at the *Zsig48* gene locus include, but are not limited to, aneuploidy, gene copy number changes, insertions, deletions, restriction

site changes and rearrangements. Of particular interest are genetic alterations that inactivate the *Zsig48* gene.

Aberrations associated with the *Zsig48* locus at chromosome 7q36.3 can be detected using nucleic acid molecules of the present invention by employing molecular genetic techniques, such as restriction fragment length polymorphism (RFLP) analysis, short tandem repeat (STR) analysis employing PCR techniques, amplification-refractory mutation system analysis (ARMS), single-strand conformation polymorphism (SSCP) detection, RNase cleavage methods, denaturing gradient gel electrophoresis, fluorescence-assisted mismatch analysis (FAMA), and other genetic analysis techniques known in the art (see, for example, Mathew (ed.), *Protocols in Human Molecular Genetics* (Humana Press, Inc. 1991), Marian, *Chest 108*:255 (1995), Coleman and Tsongalis, *Molecular Diagnostics* (Human Press, Inc. 1996), Elles (ed.) *Molecular Diagnosis of Genetic Diseases* (Humana Press, Inc. 1996), Landegren (ed.), *Laboratory Protocols for Mutation Detection* (Oxford University Press 1996), Birren *et al.* (eds.), *Genome Analysis, Vol. 2: Detecting Genes* (Cold Spring Harbor Laboratory Press 1998), Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics* (John Wiley & Sons 1998), and Richards and Ward, "Molecular Diagnostic Testing," in *Principles of Molecular Medicine*, pages 83-88 (Humana Press, Inc. 1998)).

The protein truncation test is also useful for detecting the inactivation of a gene in which translation-terminating mutations produce only portions of the encoded protein (see, for example, Stoppa-Lyonnet *et al.*, *Blood 91*:3920 (1998)). According to this approach, RNA is isolated from a biological sample, and used to synthesize cDNA. PCR is then used to amplify the *Zsig48* target sequence and to introduce an RNA polymerase promoter, a translation initiation sequence, and an in-frame ATG

triplet. PCR products are transcribed using an RNA polymerase, and the transcripts are translated *in vitro* with a T7-coupled reticulocyte lysate system. The translation products are then fractionated by SDS-PAGE to determine the lengths of the translation products. The protein truncation test is described, for example, by Dracopoli *et al.* (eds.), *Current Protocols in Human Genetics,* pages 9.11.1 - 9.11.18 (John Wiley & Sons 1998).

In a related approach, Zsig48 protein is isolated from a subject, the molecular weight of the isolated Zsig48 protein is determined, and then compared with the molecular weight a normal Zsig48 protein, such as a protein having the amino acid sequence of SEQ ID NOs:2, 3, 4 or 5. A substantially lower molecular weight for the isolated Zsig48 protein is indicative that the protein is truncated. In this context, "substantially lower molecular weight" refers to at least about 10 percent lower, and preferably, at least about 25 percent lower. The Zsig48 protein may be isolated by various procedures known in the art including immunoprecipitation, solid phase radioimmunoassay, enzyme-linked immunosorbent assay, or Western blotting. The molecular weight of the isolated Zsig48 protein can be determined using standard techniques, such as SDS-polyacrylamide gel electrophoresis.

The present invention also contemplates kits for performing a diagnostic assay for *Zsig48* gene expression or to detect mutations in the *Zsig48* gene. Such kits comprise nucleic acid probes, such as double-stranded nucleic acid molecules comprising the nucleotide sequence of SEQ ID NO:1, or a fragment thereof, as well as single-stranded nucleic acid molecules having the complement of the nucleotide sequence of SEQ ID NO:1, or a fragment thereof. Probe molecules may be DNA, RNA, oligonucleotides, and the

like. Kits may comprise nucleic acid primers for performing PCR.

Preferably, such a kit contains all the necessary elements to perform a nucleic acid diagnostic assay described above. A kit will comprise at least one container comprising a *Zsig48* probe or primer. The kit may also comprise a second container comprising one or more reagents capable of indicating the presence of *Zsig48* sequences. Examples of such indicator reagents include detectable labels such as radioactive labels, fluorochromes, chemiluminescent agents, and the like. A kit may also comprise a means for conveying to the user that the *Zsig48* probes and primers are used to detect *Zsig48* gene expression. For example, written instructions may state that the enclosed nucleic acid molecules can be used to detect either a nucleic acid molecule that encodes Zsig48, or a nucleic acid molecule having a nucleotide sequence that is complementary to an Zsig48-encoding nucleotide sequence. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

Diagnostic Application of Anti-Zsig48 Antibodies

The present invention contemplates the use of anti-Zsig48 antibodies to screen biological samples *in vitro* for the presence of Zsig48. In one type of *in vitro* assay, anti-Zsig48 antibodies are used in liquid phase. For example, the presence of Zsig48 in a biological sample can be tested by mixing the biological sample with a trace amount of labeled Zsig48 and an anti-Zsig48 antibody under conditions that promote binding between Zsig48 and its antibody. Complexes of Zsig48 and anti-Zsig48 in the sample can be separated from the reaction mixture by contacting the complex with an immobilized protein which binds with the antibody, such as an Fc antibody or

Staphylococcus protein A. The concentration of Zsig48 in the biological sample will be inversely proportional to the amount of labeled Zsig48 bound to the antibody and directly related to the amount of free Zsig48. Illustrative biological samples include blood, urine, saliva, tissue biopsy, and autopsy material.

Alternatively, *in vitro* assays can be performed in which anti-Zsig48 antibody is bound to a solid-phase carrier. For example, antibody can be attached to a polymer, such as aminodextran, in order to link the antibody to an insoluble support such as a polymer-coated bead, a plate or a tube. Other suitable *in vitro* assays will be readily apparent to those of skill in the art.

In another approach, anti- Zsig48 antibodies can be used to detect Zsig48 in tissue sections prepared from a biopsy specimen. Such immunochemical detection can be used to determine the relative abundance of Zsig48 and to determine the distribution of Zsig48 in the examined tissue. General immunochemistry techniques are well established (see, for example, Ponder, "Cell Marking Techniques and Their Application," in *Mammalian Development: A Practical Approach*, Monk (ed.), pages 115-38 (IRL Press 1987), Coligan at pages 5.8.1-5.8.8, Ausubel (1995) at pages 14.6.1 to 14.6.13 (Wiley Interscience 1990), and Manson (ed.), *Methods In Molecular Biology, Vol. 10: Immunochemical Protocols* (The Humana Press, Inc. 1992)).

Immunochemical detection can be performed by contacting a biological sample with an anti-Zsig48 antibody, and then contacting the biological sample with a detectably labeled molecule which binds to the antibody. For example, the detectably labeled molecule can comprise an antibody moiety that binds to anti-Zsig48 antibody. Alternatively, the anti-Zsig48 antibody can be conjugated

with avidin/streptavidin (or biotin) and the detectably labeled molecule can comprise biotin (or avidin/streptavidin). Numerous variations of this basic technique are well-known to those of skill in the art.

Alternatively, an anti-Zsig48 antibody can be conjugated with a detectable label to form an anti-Zsig48 immunoconjugate. Suitable detectable labels include, for example, a radioisotope, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label or colloidal gold. Methods of making and detecting such detectably-labeled immunoconjugates are well-known to those of ordinary skill in the art, and are described in more detail below.

The detectable label can be a radioisotope that is detected by autoradiography. Isotopes that are particularly useful for the purpose of the present invention are ^{3}H, ^{125}I, ^{131}I, ^{35}S and ^{14}C.

Anti-Zsig48 immunoconjugates can also be labeled with a fluorescent compound. The presence of a fluorescently-labeled antibody is determined by exposing the immunoconjugate to light of the proper wavelength and detecting the resultant fluorescence. Fluorescent labeling compounds include fluorescein isothiocyanate, rhodamine, phycoerytherin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine.

Alternatively, anti-Zsig48 immunoconjugates can be detectably labeled by coupling an antibody component to a chemiluminescent compound. The presence of the chemiluminescent-tagged immunoconjugate is determined by detecting the presence of luminescence that arises during the course of a chemical reaction. Examples of chemiluminescent labeling compounds include luminol, isoluminol,

an aromatic acridinium ester, an imidazole, an acridinium salt and an oxalate ester.

Similarly, a bioluminescent compound can be used to label anti-Zsig48 immunoconjugates of the present invention. Bioluminescence is a type of chemiluminescence found in biological systems in which a catalytic protein increases the efficiency of the chemiluminescent reaction. The presence of a bioluminescent protein is determined by detecting the presence of luminescence. Bioluminescent compounds that are useful for labeling include luciferin, luciferase and aequorin.

Alternatively, anti-Zsig48 immunoconjugates can be detectably labeled by linking an anti-Zsig48 antibody component to an enzyme. When the anti-Zsig48-enzyme conjugate is incubated in the presence of the appropriate substrate, the enzyme moiety reacts with the substrate to produce a chemical moiety which can be detected, for example, by spectrophotometric, fluorometric or visual means. Examples of enzymes that can be used to detectably label polyspecific immunoconjugates include β-galactosidase, glucose oxidase, peroxidase and alkaline phosphatase.

Those of skill in the art will know of other suitable labels which can be employed in accordance with the present invention. The binding of marker moieties to anti-Zsig48 antibodies can be accomplished using standard techniques known to the art. Typical methodology in this regard is described by Kennedy *et al.*, *Clin. Chim. Acta* *70*:1 (1976), Schurs *et al.*, *Clin. Chim. Acta* *81*:1 (1977), Shih *et al.*, *Int'l J. Cancer* *46*:1101 (1990), Stein *et al.*, *Cancer Res.* *50*:1330 (1990), and Coligan, *supra*.

Moreover, the convenience and versatility of immunochemical detection can be enhanced by using anti-

Zsig48 antibodies that have been conjugated with avidin, streptavidin, and biotin (see, for example, Wilchek *et al.* (eds.), "Avidin-Biotin Technology," *Methods In Enzymology, Vol. 184* (Academic Press 1990), and Bayer *et al.*, "Immunochemical Applications of Avidin-Biotin Technology," in *Methods In Molecular Biology, Vol. 10*, Manson (ed.), pages 149-162 (The Humana Press, Inc. 1992).

Methods for performing immunoassays are well-established. See, for example, Cook and Self, "Monoclonal Antibodies in Diagnostic Immunoassays," in *Monoclonal Antibodies: Production, Engineering, and Clinical Application*, Ritter and Ladyman (eds.), pages 180-208, (Cambridge University Press, 1995), Perry, "The Role of Monoclonal Antibodies in the Advancement of Immunoassay Technology," in *Monoclonal Antibodies: Principles and Applications*, Birch and Lennox (eds.), pages 107-120 (Wiley-Liss, Inc. 1995), and Diamandis, *Immunoassay* (Academic Press, Inc. 1996). In a related approach, biotin- or FITC-labeled Zsig48 can be used to identify cells that bind Zsig48. Such can binding can be detected, for example, using flow cytometry.

The present invention also contemplates kits for performing an immunological diagnostic assay for Zsig48 gene expression. Such kits comprise at least one container comprising an anti-Zsig48 antibody, or antibody fragment. A kit may also comprise a second container comprising one or more reagents capable of indicating the presence of Zsig48 antibody or antibody fragments. Examples of such indicator reagents include detectable labels such as a radioactive label, a fluorescent label, a chemiluminescent label, an enzyme label, a bioluminescent label, colloidal gold, and the like. A kit may also comprise a means for conveying to the user that Zsig48 antibodies or antibody fragments are used to detect Zsig48 protein. For example, written instructions may state that the enclosed antibody

or antibody fragment can be used to detect Zsig48. The written material can be applied directly to a container, or the written material can be provided in the form of a packaging insert.

Therapeutic Uses of Polypeptides Having Zsig48 Activity

Molecules of the present invention can be used to promote proliferation of peripheral blood leukocytes. This can be useful in treating cancer patients whose leukocytes have been depleted by chemotherapy, radiation or illness. Zsig48 can be administered to patients receiving bone marrow transplants to promote proliferation of leukocytes produced by the transplanted marrow. Also Zsig48 would be useful in treating immunosuppressed individuals as in the elderly or human immmundeficiency virus (HIV) infected individuals. Zsig48 can also be used as a vaccine adjuvant to be administered with vaccines.

Generally, a dosage of Zsig48 administered for inducing the proliferation of T-cells, B-cells and monocytes (or Zsig48 analog or fusion protein) will vary depending upon such factors as the patient's age, weight, height, sex, general medical condition and previous medical history. Typically, it is desirable to provide the recipient with a dosage of Zsig48 which is in the range of from about 1 pg/kg to 10 mg/kg per day (amount of agent/body weight of patient), preferably the dose will range from 4 - 100 μg/kg per day administered intravenously although a lower or higher dosage also may be administered as circumstances dictate.

Administration of a molecule having Zsig48 activity to a subject can be intravenous, intraarterial, intraperitoneal, intramuscular, subcutaneous, intrapleural, intrathecal, by perfusion through a regional

catheter, or by direct intralesional injection. When administering therapeutic proteins by injection, the administration may be by continuous infusion or by single or multiple boluses. Alternatively, Zsig48 can be administered as a controlled release formulation. For example, Cleland and Jones, *Pharm. Res. 13*:1464 (1996), describe a method for producing interferon-γ encapsulated in polylactic-coglycolic microspheres.

Additional routes of administration include oral, dermal, mucosal-membrane, pulmonary, and transcutaneous. Oral delivery is suitable for polyester microspheres, zein microspheres, proteinoid microspheres, polycyanoacrylate microspheres, and lipid-based systems [see, for example, DiBase and Morrel, "Oral Delivery of Microencapsulated Proteins," in *Protein Delivery: Physical Systems*, Sanders and Hendren (eds.), pages 255-288 (Plenum Press 1997)]. The feasibility of an intranasal delivery is exemplified by such a mode of insulin administration [see, for example, Hinchcliffe and Illum, *Adv. Drug Deliv. Rev. 35*:199 (1999)]. Dry or liquid particles comprising Zsig48 can be prepared and inhaled with the aid of dry-powder dispersers, liquid aerosol generators, or nebulizers [*e.g.*, Pettit and Gombotz, *TIBTECH 16*:343 (1998); Patton *et al.*, *Adv. Drug Deliv. Rev. 35*:235 (1999)]. This approach is illustrated by the AERX diabetes management system, which is a hand-held electronic inhaler that delivers aerosolized insulin into the lungs. Studies have shown that proteins as large as 48,000 kDa have been delivered across skin at therapeutic concentrations with the aid of low-frequency ultrasound, which illustrates the feasibility of transcutaneous administration [Mitragotri *et al.*, *Science 269*:850 (1995)]. Transdermal delivery using electroporation provides another means to administer Zsig48 (Potts *et al.*, *Pharm. Biotechnol. 10*:213 (1997)).

A pharmaceutical composition comprising a protein, polypeptide, or peptide having Zsig48 activity can be formulated according to known methods to prepare pharmaceutically useful compositions, wherein the therapeutic proteins are combined in a mixture with a pharmaceutically acceptable carrier. A composition is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient patient. Sterile phosphate-buffered saline, water, TWEEN® 80 with mannitol and sodium acetate are examples of a pharmaceutically acceptable carriers and additives. Other suitable carriers are well-known to those in the art. See, for example, Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995).

For purposes of therapy, molecules having Zsig48 activity and a pharmaceutically acceptable carrier are administered to a patient in a therapeutically effective amount. A combination of a protein, polypeptide, or peptide having Zsig48 activity and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient. In the present context, an agent is physiologically significant if its presence results in the proliferation T-cells, B-cells or monocytes.

A pharmaceutical composition comprising molecules having Zsig48 activity can be furnished in liquid form, in an aerosol, or in solid form. Proteins having Zsig48 activity, such as human or murine Zsig48, can be administered as a conjugate with a pharmaceutically acceptable water-soluble polymer moiety, as described above. As an illustration, a Zsig48-polyethylene glycol

conjugate is useful to increase the circulating half-life of the Zsig48, and to reduce the immunogenicity of the polypeptide. Liquid forms, including liposome-encapsulated formulations, are illustrated by injectable solutions and oral suspensions. Exemplary solid forms include capsules, tablets, and controlled-release forms, such as a miniosmotic pump or an implant. Other dosage forms can be devised by those skilled in the art, as shown, for example, by Ansel and Popovich, *Pharmaceutical Dosage Forms and Drug Delivery Systems*, 5th Edition (Lea & Febiger 1990), Gennaro (ed.), *Remington's Pharmaceutical Sciences*, 19th Edition (Mack Publishing Company 1995), and by Ranade and Hollinger, *Drug Delivery Systems* (CRC Press 1996).

As an illustration, Zsig48 pharmaceutical compositions may be supplied as a kit comprising a container that comprises Zsig48, a Zsig48 agonist, or a Zsig48 antagonist (*e.g.*, an anti- Zsig48 antibody or antibody fragment). Zsig48 can be provided in the form of a solution for injection for single or multiple doses, or as a sterile powder that will be reconstituted before injection. Alternatively, such a kit can include a dry-powder disperser, liquid aerosol generator, or nebulizer for administration of a therapeutic polypeptide. Such a kit may further comprise written information on indications and usage of the pharmaceutical composition. Moreover, such information may include a statement that the Zsig48 composition is contraindicated in patients with known hypersensitivity to Zsig48.

Therapeutic Uses of Zsig48 Nucleotide Sequences

Immunomodulator *Zsig48* genes can be introduced into a subject to enhance immunological responses by causing localized expression of Zsig48. As an illustration "immunomodulator gene therapy" has been

examined in model systems using vectors that express IL-2, IL-3, IL-4, IL-6, IL-10, IL-12, IL-15, interferon-γ, tumor necrosis factor-α, or granulocyte-macrophage colony-stimulating factor [see, for example, Cao *et al.*, *J. Gastroenterol. Hepatol.* *11*:1053 (1996), Tahara *et al.*, *Ann. N. Y. Acad. Sci.* *795*:275 (1996), Rakhmilevich *et al.*, *Hum. Gene Ther.* *8*:1303 (1997), and Cao *et al.*, *Transplantation* *65*:325 (1998)]. The present invention includes the use of *Zsig48* nucleotide sequences to augment the immune system to promote proliferation of leukocytes especially T-cells, B-cells or monocytes. In addition, a therapeutic expression vector can be provided that inhibits *Zsig48* gene expression, such as an anti-sense molecule, a ribozyme, or an external guide sequence molecule.

There are numerous approaches to introduce a *Zsig48* gene to a subject, including the use of recombinant host cells that express Zsig48, delivery of naked nucleic acid encoding Zsig48,use of a cationic lipid carrier with a nucleic acid molecule that encodes Zsig48, and the use of viruses that express Zsig48, such as recombinant retroviruses, recombinant adeno-associated viruses, recombinant adenoviruses, and recombinant Herpes simplex viruses [HSV] (see, for example, Mulligan, *Science* *260*:926 (1993), Rosenberg *et al.*, *Science* *242*:1575 (1988), LaSalle *et al.*, *Science* *259*:988 (1993), Wolff *et al.*, *Science* *247*:1465 (1990), Breakfield and Deluca, *The New Biologist* *3*:203 (1991)). In an *ex vivo* approach, for example, cells are isolated from a subject, transfected with a vector that expresses a *Zsig48* gene, and then transplanted into the subject.

In order to effect expression of a *Zsig48* gene, an expression vector is constructed in which a nucleotide sequence encoding a *Zsig48* gene is operably linked to a core promoter, and optionally a regulatory element, to

control gene transcription. The general requirements of an expression vector are described above.

Alternatively, a *Zsig48* gene can be delivered using recombinant viral vectors, including for example, adenoviral vectors [*e.g.*, Kass-Eisler *et al.*, *Proc. Nat'l Acad. Sci. USA 90*:11498 (1993), Kolls *et al.*, *Proc. Nat'l Acad. Sci. USA 91*:215 (1994), Li *et al.*, *Hum. Gene Ther.* 4:403 (1993), Vincent *et al.*, *Nat. Genet.* 5:130 (1993), and Zabner *et al.*, *Cell* 75:207 (1993)], adenovirus-associated viral vectors [Flotte *et al.*, *Proc. Nat'l Acad. Sci. USA 90*:10613 (1993)], alphaviruses such as Semliki Forest Virus and Sindbis Virus [Hertz and Huang, *J. Vir. 66*:857 (1992), Raju and Huang, *J. Vir. 65*:2501 (1991), and Xiong *et al.*, *Science* 243:1188 (1989)], herpes viral vectors [*e.g.*, U.S. Patent Nos. 4,769,331, 4,859,587, 5,288,641 and 5,328,688), parvovirus vectors (Koering *et al.*, *Hum. Gene Therap. 5*:457 (1994)], pox virus vectors [Ozaki *et al.*, *Biochem. Biophys. Res. Comm. 193*:653 (1993), Panicali and Paoletti, *Proc. Nat'l Acad. Sci. USA 79*:4927 (1982)], pox viruses, such as canary pox virus or vaccinia virus [Fisher-Hoch *et al.*, *Proc. Nat'l Acad. Sci. USA 86*:317 (1989), and Flexner *et al.*, *Ann. N.Y. Acad. Sci. 569*:86 (1989)], and retroviruses (*e.g.*, Baba *et al.*, *J. Neurosurg 79*:729 (1993), Ram *et al.*, *Cancer Res. 53*:83 (1993), Takamiya *et al.*, *J. Neurosci. Res 33*:493 (1992), Vile and Hart, *Cancer Res. 53*:962 (1993), Vile and Hart, *Cancer Res. 53*:3860 (1993), and Anderson *et al.*, U.S. Patent No. 5,399,346). Within various embodiments, either the viral vector itself, or a viral particle which contains the viral vector may be utilized in the methods and compositions described below.

As an illustration of one system, adenovirus, a double-stranded DNA virus, is a well-characterized gene transfer vector for delivery of a heterologous nucleic acid molecule [for a review, see Becker *et al.*, *Meth. Cell*

Biol. 43:161 (1994); Douglas and Curiel, *Science & Medicine* 4:44 (1997)]. The adenovirus system offers several advantages including: (i) the ability to accommodate relatively large DNA inserts, (ii) the ability to be grown to high-titer, (iii) the ability to infect a broad range of mammalian cell types, and (iv) the ability to be used with many different promoters including ubiquitous, tissue specific, and regulatable promoters. In addition, adenoviruses can be administered by intravenous injection, because the viruses are stable in the bloodstream.

Using adenovirus vectors where portions of the adenovirus genome are deleted, inserts are incorporated into the viral DNA by direct ligation or by homologous recombination with a co-transfected plasmid. In an exemplary system, the essential E1 gene is deleted from the viral vector, and the virus will not replicate unless the E1 gene is provided by the host cell. When intravenously administered to intact animals, adenovirus primarily targets the liver. Although an adenoviral delivery system with an E1 gene deletion cannot replicate in the host cells, the host's tissue will express and process an encoded heterologous protein. Host cells will also secrete the heterologous protein if the corresponding gene includes a secretory signal sequence. Secreted proteins will enter the circulation from tissue that expresses the heterologous gene (*e.g.*, the highly vascularized liver).

Moreover, adenoviral vectors containing various deletions of viral genes can be used to reduce or eliminate immune responses to the vector. Such adenoviruses are E1-deleted, and in addition, contain deletions of E2A or E4 (Lusky *et al.*, *J. Virol.* 72:2022 (1998); Raper *et al.*, *Human Gene Therapy* 9:671 (1998)). The deletion of E2b has also been reported to reduce

immune responses (Amalfitano *et al.*, *J. Virol.* 72:926 (1998)). By deleting the entire adenovirus genome, very large inserts of heterologous DNA can be accommodated. Generation of so called "gutless" adenoviruses, where all viral genes are deleted, are particularly advantageous for insertion of large inserts of heterologous DNA [for a review, see Yeh. and Perricaudet, *FASEB J.* 11:615 (1997)].

High titer stocks of recombinant viruses capable of expressing a therapeutic gene can be obtained from infected mammalian cells using standard methods. For example, recombinant HSV can be prepared in Vero cells, as described by Brandt *et al.*, *J. Gen. Virol.* 72:2043 (1991), Herold *et al.*, *J. Gen. Virol.* 75:1211 (1994), Visalli and Brandt, *Virology 185*:419 (1991), Grau *et al.*, *Invest. Ophthalmol. Vis. Sci.* 30:2474 (1989), Brandt *et al.*, *J. Virol. Meth.* 36:209 (1992), and by Brown and MacLean (eds.), *HSV Virus Protocols* (Humana Press 1997).

Alternatively, an expression vector comprising a *Zsig48* gene can be introduced into a subject's cells by lipofection *in vivo* using liposomes. Synthetic cationic lipids can be used to prepare liposomes for *in vivo* transfection of a gene encoding a marker [Felgner *et al.*, *Proc. Nat'l Acad. Sci. USA 84*:7413 (1987); Mackey *et al.*, *Proc. Nat'l Acad. Sci. USA 85*:8027 (1988)]. The use of lipofection to introduce exogenous genes into specific organs *in vivo* has certain practical advantages. Liposomes can be used to direct transfection to particular cell types, which is particularly advantageous in a tissue with cellular heterogeneity, such as the pancreas, liver, kidney, and brain. Lipids may be chemically coupled to other molecules for the purpose of targeting. Targeted peptides (*e.g.*, hormones or neurotransmitters), proteins such as antibodies, or non-peptide molecules can be coupled to liposomes chemically.

Electroporation is another alternative mode of administration. For example, Aihara and Miyazaki, *Nature Biotechnology 16*:867 (1998), have demonstrated the use of *in vivo* electroporation for gene transfer into muscle.

In an alternative approach to gene therapy, a therapeutic gene may encode a *Zsig48* anti-sense RNA that inhibits the expression of Zsig48. Suitable sequences for anti-sense molecules can be derived from the nucleotide sequences of Zsig48 disclosed herein.

Alternatively, an expression vector can be constructed in which a regulatory element is operably linked to a nucleotide sequence that encodes a ribozyme. Ribozymes can be designed to express endonuclease activity that is directed to a certain target sequence in a mRNA molecule (see, for example, Draper and Macejak, U.S. Patent No. 5,496,698, McSwiggen, U.S. Patent No. 5,525,468, Chowrira and McSwiggen, U.S. Patent No. 5,631,359, and Robertson and Goldberg, U.S. Patent No. 5,225,337). In the context of the present invention, ribozymes include nucleotide sequences that bind with *Zsig48* mRNA.

In another approach, expression vectors can be constructed in which a regulatory element directs the production of RNA transcripts capable of promoting RNase P-mediated cleavage of mRNA molecules that encode a *Zsig48* gene. According to this approach, an external guide sequence can be constructed for directing the endogenous ribozyme, RNase P, to a particular species of intracellular mRNA, which is subsequently cleaved by the cellular ribozyme (see, for example, Altman *et al.*, U.S. Patent No. 5,168,053, Yuan *et al.*, *Science 263*:1269 (1994), Pace *et al.*, international publication No. WO 96/18733, George *et al.*, international publication No. WO 96/21731, and Werner *et al.*, international publication No. WO 97/33991).

Preferably, the external guide sequence comprises a ten to fifteen nucleotide sequence complementary to *Zsig48* mRNA, and a 3'-NCCA nucleotide sequence, wherein N is preferably a purine. The external guide sequence transcripts bind to the targeted mRNA species by the formation of base pairs between the mRNA and the complementary external guide sequences, thus promoting cleavage of mRNA by RNase P at the nucleotide located at the 5'-side of the base-paired region.

In general, the dosage of a composition comprising a therapeutic vector having a *Zsig48* nucleotide acid sequence, such as a recombinant virus, will vary depending upon such factors as the subject's age, weight, height, sex, general medical condition and previous medical history. Suitable routes of administration of therapeutic vectors include intravenous injection, intraarterial injection, intraperitoneal injection, intramuscular injection, intratumoral injection, and injection into a cavity that contains a tumor. As an illustration, Horton *et al.*, *Proc. Nat'l Acad. Sci. USA* *96*:1553 (1999), demonstrated that intramuscular injection of plasmid DNA encoding interferon-α produces potent antitumor effects on primary and metastatic tumors in a murine model.

A composition comprising viral vectors, non-viral vectors, or a combination of viral and non-viral vectors of the present invention can be formulated according to known methods to prepare pharmaceutically useful compositions, whereby vectors or viruses are combined in a mixture with a pharmaceutically acceptable carrier. As noted above, a composition, such as phosphate-buffered saline is said to be a "pharmaceutically acceptable carrier" if its administration can be tolerated by a recipient subject. Other suitable carriers are well-known to those in the art

(see, for example, *Remington's Pharmaceutical Sciences, 19th Ed.* (Mack Publishing Co. 1995), and *Gilman's the Pharmacological Basis of Therapeutics, 7th Ed.* (MacMillan Publishing Co. 1985)).

For purposes of therapy, a therapeutic gene expression vector, or a recombinant virus comprising such a vector, and a pharmaceutically acceptable carrier are administered to a subject in a therapeutically effective amount. A combination of an expression vector (or virus) and a pharmaceutically acceptable carrier is said to be administered in a "therapeutically effective amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient subject. In the present context, an agent is physiologically significant if its presence causes proliferation T-cells, B-cells or monocytes.

When the subject treated with a therapeutic gene expression vector or a recombinant virus is a human, then the therapy is preferably somatic cell gene therapy. That is, the preferred treatment of a human with a therapeutic gene expression vector or a recombinant virus does not entail introducing into cells a nucleic acid molecule that can form part of a human germ line and be passed onto successive generations (*i.e.*, human germ line gene therapy).

Production of Transgenic Mice

Transgenic mice can be engineered to over-express the human *Zsig48* gene in all tissues or under the control of a tissue-specific or tissue-preferred regulatory element. These over-producers of Zsig48 can be used to characterize the phenotype that results from over-expression, and the transgenic animals can serve as models

for human disease caused by excess Zsig48. Transgenic mice that over-express Zsig48 also provide model bioreactors for production of Zsig48 in the milk or blood of larger animals. Methods for producing transgenic mice are well-known to those of skill in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), Monastersky and Robl (eds.), *Strategies in Transgenic Animal Science* (ASM Press 1995), and Abbud and Nilson, "Recombinant Protein Expression in Transgenic Mice," in *Gene Expression Systems: Using Nature for the Art of Expression*, Fernandez and Hoeffler (eds.), pages 367-397 (Academic Press, Inc. 1999)].

For example, a method for producing a transgenic mouse that expresses a *Zsig48* gene can begin with adult, fertile males (studs) (B6C3f1, 2-8 months of age (Taconic Farms, Germantown, NY)), vasectomized males (duds) (B6D2f1, 2-8 months, (Taconic Farms)), prepubescent fertile females (donors) (B6C3f1, 4-5 weeks, (Taconic Farms)) and adult fertile females (recipients) (B6D2f1, 2-4 months, (Taconic Farms)). The donors are acclimated for one week and then injected with approximately 8 IU/mouse of Pregnant Mare's Serum gonadotrophin (Sigma Chemical Company; St. Louis, MO) I.P., and 46-47 hours later, 8 IU/mouse of human Chorionic Gonadotropin (hCG (Sigma)) I.P. to induce superovulation. Donors are mated with studs subsequent to hormone injections. Ovulation generally occurs within 13 hours of hCG injection. Copulation is confirmed by the presence of a vaginal plug the morning following mating. Fertilized eggs are collected under a surgical scope. The oviducts are collected and eggs are released into urinanalysis slides containing hyaluronidase (Sigma). Eggs are washed once in hyaluronidase, and twice in Whitten's W640 medium (described, for example, by Menino and O'Claray, *Biol.*

Reprod. 77:159 (1986), and Dienhart and Downs, *Zygote* 4:129 (1996)) that has been incubated with 5% CO_2, 5% O_2, and 90% N_2 at 37°C. The eggs are then stored in a 37°C/5% CO_2 incubator until microinjection.

Ten to twenty micrograms of plasmid DNA containing a Zsig48 encoding sequence is linearized, gel-purified, and resuspended in 10 mM Tris-HCl (pH 7.4), 0.25 mM EDTA (pH 8.0), at a final concentration of 5-10 nanograms per microliter for microinjection. For example, the Zsig48 encoding sequences can encode the amino acid sequence of SEQ ID NOs: 2, 3, 4 or 5. Plasmid DNA is microinjected into harvested eggs contained in a drop of W640 medium overlaid by warm, CO_2-equilibrated mineral oil. The DNA is drawn into an injection needle (pulled from a 0.75mm ID, 1mm OD borosilicate glass capillary), and injected into individual eggs. Each egg is penetrated with the injection needle, into one or both of the haploid pronuclei. Picoliters of DNA are injected into the pronuclei, and the injection needle withdrawn without coming into contact with the nucleoli. The procedure is repeated until all the eggs are injected. Successfully microinjected eggs are transferred into an organ tissue-culture dish with pre-gassed W640 medium for storage overnight in a 37°C/5% CO_2 incubator.

The following day, two-cell embryos are transferred into pseudopregnant recipients. The recipients are identified by the presence of copulation plugs, after copulating with vasectomized duds. Recipients are anesthetized and shaved on the dorsal left side and transferred to a surgical microscope. A small incision is made in the skin and through the muscle wall in the middle of the abdominal area outlined by the ribcage, the saddle, and the hind leg, midway between knee and spleen. The reproductive organs are exteriorized onto a small surgical drape. The fat pad is stretched out over the surgical

drape, and a baby serrefine (Roboz, Rockville, MD) is attached to the fat pad and left hanging over the back of the mouse, preventing the organs from sliding back in. With a fine transfer pipette containing mineral oil followed by alternating W640 and air bubbles, 12-17 healthy two-cell embryos from the previous day's injection are transferred into the recipient. The swollen ampulla is located and holding the oviduct between the ampulla and the bursa, a nick in the oviduct is made with a 28 g needle close to the bursa, making sure not to tear the ampulla or the bursa.

The pipette is transferred into the nick in the oviduct, and the embryos are blown in, allowing the first air bubble to escape the pipette. The fat pad is gently pushed into the peritoneum, and the reproductive organs allowed to slide in. The peritoneal wall is closed with one suture and the skin closed with a wound clip. The mice recuperate on a 37°C slide warmer for a minimum of four hours. The recipients are returned to cages in pairs, and allowed 19-21 days gestation. After birth, 19-21 days postpartum is allowed before weaning. The weanlings are sexed and placed into separate sex cages, and a 0.5 cm biopsy (used for genotyping) is snipped off the tail with clean scissors. Genomic DNA is prepared from the tail snips using, for example, a QIAGEN DNEASY kit following the manufacturer's instructions. Genomic DNA is analyzed by PCR using primers designed to amplify a *Zsig48* gene or a selectable marker gene that was introduced in the same plasmid. After animals are confirmed to be transgenic, they are back-crossed into an inbred strain by placing a transgenic female with a wild-type male, or a transgenic male with one or two wild-type female(s). As pups are born and weaned, the sexes are separated, and their tails snipped for genotyping.

To check for expression of a transgene in a live animal, a partial hepatectomy is performed. A surgical prep is made of the upper abdomen directly below the zyphoid process. Using sterile technique, a small 1.5-2 cm incision is made below the sternum and the left lateral lobe of the liver exteriorized. Using 4-0 silk, a tie is made around the lower lobe securing it outside the body cavity. An atraumatic clamp is used to hold the tie while a second loop of absorbable Dexon (American Cyanamid; Wayne, N.J.) is placed proximal to the first tie. A distal cut is made from the Dexon tie and approximately 100 mg of the excised liver tissue is placed in a sterile petri dish. The excised liver section is transferred to a 14 ml polypropylene round bottom tube and snap frozen in liquid nitrogen and then stored on dry ice. The surgical site is closed with suture and wound clips, and the animal's cage placed on a 37°C heating pad for 24 hours post operatively. The animal is checked daily post operatively and the wound clips removed 7-10 days after surgery. The expression level of *Zsig48* mRNA is examined for each transgenic mouse using an RNA solution hybridization assay or polymerase chain reaction.

In addition to producing transgenic mice that over-express Zsig48, it is useful to engineer transgenic mice with either abnormally low or no expression of the gene. Such transgenic mice provide useful models for diseases associated with a lack of Zsig48. As discussed above, *Zsig48* gene expression can be inhibited using anti-sense genes, ribozyme genes, or external guide sequence genes. To produce transgenic mice that under-express the *Zsig48* gene, such inhibitory sequences are targeted to murine *Zsig48* mRNA. Methods for producing transgenic mice that have abnormally low expression of a particular gene are known to those in the art [see, for example, Wu *et al.*, "Gene Underexpression in Cultured Cells and Animals

by Antisense DNA and RNA Strategies," in *Methods in Gene Biotechnology*, pages 205-224 (CRC Press 1997)].

An alternative approach to producing transgenic mice that have little or no *Zsig48* gene expression is to generate mice having at least one normal *Zsig48* allele replaced by a nonfunctional *Zsig48* gene. One method of designing a nonfunctional *Zsig48* gene is to insert another gene, such as a selectable marker gene, within a nucleic acid molecule that encodes murine Zsig48. Standard methods for producing these so-called "knockout mice" are known to those skilled in the art [see, for example, Jacob, "Expression and Knockout of Interferons in Transgenic Mice," in *Overexpression and Knockout of Cytokines in Transgenic Mice*, Jacob (ed.), pages 111-124 (Academic Press, Ltd. 1994), and Wu *et al.*, "New Strategies for Gene Knockout," in *Methods in Gene Biotechnology*, pages 339-365 (CRC Press 1997)].

The present invention, thus generally described, will be understood more readily by reference to the following examples, which is provided by way of illustration and is not intended to be limiting of the present invention.

Example 1

Cloning of Zsig48

The expressed sequence tag (EST) of SEQ ID NO: 6 was discovered through the random sequencing of a mixed hematopoietic cDNA library, described in Example 2 below, and the full-length clone isolated and sequenced resulting in the sequences of SEQ ID NOs: 1 and 2.
Analysis of the 1.6 kb insert in pSLzsig48 revealed the presence of an *Eco* RI adapter sequence used for cDNA synthesis at the 5' end of the insert. At the 3' end of the insert there is a *Xho* I site. However the *Xho* I site lacks the flanking sequence that is present on the

oligonucleotide primer. This suggests that the pSLzig48 insert might be derived from a genomic contaminant that co-purified with the cDNA preparation.

Example 2
Production of a Hematopoietic Cell cDNA Library

cDNAs from human hematopoietic cell lines, K562 (ATCC #CCL243), Daudi (ATCC #CCL213, HL-60(ATCC CCL240), MOLT-4 (ATCC #CRL1582) and Raji ATCC #CCL86 were synthesized in separate reactions and size fractionated in the following manner. RNA extracted from each one of the cell lines was reversed transcribed in the following manner. The first strand cDNA reaction contained 10 µl of twice poly d(T)-selected poly $(A)^+$ mRNA from K562, Daudi, HL-60, MOLT-4 or Raji cells (Clontech, Palo Alto, CA) at a concentration of 1.0 mg/ml, and 2 µl of 20 pmole/µl first strand primer SEQ ID NO:7 (GTC TGG GTT CGC TAC TCG AGG CGG CCG CTA TTT TTT TTT TTT TTT TTT) containing an *Xho* I restriction site. The mixture was heated at 70°C for 3.0 minutes and cooled by chilling on ice. First strand cDNA synthesis was initiated by the addition of 8 µl of first strand buffer (5x SUPERSCRIPT™ buffer; Life Technologies, Gaithersburg, MD), 4.0 µl of 100 mM dithiothreitol, and 3.0 µl of a deoxynucleotide triphosphate (dNTP) solution containing 10 mM each of dTTP, dATP, dGTP and 5-methyl-dCTP (Pharmacia LKB Biotechnology, Piscataway, NJ) to the RNA-primer mixture. The reaction mixture was incubated at 37° C for 2 minutes, followed by the addition of 10 µl of 200 U/µl RNase H^- reverse transcriptase (SUPERSCRIPT II®; Life Technologies). The efficiency of the first strand synthesis was analyzed in a parallel reaction by the addition of 10 µCi of ^{32}P-αdCTP to a 5 µl aliquot from one of the reaction mixtures to label the reaction for

analysis. The reactions were incubated at 37°C for 10 minutes, 45°C for 50 minutes, then incubated at 50°C for 10 minutes. Unincorporated ^{32}P-αdCTP in the labeled reaction was removed by chromatography on a 400 pore size gel filtration column (Clontech Laboratories, Palo Alto, CA). The unincorporated nucleotides and primers in the unlabeled first strand reactions were removed by chromatography on 400 pore size gel filtration column (Clontech Laboratories, Palo Alto, CA). The length of labeled first strand cDNA was determined by agarose gel electrophoresis.

The second strand reaction contained 135 µl of the unlabeled first strand cDNA, 40 µl of 5x polymerase I buffer (125 mM Tris: HCl, pH 7.5, 500 mM KCl, 25 mM $MgCl_2$, 50mM $(NH_4)_2SO_4$)), 2.5 µl of 100 mM dithiothreitol, 5.0 µl of a solution containing 10 mM of each deoxynucleotide triphosphate, 7 µl of 5 mM β-NAD, 2.5 µl of 10 U/µl *E. coli* DNA ligase (New England Biolabs; Beverly, MA), 7 µl of 10 U/µl *E. coli* DNA polymerase I (New England Biolabs, Beverly, MA), and 2.0 µl of 2 U/µl RNase H (Life Technologies, Gaithersburg, MD). A 10 µl aliquot from one of the second strand synthesis reactions was labeled by the addition of 10 µCi ^{32}P-αdCTP to monitor the efficiency of second strand synthesis. The reactions were incubated at 16° C for two hours, followed by the addition of 1 µl of a 10 mM dNTP solution and 5.0 µl T4 DNA polymerase (10 U/µl, Boehringer Mannheim, Indianapolis, IN) and incubated for an additional 10 minutes at 16°C. Unincorporated ^{32}P-αdCTP in the labeled reaction was removed by chromatography through a 400 pore size gel filtration column (Clontech Laboratories, Palo Alto, CA) before analysis by agarose gel electrophoresis. The

reaction was terminated by the addition of 20.0 µl 0.5 M EDTA and extraction with phenol/chloroform and chloroform followed by ethanol precipitation in the presence of 3.0 M Na acetate and 2 µl of PELLET PAINT® carrier (Novagen, Madison, WI). The cDNAs were ethanol precipitated a second time to remove possible trace levels of EDTA. The yield of cDNA was estimated to be approximately 2 µg from starting mRNA template of 10 µg.

Eco RI adapters were ligated onto the 5' ends of the cDNA described above to enable cloning into an expression vector. A 12.0 µl aliquot of cDNA (~2.0 µg) and 4 µl of 69 pmole/µl of *Eco* RI adapter (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) were mixed with 2.5 µl 10x ligase buffer (660 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$), 3.0 µl of 10 mM ATP, 3.5 µl 0.1 M DTT and 1 µl of 15 U/µl T4 DNA ligase (Promega Corp., Madison, WI). The reaction was incubated in a 0° to 22° C temperature gradient for 48 hours. The reaction was terminated by the adding 65 µl H_2O and 10 µl 10X H buffer (Boehringer Mannheim, Indianapolis, IN) and incubating the mixture at 70° C for 20 minutes.

To facilitate the directional cloning of the cDNA into an expression vector, the cDNA was digested with *Xho* I, resulting in a cDNA having a 5' *Eco* RI cohesive end and a 3' *Xho* I cohesive end. The *Xho* I restriction site at the 3' end of the cDNA had been previously introduced. Restriction enzyme digestion was carried out in a reaction mixture by the addition of 1.0 µl of 40 U/µl *Xho* I (Boehringer Mannheim, Indianapolis, IN). Digestion was carried out at 37°C for one hour. The reaction was terminated by incubation at 70°C for 20 minutes and

chromatography through a 400 pore size gel filtration column (Clontech Laboratories, Palo Alto, CA).

The cDNA was ethanol precipitated, washed with 70% ethanol, air dried and resuspended in 13.5 µl water, 2 µl of 10X kinase buffer (660 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$), 0.5 µl 0.1 M DTT, 3 µl 10 mM ATP, 1.0 µl T4 polynucleotide kinase (10 U/µl, Life Technologies, Gaithersburg, MD). Following incubation at 37° C for 30 minutes, the cDNA was ethanol precipitated in the presence of 2.5 M Ammonium Acetate, and electrophoresed on a 0.8% low melt agarose gel. The contaminating adapters and cDNA below 0.5 kb in length were excised and discarded. The region of the gel containing cDNAs 0.5 to 2 kb in length was excised and was placed in an empty adjacent lane of the gel at a position identical in distance to the lane origin. The electrodes were reversed, and the cDNA was electrophoresed until the 0.5 to 2 kb length cDNA was concentrated near the lane origin. The areas of the gel containing the concentrated cDNAs were excised and placed in a microfuge tube, and the approximate volume of the gel slice was determined. An aliquot of water approximately three times the volume of the gel slice (300 µl) and 35 µl 10x β-agarose I buffer (New England Biolabs) was added to the tube, and the agarose was melted by heating to 65°C for 15 minutes. Following equilibration of the sample to 45°C, 3 µl of 1 U/µl β-agarose I (New England Biolabs, Beverly, MA) was added, and the mixture was incubated for 60 minutes at 45°C to digest the agarose. After incubation, 40 µl of 3 M Na acetate was added to the sample, and the mixture was incubated on ice for 15 minutes. The sample was centrifuged at 14,000 x g for 15 minutes at room temperature to remove undigested agarose. The cDNA was

ethanol precipitated, washed in 70% ethanol, air-dried and resuspended in 40 µl water.

Following recovery from low-melt agarose gel, the 0.5-2 kb fractions were pooled and cloned into pBLUESCRIPT (Gibco/BRL) to yield the K562L library. The pooling of the cDNAs from the five cell lines was done to increase message complexity, particularly for cDNAs encoding cytokines and cytokine receptors.

Example 3

Proliferation of Mixed Lymphocyte and Peripheral Blood Monocytes using Zsig48

The object of the present example was to test the effect of Zsig48 on peripheral blood leukocytes in a mixed leukocyte reaction.

A mixed leukocyte reaction (MLR) is induced by culturing mononuclear leukocytes (which include T cells, B cells and monocytes from one individual with mononuclear leukocytes derived from another individual. These cells are generally isolated from peripheral blood. If there are differences in the alleles of the major histocompatibility complex (MHC) genes between the two individuals the mononuclear cells will proliferate over a period of from 4-7 days. This proliferative response is measured by incorporation of ^{3}H-thymidine into DNA during cell replication. This is called the allogeneic MLR. In the present experiment the mononuclear cells from one of the individuals was rendered incapable of proliferation, by gamma irradiation prior to culture. The irradiated cells are termed the stimulators and the untreated cells, still capable of proliferation, are called the responders.

Materials Used

RPMI 1640 (Gibco) Culture Medium

FICOLL PAQUE PLUS® (Amersham-Pharmacia Biotech)

Procedure:

Blood was drawn from two healthy individuals. The blood was diluted in tissue culture medium and layered on top of a tube half full with Ficoll. Ficoll has a density greater than that of lymphocytes but less than that of red blood cells and granulocytes. After centrifugation the red blood cells and granulocytes passed down through the Ficoll to form a pellet at the bottom of the tube while the mononuclear cells, *i.e.*, T-cells, B-cells and monocytes remained at the interface of the medium and Ficoll. The layer containing the PBMNCs was removed and resuspended in culture medium at a concentration of about 5 x 10^8 cells per mL of culture medium. The cells from one of the individuals were then irradiated with 3300 rads gamma radiation. These irradiated cells were termed the stimulators. Nothing was done to the cells of the other individual. These non-irradiated cells were termed the responder cells.

A suspension of both cells in medium was made containing 1 x 10^6 cells per mL of responder cells and 0.15 X 10^6 cells per mL of stimulator cells. 100 µL aliquots of the mixed cells were placed in a series of wells of a multi-well plate. Into triplicate wells were placed aliquots of solutions of Zsig48 at the following concentrations: 0 ng/mL, 300 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL, 2 ng/mL, 0.1 ng/mL and 0.02 ng/mL.

A suspension of responder cells was also irradiated with 3300 rads of gamma radiation. A suspension of cells was then made containing 0.15 X 10^6 cells per mL of irradiated responder and 1 x 10^6 of non-irradiated responder cells. This mixture of irradiated and non-irradiated responder cells was called mock mixed leukocytes. 100 µL aliquots of the mock mixed leukocytes were placed in a series of wells of a multi-well plate. Into triplicate wells were placed aliquots of solutions of Zsig48 at the following concentrations: 0 ng/mL, 300 ng/mL, 200 ng/mL, 100 ng/mL, 50 ng/mL, 25 ng/mL, 10 ng/mL, 2 ng/mL, 0.1 ng/mL and 0.02 ng/mL.

The cultures were incubated 5days @37C with CO2. On day 5, each culture got 1µCi 3H-thymidine (Amersham-Pharmacia Biotech). Plates were incubated another 20-24h. The cells were harvested onto 96-well filter mats and the mats were dried. About 30 µL of scintillation fluid was added to each spot containing the dried cells on the mat and the radiation was detected for one minute by a scintillation counter (Packard TOPCOUNT NXT®) as counts of radiation per minute (CPM). This indicated the amount ^{3}H-thymidine which the cells took up which indicated the amount of proliferation that the leukocytes underwent. Because the wells were set up in triplicate, the results below represent the average of the three wells at each concentration of Zsig48.

Results

Mixed Leukocyte Reaction (Responder + Stimulator Cells)

Concentration of Zsig48 Added	CPM (^{3}H-Thymidine)
300 ng/mL	80,000 cpm
200 ng/mL	97,000 cpm

100 ng/mL	83,000 cpm
50 ng/mL	103,000 cpm
25 ng/mL	65,000 cpm
10 ng/mL	71,000 cpm
2 ng/mL	38,000 cpm
0.1 ng/mL	21,000 cpm
0.02 ng/mL	27,000 cpm
0.00 ng/mL	43,000 cpm

UnMixed (Mock) Leukocyte Reaction
(Responder + Irradiated Responder Cells)

Concentration of Zsig48 Added	CPM (^{3}H-Thymidine)
300 ng/mL	41,000 cpm
200 ng/mL	19,000 cpm
100 ng/mL	23,000 cpm
50 ng/mL	10,000 cpm
25 ng/mL	5,000 cpm
10 ng/mL	4,000 cpm
2 ng/mL	3,000 cpm
0.1 ng/mL	2,000 cpm
0.02 ng/mL	2,000 cpm
0.00 ng/mL	2,000 cpm

Conclusions

The data listed above shows that Zsig48 stimulates the proliferation of leukocytes in both a mixed leukocyte reaction and in an unmixed leukocyte reaction. Thus Zsig48 can be used to promote leukocyte proliferation both in the presence of antigen and in those cases where the cells have not been stimulated by antigen.

Example 4

Baculovirus Expression of zSig48

Two expression vectors were prepared to express zSig48 polypeptides in insect cells: pSig48CEE, designed to express a zSig48 polypeptide with a C-terminal GLU-GLU tag and pSig48NEE, designed to express a zSig48 polypeptide with an N-terminal GLU-GLU tag.

pSig48CEE

A 335 bp zSig48 fragment with BamHI and XbaI restriction sites on the 5' and 3' ends, respectively, was

generated by PCR amplification from zsig48/pZP9. The fragment was visualized by gel electrophoresis (1% SeaPlaque/1% NuSieve). The band was excised, diluted to 0.5% agarose with 2 mM MgCl2, melted at 65°C and ligated into an BamHI/Xba I digested baculovirus expression vector, pZBV32L (a modification of the pFastBac expression vector, the polyhedron promoter has been removed and replaced with the late activating Basic Protein Promoter and the coding sequence for the Glu-Glu tag as well as a stop signal has been inserted at the 3' end of the multiple cloning region). Forty-four point 6 nanograms of the restriction digested zsig48 insert and 215.9 ng of the corresponding vector were ligated overnight. The ligation mix was diluted 3 fold in TE (10 mM Tris-HCl, pH 7.5 and 1 mM EDTA) and 4 fmol of the diluted ligation mix was transformed into DH5a Library Efficiency competent cells (Life Technologies) according to manufacturer's direction by heat shock for 45 seconds in a 42°C waterbath. The transformed DNA and cells were diluted in 450 ml of SOC media (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 10 ml 1M NaCl, 1.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose) and plated onto LB plates containing 100 mg/ml ampicillin. Clones were analyzed by restriction digests and 1 ml of the positive clone was transformed into 20 ml DH10Bac Max Efficiency competent cells (GIBCO-BRL, Gaithersburg, MD) according to manufacturer's instruction, by heat shock for 45 seconds in a 42°C waterbath. The transformed DNA was diluted in 980 ml SOC media (2% Bacto Tryptone, 0.5% Bacto Yeast Extract, 10 ml 1M NaCl, 1.5 mM KCl, 10 mM MgCl2, 10 mM MgSO4 and 20 mM glucose) and plated onto Luria Agar plates containing 50 mg/ml kanamycin, 7 mg/ml gentamicin, 10 mg/ml tetracycline, IPTG and Bluo Gal. The cells were incubated for 48 hours at 37°C. A color selection was used to identify those cells having virus that had incorporated into the plasmid (referred to as a "bacmid"). Those colonies, which were white in color, were picked for analysis. Bacmid DNA was

isolated from positive colonies using the QiaVac Miniprep8 system (Qiagen)according the manufacturer's directions. Clones were screened for the correct insert by amplifying DNA using primers to the Basic Protein Promoter and to the SV40 terminus via PCR. Those having the correct insert were used to transfect *Spodoptera frugiperda* (Sf9) cells.

pSig48NEE

A 263 bp zSig48 fragment with BamHI and XbaI restriction sites on the 5' and 3' ends, respectively, was generated by PCR amplification from zsig48/pZP9 (described above). The fragment was visualized by gel electrophoresis and ligated into the expression vector, pZBV31L, as described above. One microliter of pSig48NEE was used to independently transform 20 ml DH10Bac Max Efficiency competent cells (GIBCO-BRL,Gaithersburg, MD) according to manufacturer's instruction, by heat shock at 42°C for 45 seconds. The transformants were then diluted in 980 ml SOC media and plated on to Luria Agar plates as described above. Bacmid DNA was isolated from positive colonies and screened for the correct insert using the PCR method as described above. Those having the correct insert were used to transfect Spodoptera frugiperda (Sf9) cells.

Transfection

Sf9 cells were seeded at 5 x 106 cells per 35 mm plate and allowed to attach for 1 hour at 27°C. Five microliters of bacmid DNA was diluted with 100 ml Sf-900 II SFM. Six ml of CellFECTIN Reagent (Life Technologies) was diluted with 100 ml Sf-900 II SFM. The bacmid DNA and lipid solutions were gently mixed and incubated 30-45 minutes at room temperature. The media from one plate of cells were aspirated, the cells were washed 1X with 2 ml fresh media. Eight hundred microliters of Sf-900 II SFM was added to the lipid-DNA mixture. The wash media was

aspirated and the DNA-lipid mix added to the cells. The cells were incubated at 27°C for 4-5 hours. The DNA-lipid mix was aspirated and 2 ml of Sf-900 II media was added to each plate. The plates were incubated at 27°C, 90% humidity, for 96 hours after which the virus was harvested.

Primary Amplification

Sf9 cells were grown in 50 ml Sf-900 II SFM in a 125 ml shake flask to an approximate density of 0.41-0.52 x 105 cells/ml. They were then infected with 100 ml of the virus stock from above and incubated at 27°C for 2-3 days after which time the virus was harvested. The virus titers for AcSig48CEE and AcSig48NEE were 1.08x108 pfu/ml and 1.84x108 pfu/ml, respectively.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. A polypeptide comprised of a sequence that is at least 90% identical with an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 3, 4, 5, 15 and 16.

2. A polypeptide containing an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 9 10, 11 and 12.

3. A polynucleotide which encodes a polypeptide that is at least 90% identical with an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 3, 4, 5, 15 and 16.

4. A polynucleotide which encodes a polypeptide containing an amino acid sequence selected from the group consisting of SEQ ID NOs: 8, 9, 10, 11 and 12.

5. An antibody which specifically binds to a polypeptide comprised of a sequence that is at least 90% identical with an amino acid sequence selected from the group consisting of SEQ ID NOs:2, 3, 4, 5, 8, 9, 10, 11, 12, 15 and 16.

6. An anti-idiotypic antibody which binds to an epitope of an antibody which specifically binds to a polypeptide comprised of a sequence selected from the group consisting of SEQ ID NOs: 2, 3, 4, 5, 8, 9, 10, 11, 12 and 15.

7. A method for promoting proliferation of leukocytes comprising bringing said leukocytes into contact with a polypeptide comprised of an amino acid sequence that is at least 90% identical with an amino acid sequence selected from the group consisting of 2, 3, 4, 5, 15 and 16.

8. The use of a polypeptide which is at least 90% identical to a polypeptide comprised of an amino acid sequence selected from the group consisting of 2, 3, 4, 5, 15 and 16 or a polynucleotide which encodes said polypeptide for the treatment of low leukocyte counts within an individual.

9. The use of a polypeptide which is at least 90% identical to a polypeptide comprised of an amino acid sequence selected from the group consisting of 2, 3, 4, 5, 15 and 16 or a polynucleotide which encodes said polypeptide for the manufacture of a medicament for the treatment of low leukocyte counts within an individual.

SEQUENCE LISTING

<110> ZymoGenetics, Inc.

<120> Secretory Protein-48

<130> 98-17PC

<150> 09/164,740
<151> 1998-10-01

<160> 17

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 1692
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (59)...(373)

<400> 1

```
ttttctaag ggatgagata agaataaata gaaattttgg catttcttct cacattag        58
atg ctg ggt tat tct gag ccc atg cca tgt gca cac cca ctt ggc ctc      106
Met Leu Gly Tyr Ser Glu Pro Met Pro Cys Ala His Pro Leu Gly Leu
 1               5                  10                  15

ttc ctc tta ggc cta cac cct gcc ctt tct ttg ccc ctt gta gtt act      154
Phe Leu Leu Gly Leu His Pro Ala Leu Ser Leu Pro Leu Val Val Thr
            20                  25                  30

gtg gct gga gtg atg agc gcc act ccc aag cat ggc ctg gaa caa tgt      202
Val Ala Gly Val Met Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys
        35                  40                  45

cct cct gcc cct cca cca gca gtg aca gga ttc act ggg gac tcg ggg      250
Pro Pro Ala Pro Pro Pro Ala Val Thr Gly Phe Thr Gly Asp Ser Gly
    50                  55                  60

gca aag gag act gtg tca caa gac aaa agg agc cag ggt cac aca tgg      298
Ala Lys Glu Thr Val Ser Gln Asp Lys Arg Ser Gln Gly His Thr Trp
```

65 70 75 80

tgt acc ctc gcc ctg cct cac cca tgg ctg aca tgg gtt gga cac ctc 346
Cys Thr Leu Ala Leu Pro His Pro Trp Leu Thr Trp Val Gly His Leu
85 90 95

aga aat cat gtg tct tca gcg agc cac tgagagttgg ggctttatct 393
Arg Asn His Val Ser Ser Ala Ser His
100 105

gttactcggc taggggtaac ctaaccgatg agactgtaac tggttactgt aaataaccaa 453
gctcccagta atagtaaacc agtgacaaaa acaattctta tccaaaaagg ttcacctttt 513
tttaaaatgt gtgaactaaa acagtcttta ttgctctaag acattaaaat ttgcactttt 573
ttgatgttga ataccactga atattttatt tttatatttt attacacaga aatacagcaa 633
ttattacaaa acgagtatta ggaatggcaa aggctttagg acagactatt agcggaaaac 693
atttggaact taaggagtgt tttacatttg gaacttactt taaggagtgt cgttcagaca 753
ctagctatat cttaacctca gtttttagaa gtaagcaagc tctcattttt tgctattcat 813
atttgaagtg attaaactca taaatttgaa atttacttttt tagagaccaa agattaaaat 873
taggtgggat gtcagctttt aaaatatact aagatttcct acaactacca atagcttatt 933
tccctgggaa acagattaca ttgtagtact taacccagaa ctcatgcagt tcatccaaaa 993
tgatggtaaa ctttttttcct cagaattacc taactttcct tgactatgaa ttcaacattc 1053
aagaatcttc ttctggtagc aggagcggca gagaggacag gcatggaaag gaggcctgtc 1113
tcccacggag aactcctcta gtgccagcag acacgcatgg tggaacacat gtgagcagga 1173
caggagggcc atctctctgg aacgcctgcc cgcacccacg cactgaccgc cagcagcgga 1233
gagaggggcc aggcagatgg agcactcctg ggtctcccgg cgcagagcct gcggcacaca 1293
ggacaggaag aggccacgcg ggttagtttc atcacagcag aaagttactt aaactgaaat 1353
gcgaaccatg tgccccgaga catgggtctt cgaaacatgc ggaagtttca ttctgtgtta 1413
aaatcacatg cattttattt atatatatac atatatatat atacacacac acatatactc 1473
tgttactcct gggaactgtg gaaagggtta gtaacccacc tgtgataagc aacatccaac 1533
aggaacttcc agaatttcaa actgaaggga cctttgccgt caccctaaag cccatgagga 1593
aagtcctacc acaggtgcag gggcagctag ggcagcggtt accccaggcc tgacactcct 1653
aggcttccca aagtgagtcc tcgacctccc ccgctcgag 1692

<210> 2
<211> 105
<212> PRT
<213> Homo sapiens

<400> 2
Met Leu Gly Tyr Ser Glu Pro Met Pro Cys Ala His Pro Leu Gly Leu
1 5 10 15
Phe Leu Leu Gly Leu His Pro Ala Leu Ser Leu Pro Leu Val Val Thr
20 25 30

Val Ala Gly Val Met Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys

```
        35                  40                  45
Pro Pro Ala Pro Pro Pro Ala Val Thr Gly Phe Thr Gly Asp Ser Gly
    50                  55                  60
Ala Lys Glu Thr Val Ser Gln Asp Lys Arg Ser Gln Gly His Thr Trp
65                  70                  75                  80
Cys Thr Leu Ala Leu Pro His Pro Trp Leu Thr Trp Val Gly His Leu
                85                  90                  95
Arg Asn His Val Ser Ser Ala Ser His
            100                 105
```

<210> 3
<211> 79
<212> PRT
<213> Homo sapiens

<400> 3

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

<210> 4
<211> 77
<212> PRT
<213> Homo sapiens

<400> 4

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

<210> 5

<211> 65
<212> PRT
<213> Homo sapiens

<400> 5

```
Pro Lys His Gly Leu Glu Gln Cys Pro Pro Ala Pro Pro Pro Ala Val
 1               5                  10                  15
Thr Gly Phe Thr Gly Asp Ser Gly Ala Lys Glu Thr Val Ser Gln Asp
            20                  25                  30
Lys Arg Ser Gln Gly His Thr Trp Cys Thr Leu Ala Leu Pro His Pro
        35                  40                  45
Trp Leu Thr Trp Val Gly His Leu Arg Asn His Val Ser Ser Ala Ser
    50                  55                  60
His
65
```

<210> 6
<211> 384
<212> DNA
<213> Homo sapiens

<220>
<221> variation
<222> (1)...(384)
<223> n is any nucleotide

<221> misc_feature
<222> (1)...(384)
<223> n = A,T,C or G

<400> 6

```
tttttctaag ggatgagata agaataaata gaaattttgg catttcttct cacattagat      60
gctgggttat tctgagccca tgccatgtgc acacccactt ggcctcttcc tcttaggcct     120
acaccctgcc ctttctttgc cccttgtagt tactgtggct ggagtgatga gcgccactcc     180
caagcatggc ctggaacaat gtcctcctgc ccctccacca gcagtgacag gattcactgg     240
ggactcgggg gcaaaggaga ctgtgtcaca agacaaaagg agccagggtc acacatggtg     300
ttacctcgcc ctgcctcacc catgggtgac atgggttgga cacctcanaa atcntgtttc     360
ttcaccganc cactgaaaat tggg                                            384
```

<210> 7
<211> 48
<212> DNA
<213> Homo sapiens

<400> 7

gtctgggttc gctactcgag gcggccgcta ttttttttt ttttttt 48

<210> 8
<211> 20
<212> PRT
<213> Homo sapiens

<400> 8
Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys Pro Pro Ala Pro Pro
1 5 10 15
Pro Ala Val Thr
20

<210> 9
<211> 42
<212> PRT
<213> Homo sapiens

<400> 9
Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys Pro Pro Ala Pro Pro
1 5 10 15
Pro Ala Val Thr Gly Phe Thr Gly Asp Ser Gly Ala Lys Glu Thr Val
20 25 30
Ser Gln Asp Lys Arg Ser Gln Gly His Thr
35 40

<210> 10
<211> 65
<212> PRT
<213> Homo sapiens

<400> 10
Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys Pro Pro Ala Pro Pro
1 5 10 15
Pro Ala Val Thr Gly Phe Thr Gly Asp Ser Gly Ala Lys Glu Thr Val
20 25 30
Ser Gln Asp Lys Arg Ser Gln Gly His Thr Trp Cys Thr Leu Ala Leu
35 40 45
Pro His Pro Trp Leu Thr Trp Val Gly His Leu Arg Asn His Val Ser
50 55 60
Ser
65

<210> 11
<211> 20

<212> PRT
<213> Homo sapiens

<400> 11
Thr Gly Asp Ser Gly Ala Lys Glu Thr Val Ser Gln Asp Lys Arg Ser
1 5 10 15
Gln Gly His Thr
20

<210> 12
<211> 43
<212> PRT
<213> Homo sapiens

<400> 12
Thr Gly Asp Ser Gly Ala Lys Glu Thr Val Ser Gln Asp Lys Arg Ser
1 5 10 15
Gln Gly His Thr Trp Cys Thr Leu Ala Leu Pro His Pro Trp Leu Thr
20 25 30
Trp Val Gly His Leu Arg Asn His Val Ser Ser
35 40

<210> 13
<211> 12001
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (10258)...(10572)

<400> 13
actttgtgca aaattccttc attaagcctt agcttcctta tctgtaatac agtgatagta 60
tcatcttcct gttagggttt ttgtgaagat caacggaaat aattctgtaa gatccttagc 120
atagcgcctg gcacatccta agaactcagt aaatattagc ccctttatta tgacgatggt 180
ggtcatggtg gtggtgagga tgatacggtg tgaaaagctt atctcttggt aataatacct 240
tttagttaaa gctttttga ggcttggatt ttgcaagtat taggctaacc cataagtctc 300
ttcattaagc cagagaataa attcaagatg aaaacgttag cattcttggc attgatgtaa 360
tagaagagag gggatttact gttatgtgtt ccaagagtca catgtattgt aatggtgtta 420
aaaacgggta ggtttagcta aagggtacaa acgtaaccta tgaatgtatt tttatgctta 480
tttccacatt agtgctaaac atatttcaag ttttatactt taaaaatacc aggacaaagt 540
aaattatctt ggtttggggt gggaggggt tgtaatttta tgacagaaga agggaaaggc 600
agtgacttct tgtagaaaat ttttaaaaat cctgacatta gctcatttac ctgagttgac 660
atgatttgaa tgcatatgac tccatactgg ggcttttagc tattgtaaaa ggccacatac 720
tgatggattc attaaggtcc agttttcaga taacttaaac gatatgagca gcaataaagc 780

ttctcagatc accaggcctt tccaaccttg atgtttgaga gggtgacctt tgggaggcac 840
aaaagatttc agatgagctg tccatatgta ttttactttg aatatgccct gggaggggat 900
ggctcatcaa atattgcaat gcctgacagg aaaaagtcac agctcatttc agctgacaca 960
ccagataact tatacctttt aatgcttagg tttaataaag ctggcccaac ttgaagtagg 1020
aatcaaacag tccttttttat cagatgtcta gcattaaaac ttaattttta agcctgttat 1080
aatatcagca agattagtta gccatggttt cagataaatt tccactttcc attcgctaaa 1140
tgagatggtt gcaaatgaac tgccgtaact ttagcttttg aattaggtat tctggacatc 1200
attttgctaa gaaagccttt attaaagtaa taaaacataa cctgatataa aaggccttat 1260
atgcatgtca gttccttgac cataagagag agtagaatta gcaagagttg tataaaacta 1320
cctaatagat acatttactt ttcttcccca gtgtttttca gtattctttg gggtgtgcta 1380
cggggcaatt tatacataga aaaagagtct tattaagtat atgtaatgtt tgaatgatct 1440
gagatcttaa cagggattta gctgagactt gtaatttgat tgtaaagtag ctatcccctt 1500
tctttctttt tttttgtaga gatttttttcc cctctgctac tctgcccatt gataataata 1560
gtatccatct cagagaatat ctagcacata gtaaacacta gaaatttagc tgtggtgatg 1620
gtggtaatgg acgggattgt ttctggagtt gtctgcagaa gagacacatc agaacgtttc 1680
agaatgcata acctacatga cagccaagtt ttaggcgtga actcagataa tcgatcctaa 1740
aagggtgcct tcatttcagc tcagtcagtg ggtaccgcag tgatcctctg tcttcactca 1800
gtccctttttc taacaagctt gattttagca cacttcctca actccagcag ctgtgggttc 1860
ctattgtcat tcctgggacc tgaccatttt ttgtgttgga ttgatttctt ttttcccttc 1920
ccattcaaac ataggctcag ttttaccttt tctttcatta agatatgcag gtgacaggaa 1980
gattaaaatt tggagtgcta tattagttta tgagtgatgt aaaactgcct aataggtcag 2040
ttaccatgtg aaattttagg gagaaaaatc ttttccaagt aagttgttga gatgccagaa 2100
aggtctgagc ttcgtgaaaa gtattttctc aacatctgcc ctgtggaagt gctgctgtgt 2160
ggccataggc cacatgatga tcatttggtc aataacagac cacttgccta tgtgacagtg 2220
gccacagcag atgatcatgg agctgaaccg tttctgccag cagtggcgtg gcggctgttg 2280
taatgtcctg gcgccacgca tcagcttttc taggttcata tgtttctaga tgcacagata 2340
tgtgccatgg tgccgcagtg gctgcagcac tcaggacagt gacatgctgt ctgcttggta 2400
actgaggagc agccggccac accctgcagc ctagggatgg catggctgtg ccctctgggt 2460
gtatagatac actctatgat ggcatcacag cgacagaatc gctcgacagc gcatttctca 2520
gaatgcattc ctgttgttcg gcaacacctg actgtgttgc tgtatgtcat tgtgtttcat 2580
ttttattaaa gtgcttgacg ctccagtgcc acagaagctc ttacattttc ttctgccctt 2640
tctccctgtg agagggcaat gttggtctgt gtatcaggta tattatgtaa ataattgttt 2700
tatgatacag agagaaataa atgtaacttt aaaagatagt gatagttttt tattctgtga 2760
aataccttg ggtagtgaga tattatttat gttcagttta ttcttttgtc atttctttat 2820
tttttagtaa tgttttccat tcttttcttt ttaattacat attaatattt ggctgtggct 2880
ttccctacat tttcctgcat gtgtgtaagg ggcaatttat tttactttat ttaaaatttt 2940
tttgagacag ggtctcctct gtcacccagg ctggagtgca gtggtgtgat cacatctcac 3000
tgcagtcaca acctgctgag ctcaaggaat tctcccacct cagcctccca gagtgctggg 3060
attataggca tgagccactg cagccagcta atttttgtgt ttttcatgaa catggggttt 3120
cacaatgttg cccaggcctg tctcaaaccc ctcagctcaa acgatccttc cacctcggcc 3180
tcctgaagtg ctgggatgac aggcgtgagc caccacgccc agcctgtgtg tgtgcatttt 3240
aaaatgcaag tgggaacgta tctgtctcgg gttaggttcc ctgagaagca cacttcagat 3300
gggaatctgc ttgccaggaa gaggtttatt agccgggttg ggacaacgga agaggaaggg 3360
gctgtgcaga gggaggaact gtgccgtgat gcagtcacac tggaagcctc cgctggccct 3420
cggggatgtt gtgacttcga tggcccttca gagctccact gaactgagaa gaagggacag 3480

gctcttagga ccctcacgtg ctgtcctgga aggggcagga cttccgcctt gcagcagtct 3540
tcagcttagg ccatccccaa agggaggttg ccagtaggga gaagaaatca ttcattcctc 3600
aaggggatct ggccagcacc tcacagcttt ccaaataaca cttagctttg taaatctcct 3660
cttctcccta ccctaaccct ttcccagcca ctcagcaata tatcatttct caccattttc 3720
ttaatttttt tgcagggctt cataaacttc acttaccaaa tacttcaagg gattcagaaa 3780
cagccaagcc ttctgtaaat gggcatcaga aagcactgtg agacgcacag acggcgtctt 3840
ctgccaccaa gagacccgag aactccagat tcacgacatt cctgtcccat gtagaagcat 3900
ttccattcaa ccgtggcccc tcttcagaac ctagacctat cagtgccatt ttttttcat 3960
aatctacgaa gaacttggct atggctgatc ttttttaaat ttaactttct gatggaccct 4020
gtagtttcca gttaagtgca gattccttac agacatatag aacagcgcat tcttctgtag 4080
acatttgctc atgttggtaa atacaatcac ccatatgaaa aaattgtttt cacctgatat 4140
gaaaatgtta gaaaaggcaa actccgggac ttctaaagat ttacttaaat cccattatgt 4200
actttattca gaatgtagaa gctgacttga aaggcatcct tggtactaag tgaagcttat 4260
tcagaaaatg catttttcaa atgcaatggc aactgcttgt agatatcatt tttgcagtgt 4320
atgttggagc tgtaatggtt gcaattatgt ttcttatttc cttaaaagca aaaagcgtag 4380
tttctgattt atgttataga atgatactga ttagactttg agccaagggg aaaatactaa 4440
attcttttaa acctggagcc ttagagagcc acaggaatat cttctgttgt acagtctaat 4500
aagctgtggt aggaagtatc atgtaatcac agtttaatga cagtttatgt atatatataa 4560
ttcagtattc cctctgataa catagttgcc agtgtttaat acacttgtaa cttggatttt 4620
taccttatag gctatatgta tactcagttt tttaaagcat ttttttcaga gatcacttaa 4680
ttccccatgc ttctgcaatg catataaaaa ctataaatgc cgagtggtag aaactcctct 4740
ttcttcatag tcctcaggct ttggttacat ttgcatatgc catttgaagc ctccagcttt 4800
taccagttta acatccaaag ttcacagcat cagcattcat ggtgtaagaa cagttttgca 4860
gtataacacg atctgataat cattcagtta ttaaattgta aataattatt gggatggttt 4920
cttggcttta agtccactga ataaaaacta tgaaattgca ctctgtgtca accatccact 4980
aggatagaat accgaaatct gtgcatgcaa aaataggaga tgggcccatt tgcacacaat 5040
tcgtagttat gcagtctgct atataaatat gttcacatgc actgtgtgta tgaaaataga 5100
tggtctgtgt tcagacaaaa gtaaaacatt tttttcaaat tgttacattt aaaggttttc 5160
tgggagaaat ttatgaaacg caggctgtgt ctatttgaca tcagaaattt ccactttaaa 5220
ccaaaataat aagaaacttt aatctgtata tttacaacct ttgttgagta cacttccccc 5280
ttatttatac gtctgcattt ccttccgagc ttcacatctt tctaaaatgc agcttggttt 5340
taaaataaaa gaacattcat tttgtgattc taaacaagct tcagtaaata ccaccagtat 5400
agtactggtg aatttctcag cataaaatcg acatacctaa aaagttaata aaattcagct 5460
cttttccaat ttcattgtta tgcctattga agtattaatt gccaggtttg atttttagtg 5520
aagcttggag tccatacttt gagcagacca agtgaaggga agaacagaaa gaaactcagg 5580
agtagagtaa tatcacttct cacttacacc actttcaggc acatccaaag agttcctaga 5640
tacttggaaa atgtctgaaa atttttaagt aaaatactaa acttttcagt gtttagctca 5700
acttttttgtt catttggaag tttctctcca tccgaggact taagccagtt ttggatttgt 5760
aagccctgag tacaatacac ttcctggagg catcctcact gctgttgaag caaaggatat 5820
gcatggggtg gaaggacggc ttcgaacctg ggactcatat gccttgagaa caaatagatt 5880
gttacagcct tgggctgctg cgtaatcacg gttcctcgag gctcttcctg agcacatgcc 5940
caagcatctg cctctggaga gactgactcc aaatgcaggt gcttccattg gagctaggtc 6000
ggaggctgct ttatatgacg aactccagaa atggatgcca gaatacggag gccaaacgtt 6060
ctgagtcctg gtaaggacag tcgctctggg ggtcctcatt ttactgcagt tcctgcacgc 6120
cagtgaaaga gaggagatag accctggaag gcagagctgc agatgctcat catcaggtca 6180

attctggagc tacagttttg tttctgactg gatagggatg caccagtgac tgtcacatca 6240
agcagtcctt ttattctctc tcctttagta tcgattttaa agggcattag gcactatggt 6300
tccagagttt cttggggaaa acttgcagat tcttattaat tggttctgca atacttaaat 6360
aaattatttt acaattataa gttttcagat tataacattt gtattaattt ttactgattt 6420
tccaagatac ttcttagatt tactatttac gtagctttat gtacattctc tgtaaaaata 6480
gacctctaaa tatgaggctt tacatgaaat ttgtacacac atacacacta atgttagctc 6540
cttaaattgc tgcactaagg tgctggttag tagagatgga cggagcctct cgcgttttgc 6600
tctcagatgt gttaaaggcg cacgtgtacc tgctctcagc ggcagtgcgg cctccccatc 6660
tgctgggtgc ccatggccct ccctgcagcc tcagtgatga cctcgtctgc cagggacaca 6720
ggttttcatc atttacaggc tcttatgtgc tagttttgtt ggtagcacgt tatttaatgc 6780
ataaaggcag aattcttaca agtttttttt tttaatgtga acatagatgc agcaccgact 6840
ttttaaactt gaaaaaactg gtataatgtt aacttttaaa aataacattt ggacacacta 6900
gtaattgatt tttgtttaca gattgttttg tttacaaatt gttagtcttt gtttctatga 6960
gatactttta gtgtgacttt ttaaatgtct tagaaattaa aagttgtaca aaaagtgatt 7020
tcatatttgg tttataagca tttatatgtg gggtttattt gttcttttgt tttttccatc 7080
ttaaatatta tcatggctaa aacttaaggg tatttatagt ttaattccat ttcagtttta 7140
tagagggcag taattattct gatgaatgtt gaattaagaa atggatattt tctttctctg 7200
ttgtgcagtt attggtagat caatttctta taacccacaa tgtagcatca ataattgata 7260
gcatgtattt tatttaatta cttgaattat ttagacttga tttctctaat tttttccata 7320
aaaggactga acagcaccta cttgtggtct ggacagctta acccagagtt cctggaagaa 7380
taaatgctgt tagcatctgg ttaatttact ggcagacaga agcctactta cagtggcttt 7440
caacttttttg accacagtaa gaaataaccc attacacaca cacacacaca cacactctct 7500
ctctctctct ctctctctct ctgtctctca gatacgtata agcaaaaatt taacaagaca 7560
gtacttgttt ttcctaagtg tgcatgctgg catcttctgt tttattattt ttaaaaatac 7620
tagacacaga cactaagtta ctttgtggcc tcctaataga tggcagactt cagtttgaaa 7680
agcatccctt tggaatgtgg tttaaaagaa gaaataatac aaagaccttt ttggagtttt 7740
ttttttttt ggtttttgaa tattgtttag gtaaaaatta ttgctgcaaa cacaactgaa 7800
gcaaaagtac tgtgtacgca aattacttgt tgcactgtag aaaatgtaat atcaggagaa 7860
gttcctaaaa ctcacaaata tatacaaaat ttcaaaactt ggtgattgat ccatatgtct 7920
tgaaggaata ttaggcaata aacttacctt ttcaagagaa gttatttaac ttccaattct 7980
gtgttcttta attaggaaaa aatttttca aggttaacta taatcaaata ggaacttcac 8040
acaaaacctc attattcttg gatatggatt tggtgttctg ctgttacttc taaaggtggt 8100
acagaagtca agtttatgag cctggattat gaacagcagg gcggccctga tgtgagtggt 8160
tgtggaggac tagagtgatg tagaagaatc tgttgtgtct gagtgtgagg aggtggagag 8220
tcacgtgtgg gctatgggtc tgagaactga agttttaaca gttactaaaa ctgttttgtg 8280
gctgttgtaa tgtcttctcc tttcctttcc tttgaaaagc tgttactgaa tgaacgatgc 8340
cgcttacaaa cactgacact tcagaaccaa gcatatttga tactgctgta gctatttata 8400
ttgattctgc aacttctctt taacagcatc ttgaagtcat ccacagttgt tctatatgga 8460
ctccacagta gagtcagaga actaagtgac aagtggagca tcgtcctcct cctgctcact 8520
tcctcctcgc cacctctctg gcctggctat ccttcagtct gaagagtgat tcacttttc 8580
ttttcttttt agtctttact gtggaatctc atcaaagact tggaagcagg ggtggtgaga 8640
ggggcctggg gcgtggtgtg gagggtcgag ttctttgtcg tcccacagtc acccccagga 8700
ccaccttgcg aagccgaaaa tacacatctg cattttaccg acaaggaacg ccactgagaa 8760
agctgcagac atttgcctgg ggtcacgctt acagtgggca gatgaactgg atgtaaatac 8820
aggccagtac aaccgcagtc tcatttttc tttgttttag gttttcagtt tcttgttcat 8880

```
tcatgtacct tacagttcta ttatctggtt gataaagatt catgacaggt atcctcattc   8940
tagattataa cctttattct tatcaaatgc ctccagtccc acaattcgct aagaattctc   9000
tttctggact tgatagtgat ttaatgattc cccccgccca tggaatggta ggctcctaat   9060
taagtggatt taaaggcaaa aagggatgtg tgagaaggca caagaagtgg cttcatcttg   9120
caaggtgact gtcagacaag tgggttccaa cctgttaaaa taagggagga atggccacat   9180
cccgtaggga ctgctgtagc tgaccaggaa gacacaatga gggcagcaga cagggctctc   9240
cctgtccgct gagtcctccc atgaactcat gtttccaaag gccccactct ttcttggtca   9300
gtgtcccagt ttttatagaa gagacctgat gaggctgtgg agtaattgta taaaagcttg   9360
cactgttttc ctgacttgac ctgacaatgt ggcgttatct tcaaattgtc cagagaagta   9420
gctacttcat tcaggtcctt tttagcgttc tgtggttgca acttggtctc gcaagctttc   9480
gatgggcgcg ccattccatg gccacttttc agtaagaaaa ttgcctgatt ttctgttaac   9540
tgtcacaggc tgccctggac cattcctcag aactcatggg attttcgcga ccctcatcct   9600
cagtgaagtt agatgaccat tcctgccttt catcccaccg attttcctaa gtatctgttg   9660
cctgcaacta acactagttc ctgtgtcagt aaagtactgc acttggttgc aaagaataga   9720
gatggactct gccaacatag gcacggaagg tttacggggt ggatggcatg gctcacagat   9780
gtgatgagaa agccccggaa gaagtgttgg gcacagcctt aggcacaacc tttcctgggc   9840
tcttcccatc actgaatgat ccatgtatct ccgggactct ccttctagat ccaaagttta   9900
gggcggcgga atcggactgc tctggccttt gacacgtgcc tgtgctttgc ctgtcaacgc   9960
tatggggaga gagatgtcag cttacagaaa attgaggttc tgtgattcag tttccctctg  10020
ctctcttaga ataaaagtgc ttactcattg tttaaagcat tctccaaatt agtctgcaaa  10080
ttttttgata gtatacatcc ttaaaaaatg agcacatccc aatatatgca tatttattca  10140
taaatgatac atacatatgt agctatataa tgtgtacatc acaaaacata ggagagtatt  10200
ttttctaagg gatgagataa gaataaatag aaattttggc atttcttctc acattag atg  10260
                                                              Met
                                                               1

ctg ggt tat tct gag ccc atg cca tgt gca cac cca ctt ggc ctc ttc    10308
Leu Gly Tyr Ser Glu Pro Met Pro Cys Ala His Pro Leu Gly Leu Phe
            5                  10                  15

ctc tta ggc cta cac cct gcc ctt tcc ttg ccc ctt gta gtt act gtg    10356
Leu Leu Gly Leu His Pro Ala Leu Ser Leu Pro Leu Val Val Thr Val
        20                  25                  30

gct gga gtg atg agc gcc act ccc aag cat ggc ctg gaa caa tgt cct    10404
Ala Gly Val Met Ser Ala Thr Pro Lys His Gly Leu Glu Gln Cys Pro
    35                  40                  45

cct gcc cct cca cca gca gtg aca gga ttc act ggg gac tcg ggg gca    10452
Pro Ala Pro Pro Pro Ala Val Thr Gly Phe Thr Gly Asp Ser Gly Ala
50                  55                  60                  65

aag gag act gtg tca caa gac aaa agg agc cag ggt cac aca tgg tgt    10500

Lys Glu Thr Val Ser Gln Asp Lys Arg Ser Gln Gly His Thr Trp Cys
```

70 75 80

acc ctc gcc ctg cct cac cca tgg ctg aca tgg gtt gga cac ctc aga 10548
Thr Leu Ala Leu Pro His Pro Trp Leu Thr Trp Val Gly His Leu Arg
85 90 95

aat cat gtg tct tca gcg agc cac tgagagttgg ggctttatct gttactcggc 10602
Asn His Val Ser Ser Ala Ser His
100 105

taggggtaac ctaaccgatg agactgtaac tggttactgt aaataaccaa gctcccagta 10662
atagtaaacc agtgacaaaa acaattctta tccaaaaagg ttcacttttt tttaaaatgt 10722
gtgaactaaa acagtcttta ttgctctaag acattaaaat ttgcactttt ttgatgttga 10782
ataccactga atattttatt tttatatttt attacacaga aatacagcaa ttattacaaa 10842
acgagtatta ggaatggcaa aggctttagg acagactatt agcggaaaac atttggaact 10902
taaggagtgt tttacatttg gaacttactt taaggagtgt cgttcagaca ctagctatat 10962
cttaacctca atttttagaa gtaagcaagc tctcattttt tgctattcat atttgaagtg 11022
attaaactca taaatttgaa atttactttt tagagaccaa agattaaaat taggtgggat 11082
gtcagctttt aaaatatact aagatttcct acaactacca atagcttatt tccctgggaa 11142
acagattaca ttgtagtact taacccagaa ctcatgcagt tcatccaaaa tgatggtaaa 11202
ctttttttcct cagaattacc taactttcct tgactatgaa ttcaacattc aagaatcttc 11262
ttctggtagc aggagcggca gagaggacag gcatggaaag gaggcctgtc tcccacggag 11322
aactcctcta gtgccagcag acacgcatgg tggaacacat gtgagcagga caggagggcc 11382
atctctctgg aacgcctgcc tgcacccacg cgctgaccgc cagcagcgga gagaggggcc 11442
aggcagatgg agcactcgtg ggtctcccgg cgcagagcct gcggcacaca ggacgggaag 11502
aggccacgcg ggttagtttc atcacagcag aaagttactt aaactgaaat gcgaaccatg 11562
tgccccgaga catgggtctt cgaaacatgc ggaagtttca ttctgtgtta aaatcacatg 11622
cattttattt atatatatac atatatatat acacacacat atactctgtt actcctggga 11682
actgtggaaa gggttagtaa cccacctgtg ataagcaaca tccaacagga acttccagaa 11742
tttcaaactg aagggacctt tgccgtcacc ctaaagccca tgaggaaagt cctaccacag 11802
gtgcaggggc agctagggca gcggttaccc cgggactgac actcctaggc ttcccaaagt 11862
gagtcctcga cctcccccga catgccaccc ccacagcctc cacggctgca ccccgggcct 11922
gacactccta ggctccccaa agtgagtcct cgacctcccc ccacatgcca ctccccacag 11982
cctctgcagc tgcagctga 12001

<210> 14
<211> 105
<212> PRT
<213> Homo sapiens

<400> 14
Met Leu Gly Tyr Ser Glu Pro Met Pro Cys Ala His Pro Leu Gly Leu
1 5 10 15

Phe Leu Leu Gly Leu His Pro Ala Leu Ser Leu Pro Leu Val Val Thr

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

<210> 15
<211> 89
<212> PRT
<213> Homo sapiens

<400> 15

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

<210> 16
<211> 80
<212> PRT
<213> Homo sapiens

<400> 16

```
Ser Leu Pro Leu Val Val Thr Val Ala Gly Val Met Ser Ala Thr Pro
 1               5                  10                  15
Lys His Gly Leu Glu Gln Cys Pro Pro Ala Pro Pro Pro Ala Val Thr
            20                  25                  30
Gly Phe Thr Gly Asp Ser Gly Ala Lys Glu Thr Val Ser Gln Asp Lys
        35                  40                  45
Arg Ser Gln Gly His Thr Trp Cys Thr Leu Ala Leu Pro His Pro Trp
    50                  55                  60
```

Leu Thr Trp Val Gly His Leu Arg Asn His Val Ser Ser Ala Ser His
65 70 75 80

<210> 17
<211> 16
<212> PRT
<213> Homo sapiens

<400> 17
Gly Gly Ser Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15