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PLACENTAL GROWTH FACTOR SPLICE VARIANTS AND THEIR USES

FIELD OF THE INVENTION

The present invention relates to nucleic acid and amino acid sequences of human Placental Growth Factor (PIGF) splice variants designated herein as LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 and to the use of these sequences for the treatment of cardiovascular diseases, tumorous cancers, inflammation, neuropathy, and liver regeneration. The present invention also relates to vectors and host cells and antibodies directed to these polypeptides.

BACKGROUND OF THE INVENTION

Angiogenesis, the formation of new blood vessels, is a crucial process during development, certain periods of adult life, and tumorigenesis. It is tightly regulated by a network of growth factors and growth factor receptors. Placental Growth Factor (PIGF), a vascular endothelial growth factor (VEGF)-related factor, has been shown to play an important role in pathological VEGF-driven angiogenesis. PIGF has been reported to benefit tumor vasculature by inducing angiogenesis, leading to increased vascularity and tumor growth. Conversely, withdrawal of PIGF leads to increased apoptosis and necrosis. In particular, both vascular cells and tumor macrophages appear dependent on PIGF for survival [Adini, et al., Cancer Res. 62(10):2249-52 (200)].

VEGF and PIGF constitute a family of regulatory peptides capable of controlling blood vessel formation and permeability by interacting with 2 endothelial tyrosine kinase receptors, FLT1 (VEGFR1) and KDR/FLK1.

Luttun et al. reported experiments bearing on the therapeutic potential of PIGF and its receptor FLT1 in angiogenesis. [Nat. Med.8(8):831-40 (2000)] They reported that PIGF induced angiogenesis and collateral growth in ischemic hearts and limbs with at least a comparable efficiency to VEGF. An antibody against FLT1 suppressed neovascularization in tumors and ischemic retina, as well as angiogenesis and inflammatory joint destruction in autoimmune arthritis. Anti-FLT1 also reduced atherosclerotic plaque growth and vulnerability, but the atheroprotective effect was not attributable to reduced plaque neovascularization. Inhibition of VEGF receptor FLK1 did not affect arthritis or atherosclerosis, indicating that inhibition of FLK1-driven
angiogenesis alone is not sufficient to halt disease progression. The anti-inflammatory effects of anti-FLT1 were attributable to reduced mobilization of bone marrow-derived myeloid progenitors into the peripheral blood, impaired infiltration of FLT1-expressing leukocytes in inflamed tissues, and defective activation of myeloid cells. Thus, PIGF and FLT1 were considered potential candidates for therapeutic modulation of angiogenesis and inflammation.

There are three reported isoforms of the PIGF gene (PIGF-1, PIGF-2 and PIGF-3). Alternative splicing of PIGF mRNA produces polypeptides with different secretion patterns, heparin-binding affinity and dimerization properties. LP2017 is a splice variant of PIGF-2; LP2022 is a splice variant of PIGF-2; LP2023 is a splice variant of PIGF-2; LP2024 is a splice variant of PIGF-2; LP2025 is a splice variant of PIGF-2; LP2026 is a splice variant of PIGF-1; LP2027 is a splice variant of PIGF-2; and LP2029 is a splice variant of PIGF-2.

SUMMARY OF THE INVENTION

The present invention relates to novel splice variants of PIGF and a method of using PIGF splice variants to treat PIGF-mediated disorders.

The present invention provides isolated LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides encoding nucleic acids and the polypeptides encoded thereby, including fragments and/or specified variants thereof. Contemplated by the present invention are LP probes, primers, recombinant vectors, host cells, transgenic animals, chimeric antibodies and constructs, LP polypeptide antibodies, as well as methods of making and using them diagnostically and therapeutically as described and enabled herein.

The present invention includes isolated nucleic acid molecules comprising polynucleotides that encode LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides as defined herein, as well as fragments and/or specified variants thereof, or isolated nucleic acid molecules that are complementary to polynucleotides that encode such LP polypeptides, or fragments and/or specified variants thereof as defined herein.
A polypeptide of the present invention includes an isolated LP polypeptide comprising at least one fragment, domain, or specified variant of at least 95-100% of the contiguous amino acids of at least one portion of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16. See Table 1.

The present invention also provides an isolated LP polypeptide as described herein, wherein the polypeptide further comprises at least one specified substitution, insertion, or deletion corresponding to portions or specific residues of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16.

The present invention also provides an isolated nucleic acid probe, primer, or fragment, as described herein, wherein the nucleic acid comprises a polynucleotide of at least 10 nucleotides, corresponding or complementary to at least 10 nucleotides of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15. See Table 1.

The present invention also provides compositions, including pharmaceutical compositions, comprising an LP polypeptide, an LP polypeptide-encoding polynucleotide, an LP polynucleotide, and/or an LP polypeptide antibody, wherein the composition has a measurable effect on an activity associated with a particular LP polypeptide as disclosed herein. A method of treatment or prophylaxis based on an LP polypeptide associated activity as disclosed herein can be effected by administration of one or more of the polypeptides, nucleic acids, antibodies, vectors, host cells, transgenic cells, and/or compositions described herein to a mammal in need of such treatment or prophylactic. Accordingly, the present invention also includes methods for the prophylaxis or treatment of a patho-physiological condition in which at least one cell type involved in said condition is sensitive or responsive to an LP polypeptide, LP polypeptide-encoding polynucleotide, LP nucleic acid, LP polypeptide antibody, host cell, transgenic cell, and/or composition of the present invention.

The present invention also provides an article of manufacture comprising a container, holding a composition effective for treating a condition disclosed herein, and a label.

The present invention also provides a method for identifying compounds that bind an LP polypeptide, comprising:

a) admixing at least one isolated LP polypeptide as described herein with a test compound or composition; and
b) detecting at least one binding interaction between the polypeptide and
the compound or composition, optionally further comprising detecting a change in
biological activity, such as a reduction or increase.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

No previous reports have suggested the existence of PIGF splice variants, such as
e.g., due to alternate mRNA splicing events. Applicants' invention is the first to do so.

The present invention provides the amino acid sequences and DNA sequences of
mammalian, herein primate, preferably human, PIGF receptors designated herein as
LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029. LP2017,
LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 have particular defined
properties, both structural and biological. The cDNA encoding these molecules was
obtained from primate. Other primate or mammalian counterparts are also contemplated.

This invention is not limited to the particular compositions, methods, and
techniques described herein, as such may vary. The terminology used herein is for the
purpose of describing particular embodiments and is not intended to limit the scope of the
present invention that is only limited by the appended terms.

LP2017 polypeptides comprising the amino acid sequence of the open reading
frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 1 are
contemplated as one embodiment of the present invention. Specifically, polypeptides of
the present invention comprise the amino acid sequence as shown in SEQ ID NO: 2, as
well as fragments, variants, and derivatives thereof. Accordingly, LP2017
polynucleotides encoding the LP2017 polypeptides of the present invention are also
contemplated by the present invention.

Sequence analysis of LP2017 indicates that it lacks the 5th of the 7 exons of
PIGF-2. The 480 bp LP2017 open reading frame (ORF) encodes for a 160 amino acid
copolyptide.

LP2022 polypeptides comprising the amino acid sequence of the open reading
frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 3 are
contemplated as one embodiment of the present invention. Specifically, polypeptides of
the present invention comprise the amino acid sequence as shown in SEQ ID NO: 4, as
well as fragments, variants, and derivatives thereof. Accordingly, LP2022 polynucleotides encoding the LP2022 polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2022 indicates that, relative to PIGF-2, LP2022 contains an additional 31 base pairs at the 5’ end of exon 6 as a result of the use of an alternative spliceosome acceptor site during RNA processing. The additional 31 nucleotides included in the mature LP2022 mRNA results in a frame shift in the sixth exon, and the use of an alternative stop codon in the seventh exon. The 609 nucleotide open reading frame encodes a 203 amino acid polypeptide.

LP2023 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 5 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 6, as well as fragments, variants, and derivatives thereof. Accordingly, LP2023 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2023 indicates that it is a protein approximately 181 amino acids in length. LP2023, a splice variant of PIGF-2, contains an exon insertion between PIGF-2’s fourth and fifth exons. The novel 164 base exon processed into the mature LP2023 mRNA, includes a stop codon. LP2023 has a 543 base pair open reading frame encoding for a 181 amino acid polypeptide.

LP2024 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 7 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 8, as well as fragments, variants, and derivatives thereof. Accordingly, LP2024 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2024 indicates that it is a splice variant of the publicly reported PIGF-2 and PIGF-3 isoforms. The mature LP2024 mRNA includes all the exons of PIGF-2 as well as an in-frame insertion of an exon encoding 72 amino acids unique to PIGF-3, encoding for a 242 amino acid polypeptide.
LP2025 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 9 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 10, as well as fragments, variants, and derivatives thereof. Accordingly, LP2025 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2025 indicates that it is a splice variant of the publicly reported PIGF-2 that uses an alternative spliceosome acceptor site in the second intron, resulting in a 14 base pair deletion relative to PIGF-2. This deletion results in a frame shift of the ORF and a truncated protein product. The ORF of the mature LP2025 mRNA encodes an 84 amino acid polypeptide.

LP2026 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 11 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 12, as well as fragments, variants, and derivatives thereof. Accordingly, LP2026 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2026 indicates that it is a protein approximately 195 amino acids in length. LP2026 has the same genomic structure as PIGF-1 except for an additional 70 base pairs in the fourth exon, which is due to an alternative acceptor spliceosome site in the third intron. This results in a frame shift, which results in an ORF that encodes for a 195 amino acid polypeptide.

LP2027 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 13 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 14, as well as fragments, variants, and derivatives thereof. Accordingly, LP2027 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.
Sequence analysis of LP2027 indicates that it is a protein approximately 216 amino acids in length. LP2027 has the same genomic structure as PIGF-2, with the exception of 70 additional base pairs in the fourth exon due to the use of an alternative acceptor splicesome site in the third intron. This results in a frame shift and the use of an alternative stop codon in the seventh exon. The 648 base pair ORF of LP2027 encodes for a 216 amino acid polypeptide.

LP2029 polypeptides comprising the amino acid sequence of the open reading frame encoded by the polynucleotide sequence as shown in SEQ ID NO: 15 are contemplated as another embodiment of the present invention. Specifically, polypeptides of the present invention comprise the amino acid sequence as shown in SEQ ID NO: 16, as well as fragments, variants, and derivatives thereof. Accordingly, LP2029 polynucleotides encoding the polypeptides of the present invention are also contemplated by the present invention.

Sequence analysis of LP2029 indicates that it is a splice variant of the publicly reported PIGF-2 isoform that lacks the second exon of the mature PIGF-2 mRNA. This deletion results in a frame shift of the open reading frame, which encodes for a 92 amino acid polypeptide.

The LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides of the present invention can be prepared in any manner suitable to those known in the art. Such polypeptides include, e.g., naturally occurring polypeptides that are isolated, recombinantly produced polypeptides, synthetically produced polypeptides, or polypeptides produced by any combination of these methods. Means for preparing such polypeptides are well understood in the art. Throughout the present application, it is understood that an LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptide of the invention may be in the form of a full-length polypeptide with a sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 and 16; a mature, a secreted protein (including the mature form), or it may be comprised within a larger polypeptide or protein, such as, e.g., a fusion protein, or it may be a variant with at least 95%, 96%, 97%, 98%, 99% or greater than 99% sequence identity to the sequences shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 and 16 or the mature form thereof.

It is often advantageous to include with an LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptide, e.g., additional amino acid sequence...
that contains, e.g., secretory or leader sequences, pro-sequences, sequences that aid in
purification, such as, e.g., multiple histidine residues, or an additional sequence for
stability during recombinant production. Such modified forms of LP2017, LP2022,
LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 are also encompassed herein.

An LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029
polypeptide is preferably provided in a purified and isolated or recombinant form. A
recombinantly produced version of an LP2017, LP2022, LP2023, LP2024, LP2025,
LP2026, LP2027 and LP2029 polypeptide of the present invention, including a secreted
polypeptide, can be purified using techniques described herein or otherwise known in the
art, such as, e.g., the single-step purification method (Smith and Johnson (1988) Gene
67:31). An LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029
polypeptide can also be purified from natural, synthetic or recombinant sources using
techniques described herein or otherwise known in the art, such as, e.g., using an antibody
of the invention raised against a secreted protein.

Polynucleotides encoding a polypeptide comprising, or alternatively consisting of,
a polypeptide sequence of SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16 are also encompassed
by the invention.

The present invention provides an isolated or recombinant LP polynucleotide
comprising, or alternatively consisting of, a nucleic acid molecule having a mature
polynucleotide sequence of SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15 wherein said
polynucleotide sequence or said cDNA encodes a mature polypeptide of SEQ ID NO: 1,
3, 5, 7, 9, 11, 13 or 15.

An LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029
polynucleotide sequence can be composed of any polyribonucleotide or
polideoxribonucleotide, which may be unmodified RNA or DNA or modified RNA or
DNA. For example, polynucleotides can be composed of single- and double-stranded
DNA, DNA that is a mixture of single- and double-stranded regions, single- and
double-stranded RNA, and RNA that is mixture of single- and double-stranded regions,
hybrid molecules comprising DNA and RNA that may be single-stranded or, more
typically, double-stranded or a mixture of single-and double-stranded regions.

The invention encompasses variants of polynucleotide sequences encoding
LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029. In
particular, such variant polynucleotide sequences will have at least about 95%, more preferably at least 96%, 97%, 98%, 99% or greater than 99% sequence identity to the polynucleotide sequences as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 and 15 respectively. It will be appreciated by those skilled in the art that as a result of the degeneracy of the genetic code, a multitude of polynucleotide sequences encoding LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides, some bearing minimal homology to the polynucleotide sequences of any known and naturally occurring gene may be produced. Thus, the invention contemplates each and every possible variation of polynucleotide sequences that could be made by selecting combinations based on possible codon choices. These combinations are made in accordance with the standard triplet genetic code as applied to the polynucleotide sequences of naturally occurring LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029, and all such variations are to be considered as being specifically disclosed.

The invention also encompasses production of DNA sequences, which encode mature or full-length LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029, or LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 variants, by synthetic chemistry. After production, the synthetic sequence may be inserted into any of the many available expression vectors and cell systems using reagents that are well known in the art. Moreover, synthetic chemistry may be used to introduce mutations into a sequence encoding LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029.

An algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul, et al. (1990) J. Mol. Biol. 215:403. Software for performing BLAST analysis is publicly available through the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence
for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff and Henikoff (1989) PNAS 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=4, and a comparison of both strands.

Many proteins (and translated DNA sequences) contain regions where the amino acid composition is highly biased toward a small subset of the available residues. For example, membrane spanning domains and signal peptides (that also are membrane spanning) typically contain long stretches where Leucine (L), Valine (V), Alanine (A), and Isoleucine (I) predominate. Poly-Adenosine tracts (polyA) at the end of cDNAs appear in forward translations as poly-Lysine (poly-K) and poly-Phenylalanine (poly-F) when the reverse complement is translated. These regions are often referred to as “low complexity” regions. Such regions can cause database similarity search programs such as BLAST to find high-scoring sequence matches that do not imply true homology. The problem is exacerbated by the fact that most weight matrices (used to score the alignments generated by BLAST) give a match between any of a group of hydrophobic amino acids (L, V and I) that are commonly found in certain low complexity regions almost as high a score as for exact matches. To compensate for this, BLASTX2 (version 2.0 aSMP-WashU) employs filters (designated “seg” and “xnu”) that “mask” the low complexity regions in a particular sequence. These filters parse the sequence for such regions, and create a new sequence in which the amino acids in the low complexity region have been replaced with the character “X”. This is then used as the input sequence (sometimes referred to herein as “Query” and/or “Q”) to the BLASTX program. While this regime helps to ensure that high-scoring matches represent true homology, there is a negative consequence in that the BLASTX program uses the query sequence that has been masked by the filters to draw alignments. Thus, a stretch of “X”s in an alignment shown in the following application does not necessarily indicate that either the underlying DNA sequence or the translated protein sequence is unknown or uncertain. Nor is the
presence of such stretches meant to indicate that the sequence is identical or not identical to the sequence disclosed in the alignment of the present invention. Such stretches may simply indicate that the BLASTX program masked amino acids in that region due to the detection of a low complexity region, as defined above.

A further indication that two nucleic acid sequences are substantially identical for purposes of the invention is that the polypeptide encoded by the first nucleic acid is immunologically cross reactive with the polypeptide encoded by the second nucleic acid, as described below. Thus, a polypeptide is typically substantially identical to a second polypeptide, for example, where the two peptides differ only by conservative substitutions. Another indication that two nucleic acid sequences are substantially identical is that the two molecules hybridize to each other under stringent conditions, as described below.

Further, LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides encompasses polypeptide sequences that are pre- or pro-proteins of LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029. Moreover, the present invention encompasses mature LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 proteins, including polypeptides or proteins that are capable of being directed to the endoplasmic reticulum (ER), a secretory vesicle, a cellular compartment, or an extracellular space typically, e.g., as a result of a signal sequence, however, proteins released into an extracellular space without necessarily having a signal sequence are also encompassed. Generally, the polypeptides undergo processing, e.g., cleavage of a signal sequence, modification, folding, etc., resulting in a mature form (see, e.g., Alberts, et al. (1994) Molecular Biology of The Cell, Garland Publishing, New York, NY, pp. 557-592.). If an LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 or LP2029 polypeptide is released into the extracellular space, it can undergo extracellular processing to produce a “mature” protein. Release into the extracellular space can occur by many mechanisms, including, e.g., exocytosis, and proteolytic cleavage.

The LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 proteins may also be “altered,” resulting in “variations,” and may contain deletions, insertions, or substitutions of amino acid residues that produce a silent change and result in functionally equivalent proteins. Deliberate amino acid substitutions may be made
based on similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipathic nature of the residues, as long as the biological or immunological activity of the LP polypeptide is retained. For example, negatively charged amino acids may include aspartic acid and glutamic acid, and positively charged amino acids may include lysine and arginine. Amino acids with uncharged polar side chains having similar hydrophilicity values may include: asparagine and glutamine; and serine and threonine. Amino acids with uncharged side chains having similar hydrophilicity values may include: leucine, isoleucine, and valine; glycine and alanine; and phenylalanine and tyrosine.

The polynucleotides and polypeptides of the present invention are designated herein as “LP polynucleotides” or “LP polypeptide-encoding polynucleotides” and “LP polypeptides.” When immediately followed by a numerical designation (i.e., LP2017), the term “LP” refers to a specific group of molecules as defined herein. A complete designation wherein the term “LP” is immediately followed by a numerical designation and a molecule type (i.e., LP2017 polypeptide) refers to a specific type of molecule within the designated group of molecules as designated herein.

The terms “LP polypeptide-encoding polynucleotides” or “LP polynucleotides” and “LP polypeptides” wherein the term “LP” is followed by an actual numerical designation as used herein encompass novel polynucleotides and polypeptides, respectively, which are further defined herein. The LP molecules described herein may be isolated from a variety of sources including, but not limited to human tissue types, or prepared by recombinant or synthetic methods.

One aspect of the present invention provides an isolated nucleic acid molecule comprising polynucleotides, which encode LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptides as defined herein. In a preferred embodiment of the present invention, the isolated nucleic acid comprises 1) a polynucleotide encoding an LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptide having an amino acid sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16, respectively; 2) a polynucleotide complementary to such encoding nucleic acid sequences, and which remain stably bound to them under at least moderate, and optionally, high stringency conditions; or 3) any fragment and/or variant of 1) or 2).
The term “LP polypeptide” specifically encompasses truncated or secreted forms of an LP polypeptide, (e.g., soluble forms containing an extracellular domain sequence), variant forms (e.g., alternatively spliced forms) and allelic variants of an LP polypeptide.

In one embodiment of the invention, the native sequence LP polypeptide is a full-length or mature LP polypeptide comprising amino acids as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16. Also, while the LP polypeptides disclosed herein are shown to begin with a methionine residue designated as amino acid position 1 it is conceivable and possible that another methionine residue located either upstream or downstream from amino acid position 1 may be employed as the starting amino acid residue.

“LP polypeptide variant” is intended to refer to an “active” LP polypeptide, wherein activity is as defined herein, having at least about 95% amino acid sequence identity with an LP polypeptide having a deduced amino acid sequences as shown herein. Such LP polypeptide variants include, for instance, LP polypeptides, wherein one or more amino acid residues are added, substituted or deleted, at the N- or C-terminus or within the sequences shown. Ordinarily, an LP polypeptide variant will have at least about 95% amino acid sequence identity, preferably at least about 96% sequence identity, more preferably at least about 97% sequence identity, yet more preferably at least about 98% sequence identity, yet more preferably at least about 99% amino acid sequence identity with the amino acid sequence described, with or without the signal peptide.

The term “similar” or “similarity” as used herein describes the relationship between different nucleic acid compounds or amino acid sequences in which said sequences or molecules are related by partial sequence identity or sequence similarity at one or more blocks or regions within said molecules or sequences.

In referring to amino acid sequences, the term “similar” or “similarity” describes amino acid residues which are either identical between different amino acid sequences, or represent conservative amino acid substitutions between different sequences. Conservative amino acid substitutions are discussed infra.

The term “identity” describes amino acid residues, which are identical between different amino acid sequences. Amino acid sequence similarity or identity with respect to each LP amino acid sequence identified herein is defined as the percentage of amino acid residues in a candidate sequence that are similar or identical with the amino acid
residues in an LP polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence similarity or identity.

"Percent (%) amino acid sequence identity" with respect to the LP amino acid sequences identified herein is defined as the percentage of amino acid residues in a candidate sequence that are identical with the amino acid residues in an LP polypeptide sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN, ALIGN-2, Megalign (DNASTAR) or BLAST (e.g., Blast, Blast-2, WU-Blast-2) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For example, the percent identity values used herein are generated using WU-BLAST-2 [Altschul, et al., Methods in Enzymology 266:460-80 (1996)]. Most of the WU-BLAST-2 search parameters are set to the default values. Those not set to default values, i.e., the adjustable parameters, are set with the following values: overlap span = 1; overlap fraction = 0.125; word threshold (T) = 11; and scoring matrix = BLOSUM 62. For purposes herein, a percent amino acid sequence identity value is determined by dividing (a) the number of matching identical amino acid residues between the amino acid sequence of the LP polypeptide of interest and the comparison amino acid sequence of interest (i.e., the sequence against which the LP polypeptide of interest is being compared) as determined by WU-BLAST-2, by (b) the total number of amino acid residues of the LP polypeptide of interest, respectively.

An "LP variant polynucleotide," "LP polynucleotide variant," or "LP variant nucleic acid sequence" is intended to refer to a nucleic acid molecule as defined below having at least about 75% nucleic acid sequence identity with the polynucleotide sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15. Ordinarily, an LP polynucleotide variant will have at least about 75% nucleic acid sequence identity, more preferably at least about 80% nucleic acid sequence identity, yet more preferably at least about 81% nucleic acid sequence identity, yet more preferably at least about 82% nucleic acid sequence identity, yet more preferably at least about 83% nucleic acid sequence
identity, yet more preferably at least about 84% nucleic acid sequence identity, yet more preferably at least about 85% nucleic acid sequence identity, yet more preferably at least about 86% nucleic acid sequence identity, yet more preferably at least about 87% nucleic acid sequence identity, yet more preferably at least about 88% nucleic acid sequence identity, yet more preferably at least about 89% nucleic acid sequence identity, yet more preferably at least about 90% nucleic acid sequence identity, yet more preferably at least about 91% nucleic acid sequence identity, yet more preferably at least about 92% nucleic acid sequence identity, yet more preferably at least about 93% nucleic acid sequence identity, yet more preferably at least about 94% nucleic acid sequence identity, yet more preferably at least about 95% nucleic acid sequence identity, yet more preferably at least about 96% nucleic acid sequence identity, yet more preferably at least about 97% nucleic acid sequence identity, yet more preferably at least about 98% nucleic acid sequence identity, yet more preferably at least about 99% nucleic acid sequence identity with the nucleic acid sequences shown above. Variants specifically exclude or do not encompass the native nucleotide sequence, as well as those prior art sequences that share 100% identity with the nucleotide sequences of the invention.

"Percent (%) nucleic acid sequence identity" with respect to the LP polynucleotide sequences identified herein is defined as the percentage of nucleotides in a candidate sequence that are identical with the nucleotides in the LP polynucleotide sequence after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity. Alignment for purposes of determining percent nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software such as ALIGN, Align-2, Megalign (DNASTAR), or BLAST (e.g., Blast, Blast-2) software. Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, however, % nucleic acid identity values are generated using the WU-BLAST-2 (BlastN module) program [Altschul, et al., Methods in Enzymology 266:460-80 (1996)]. For purposes herein, a percent nucleic acid sequence identity value is determined by dividing (a) the number of matching identical nucleotides between the nucleic acid sequence of the LP polypeptide-encoding nucleic acid molecule of interest and the comparison nucleic acid molecule of interest (i.e., the sequence against
which the LP polypeptide-encoding nucleic acid molecule of interest is being compared) as determined by WU-BLAST-2, by (b) the total number of nucleotides of the LP polypeptide-encoding nucleic acid molecule of interest.

In other embodiments, the LP variant polypeptides are encoded by nucleic acid molecules which are capable of hybridizing, preferably under stringent hybridization and wash conditions, to nucleotide sequences encoding the full-length LP polypeptide as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16. This scope of variant polynucleotides specifically excludes those sequences that are known as of the filing and/or priority dates of the present application.

The term "mature protein" or "mature polypeptide" as used herein refers to the form(s) of the protein produced by expression in a mammalian cell. It is generally hypothesized that once export of a growing protein chain across the rough endoplasmic reticulum has been initiated, proteins secreted by mammalian cells have a signal peptide (SP) sequence which is cleaved from the complete polypeptide to produce a "mature" form of the protein. Oftentimes, cleavage of a secreted protein is not uniform and may result in more than one species of mature protein. The cleavage site of a secreted protein is determined by the primary amino acid sequence of the complete protein.

The term "inducing angiogenesis" is used to indicate that angiogenesis is initiated and/or enhanced.

"Isolated," when used to describe the various polypeptides disclosed herein, means a polypeptide that has been identified and separated and/or recovered from a component of its natural environment. Preferably, the isolated polypeptide is free of association with all components with which it is naturally associated. Contaminant components of its natural environment are materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide, and may include enzymes, hormones, and other proteinaceous or non-proteinaceous solutes. In preferred embodiments, the polypeptide will be purified (1) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or, preferably, silver stain. Isolated polypeptide includes polypeptide in situ within recombinant cells, since at least one component of the LP polypeptide natural
environment will not be present. Ordinarily, however, isolated polypeptide will be prepared by at least one purification step.

An "isolated LP polypeptide-encoding nucleic acid" or "isolated LP nucleic acid" is a nucleic acid molecule that is identified and separated from at least one contaminant nucleic acid molecule with which it is ordinarily associated in the natural source of the nucleic acid. Such an isolated nucleic acid molecule is other than in the form or setting in which it is found in nature. Isolated nucleic acid molecules therefore are distinguished from the nucleic acid molecule as it exists in natural cells. However, an isolated LP polypeptide-encoding nucleic acid molecule includes LP polypeptide-encoding nucleic acid molecules contained in cells that ordinarily express LP polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

Nucleic acid is "operably linked" when it is placed into a functional relationship with another nucleic acid sequence. For example, DNA for a presequence or secretory leader is operably linked to DNA for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, the synthetic oligonucleotide adapters or linkers are used in accordance with conventional practice.

The term "amino acid" is used herein in its broadest sense, and includes naturally occurring amino acids as well as non-naturally occurring amino acids, including amino acid analogs and derivatives. The latter includes molecules containing an amino acid moiety. One skilled in the art will recognize, in view of this broad definition, that reference herein to an amino acid includes, for example, naturally occurring proteogenic L-amino acids; D-amino acids; chemically modified amino acids such as amino acid analogs and derivatives; naturally-occurring non-proteogenic amino acids such as norleucine, beta-alanine, ornithine, etc.; and chemically synthesized compounds having properties known in the art to be characteristic of amino acids. As used herein, the term
"proteogenic" indicates that the amino acid can be incorporated into a peptide, polypeptide, or protein in a cell through a metabolic pathway.

The incorporation of non-natural amino acids, including synthetic non-native amino acids, substituted amino acids, or one or more D-amino acids into the LP peptides, polypeptides, or proteins of the present invention ("D-LP polypeptides") is advantageous in a number of different ways. D-amino acid-containing peptides, polypeptides, or proteins exhibit increased stability in vitro or in vivo compared to L-amino acid-containing counterparts. Thus, the construction of peptides, polypeptides, or proteins incorporating D-amino acids can be particularly useful when greater intracellular stability is desired or required. More specifically, D-peptides, polypeptides, or proteins are resistant to endogenous peptidases and proteases, thereby providing improved bioavailability of the molecule and prolonged lifetimes in vivo when such properties are desirable. When it is desirable to allow the peptide, polypeptide, or protein to remain active for only a short period of time, the use of L-amino acids therein will permit endogenous peptidases, proteases, etc., in a cell to digest the molecule in vivo, thereby limiting the cell's exposure to the molecule. Additionally, D-peptides, polypeptides, or proteins cannot be processed efficiently for major histocompatibility complex class II-restricted presentation to T helper cells, and are therefore less likely to induce humoral immune responses in the whole organism.

In addition to using D-amino acids, those of ordinary skill in the art are aware that modifications in the amino acid sequence of a peptide, polypeptide, or protein can result in equivalent, or possibly improved, second generation peptides, polypeptides, or proteins, that display equivalent or superior functional characteristics when compared to the original amino acid sequences. Alterations in the LP peptides, polypeptides, or proteins of the present invention can include one or more amino acid insertions, deletions, substitutions, truncations, fusions, shuffling of subunit sequences, and the like, either from natural mutations or human manipulation, provided that the sequences produced by such modifications have substantially the same (or improved or reduced, as may be desirable) activity(ies) as the naturally-occurring counterpart sequences disclosed herein.

One factor that can be considered in making such changes is the hydropathic index of amino acids. The importance of the hydropathic amino acid index in conferring
interactive biological function on a protein has been discussed by Kyte and Doolittle [J. Mol. Biol. 157:105-32 (1982)]. It is accepted that the relative hydrophobic character of amino acids contributes to the secondary structure of the resultant protein. This, in turn, affects the interaction of the protein with molecules such as enzymes, substrates, receptors, ligands, DNA, antibodies, antigens, etc. Based on its hydrophobicity and charge characteristics, each amino acid has been assigned a hydrophobic index as follows: isoleucine (+4.5); valine (+4.2); leucine (+3.8); phenylalanine (+2.8); cysteine/cystine (+2.5); methionine (+1.9); alanine (+1.8); glycine (-0.4); threonine (-0.7); serine (-0.8); tryptophan (-0.9); tyrosine (-1.3); proline (-1.6); histidine (-3.2); glutamate/glutamine/aspartate/asparagine (-3.5); lysine (-3.9); and arginine (-4.5).

As is known in the art, certain amino acids in a peptide, polypeptide, or protein can be substituted for other amino acids having a similar hydrophobic index or score and produce a resultant peptide, polypeptide, or protein having similar biological activity, i.e., which still retains biological functionality. In making such changes, it is preferable that amino acids having hydrophobic indices within ±2 are substituted for one another. More preferred substitutions are those wherein the amino acids have hydrophobic indices within ±1. Most preferred substitutions are those wherein the amino acids have hydrophobic indices within ±0.5.

Like amino acids can also be substituted on the basis of hydrophilicity. U.S. Patent 4,554,101 discloses that the greatest local average hydrophilicity of a protein, as governed by the hydrophilicity of its adjacent amino acids, correlates with a biological property of the protein. The following hydrophilicity values have been assigned to amino acids: arginine/lysine (+3.0); aspartate/glutamate (+3.0±1); serine (+0.3); asparagine/glutamine (+0.2); glycine (0); threonine (-0.4); proline (-0.5±1); alanine/histidine (-0.5); cysteine (-1.0); methionine (-1.3); valine (-1.5); leucine/iso-leucine (-1.8); tyrosine (-2.3); phenylalanine (-2.5); and tryptophan (-3.4). Thus, one amino acid in a peptide, polypeptide, or protein can be substituted by another amino acid having a similar hydrophilicity score and still produce a resultant peptide, polypeptide, or protein having similar biological activity, i.e., still retaining correct biological function. In making such changes, amino acids having hydrophobic indices
within \pm 2 are preferably substituted for one another, those within \pm 1 are more preferred, and those within \pm 0.5 are most preferred.

It should be noted that changes which are not expected to be advantageous can also be useful if these result in the production of functional sequences. Since small peptides, polypeptides, and some proteins can be easily produced by conventional solid phase synthetic techniques, the present invention includes peptides, polypeptides, or proteins such as those discussed herein, containing the amino acid modifications discussed above, alone or in various combinations. To the extent that such modifications can be made while substantially retaining the activity of the peptide, polypeptide, or protein, they are included within the scope of the present invention. The utility of such modified peptides, polypeptides, or proteins can be determined without undue experimentation by, for example, the methods described herein.

While biologically functional equivalents of the present LP polypeptides can have any number of conservative or non-conservative amino acid changes that do not significantly affect their activity(ies), or that increase or decrease activity as desired, the number of changes preferred may be 40, 30, 20, 10, 5, 3 or fewer changes, or any range or value therein, such as 1 to 30 changes. In particular, ten or fewer amino acid changes may be preferred. More preferably, seven or fewer amino acid changes may be preferred; most preferably, five or fewer amino acid changes may be preferred. The encoding nucleotide sequences (gene, plasmid DNA, cDNA, synthetic DNA, or mRNA, for example) will thus have corresponding base substitutions, permitting them to code expression for the biologically functional equivalent forms of the LP polypeptides. In any case, the LP peptides, polypeptides, or proteins exhibit the same or similar biological or immunological activity(ies) as that(those) of the LP polypeptides specifically disclosed herein, or increased or reduced activity, if desired. The activity(ies) of the variant LP polypeptides can be determined by the methods described herein. Variant LP polypeptides biologically functionally equivalent to those specifically disclosed herein have activity(ies) differing from those of the presently disclosed molecules by about \pm 50% or less, preferably by about \pm 40% or less, more preferably by about \pm 30% or less, more preferably by about \pm 20% or less, and even more preferably by about \pm 10% or less, when assayed by the methods disclosed herein.
Amino acids in an LP polypeptide of the present invention that are essential for activity can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis [Cunningham and Wells, Science 244:1081-5 (1989)]. The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity. Sites that are critical for ligand-protein binding can also be identified by structural analysis such as crystallization, nuclear magnetic resonance, or photoaffinity labeling [Smith, et al., J. Mol. Biol. 224:899-904 (1992); de Vos, et al., Science 255:306-12 (1992)].

"Stringency" of hybridization reactions is readily determinable by one of ordinary skill in the art, and generally is an empirical calculation dependent upon probe length, washing temperature, and salt concentration. In general, longer nucleic acid probes required higher temperatures for proper annealing, while shorter nucleic acid probes need lower temperatures. Hybridization generally depends on the ability of denatured DNA to reanneal when complementary strands are present in an environment below their melting temperature. The higher the degree of desired complementarity between the probe and hybridizable sequence, the higher the relative temperature that can be used. As a result, it follows that higher relative temperatures would tend to make the reactions more stringent, while lower temperatures less so. For additional details and explanation of stringency of hybridization reactions, see Ausubel, et al., Current Protocols in Molecular Biology, Wiley Interscience Publishers (1995).

"Stringent conditions" or "high stringency conditions," as defined herein, may be identified by those that (1) employ low ionic strength and high temperature for washing, for example, 15 mM sodium chloride/1.5 mM sodium citrate/0.1% sodium dodecyl sulfate at 50 degrees C; (2) employ during hybridization a denaturing agent, such as formamide, for example, 50% (v/v) formamide with 0.1% bovine serum albumin/0.1% ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM sodium chloride/75 mM sodium citrate at 42 degrees C; or (3) employ 50% formamide, 5X SSC (750 mM sodium chloride, 75 mM sodium citrate), 50 mM sodium phosphate (pH 6.8), 0.1% sodium pyrophosphate, 5X Denhardt's solution, sonicated salmon sperm DNA (50 µg/mL), 0.1% SDS, and 10% dextran sulfate at 42 degrees C with washes at 42 degrees C in 0.2X SSC (30 mM sodium chloride/3 mM sodium citrate) and 50% formamide at
55 degrees C, followed by a high-stringency wash consisting of 0.1X SSC containing EDTA at 55 degrees C.

As used herein, the term “immunoadhesin,” sometimes referred to as an Fc fusion, designates antibody-like molecules that combine the binding specificity of a heterologous protein (an “adhesin”) with the effector functions of immunoglobulin constant domains. Structurally, the immunoadhesins comprise a fusion of an amino acid sequence with the desired binding specificity which is other than the antigen recognition and binding site of an antibody (i.e., is “heterologous”), and an immunoglobulin constant domain sequence. The adhesin part of an immunoadhesin molecule typically is a contiguous amino acid sequence comprising at least the binding site of a receptor or a ligand. The immunoglobulin constant domain sequence in the immunoadhesin may be obtained from any immunoglobulin, such as IgG-1, IgG-2, IgG-3 or IgG-4 subtypes, IgA (including IgA-1 and IgA-2), IgE, IgD or IgM.

“Active” or “activity” for the purposes herein refers to form(s) of LP polypeptide which retain all or a portion of the biologic and/or immunologic activities of native or naturally occurring LP polypeptide. Elaborating further, “biological” activity refers to a biological function (either inhibitory or stimulatory) caused by a native or naturally occurring LP polypeptide other than the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally-occurring LP polypeptide. An “immunological” activity refers only to the ability to induce the production of an antibody against an antigenic epitope possessed by a native or naturally occurring LP polypeptide.

“Antibodies” (Abs) and “immunoglobulins” (Igs) are glycoproteins having the same structural characteristics. While antibodies exhibit binding specificity to a specific antigen, immunoglobulins include both antibodies and other antibody-like molecules that lack antigen specificity. Polypeptides of the latter kind are, for example, produced at low levels by the lymph system and at increased levels by myelomas. The term “antibody” is used in the broadest sense and specifically covers, without limitation, intact monoclonal antibodies, polyclonal antibodies, multispecific antibodies (e.g., bispecific antibodies) formed from at least two intact antibodies, and antibody fragments so long as they exhibit the desired biological activity.
The terms “treating,” “treatment,” and “therapy” as used herein refer to curative therapy, prophylactic therapy, and preventive therapy. An example of “preventive therapy” is the prevention or lessened targeted pathological condition or disorder. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in whom the disorder is to be prevented.

“Chronic” administration refers to administration of the agent(s) in a continuous mode as opposed to an acute mode, so as to maintain the initial therapeutic effect (activity) for an extended period of time. “Intermittent” administration is treatment that is not consecutively done without interruption but, rather, is cyclic in nature.

Administration “in combination with” one or more further therapeutic agents includes simultaneous (concurrent) and consecutive administration in any order.

A “therapeutically-effective amount” is the minimal amount of active agent (e.g., an LP polypeptide, antagonist or agonist thereof) which is necessary to impart therapeutic benefit to a mammal. For example, a “therapeutically-effective amount” to a mammal suffering or prone to suffering or to prevent it from suffering is such an amount which induces, ameliorates, or otherwise causes an improvement in the pathological symptoms, disease progression, physiological conditions associated with or resistance to succumbing to the aforesaid disorder.

“Carriers” as used herein include pharmaceutically acceptable carriers, excipients, or stabilizers which are nontoxic to the cell or mammal being exposed thereto at the dosages and concentrations employed. Often the physiologically acceptable carrier is an aqueous pH buffered solution. Examples of physiologically acceptable carriers include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecule weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; salt-forming counterions such as sodium; and/or nonionic surfactants such as TWEEN®, polyethylene glycol (PEG), and PLURONIC®.

“Antibody fragments” comprise a portion of an intact antibody, preferably the antigen binding or variable region of the intact antibody. Examples of antibody
fragments include Fab, Fab', F(ab')₂ and Fv fragments; diabodies; linear antibodies
and multispecific antibodies formed from antibody fragments.

An “isolated” antibody is one which has been identified and separated and/or
recovered from a component of its natural environment. Contaminant components of its
natural environment are materials which would interfere with diagnostic or therapeutic
uses for the antibody, and may include enzymes, hormones, and other proteinaceous or
non-proteinaceous solutes. In preferred embodiments, the antibody will be purified (1) to
greater than 95% by weight of antibody as determined by the Lowry method, and most
preferably more than 99% by weight, (2) to a degree sufficient to obtain at least 15
residues of N-terminal or internal amino acid sequence by use of a spinning cup
sequenator, or (3) to homogeneity by SDS-PAGE under reducing or nonreducing
conditions using Coomassie blue, or preferably, silver stain. Isolated antibody includes
the antibody *in situ* within recombinant cells since at least one component of the
antibody’s natural environment will not be present. Ordinarily, however, isolated
antibody will be prepared by at least one purification step.

An “LP polypeptide antibody” or “LP antibody” refers to an antibody as defined
herein that recognizes and binds at least one epitope of an LP polypeptide of the present
invention. The term “LP polypeptide antibody” or “LP antibody” wherein the term “LP”
is followed by a numerical designation refers to an antibody that recognizes and binds to
at least one epitope of that particular LP polypeptide as disclosed herein.

A “liposome” is a small vesicle composed of various types of lipids,
phospholipids and/or surfactant which is useful for delivery of a drug (such as an LP
polypeptide or antibody thereto) to a mammal. The components of the liposome are
commonly arranged in a bilayer formation, similar to the lipid arrangement of biological
membranes.

A “small molecule” is defined herein to have a molecular weight below about 500
daltons.

The term “modulate” means to affect (e.g., either upregulate, downregulate or
otherwise control) the level of a signaling pathway. Cellular processes under the control
of signal transduction include, but are not limited to, transcription of specific genes,
normal cellular functions, such as metabolism, proliferation, differentiation, adhesion,
apoptosis and survival, as well as abnormal processes, such as transformation, blocking of differentiation and metastasis.

An LP polynucleotide can be composed of any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified RNA or DNA or modified RNA or DNA. For example, the LP polynucleotides can be composed of single- and double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, LP polynucleotides can be composed of triple-stranded regions comprising RNA or DNA or both RNA and DNA. LP polynucleotides may also contain one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. “Modified” bases include, for example, tritylated bases and unusual bases such as inosine. A variety of modifications can be made to DNA and RNA; thus, “polynucleotide” embraces chemically, enzymatically, or metabolically modified forms.

LP polypeptides can be composed of amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain amino acids other than the gene-encoded amino acids. The LP polypeptides may be modified by either natural processes, such as post-translational processing, or by chemical modification techniques which are well known in the art. Such modifications are well described in basic texts and in more detailed monographs, as well as in a voluminous research literature. Modifications can occur anywhere in the LP polypeptides, including the peptide backbone, the amino acid side-chains and the amino or carboxyl termini. It will be appreciated that the same type of modification may be present in the same or varying degrees at several sites in a given LP polypeptide. Also, a given LP polypeptide may contain many types of modifications. LP polypeptides may be branched, for example, as a result of ubiquitination, and they may be cyclic, with or without branching. Cyclic, branched, and branched cyclic LP polypeptides may result from post-translation natural processes or may be made by synthetic methods. Modifications include acetylation, acylation, ADP-ribosylation, amidation, covalent attachment of flavin, covalent attachment of a heme moiety, covalent attachment of a nucleotide or nucleotide derivative, covalent attachment of a lipid or lipid derivative, covalent attachment of

Variations in the full-length sequence LP polypeptide or in various domains of the LP polypeptide described herein can be made, for example, using any of the techniques and guidelines for conservative and non-conservative mutations set forth, for instance, in U.S. Patent 5,364,934. Variations may be a substitution, deletion or insertion of one or more codons encoding LP polypeptide that results in a change in the amino acid sequence of the LP polypeptide as compared with the native sequence LP polypeptide or an LP polypeptide as disclosed herein. Optionally the variation is by substitution of at least one amino acid with any other amino acid in one or more of the domains of the LP polypeptide. Guidance in determining which amino acid residue may be inserted, substituted or deleted without adversely affecting the desired activity may be found by comparing the sequence of the LP polypeptide with that of homologous known protein molecules and minimizing the number of amino acid sequence changes made in regions of high identity and/or similarity. Amino acid substitutions can be the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with a serine, i.e., conservative amino acid replacements. Insertions or deletions may optionally be in the range of one to five amino acids. The variation allowed may be determined by systematically making insertions, deletions or substitutions of amino acids in the sequence and testing the resulting variants for activity (such as in any of the *in vitro* assays described herein) for activity exhibited by the full-length or mature polypeptide sequence.

Covalent modifications of LP polypeptides are included within the scope of this invention. One type of covalent modification includes reacting targeted amino acid
residues of an LP polypeptide with an organic derivatizing agent that is capable of reacting with selected side chains or the N- or C-terminal residues of an LP polypeptide. Derivatization with bifunctional agents is useful, for instance, for crosslinking LP polypeptide to a water-insoluble support matrix or surface for use in the method for purifying anti-LP polypeptide antibodies, and vice-versa. Commonly used crosslinking agents include, e.g., 1,1-bis(diazoacetyl)-2-phenylethane, glutaraldehyde, N-hydroxysuccinimide esters, for example, esters with 4-azidosalicylic acid, homobifunctional imidoesters, including disuccinimidyl esters such as 3,3'-dithiobis-(succinimidylpropionate), bifunctional maleimides such as bis-N-maleimido-1,8-octane and agents such as methyl-3-[(p-azidophenyl)dithiolpropioimidate.

Other modifications include deamidation of glutaminyl and asparaginyl residues to the corresponding glutamyl and aspartyl residues, respectively, hydroxylation of proline and lysine, phosphorylation of hydroxyl groups of seryl or threonyl residues, methylation of the alpha-amino groups of lysine, arginine, and histidine side chains [Creighton, *Proteins: Structure and Molecular Properties*, W.H. Freeman & Co., San Francisco, pp. 79-86 (1983)], acetylation of the N-terminal amine, and amidation of any C-terminal carboxyl group.

Another type of covalent modification of the LP polypeptides included within the scope of this invention comprises altering the native glycosylation pattern of the polypeptide. “Altering the native glycosylation pattern” is intended for purposes herein to mean deleting one or more carbohydrate moieties found in native sequence LP polypeptide and/or adding one or more glycosylation sites that are not present in the native sequences of LP polypeptides. Additionally, the phrase includes qualitative changes in the glycosylation of the native proteins, involving a change in the nature and proportions of the various carbohydrate moieties present.

Addition of glycosylation sites to LP polypeptides may be accomplished by altering the amino acid sequence thereof. The alteration may be made, for example, by the addition of, or substitution by, one or more serine or threonine residues to the native sequences of LP polypeptides (for O-linked glycosylation sites). The LP amino acid sequences may optionally be altered through changes at the DNA level, particularly by mutating the DNA encoding the LP polypeptides at preselected bases such that codons are generated that will translate into the desired amino acids.
Another means of increasing the number of carbohydrate moieties on the LP polypeptides is by chemical or enzymatic coupling of glycosides to the polypeptide. Such methods are described in the art, e.g., in WO 87/05330, and in Aplin and Wriston, *CRC Crit. Rev. Biochem.*, pp. 259-306 (1981).


Another type of covalent modification of LP comprises linking any one of the LP polypeptides to one of a variety of non-proteinaceous polymers (e.g., polyethylene glycol, polypropylene glycol, or polyoxyalkylenes) in the manner set forth in U.S. Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192, or 4,179,337.

LP polypeptides of the present invention may also be modified in a way to form chimeric molecules comprising an LP polypeptide fused to another heterologous polypeptide or amino acid sequence. In one embodiment, such a chimeric molecule comprises a fusion of an LP polypeptide with a tag polypeptide which provides an epitope to which an anti-tag antibody can selectively bind. The epitope tag is generally placed at the amino- or carboxyl- terminus of an LP2017, LP2022, LP2023, LP2024, LP2025, LP2026, LP2027 and LP2029 polypeptide. The presence of such epitome-tagged forms of an LP polypeptide can be detected using an antibody against the tag polypeptide. Also, provision of the epitome tag enables an LP polypeptide to be readily purified by affinity purification using an anti-tag antibody or another type of affinity matrix that binds to the epitome tag.

In an alternative embodiment, the chimeric molecule may comprise a fusion of an LP polypeptide with an immunoglobulin or a particular region of an immunoglobulin.

For a bivalent form of the chimeric molecule, such a fusion could be to the Fc region of an IgG molecule. The Ig fusions preferably include the substitution of a soluble transmembrane domain deleted or inactivated form of an LP polypeptide in place of at
least one variable region within an Ig molecule. In a particularly preferred embodiment, the immunoglobulin fusion includes the hinge, CH2 and CH3 or the hinge, CH1, CH2 and CH3 regions of an IgG1 molecule. For the production of immunoglobulin fusions, see also U.S. Patent 5,428,130.

In yet a further embodiment, the LP polypeptides of the present invention may also be modified in a way to form a chimeric molecule comprising an LP polypeptide fused to a leucine zipper. Various leucine zipper polypeptides have been described in the art. See, e.g., Landschulz, et al., Science 240(4860):1759-64 (1988); WO 94/10308; Hoppe, et al., FEBS Letters 344(2-3):191-5 (1994); Abel, et al., Nature 341(6237):24-5 (1989). It is believed that use of a leucine zipper fused to an LP polypeptide may be desirable to assist in dimerizing or trimerizing soluble LP polypeptide in solution. Those skilled in the art will appreciate that the zipper may be fused at either the N- or C-terminal end of an LP polypeptide.

The description below relates primarily to production of LP polypeptides by culturing cells transformed or transfected with a vector containing an LP polypeptide-encoding nucleic acid. It is, of course, contemplated that alternative methods, which are well known in the art, may be employed to prepare LP polypeptides. For instance, the LP polypeptide sequence, or portions thereof, may be produced by direct peptide synthesis using solid-phase techniques [see, e.g., Stewart, et al., Solid-Phase Peptide Synthesis, W.H. Freeman & Co., San Francisco, CA (1969); Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963)]. In vitro protein synthesis may be performed using manual techniques or by automation. Automated synthesis may be accomplished, for instance, using an Applied Biosystems Peptide Synthesizer (Foster City, CA) using manufacturer’s instructions. Various portions of an LP polypeptide may be chemically synthesized separately and combined using chemical or enzymatic methods to produce a full-length LP polypeptide.

DNA encoding an LP polypeptide may be obtained from a cDNA library prepared from tissue believed to possess the LP polypeptide-encoding mRNA and to express it at a detectable level. Libraries can be screened with probes (such as antibodies to an LP polypeptide or oligonucleotides of at least about 20 to 80 bases) designed to identify the gene of interest or the protein encoded by it. Screening the cDNA or genomic library with the selected probe may be conducted using standard procedures, such as described in

Nucleic acids having protein coding sequence may be obtained by screening selected cDNA or genomic libraries using the deduced amino acid sequence disclosed herein for the first time and, if necessary, using conventional primer extension procedures as described in Sambrook, et al., supra, to detect precursors and processing intermediates of mRNA that may not have been reverse-transcribed into cDNA.

Host cells are transfected or transformed with expression or cloning vectors described herein for LP polypeptide production and cultured in conventional nutrient media modified as appropriate for inducing promoters, selecting transformants, or amplifying the genes encoding the desired sequences. The culture conditions, such as media, temperature, pH and the like, can be selected by the skilled artisan without undue experimentation. In general, principles, protocols, and practical techniques for maximizing the productivity of cell cultures can be found in Mammalian Cell Biotechnology: A Practical Approach, Butler, ed. (IRL Press, 1991) and Sambrook, et al., supra. Methods of transfection are known to the ordinarily skilled artisan, for example, calcium phosphate and electroporation. General aspects of mammalian cell host system transformations have been described in U.S. Patent 4,399,216.

Transformations into yeast are typically carried out according to the method of van Solingen, et al., J. Bact. 130(2):946-7 (1977) and Hsiao, et al., Proc. Natl. Acad. Sci. USA 76(8):3829-33 (1979). However, other methods for introducing DNA into cells, such as by nuclear microinjection, electroporation, bacterial protoplast fusion with intact cells, or polycations, e.g., polybrene or polyornithine, may also be used. For various techniques for transforming mammalian cells, see Keown, et al., Meths. in Enzymology 185:527-37 (1990) and Mansour, et al., Nature 336(6197):348-52 (1988).

Suitable host cells for cloning or expressing the nucleic acid (e.g., DNA) in the vectors herein include prokaryote, yeast, or higher eukaryote cells. Suitable prokaryotes include but are not limited to eubacteria, such as Gram-negative or Gram-positive organisms, for example, Enterobacteriacea such as E. coli. Various E. coli strains are publicly available, such as E. coli K12 strain MM294 (ATCC 31,446); E. coli strain
X1776 (ATCC 31,537); E. coli strain W3110 (ATCC 27,325) and K5 772 (ATCC 53,635). Other suitable prokaryotic host cells include Enterobacteriaceae such as Escherichia, e.g., E. coli, Enterobacter, Erwinia, Klebsiella, Proteus, Salmonella, e.g., Salmonella typhimurium, Serratia, e.g., Serratia marcescans, and Shigella, as well as Bacilli such as B. subtilis and B. licheniformis (e.g., B. licheniformis 41P disclosed in DD 266,710, published 12 April 1989), Pseudomonas such as P. aeruginosa, and Streptomyces. These examples are illustrative rather than limiting. Strain W3110 is one particularly preferred host or parent host because it is a common host strain for recombinant DNA product fermentations. Preferably, the host cell secretes minimal amounts of proteolytic enzymes. For example, strain W3110 may be modified to effect a genetic mutation in a gene encoding proteins endogenous to the host, with examples of such hosts including E. coli W3110 strain 1A2, which has the complete genotype tonAD; E. coli W3110 strain 9E4, which has the complete genotype tonAD ptr3; E. coli W3110 strain 27C7 (ATCC 55,244), which has the complete genotype tonAD ptr3 phoADE15 D(argF-lac)169 ompTD degP41kanR; E. coli W3110 strain 37D6, which has the complete genotype tonAD ptr3 phoADE15 D(argF-lac)169 ompTD degP41kanR rbs7D ilvG; E. coli W3110 strain 40B4, which is strain 37D6 with a non-kanamycin resistant degP deletion mutation; and an E. coli strain having mutant periplasmic protease as disclosed in U.S. Patent 4,946,783 issued 7 August 1990. Alternatively, in vivo methods of cloning, e.g., PCR or other nucleic acid polymerase reactions, are suitable.


Suitable host cells for the expression of glycosylated LP polypeptides are derived from multicellular organisms. Examples of invertebrate cells include insect cells such as Drosophila S2 and Spodoptera Sp, Spodoptera high5 as well as plant cells. Examples of useful mammalian host cell lines include Chinese hamster ovary (CHO) and COS cells. More specific examples include monkey kidney CV1 line transformed by SV40 (COS-7, ATCC CRL 1651); human embryonic kidney line [293 or 293 cells subcloned for growth in suspension culture, Graham, et al., J. Gen Virol., 36(1):59-74 (1977)]; Chinese hamster ovary cells/DHFR [CHO, Urlaub and Chasin, Proc. Natl. Acad. Sci. USA, 77(7):4216-20 (1980)]; mouse sertoli cells [TM4, Mather, Biol. Reprod. 23(1):243-52 (1980)]; human lung cells (W138, ATCC CCL 75); human liver cells (Hep G2, HB 8065); and mouse mammary tumor (MMT 060562, ATCC CCL51). The selection of the appropriate host cell is deemed to be within the skill in the art.

Both expression and cloning vectors contain a nucleic acid sequence that enables the vector to replicate in one or more selected host cells. Such sequences are well known for a variety of bacteria, yeast, and viruses. The origin of replication from the plasmid pBR322 is suitable for most Gram-negative bacteria, the 2μ plasmid origin is suitable for yeast, and various viral origins (SV40, polyoma, adenovirus, VSV or BPV) are useful for cloning vectors in mammalian cells.

Expression and cloning vectors will typically contain a selection gene, also termed a selectable marker. Typical selection genes encode proteins that (a) confer
resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli.

An example of suitable selectable markers for mammalian cells are those that enable the identification of cells competent to take up the LP polypeptide-encoding nucleic acid, such as DHFR or thymidine kinase. An appropriate host cell when wild-type DHFR is employed is the CHO cell line deficient in DHFR activity, prepared and propagated as described Urlaub and Chasin, *Proc. Natl. Acad. Sci. USA*, 77(7):4216-20 (1980). A suitable selection gene for use in yeast is the trpl gene present in the yeast plasmid YRp7 [Stinchcomb, et al., *Nature* 282(5734):39-43 (1979); Kingsman, et al., *Gene* 7(2):141-52 (1979); Tschumper, et al., *Gene* 10(2):157-66 (1980)]. The trpl gene provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example, ATCC No. 44076 or PEPC1 [Jones, *Genetics* 85:23-33 (1977)].


Other yeast promoters, which are inducible promoters having the additional advantage of transcription controlled by growth conditions, are the promoter regions for
alcohol dehydrogenase 2, isocitrate C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, metallothionein, glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose utilization. Suitable vectors and promoters for use in yeast expression are further described in EP 73,657. LP transcription from vectors in mammalian host cells is controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40), from heterologous mammalian promoters, e.g., the actin promoter or an immunoglobulin promoter, and from heat-shock promoters, provided such promoters are compatible with the host cell systems.

Transcription of a polynucleotide encoding an LP polypeptide by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, that act on a promoter to increase its transcription. Many enhancer sequences are now known from mammalian genes (globin, elastase, albumin, alpha-ketoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples include the SV40 enhancer on the late side of the replication origin (bp 100-270), the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the vector at a position 5’ or 3’ to the LP polypeptide coding sequence but is preferably located at a site 5’ from the promoter.

Expression vectors used in eukaryotic host cells (yeast, fungi, insect, plant, animal, human, or nucleated cells from other multicellular organisms) will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5’ and occasionally 3’ untranslated regions of eukaryotic or viral DNAs or cDNAs. These regions contain nucleotide segments transcribed as polyadenylated fragments in the untranslated portion of the mRNA encoding LP.

Gene amplification and/or expression may be measured in a sample directly, for example, by conventional Southern blotting, Northern blotting to quantitate the
transcription of mRNA [Thomas, Proc. Natl. Acad. Sci. USA 77(9):5201-5 (1980)], dot blotting (DNA analysis), or in situ hybridization, using an appropriately labeled probe, based on the sequences provided herein. Alternatively, antibodies may be employed that can recognize specific duplexes, including DNA duplexes, RNA duplexes, and DNA-RNA hybrid duplexes or DNA-protein duplexes. The antibodies in turn may be labeled and the assay may be carried out where the duplex is bound to a surface, so that upon the formation of duplex on the surface, the presence of antibody bound to the duplex can be detected.

Gene expression, alternatively, may be measured by immunological methods, such as immunohistochemical staining of cells or tissue sections and assay of cell culture or body fluids, to quantitate directly the expression of gene product. Antibodies useful for immunohistochemical staining and/or assay of sample fluids may be either monoclonal or polyclonal and may be prepared in any mammal. Conveniently, the antibodies may be prepared against a native sequence provided herein or against exogenous sequence fused to an LP polypeptide-encoding DNA and encoding a specific antibody epitope.

Various forms of an LP polypeptide may be recovered from culture medium or from host cell lysates. If membrane-bound, it can be released from the membrane using a suitable detergent solution (e.g., Triton X-100™) or by enzymatic cleavage. Cells employed in expression of an LP polypeptide can be disrupted by various physical or chemical means, such as freeze-thaw cycling, sonication, mechanical disruption, or cell lysing agents.

It may be desireable to purify LP polypeptides from recombinant cell proteins or polypeptides. The following procedures are exemplary of suitable purification procedures: by fractionation on an ion-exchange column; ethanol precipitation; reversed-phase HPLC; chromatography on silica or on a cation-exchange resin such as DEAE; chromatofocusing; SDS-PAGE; ammonium sulfate precipitation; gel filtration using, for example, Sephadex® G-75; protein A Sepharose® columns to remove contaminants such as IgG; and metal chelating columns to bind epitope-tagged forms of an LP polypeptide. Various methods of protein purification may be employed and such methods are known in the art and described, for example, in Deutscher, Methods in Enzymology 182:83-9 (1990) and Scopes, Protein Purification: Principles and Practice, Springer-Verlag, NY
(1982). The purification step(s) selected will depend, for example, on the nature of the production process used and the particular LP polypeptide produced.

Nucleotide sequences (or their complement) encoding LP polypeptides have various applications in the art of molecular biology, including uses as hybridization probes, in chromosome and gene mapping and in the generation of anti-sense RNA and DNA. LP polypeptide-encoding nucleic acids will also be useful for the preparation of LP polypeptides by the recombinant techniques described herein.

The full-length LP polypeptide-encoding nucleotide sequence (e.g., SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15), or portions thereof, may be useful as hybridization probes for probing a cDNA or genomic library to isolate the full-length LP polypeptide-encoding cDNA or genomic sequences including promoters, enhancer elements and introns of native sequence LP polypeptide-encoding DNA or to isolate still other genes (for instance, those encoding naturally-occurring variants of LP polypeptides or the same from other species) which have a desired sequence identity to the LP polypeptide-encoding nucleotide sequence disclosed in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15. Hybridization techniques are well known in the art and some of which are described in further detail in the Examples below.

Other useful fragments of the LP polypeptide-encoding nucleic acids include anti-sense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target LP polypeptide-encoding mRNA (sense) of LP polypeptide-encoding DNA (anti-sense) sequences. Anti-sense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of LP polypeptide-encoding DNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to 30 nucleotides. The ability to derive an anti-sense or a sense oligonucleotide, based upon a cDNA sequence encoding a given protein is described in, for example, Stein and Cohen, Cancer Res. 48(10):2659-68 (1988) and van der Krol, et al., Bio/Techniques 6(10):958-76 (1988).

Binding of anti-sense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block transcription or translation of the target sequence by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The anti-sense oligonucleotides thus may be used to block expression of LP mRNA and therefore any
LP polypeptide encoded thereby. Anti-sense or sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester linkages (or other sugar linkages, such as those described in WO 91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable \textit{in vivo} (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences.

Other examples of sense or anti-sense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increase affinity of the oligonucleotide for a target nucleic acid sequence, such poly-L-lysine. Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or anti-sense oligonucleotides to modify binding specificities of the anti-sense or sense oligonucleotide for the target nucleotide sequence.

Anti-sense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, calcium phosphate-mediated DNA transfection, electroporation, or by using gene transfer vectors such as Epstein-Barr virus. In a preferred procedure, an anti-sense or sense oligonucleotide is inserted into a suitable retroviral vector. A cell containing the target nucleic acid sequence is contacted with the recombinant retroviral vector, either \textit{in vivo} or \textit{ex vivo}. Suitable retroviral vectors include, but are not limited to, those derived from the murine retrovirus M-MSV, N2 (a retrovirus derived from M-MuLV), or the double copy vectors designated CDTSA, CTSB and DCTSC (see WO 90/13641).

Alternatively, a sense or an anti-sense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or anti-sense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase. When the amino acid sequence for an LP polypeptide encodes a protein which binds to another protein (for example, where the LP polypeptide functions as a receptor), the LP polypeptide can be used in assays to identify the other proteins or molecules involved in the binding interaction. By such methods, inhibitors of the receptor/ligand binding interaction can be identified. Proteins involved in such binding interactions can also be used to screen for peptide or small molecule inhibitors or agonists of the binding
interaction. Also, the receptor LP polypeptide can be used to isolate correlative ligand(s). Screening assays can be designed to find lead compounds that mimic the biological activity of the LP polypeptides disclosed herein or a receptor for such LP polypeptides. Typical screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates. Small molecules contemplated include synthetic organic or inorganic compounds. The assays can be performed in a variety of formats, including protein-protein binding assays, biochemical screening assays, immunoassays and cell based assays, which are well characterized in the art.

"Gene therapy" includes both conventional gene therapy, where a lasting effect is achieved by a single treatment, and the administration of gene therapeutic agents, which involves the one time or repeated administration of a therapeutically effective DNA or mRNA. Anti-sense RNAs and DNAs can be used as therapeutic agents for blocking the expression of certain genes in vivo. It has already been shown that short anti-sense oligonucleotides can be imported into cells where they act as inhibitors, despite their low intracellular concentrations caused by their restricted uptake by the cell membrane [Zamecnik, et al., Proc. Natl. Acad. Sci. USA 83(12):4143-6 (1986)]. The oligonucleotides can be modified to enhance their uptake, e.g., by substituting their negatively charged phosphodiester groups with uncharged groups.

administration \textit{in situ}. Well-known viral delivery techniques include the use of adenovirus, retrovirus, lentivirus, foamy virus, herpes simplex virus, and adeno-associated virus vectors. Exemplary non-viral techniques include the use of naked DNA; DNA complexed with cationic lipids, alone or in combination with cationic polymers; anionic and cationic liposomes; DNA-protein complexes and particles comprising DNA condensed with cationic polymers such as heterogeneous polylysine, defined-length oligopeptides, and polyethylene imine, in some cases contained in liposomes; and the use of ternary complexes comprising a virus and polylysine-DNA. Physical methods include the use of needle-free injectors, such as "gene gun" devices and devices using liquid under high pressure for delivery into interstitial spaces, and electroporation.


In some situations it is desirable to provide the nucleic acid source with an agent that targets the target cells, such as an antibody specific for a cell surface membrane.
protein or the target cell, a ligand for a receptor on the target cells, etc. Where liposomes are employed, proteins which bind to a cell surface membrane protein associated with endocytosis may by used for targeting and/or to facilitate uptake, e.g., capsid proteins or fragments thereof trophic for a particular cell type, antibodies for proteins which undergo internalization in cycling, proteins that target intracellular localization and enhance intracellular half-life. The technique of receptor-mediated endocytosis is described, for example, by Wu, et al., J. Biol. Chem. 262(10):4429-32 (1987); and Wagner, et al., Proc. Natl. Acad. Sci. USA 87(9):3410-4 (1990). For a review of gene marking and gene therapy protocols, see Anderson, Science 256(5058):808-13 (1992).

The nucleic acid molecules encoding LP polypeptides or fragments thereof described herein are useful for chromosome identification. In this regard, there exists an ongoing need to identify new chromosome markers, since relatively few chromosome marking reagents, based upon actual sequence data, are presently available. Each LP polypeptide-encoding nucleic acid molecule of the present invention can be used as a chromosome marker. An LP polypeptide-encoding nucleic acid or fragments thereof can also be used for chromosomal localization of the gene encoding that LP polypeptide.

The present invention further provides anti-LP polypeptide antibodies. Exemplary antibodies include polyclonal, monoclonal, humanized, bispecific, and heteroconjugate antibodies.

The anti-LP polypeptide antibodies of the present invention may comprise polyclonal antibodies. Methods of preparing polyclonal antibodies are known to the skilled artisan. Polyclonal antibodies can be raised in a mammal, for example, by one or more injections of an immunizing agent and, if desired, an adjuvant. Typically, the immunizing agent and/or adjuvant will be injected in the mammal by multiple subcutaneous or intraperitoneal injections. The immunizing agent may include the LP polypeptide or a fusion protein thereof. It may be useful to conjugate the immunizing agent to a protein known to be immunogenic in the mammal being immunized. Examples of such immunogenic proteins include, but are not limited to, keyhole limpet hemocyanin, serum albumin, bovine thyroglobulin, and soybean trypsin inhibitor.

Examples of adjuvants which may be employed include Freund’s complete adjuvant and MPL-TDM adjuvant (monophosphoryl Lipid A, synthetic trehalose dicorynomycolate).
The immunization protocol may be selected by one skilled in the art without undue experimentation.

The anti-LP polypeptide antibodies may, alternatively, be monoclonal antibodies. Monoclonal antibodies may be prepared using hybridoma methods, such as those described by Kohler and Milstein, *Nature* 256(5517):495-7 (1975). In a hybridoma method, a mouse, hamster, or other appropriate host animal is typically immunized with an immunizing agent to elicit lymphocytes that produce or are capable of producing antibodies that will specifically bind to the immunizing agent. Alternatively, the lymphocytes may be immunized *in vitro*.

The immunizing agent will typically include an LP polypeptide or a fusion protein thereof. Generally, either peripheral blood lymphocytes ("PBLs") are used, if cells of human origin are desired, or spleen cells or lymph node cells are used, if non-human mammalian sources are desired. The lymphocytes are then fused with an immortalized cell line using a suitable fusing agent, such as polyethylene glycol, to form a hybridoma cell [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, pp. 59-103 (1986)]. Immortalized cell lines are usually transformed mammalian cells, particularly myeloma cells of rodent, bovine and human origin. Usually, rat or mouse myeloma cell lines are employed. The hybridoma cells may be cultured in a suitable culture medium that preferably contains one or more substances that inhibit the growth or survival of the unfused, immortalized cells. For example, if the parental cells lack the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT or HPRT), the culture medium for the hybridomas typically will include hypoxanthine, aminopterin, and thymidine ("HAT medium"), which prevents the growth of HGPRT-deficient cells.

Preferred immortalized cell lines are those that fuse efficiently, support stable high level expression of antibody by the selected antibody-producing cells, and are sensitive to a medium such as HAT medium. More preferred immortalized cell lines are murine myeloma lines, which can be obtained, for instance, from the Salk Institute Cell Distribution Center, San Diego, CA, and the American Type Culture Collection (ATCC), Rockville, MD. Human myeloma and mouse-human heteromyeloma cell lines also have been described for the production of human monoclonal antibodies [Kozbor, *J. Immunol.* 133(6):3001-5 (1984); Brodeur, *et al.*, *Monoclonal Antibody Production Techniques and Applications*, Marcel Dekker, Inc., NY, pp. 51-63 (1987)].
The culture medium in which the hybridoma cells are cultured can then be assayed for the presence of monoclonal antibodies directed against an LP polypeptide. Preferably, the binding specificity of monoclonal antibodies produced by the hybridoma cells is determined by immunoprecipitation or by an *in vitro* binding assay, such as radioimmunoassay (RIA) or enzyme-linked immunoabsorbent assay (ELISA). Such techniques and assays are known in the art. The binding affinity of the monoclonal antibody can, for example, be determined by the Scatchard analysis of Munson and Rodbard, *Anal. Biochem.* 107(1):220-39 (1980).

After the desired hybridoma cells are identified, the clones may be subcloned by limiting dilution procedures and grown by standard methods [Goding, *Monoclonal Antibodies: Principles and Practice*, Academic Press, pp. 59-103 (1986)]. Suitable culture media for this purpose include, for example, Dulbecco’s Modified Eagle’s Medium and RPMI-1640 medium. Alternatively, the hybridoma cells may be grown *in vivo* as ascites in a mammal.

The monoclonal antibodies may also be made by recombinant DNA methods, such as those described in U.S. Patent 4,816,567. DNA encoding the monoclonal antibodies of the invention can be readily isolated and sequenced using conventional procedures (e.g., by using oligonucleotide probes that are capable of binding specifically to genes encoding the heavy and light chains of murine antibodies). The hybridoma cells of the invention serve as a preferred source of such DNA. Once isolated, the DNA may be placed into expression vectors, which are then transfected into host cells such as simian COS cells, Chinese hamster ovary (CHO) cells, or myeloma cells that do not otherwise produce immunoglobulin protein, to obtain the synthesis of monoclonal antibodies in the recombinant host cells. The DNA also may be modified, for example, by substituting the coding sequence for human heavy and light chain constant domains in place of the homologous murine sequences [U.S. Patent 4,816,567; Morrison, *et al.*, *Proc. Natl. Acad. Sci. USA* 81(21):6851-5 (1984)] or by covalently joining to the immunoglobulin coding sequence all or part of the coding sequence for a non-immunoglobulin polypeptide. Such a non-immunoglobulin polypeptide can be substituted for the constant domains of an antibody of the invention or can be substituted for the variable domains of one antigen-combining site of an antibody of the invention to create a chimeric bivalent antibody.
The antibodies may be monovalent antibodies. Methods for preparing monovalent antibodies are well known in the art. For example, one method involves recombinant expression of immunoglobulin light chain and modified heavy chain. The heavy chain is truncated generally at any point in the Fc region so as to prevent heavy chain crosslinking. Alternatively, the relevant cysteine residues are substituted with another amino acid residue or are deleted so as to prevent crosslinking.

*In vitro* methods are also suitable for preparing monovalent antibodies. Digestion of antibodies to produce fragments thereof, particularly Fab fragments, can be accomplished using routine techniques known in the art.

The anti-LP polypeptide antibodies of the invention may further comprise humanized antibodies or human antibodies. Humanized forms of non-human (e.g., murine) antibodies are chimeric immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab')2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues from a complementary-determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin, and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones, *et al.*, *Nature* 321(6069):522-5 (1986); Riechmann, *et al.*, *Nature* 332(6162):323-7 (1988); and Presta, *Curr. Op. Struct. Biol.* 2:593-6 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often
referred to as "import" residues, which are typically taken from an "import" variable domain. Humanization can be essentially performed following the method of Winter and co-workers [Jones, et al., *Nature* 321(6069):522-5 (1986); Riechmann, et al., *Nature* 332(6162):323-7 (1988); Verhoeyen, et al., *Science* 239(4847):1534-6 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such "humanized" antibodies are chimeric antibodies (U.S. Patent 4,816,567) wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.


Bispecific antibodies are monoclonal, preferably human or humanized, antibodies that have binding specificities for at least two different antigens. In the present case, one of the binding specificities is for an LP polypeptide, the other one is for any other antigen, and preferably for a cell-surface protein or receptor or receptor subunit.
Methods for making bispecific antibodies are known in the art. Antibodies with more than two valencies are contemplated. For example, trispecific antibodies can be prepared [Tutt, et al., J Immunol. 147(1):60-9 (1991)].

Heteroconjugate antibodies are also within the scope of the present invention. Heteroconjugate antibodies are composed of two covalently joined antibodies. Such antibodies have, for example, been proposed to target immune system cells to unwanted cells [U.S. Patent 4,676,980], and for treatment of HIV infection [WO 91/00360; WO 92/20373]. It is contemplated that the antibodies may be prepared in vitro using known methods in synthetic protein chemistry, including those involving crosslinking agents. For example, immunotoxins may be constructed using a disulfide exchange reaction or by forming a thioether bond. Examples of suitable reagents for this purpose include iminothiolate and methyl-4-mercaptopbutyrimidate and those disclosed, for example, in U.S. Patent 4,676,980.

The invention also pertains to immunoconjugates comprising an antibody conjugated to a cytotoxic agent such as a chemotherapeutic agent, toxin (e.g., an enzymatically active toxin of bacterial, fungal, plant or animal origin, or fragments thereof, or a small molecule toxin), or a radioactive isotope (i.e., a radioconjugate).

Conjugates of the antibody and cytotoxic agent are made using a variety of bifunctional protein coupling agents such as N-succinimidyl-3-(2-pyridyldithiol) propionate (SPDP), iminothiolane (IT), bifunctional derivatives of imidoesters (such as dimethyl adipimidate HCL), active esters (such as disuccinimidyl suberate), aldehydes (such as glutaraldehyde), bis-azido compounds, bis-diazenium derivatives (such as bis-2-diazeniumbenzoyl)-ethylendiamine) diisocyanates (such as tolylene-2,6-diisocyanate), and bioactive fluorine compounds. For example, a ricin immunotoxin can be prepared as described in Vitetta, et al., Science 238(4830):1098-104 (1987). Carbon-14-labeled 1-isothiocyanatobenzyl-3-methyldiethylene triaminepentaacetic acid (MX-DTPA) is an exemplary chelating agent for conjugation of radionucleotide to the antibody.

In another embodiment, the antibody may be conjugated to a “receptor” (such as streptavidin) for utilization in tumor pretargeting wherein the antibody-receptor conjugate is administered to the patient, followed by removal of unbound conjugate from the circulation using a clearing agent, and then administration of a “ligand” (e.g., avidin) which is conjugated to a cytotoxic agent (e.g., a radionucleotide).
The antibodies disclosed herein may also be formulated as immunoliposomes. Liposomes containing the antibody are prepared by methods known in the art, such as described in Eppstein, et al., *Proc. Natl. Acad. Sci. USA* 82:3688-92 (1985); Hwang, et al., *Proc. Natl. Acad. Sci. USA* 77(7):4030-4 (1980); and U.S. Patents 4,485,045 and 4,544,545. Liposomes with enhanced circulation time are disclosed in U.S. Patent 5,013,556.

Particularly useful liposomes can be generated by the reverse phase evaporation method with a lipid composition comprising phosphatidylcholine, cholesterol and PEG-derivatized phosphatidylethanolamine (PEG-PE). Liposomes are extruded through filters of defined pore size to yield liposomes with the desired diameter. Fab' fragments of the antibody of the present invention can be conjugated to the liposomes as described in Martin, et al., *J. Biol. Chem.* 257(1):286-8 (1982) via a disulfide interchange reaction. A chemotherapeutic agent (such as Doxorubicin) is optionally contained within the liposome. See Gabizon, et al., *J. National Cancer Inst.* 81(19):484-8 (1989).

Antibodies specifically binding an LP polypeptide identified herein, as well as other molecules identified by the screening assays disclosed hereinbefore, can be administered for the treatment of various disorders in the form of pharmaceutical compositions.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Alternatively, or in addition, the composition may comprise an agent that enhances its function, such as, for example, a cytotoxic agent, cytokines, chemotherapeutic agent, or growth-inhibitory agent. Such molecules are suitable present in combination in amounts that are effective for the purpose intended. The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles, and nanocapsules) or in macroemulsions. Such techniques are disclosed in *Remington's Pharmaceutical Sciences* 16th edition (1980).
The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the antibody, which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol), polylactides (U.S. Patent 3,773,919), copolymers of L-glutamic acid gamma-ethyl-L-glutamate, non-degradable ethylene-vinylacetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid. While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 degrees C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilization depending on the mechanisms involved. For example, if the aggregation mechanism is discovered to be intermolecular S-S bond formation through thiosulfide interchange, stabilization may be achieved by modifying sulfhydryl residues, lyophilizing from acidic solutions, controlling moisture content, using appropriate additives, and developing specific polymer matrix compositions.

The anti-LP polypeptide antibodies of the present invention have various utilities. For example, such antibodies may be used in diagnostic assays for LP polypeptide expression, e.g., detecting expression in specific cells, tissues, or serum. Various diagnostic assay techniques known in the art may be used, such as competitive binding assays, direct or indirect sandwich assays and immunoprecipitation assays conducted in either heterogeneous or homogeneous phases [Zola, Monoclonal Antibodies: A Manual of Techniques, CRC Press, Inc., pp. 147-158 (1987)]. The antibodies used in the assays can be labeled with a detectable moiety. The detectable moiety should be capable of producing, either directly or indirectly, a detectable signal. For example, the detectable moiety may be a radioisotope, such as ³H, ¹⁴C, ³²P, ³⁵S, or ¹²⁵I, a fluorescent or chemiluminescent compound, such as fluorescein isothiocyanate, rhodamine, or luciferin,
or an enzyme, such as alkaline phosphatase, beta-galactosidase or horseradish peroxidase. Any method known in the art for conjugating the antibody to the detectable moiety may be employed, including those methods described by Hunter, et al., Nature 144:945 (1962); David, et al., Biochemistry 13(5):1014-21 (1974); Pain, et al., J Immunol. Meth., 40(2):219-30 (1981); and Nygren, J. Histochem. Cytochem. 30(5):407-12 (1982).

Anti-LP polypeptide antibodies also are useful for affinity purification from recombinant cell culture or natural sources. In this process, the antibodies are immobilized on a suitable support, such a Sephadex® resin or filter paper, using methods well known in the art. The immobilized antibody is then contacted with a sample containing the LP polypeptide to be purified, and thereafter the support is washed with a suitable solvent that will remove substantially all the material in the sample except the LP polypeptide, which is bound to the immobilized antibody. Finally, the support is washed with another suitable solvent that will release the desired polypeptide from the antibody.

This invention encompasses methods of screening compounds to identify those that mimic the activity of the LP polypeptide (agonists) disclosed herein or prevent the effects of the LP polypeptide (antagonists). Screening assays for antagonist drug candidates are designed to identify compounds that bind or complex with an LP polypeptide encoded by the genes identified herein or otherwise interfere with the interaction of LP polypeptides with other cellular proteins. Such screening assays will include assays amenable to high-throughput screening of chemical libraries, making them particularly suitable for identifying small molecule drug candidates.

The assays can be performed in a variety of formats. In binding assays, the interaction is binding, and the complex formed can be isolated or detected in the reaction mixture. In a particular embodiment, an LP polypeptide encoded by a gene identified herein or the drug candidate is immobilized on a solid phase, e.g., on a microtiter plate, by covalent or non-covalent attachments. Non-covalent attachment generally is accomplished by coating the solid surface with a solution comprising LP polypeptide and drying. Alternatively, an immobilized antibody, e.g., a monoclonal antibody, specific for the polypeptide to be immobilized can be used to anchor it to a solid surface. The assay is performed by adding the non-immobilized component, which may be labeled by a detectable label, to the immobilized component, e.g., the coated surface containing the anchored component. When the reaction is complete, the non-reacted components are
removed, e.g., by washing, and complexes anchored on the solid surface are detected. When the originally non-immobilized component carries a detectable label, the detection of label immobilized on the surface indicates that complexing occurred. Where the originally non-immobilized component does not carry a label, complexing can be detected, for example, by using a labeled antibody specifically binding the immobilized complex.

If the candidate compound interacts with but does not bind to an LP polypeptide, its interaction with that polypeptide can be assayed by methods well known for detecting protein-protein interactions. Such assays include traditional approaches, such as, e.g., cross-linking, co-immunoprecipitation, and co-purification through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored through gradients or chromatographic columns. In addition, protein-protein interactions can be monitored by using a yeast-based genetic system described by Fields and co-workers [Fields and Song, *Nature* 340(6230):245-6 (1989); Chien, *et al.*, *Proc. Natl. Acad. Sci. USA* 88(21):9578-82 (1991); Chevray and Nathans, *Proc. Natl. Acad. Sci. USA* 89(13):5789-93 (1992)]. Many transcriptional activators, such as yeast GAL4, consist of two physically discrete modular domains, one acting as the DNA-binding domain, while the other functions as the transcription-activation domain. The yeast expression system described in the foregoing publications (generally referred to as the "two-hybrid system") takes advantage of this property, and employs two hybrid proteins, one in which the target protein is fused to the DNA-binding domain of GAL4, and another in which candidate activating proteins are fused to the activation domain. The expression of GAL1-lacZ reporter gene under control of a GAL4-activated promoter depends on reconstitution of GAL4 activity via protein-protein interaction. Colonies containing interacting polypeptides are detected with chromogenic substrate for beta-galactosidase. A complete kit (MATCHMAKER™) for identifying protein-protein interactions between two specific proteins using the two-hybrid technique is commercially available from Clontech. This system can also be extended to map protein domains involved in specific protein interactions as well as to pinpoint amino acid residues that are crucial for these interactions.

Compounds that interfere with the interaction of an LP polypeptide identified herein and other intra- or extracellular components can be tested as follows: usually a
reaction mixture is prepared containing the product of the gene and the intra- or extracellular component under conditions and for a time allowing for the interaction and binding of the two products. To test the ability of a candidate compound to inhibit binding, the reaction is run in the absence and in the presence of the test compound. In addition, a placebo may be added to a third reaction mixture to serve as a positive control. The binding (complex formation) between the test compound and the intra- or extracellular component present in the mixture is monitored as described hereinabove. The formation of a complex in the control reaction(s) but not in the reaction mixture containing the test compound indicates that the test compound interferes with the interaction of the test compound and its reaction partner.

Antagonists may be detected by combining at least one LP polypeptide and a potential antagonist with a membrane-bound or recombinant receptor for that LP polypeptide under appropriate conditions for a competitive inhibition assay. The LP polypeptide can be labeled, such as by radioactivity, such that the number of LP polypeptide molecules bound to the receptor can be used to determine the effectiveness of the potential antagonist. The gene encoding the receptor for an LP polypeptide can be identified by numerous methods known to those of skill in the art, for example, ligand panning and FACS sorting. See Coligan, et al., Current Protocols in Immunology l(2):Ch. 5 (1991). Preferably, expression cloning is employed such that polyadenylated mRNA is prepared from a cell responsive to the secreted form of a particular LP polypeptide, and a cDNA library created from this mRNA is divided into pools and used to transfect COS cells or other cells that are not responsive to the secreted LP polypeptide. Transfected cells that are grown on glass slides are exposed to the labeled LP polypeptide. The LP polypeptide can be labeled by a variety of means including iodination or inclusion of a recognition site for a site-specific protein kinase. Following fixation and incubation, the slides are subjected to autoradiographic analysis. Positive pools are identified and sub-pools are prepared and re-transfected using an interactive sub-pooling and re-screening process, eventually yielding a single clone that encodes the putative receptor.

As an alternative approach for receptor identification, a labeled LP polypeptide can be photoaffinity-linked with cell membrane or extract preparations that express the receptor molecule. Cross-linked material is resolved by PAGE and exposed to X-ray
film. The labeled complex containing the receptor can be excised, resolved into peptide fragments, and subjected to protein micro-sequencing. The amino acid sequence obtained from micro-sequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the gene encoding the putative receptor.

In another assay for antagonists, mammalian cells or a membrane preparation expressing the receptor would be incubated with a labeled LP polypeptide in the presence of the candidate compound. The ability of the compound to enhance or block this interaction could then be removed.

Alternatively, a potential antagonist may be a closely related protein, for example, a mutated form of the LP polypeptide that recognizes the receptor but imparts no effect, thereby competitively inhibiting the action of the polypeptide.

Another potential LP antagonist is an anti-sense RNA or DNA construct prepared using anti-sense technology, where, e.g., an anti-sense RNA or DNA molecule acts to block directly the translation of mRNA by hybridizing to targeted mRNA and prevent its translation into protein. Anti-sense technology can be used to control gene expression through triple-helix formation or anti-sense DNA or RNA, both of which methods are based on binding of a polynucleotide to DNA or RNA. For example, the 5' coding portion of the polynucleotide sequence, which encodes the mature form of an LP polypeptide can be used to design an anti-sense RNA oligonucleotide sequence of about 10 to 40 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription [triple helix; see Lee, et al., Nucl. Acids Res 6(9):3073-91 (1979); Cooney, et al., Science 241(4864):456-9 (1988); Beal and Dervan, Science 251(4999):1360-3 (1991)], thereby preventing transcription and production of the LP polypeptide. The anti-sense RNA oligonucleotide hybridizes to the mRNA in vivo and blocks translation of the mRNA molecules [anti-sense; see Okano, J. Neurochem. 56(2):560-7 (1991); Oligodeoxynucleotides as Anti-sense Inhibitors of Gene Expression (CRC Press: Boca Raton, FL 1988)]. The oligonucleotides described above can also be delivered to cells such that the anti-sense RNA or DNA may be expressed in vivo to inhibit production of the LP polypeptide. When anti-sense DNA is used, oligodeoxyribonucleotides derived from the translation-initiation site, e.g., between about -10 and +10 positions of the target gene nucleotide sequence, are preferred.
Potential antagonists include small molecules that bind to the active site, the receptor binding site, or growth factor or other relevant binding site of the LP polypeptide, thereby blocking the normal biological activity of the LP polypeptide. Examples of small molecules include, but are not limited to, small peptides or peptide-like molecules, preferably soluble peptides, and synthetic non-peptidyl organic or inorganic compounds.

Ribozymes are enzymatic RNA molecules capable of catalyzing the specific cleavage of RNA. Ribozymes act by sequence-specific hybridization to the complementary target RNA, followed by endonucleolytic cleavage. Specific ribozyme cleavage sites within a potential RNA target can be identified by known techniques. For further details, see, e.g., Rossi, *Current Biology* 4(5):469-71 (1994) and PCT publication No. WO 97/33551 (published September 18, 1997).

Nucleic acid molecules in triple-helix formation used to inhibit transcription should be single-stranded and composed of deoxynucleotides. The base composition of these oligonucleotides is designed such that it promotes triple-helix formation via Hoogsteen base-pairing rules, which generally require sizeable stretches of purines or pyrimidines on one strand of a duplex. For further details see, e.g., PCT publication No. WO 97/33551, supra.

Another use of the compounds of the invention (e.g., LP polypeptides, fragments and variants thereof and LP antibodies directed thereto) described herein is to help diagnose whether a disorder is driven to some extent by the modulation of signaling by an LP polypeptide

A diagnostic assay to determine whether a particular disorder is driven by LP polypeptide dependent signaling can be carried out using the following steps:

a) culturing test cells or tissues expressing an LP polypeptide;

b) administering a compound which can inhibit LP polypeptide dependent signaling; and

c) measuring LP polypeptide mediated phenotypic effects in the test cells.

The steps can be carried out using standard techniques in light of the present disclosure. Appropriate controls take into account the possible cytotoxic effect of a compound, such as treating cells not associated with a cell proliferative disorder (e.g., control cells) with a test compound and can also be used as part of the diagnostic assay.
The diagnostic methods of the invention involve the screening for agents that modulate the effects of LP polypeptide-associated disorders.

The LP polypeptides or antibodies thereto as well as LP polypeptide antagonists or agonists can be employed as therapeutic agents for the treatment of cardiovascular diseases, tumorous cancers and inflammation. For example, the LP polypeptides of the present invention may be used for the treatment of ischemia by inducing angiogenesis. Such therapeutic agents are formulated according to known methods to prepare pharmaceutically useful compositions, whereby the LP polypeptide or agonist or antagonist thereof is combined in a mixture with a pharmaceutically acceptable carrier.

In the case of LP polypeptide antagonistic or agonistic antibodies, if the LP polypeptide is intracellular and whole antibodies are used as inhibitors, internalizing antibodies are preferred. However, lipofection or liposomes can also be used to deliver the antibody, or an antibody fragment, into cells. Where antibody fragments are used, the smallest inhibitory fragment which specifically binds to the binding domain of the target protein is preferred. For example, based upon the variable region sequences of an antibody, peptide molecules can be designed which retain the ability to bind the target protein sequence. Such peptides can be synthesized chemically and/or produced by recombinant DNA technology [see, e.g., Marasco, et al., Proc. Natl. Acad. Sci. USA 90(16):7889-93 (1993)].

Therapeutic formulations are prepared for storage by mixing the active ingredient having the desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers [Remington's Pharmaceutical Sciences 16th edition (1980)], in the form of lyophilized formulations or aqueous solutions.

The formulation herein may also contain more than one active compound as necessary for the particular indication being treated, preferably those with complementary activities that do not adversely affect each other. Such molecules are suitably present in combination in amounts that are effective for the purpose intended.

The active ingredients may also be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, hydroxymethylcellulose or gelatin-microcapsules and poly-(methylnmethacrylate) microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules) or in
macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, supra.

The formulations to be used for in vivo administration must be sterile. This is readily accomplished by filtration through sterile filtration membranes.

Therapeutic compositions herein generally are placed into a container having a sterile access port, for example, and intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

Sustained-release preparations may be prepared. Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the therapeutic agent(s), which matrices are in the form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels [for example, poly(2-hydroxyethyl-methacrylate), or poly(vinylalcohol)], polylactides, copolymers of L-glutamic acid and gamma-ethyl-L-glutamate, non-degradable ethylene-vinyl acetate, degradable lactic acid-glycolic acid copolymers such as the LUPRON DEPOT™ (injectable microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D(-)-3-hydroxybutyric acid. Microencapsulation of recombinant proteins for sustained release has been successfully performed with human growth hormone (rhGH), interferon, and interleukin-2. Johnson, et al., Nat. Med. 2(7):795-9 (1996); Yasuda, et al., Biomed. Ther. 27:1221-3 (1993); Hora, et al., Bio/Technology 8(8):755-8 (1990); Cleland, “Design and Production of Single Immunization Vaccines Using Polylactide Polylglycolide Microsphere Systems” in Vaccine Design: The Subunit and Adjuvant Approach, Powell and Newman, Eds., Plenum Press, NY, 1995, pp. 439-462 WO 97/03692; WO 96/40072; WO 96/07399; and U.S. Patent 5,654,010.

The sustained-release formulations of these proteins may be developed using polylactic-coglycolic acid (PLGA) polymer due to its biocompatibility and wide range of biodegradable properties. The degradation products of PLGA, lactic and glycolic acids, can be cleared quickly within the human body. Moreover, the degradability of this polymer can be adjusted from months to years depending on its molecular weight and composition. See Lewis, “Controlled release of bioactive agents from lactide/glycolide polymer” in Biodegradable Polymers as Drug Delivery Systems (Marcel Dekker; New York, 1990), M. Chasin and R. Langer (Eds.) pp. 1-41.
While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release proteins for shorter time periods. When encapsulated antibodies remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37 degrees C, resulting in a loss of biological activity and possible changes in immunogenicity.

It is contemplated that the compounds, including, but not limited to, antibodies, small organic and inorganic molecules, peptides, anti-sense molecules, ribozymes, etc., of the present invention may be used to treat various conditions including those characterized by overexpression and/or activation of the disease-associated genes identified herein. The active agents of the present invention (e.g., antibodies, polypeptides, nucleic acids, ribozymes, small organic or inorganic molecules) are administered to a mammal, preferably a human, in accord with known methods, such as intravenous administration as a bolus or by continuous infusion over a period of time, by intramuscular, intraperitoneal, intracerebral, intracerebrospinal, subcutaneous, intra-articular, intrasynovial, intrathecal, intraocular, intrallesional, oral, topical, inhalation, pulmonary, and/or through sustained release.

Other therapeutic regimens may be combined with the administration of LP polypeptide antagonists or antagonists, anti-cancer agents, e.g., antibodies of the instant invention.

For the prevention or treatment of disease, the appropriate dosage of an active agent, (e.g., an antibody, polypeptide, nucleic acid, ribozyme, or small organic or inorganic molecule) will depend on the type of disease to be treated, as defined above, the severity and course of the disease, whether the agent is administered for preventive or therapeutic purposes, previous therapy, the patient's clinical history and response to the agent, and the discretion of the attending physician. The agent is suitably administered to the patient at one time or over a series of treatments.

Dosages and desired drug concentration of pharmaceutical compositions of the present invention may vary depending on the particular use envisioned. The determination of the appropriate dosage or route of administration is well within the skill of an ordinary artisan. Animal experiments provide reliable guidance for the determination of effective doses for human therapy. Interspecies scaling of effective doses can be performed following the principles laid down by Mordenti and Chappell,

When *in vivo* administration of a composition comprising an LP polypeptide, an LP polypeptide antibody, an LP polypeptide-encoding nucleic acid, ribozyme, or small organic or inorganic molecule is employed, normal dosage amounts may vary from about 1 ng/kg up to 100 mg/kg of mammal body weight or more per day, preferably about 1 pg/kg/day up to 100 mg/kg of mammal body weight or more per day, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature; see, for example, U.S. Patents 4,657,760, 5,206,344 or 5,225,212. It is within the scope of the invention that different formulations will be effective for different treatment compounds and different disorders, that administration targeting one organ or tissue, for example, may necessitate delivery in a manner different from that to another organ or tissue. Moreover, dosages may be administered by one or more separate administrations or by continuous infusion. For repeated administrations over several days or longer, depending on the condition, the treatment is sustained until a desired suppression of disease symptoms occurs. However, other dosage regimens may be useful. The progress of this therapy is easily monitored by conventional techniques and assays.

In another embodiment of the invention, an article of manufacture containing materials useful for the diagnosis or treatment of the disorders described above is provided. The article of manufacture comprises a container and a label. Suitable containers include, for example, bottles, vials, syringes, and test tubes. The containers may be formed from a variety of materials such as glass or plastic. The container holds a composition which is effective for diagnosing or treating the condition and may have a sterile access port (for example, the container may be an intravenous solution bag or a vial having a stopper pierceable by a hypodermic injection needle). The active agent in the composition is typically an LP polypeptide, antagonist or agonist thereof. The label on, or associated with, the container indicates that the composition is used for diagnosing or treating the condition of choice. The article of manufacture may further comprise a second container comprising a pharmaceutically-acceptable buffer, such as phosphate-buffered saline, Ringer’s solution and dextrose solution. It may further include other
materials desirable from a commercial and user standpoint, including other buffers, diluents, filters, needles, syringes, and package inserts with instructions for use.

In another embodiment of the invention, therapeutic utility of the LP polypeptide is determined by measuring phosphorylation of tyrosine residues on specific cell lines. The early cellular response of cells stimulated with the majority of proteins is protein phosphorylation of the tyrosine residues. This response includes autophosphorylation of corresponding receptors, which thereby leads to the activation of catalytic properties and the initiation of intracellular pathways specific to the cell type. Moreover, signaling downstream of receptors requires phosphorylation of specific kinases inside the cell and other intracellular enzymes of different origin as well as the phosphorylation of multiple adapter/scaffold, structural proteins and transcriptional factors. Therefore, diverse protein-induced cell responses can be visualized by monitoring the state of protein phosphorylation after cell stimulation.

Immunodetection is used to detect the protein phosphorylation of the stimulated cell. Several antibodies that are directed against specific phosphorylated epitopes in signaling molecules are readily available. Two specific antibodies are used: phosphospecific anti-MAPK and anti-AKT antibodies. Additionally, non-specific anti-phosphotyrosine antibodies, which recognize tyrosine-phosphorylated proteins, are used. While anti-phosphotyrosine antibodies allow detection of diverse tyrosine phosphorylated proteins without directly addressing the nature of their identity, the phosphospecific anti-MAPK and anti-AKT antibodies recognize only the corresponding proteins in their phosphorylated form.

Another assay to determine utility of LP polypeptides involves transfection of cell lines with reporter plasmids followed by cell stimulation with an LP polypeptide. Each reporter consists of a defined element, responsive to specific intracellular signaling pathways, upstream of a sequence involving a reporter protein such as luciferase. Upon stimulation of the element, reporter transcription and translation ensues, and the resulting protein levels can be detected using an assay such as a luminescence assay. The cell stimulation period depends on the reporter plasmid used.

For each reporter used, positive controls are designed in the form of agonist cocktails which include approximately maximal stimulatory doses of several ligands known to stimulate the represented signaling pathway. Using this design, the chances of
finding a positive stimulus for each cell line is maximized. The caveat, however, is that some cell line/reporter combinations will not be stimulated by the specific agonist cocktail, due to lack of an appropriate ligand in the cocktail. Alternately, the lack of signal induction by an agonist cocktail may be the lack of all signaling components within a particular cell line to activate the transcriptional element. Cell line/reporter combinations with no exogenous stimulus added make up the negative controls.

Another assay to determine utility of LP polypeptides involves transfection of cell lines with reporter plasmids followed by cell stimulation with an LP polypeptide. Each reporter consists of a defined element, responsive to specific intracellular signaling pathways, upstream of a sequence involving a reporter protein such as luciferase. Upon stimulation of the element, reporter transcription and translation ensues, and the resulting protein levels can be detected using an assay such as a luminescence assay. The cell stimulation period depends on the reporter plasmid used.

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In another assay, utility of LP polypeptide is determined by proliferation of cells. In this assay, gross changes in the number of cells remaining in a culture are monitored after exposure to an LP polypeptide for three days. The cells are incubated in an appropriate assay medium to produce a sub-optimal growth rate. For example, usually a 1:10 or 1:20 dilution of normal culture medium results in a 40 to 60% reduction in cell number compared to the undiluted culture medium. This broad growth zone is chosen so that if an LP polypeptide is a stimulator of growth, the cells have room to expand, and conversely, if the LP polypeptide is deleterious, a reduction in cell density can be detected. After a period of exposure, the assay media is replaced with media containing a
detection agent such as Calcein AM, a membrane-permeant fluorescent dye, allowing quantification of the cell number.

For use in another assay, a FLAG-HIS (FLIS)-tagged version of the LP polypeptide is expressed using mammalian cells such as HEK-293EBNA, COS-7, or HEK293T. The coding region of the cDNA is amplified by PCR of a vector containing a fragment encoding the LP polypeptide. The PCR-generated fragment is cleaved with restriction enzymes and gel-purified. The fragment is then ligated into a mammalian expression vector containing the FLIS epitope tag fused to the C-terminus. Protein expressed by this plasmid construct includes both the FLAG tag (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) and the 6X His tag at the COOH-terminus of the protein. This tag provides epitopes for commercially available tag-specific antibodies, enabling detection of the protein.

To determine expression of the LP polypeptide in tissues, a protein-binding assay is performed. The fixed tissue sample is exposed to the FLIS-tagged LP polypeptide, followed by exposure to a primary antibody and a secondary antibody containing a fluorescent dye. Tagged LP polypeptide that binds to the antigens in the tissue will fluoresce. Binding of the protein to an antigen in the tissue suggests that the protein is expressed in that tissue. Thus, protein expression can be determined by measuring which tissues fluoresce.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

**EXAMPLES**

**Example 1**

Expressing and Purification of PIGF Splice Variants

Recombinant PIGF splice variants containing a C-terminal FLAG tag (Eastman Kodak, Rochester, NY) followed by six histidine residues are produced in 293EBNA1 cells.

For purification, cell culture media containing PIGF splice variants (secreted from cells expressing FLIS-tagged-PIGF splice variants) is concentrated in an Amicon ProFlux M12 tangential filtration system using an Amicon S3Y10 UF membrane. The
concentrated media is passed over an immobilized metal-affinity chromatography column (Pharmacia) at a flow rate of 2 ml/min. The column is washed with buffer A (PBS (1 mM potassium phosphate, 3 mM sodium phosphate), 0.15 M NaCl, pH 7.4 containing 50 mM Imidazol) until the absorbance returns to baseline. The bound polypeptides are eluted with a gradient from 100% Buffer A to 55% Buffer A developed over 70 min. The gradient is then stepped to 100% Buffer B (Buffer A containing 0.5 M Imidazol) for 20 min. Fractions containing PIGF splice variants are pooled and concentrated using an Ultrafree centrifugal filter unit (Millipore, 10 kDa molecular weight cut-off) to 14 ml. This material is passed over a Superdex 75 (Pharmacia, 26/60) sizing column equilibrated with PBS, 0.5 M NaCl, pH 7.4, at a flow rate of 3 ml/min. Fractions containing PIGF splice variants are analyzed by SDS-PAGE. The N-termination sequence of PIGF splice variants is confirmed on the purified polypeptide using standard techniques.

Example 2

Expression of PIGF Splice Variants in E.coli

Production of PIGF splice variants is performed by cloning the human PIGF splice variant’s gene into the pHMM176 expression construct and expressing that gene in the RQ228 E. coli production line. Yields from this production are usually no more than double over the steady-state levels of the protein in uninduced cells. Multiple variations are made on the protocol in an attempt to optimize production, including variations in growth temperature, inducer concentration, induction length, and E.coli strain.

Example 3

PIGF Activity Assay

Biological activity of PIGF-1 and splice variants is determined using cells transfected with PIGF receptor flt. HEK 293 cells are co-transfected with separate plasmids containing flt, KDR, and Elk-luciferase for 24 hours. Cells are then treated with adenoviral vector containing PIGF gene. The luciferase light signal generated by PIGF – flt mediated activation of Elk is determined using a luminometer. The data are expressed as light unit generated by indicated virus pfu containing the respective PIGF gene.
Control virus or cells in the absence of flt are used as experimental negative controls. The assay is calibrated using purified PlGF protein.

**Table 1.**

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Example 4

PIGF Splice Variants Transgenic Rodent Development

A. Transgene construction.

Polymerase chain reaction (PCR) primers are synthesized and are used to amplify the PIGF splice variants’ coding region from a plasmid containing the full length coding region plus surrounding sequences. The 5’ oligonucleotide incorporates an Ascl restriction enzyme site and Kozak sequence while the 3’ primer incorporates a NotI restriction enzyme site to facilitate cloning. The amplified ~0.7 kb fragment is ligated into the multiple cloning site (Ascl -NotI) of plasmid pK409, a derivative of the pFastBac...
expression vector (Gibco BRL) with the altered cloning vector. The constructed vector with PIGF splice variants Flag [pEW1943] is subsequently digested with Ascl-XhoI and cloned into pLIV7 (provided by John Taylor, The J. David Gladstone Institutes) at the MluI-XhoI sites generating plasmid pLIV7/PIGF splice variants Flag-pEW3033.

B. Transgenic animal development.

Transgenic mice are generated using established techniques [Hogan, B. et al. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual*. Cold Spring Harbor Laboratory, NY] as modified by Fox and Solter (Mol. Cell. Biol. 8: 5470, 1988). Briefly, a 6.4 kb DNA fragment encompassing the human apolipoprotein E (hApoE) gene promoter-5' hApoE untranscribed region-PIGF splice variants/FLAG-hepatic control region (HCR) fusion gene is excised from plasmid pLIV7-PIGF splice variants by digestion with SalI and SpeI and purified by gel electrophoresis and glass bead extraction. The purified DNA fragment encompassing the hApoE gene promoter-5' hApoE untranslated region-PIGF splice variants-HCR fusion gene is microinjected into the male pronuclei of newly fertilized one-cell-stage embryos (zygotes) of the FVB/N strain. The embryos are cultured *in vitro* overnight to allow development to the two-cell-stage. Two-cell embryos are then transplanted into the oviducts of pseudopregnant ICR strain mice to allow development to term. To test for the presence of the transgene in the newborn mice, a small piece of toe is removed from each animal and digested with proteinase K to release the nucleic acids. A sample of the toe extract is subsequently subjected to PCR analysis using primers specific for the hApoE untranscribed region to identify transgene containing mice. Five founder transgenic mice are identified designated 14074, 14076, 14282, 14283 and 14379. Each of these founders is bred to produce F1 and F2 progeny.

**Example 5**

*In Vitro Testing PIGF Splice Variant Effect On Bone Marrow Cells*

A. *In vitro transmigration.*

Human CD34+ cells (purity>95%) are added to 8-µm-pore Matrigel-coated transwell inserts (Costar, Cambridge, Massachusetts). Recombinant PIGF splice variants (100 ng/ml) are added to the lower chamber with/without neutralizing anti-VEGFR1
(1 μg/ml), which is added to both chambers. Transmigrated cells are placed in cultures to measure the number of colony forming unites by Colony assay.

B. Colony Assays for Human and Murine Progenitors

CD34+ human bone marrow cells (Poietics, Inc.) are added to methylcellulose (Stem Cell Technologies, Vancouver, Canada) or agar culture medium. Colony growth is stimulated with the following human recombinant cytokines in the presence or absence of 200 ng/ml rhuIL-20: 1) Control Medium; 2) Epo (1-2 U/ml); 3) Epo + IL-3 (10 ng/ml); 4) Epo + IL-3 + GM-CSF (10ng/ml) + SLF (50ng/ml); 5) GM-CSF; 6) GM-CSF + SLF; 7) M-CSF (10ng/ml); 8) M-CSF + SLF; 9) G-CSF (10 ng/ml); 10) G-CSF + SLF; 11) Epo + SLF. In addition, three different populations of cord blood cells are used for the colony formation assays: low- density (less than 1.077 gm/cm³) mononuclear cells, CD34+ cells isolated via magnetic beads, and FACS sorted CD34+ cells. After culture at 37°C with 5% CO₂, lowered (5%) oxygen and 98% humidity for 2 weeks, colonies in each dish are counted and categorized under an inverted microscope.

C. Proliferation/Differentiate Activity of PIGF Splice Variants Agonists on Human Bone Marrow Liquid Culture

CD34+ human bone marrow cells are purchased from Poietic, BioWhiteeaker and are incubated in the following culture medium: Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 30% fetal bovine serum, antibiotics, 2 mM Glutamine, 2-mercapto-ethanol (10⁻⁴ M), and various concentration of human stem cell factor, IL-3, Epo and/or GM-CSF. CD34+ cells are plated in polypropylene U-bottomed 96 well plates at 5000 cells/well and are cultured at 37 C, 5% CO₂ for 10 days with a breathable membrane to prevent evaporation. Feeding occurs at days 4 and 7 by replacing 80% of the medium with fresh medium. At day 10, the cells are transferred to V-bottomed plates and stained for CD41 (FITC) and CD36 (PE). Cells are then acquired on a flow cytometer in timed acquisition mode and compared to the negative controls. PIGF splice variants stimulate the proliferation of CD34+ hematopoietic cells.
Example 6

*In Vivo* Testing for PIGF Splice Variants on Bone Marrow Cells

5 **A. Assays for Recovery of Blood Cells after Bone Marrow Transplantation.**

Bone marrow is harvested by gentle flushing the hind limbs of normal 8- to 10-week-old Balb C mice (purchased from Harlan Sprague Dawley, Indianapolis, IN) using RPMI medium ( Gibco, Grand Island, NY) containing 10% fetal calf serum (Gibco). For some experiments, donor mice are pretreated with 5-fluorouracil (5-FU) at 150-mg/kg-body weight intraperitoneally (IP) 3 days before harvesting BM for infusion. After total body irradiation with 10.8 Gy ($^{137}$Cs at 126cGy/min, split dose with a minimum of 3 hours between doses), 1X 10$^6$ bone marrow cells are injected intravenously (IV) into lethal irradiated mice. PIGF splice variants (250 μg/kg body weight) are diluted in PBS and are injected subcutaneously in 0.2-ml volume daily starting on the same day as irradiation and infusion of donor bone marrow cells. Control mice received the same volume of PBS. PIGF splice variants administration last 14-18 day. Mice are weighed every 2 to 4 days during the post-transplantation period. Hematologic analysis of Leukocyte cell counts and platelete counts are performed on orbit bleeds on a CDC Mascot™ machine. Blood smears are stained with Wright-Giemsia using standard methods and examined at 100X for differentiation analysis. Peripheral blood hematocrits are performed by spinning capillary tubes for 5 minutes in a Model MB Micro-Capillary Centrifuge. PIGF splice variants can be used for acceleration of recovery of peripheral blood cell counts.

25 **B. Assays for Recovery of Blood Cells After Combined Chemo-/Radiation Therapy**

Eight to ten-week old Balb/C mice (purchased from Harlan Sprague Dawley, Indianapolis, IN) are administered 5-fluorouracil (5-FU) at 150-mg/kg body weights intraperitoneally (IP) 3 days before sublethal irradiation (0.6 Gy total body irradiation for 20-22 mg mouse). PIGF splice variants are injected subcutaneously in 0.2 ml volume daily starting on the same day as irradiation. Negative control mice receive the same volume of PBS. PIGF splice variants administration lasts for 14 days. The mice are analyzed at 7 days and 14 days post-radiation. Mice are weighed every 2 to 4 days during
the post-radiation period. Hematologic analysis of Leukocyte cell counts and platelete counts are performed on orbit bleeds on a CDC Mascot™ machine. Blood smears are stained with Wright-Giemsa using standard methods and examined at 100X for differentiation analysis. Peripheral blood hematocrits are performed by spinning capillary tubes for 5 minutes in a Model MB Micro-Capillary Centrifuge. PLGF splice variants can be used for acceleration of recovery of peripheral blood cell counts.

Example 7
Testing PLGF Variants In Mouse Matrigel and Corneal Micropocket Assays

Matrigel and mouse corneal micropocket assays with PLGF variants with addition of 1 μg control IgG or anti-Flt1 are performed as previously described. 2 × 10⁶ A431 or PLGF variants-overexpressing rat C6 glioma tumor cells are subcutaneously injected in nude mice. Groups of 10 mice with tumors of comparable size (150–200 mm) receive intraperitoneal injections of anti-Flt1 (200 or 1,000 μg), anti-Flk1 (800 μg) or control IgG (1,000 μg) every 3 days. Tumors are measured with calipers and tumor volumes calculated using the formula \[\frac{\pi}{6} (w_1 \times w_2 \times w_3)\], where 'w_1' and 'w_3' represent the largest and smallest tumor diameter, respectively. Tumor vessel density and size are determined on CD31-stained sections. Ischemic retinal neovascularization with control IgG or anti-Flt1 (400 μg per pup; administered from P12 onwards) is analyzed as described.

Example 8
Testing PLGF In Mouse Model of Myocardial and Limb Ischemia

For therapeutic angiogenesis, a subcutaneously implanted osmotic minipump (Alzet, type 2001, Iffa Credo, Belgium) continuously delivers a daily dose of hPLGF-variants over 7 days. Thereafter, infarcted hearts are processed for morphometric analysis after immunostaining for endothelial thrombomodulin (all vessels) or for smooth muscle α-actin (mature SMC-covered vessels). To induce limb ischemia, unilateral right or bilateral ligations of the femoral artery and vein (proximal to the popliteal artery) and the cutaneous vessels branching from the caudal femoral artery side branch are performed without damaging the nervus femoralis. rhPIGF-2 and rhVEGF₁₆₅ are administered as described above. Two superficial preexisting collateral arterioles, connecting the femoral and saphenous artery, are used for analysis. Functional perfusion measurements of the
collateral region are performed using a Lisca PIM II camera (Gambro, Breda, the Netherlands) and analyzed as described. Perfusion, average from 3 images per mouse in the upper hindlimb (adductor region where collaterals enlarge) or in total hind limb, is expressed as a ratio of right (ischemic) to left (normal) limb. Spontaneous mobility was scored by monitoring the gait abnormalities, the position of right foot in rest and after manipulation, and the 'tail-abduction-reflex'. Mice are scored 0 when one observation is abnormal and 1 when normal. An endurance exercise swim test for mice is developed. Mice are conditioned for 9 days to swim in a 31 °C controlled swimming pool in non-stressed conditions. At day 10, baseline exercise time for each mouse is determined using a counter-current swimming pool (31 °C; flow at 0.2 m/s). For determining maximal endurance exercise, the total swimming period until fatigue, defined as the failure to rise to the surface of the water to breathe within 7 seconds, is assessed. At day 11, the femoral artery is occluded, and at day 18, minipumps are removed under isoflurane anesthesia before endurance exercise. Recovery of functionality is expressed as a ratio to the baseline exercise time. Fluorescent microspheres (yellow-green, 15 μm, 1 × 10^6 beads per ml, Molecular Probes, Eugene, Oregon) are administered after maximal vasodilation (sodium nitroprusside, 50 ng/ml, Sigma), processed and flow is calculated as described. Bismuth gelatino-angiography is performed as described and photo-angiographs (Nikon D1 digital camera) are analyzed in a blinded manner. Collateral side branches are categorized as follows: second-generation collateral arterioles directly branched off from the main collateral, whereas third-generation collateral arterioles are orientated perpendicular to the second-generation branches. The number of collateral branches per cm length of the primary collateral arteriole is counted. Fluoroangiography is performed with a modified version of a described protocol. Images are reconstructed with a Zeiss LSM510 confocal laser microscope. After perfusion-fixation, the 2 superficial collateral arterioles are post-fixed in paraformaldehyde 1% and paraffin-embedded. Twelve 5 μm cross-sections per superficial collateral, starting from the midzone and ranging over 1.95 mm to each end, are morphometrically analyzed. Collateral side branches are categorized as second generation (luminal area > 300 μm²) or third generation (< 300 μm²). Total perfusion area is calculated using the total sum of the side branch luminal areas. Capillary density is determined by immunostaining for thrombomodulin. Wall thickness of fully SMC-covered vessels is morphometrically measured on histological sections,
after smooth muscle α-actin staining. For all treatment groups, 6 cross-sections (150 μm apart) are analyzed per main collateral. Only second-generation collateral arterioles larger than 300 μm², are included in the analysis. At least 10 measurements of wall thickness of the second-generation collateral arterioles are obtained.

Example 9

Testing PI GF In vitro Angiogenesis Assays

A. Tube Forming Assay Using HUVEC

96 well plates are coated with 50 μl per well of Matrigel respectively without introducing air bubbles. The plates are allowed to sit at room temperature for 15 min to allow gelling of Matrigel. 2 x 10⁴ HUVEC cells are prepared in 100 μl for each well. PI GF splice variants are added into the cell mixture. PI GF splice variants are gently mixed with the cells. The cell or cell/compound mixture is plated into each Matrigel-coated well. Plates are incubated overnight at 37°C in a 5% CO₂/95% air incubator. Cells are labeled with fluorescent dye (Calcein AM) for 30 min. Full images are acquired under a microscope with a 2x objective lens at 16 hours. Tube length measurement and quantification are obtained.

B. Capillary Outgrowth From Cultured Rings of Mouse Aorta

Capillary outgrowth from cultured rings of mouse aorta in gels of rat-tail interstitial collagen (1.5 mg/ml) can be performed as described for the rat, except that 2.5% mouse serum is used. To quantify the number, length and branching of vessels, a scanned image of the aortic ring is converted to a binary image (aortic ring and capillaries in blue with black background; fibroblasts are subtracted). To obtain mouse capillary endothelial cells, mice are injected subcutaneously with 500 ml of Matrigel containing bFGF (100 ng/ml) and heparin (100 μg/ml). After 7 days, the Matrigel pellet is enzymatically dispersed, and endothelial cells are cultured in M131 medium supplemented with 5% MVGS (Gibco-BRL). Cells are starved in medium with 0.5% serum for 24 hours, after which they are stimulated with human PI GF splice variants, or bFGF (all from R&D) for 24 hours. Cultures are then analyzed for the total cell number (proliferation) or the number of cells migrated after scrape-wounding (migration).
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Example 10

Testing PIGF Variants In Vascular Permeability

Mustard oil is painted on the ears of Swiss mice, and extravasation of Evans blue determined. PIGF splice variants can be injected (1–5 mg/Kg) i.v 30 min before injection of Evans blue and application of mustard oil. For intravital analysis, the mouse cremaster muscle is exposed, and a flexible ovoid ring is introduced into the cremaster pouch to allow transillumination of the muscle, covered with carbogen-bubbled prewarmed mineral oil. Within 10 min after i.v. injection of FITC-labeled dextran, 150,000 (100 mg/kg), 10 µl of VEGF165 (260 nM) or histamine (500 nM) is topically administered. Video images of fluorescent microcirculation are recorded throughout the experiment for off-line analogous video image processing of the light intensity in a selected area of the video picture, expressed as gray values (arbitrary units).

Example 11

Production of an PIGF Splice Variants Antibody

Substantially pure PIGF splice variants protein or any fragment thereof is isolated from transfected or transformed cells using any of the well known methods in the art, or by a method specifically disclosed herein. Concentration of protein in a final preparation is adjusted, for example, by filtration through an Amicon filter device such that the level is about 1 to 5 µg/ml.

Monoclonal or polyclonal antibodies can be prepared as follows. A monoclonal antibody can be prepared from murine hybridomas according to the method of Kohler and Milstein (Nature, 256, 495, 1975), or a modified method thereof. Briefly, a mouse is repetitively inoculated with a few micrograms of the protein or fragment thereof, or fusion peptide thereof, over a period of a few weeks. The mouse is then sacrificed and the antibody producing cells of the spleen isolated. The spleen cells are fused by means of polyethylene glycol with mouse myeloma cells. Fused cells that produce antibody are identified by any suitable immunoassay, for example, ELISA, as described in E. Engvall, Meth. Enzymol., 70:419, 1980.

Polyclonal antiserum can be prepared by well known methods (See e.g. J. Vaitukaitis et al., Clin. Endocrinol. Metab. 33:988, 1971) that involve immunizing
suitable animals with the proteins, fragments thereof, or fusion proteins thereof, disclosed herein. Small doses (e.g. nanogram amounts) of antigen administered at multiple intradermal sites appear to be the most reliable method.

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WE CLAIM:

1. An isolated nucleic acid comprising a polynucleotide selected from the group consisting of:
   (a) a polynucleotide having a nucleotide sequence as shown in SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15; and
   (b) a polynucleotide encoding a polypeptide having the amino acid sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16.

2. An isolated nucleic acid comprising a polynucleotide having a nucleotide sequence selected from the group consisting of: SEQ ID NO: 1, 3, 5, 7, 9, 11, 13 or 15.

3. A vector comprising the nucleic acid molecule of any of Claim 1.

4. The vector of Claim 3, wherein said nucleic acid molecule is operably linked to control sequences recognized by a host cell transformed with the vector.

5. A host cell comprising the vector of Claim 4.

6. An isolated polypeptide comprising an amino acid sequence having at least 95% sequence identity to a polypeptide having the amino acid sequence as shown in SEQ ID NO: 2, 4, 6, 8, 10, 12, 14 or 16, respectively.

7. A method of treating a mammal suffering from a PIGF mediated disease, condition, or disorder comprising administering a therapeutically effective amount the polypeptides of Claim 6.

8. A method for inducing angiogenesis comprising administering to a patient in need thereof an effective amount of a polypeptide selected from the group consisting of SEQ ID NO.: 2, 4, 6, 8, 10, 12, 14 and 16.
SEQUENCE LISTING

Eli Lilly and Company

PLACENTAL GROWTH FACTOR SPLICE VARIANTS AND THEIR USES

X-16010_WO

US 60/555,272
2004-03-22

16

PatentIn version 3.3

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ttg tct gct ggg aac gcc tgg tca gag gtg gaa gtg gta ccc ttc cag
Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln
30 35 40

gaa gtg tgg gcc agc tac tgc cgg ggc ctg gag agg ctg gtg gac
Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp
45 50 55 60

gtc gtg tcc gag tac ccc agc gag gtg gag cac atg ttc agc cca ttc
Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser
65 70 75

tgt gtc tcc ctt gtc cgc tgc acc ggc tgc ggc gat gag aat ctg
Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu
80 85 90

cac tgt gtg cgc ggc gag acg gcc aat gtc acc atg cag ctc cta aag
His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Glu Leu Leu Lys
95 100 105

atc cgt tct ggg gac cgg ccc tcc tac gtg gac ctg gag ctg acg ttc tct cag
Ile Arg Ser Gly Asp Pro Ser Tyr Val Leu Thr Phe Ser Gln
110 115 120

cac gtt cgc tgc gaa tgc cgc cac agc cct ggg agg cag agg cgc cca gag
His Val Arg Cys Glu Cys Arg His Ser Pro Gly Arg Gln Ser Pro Asp
125 130 135 140

atg cct ctc gac ttc agg gct gat gct ccc tcc ttc ctc cca ccc cgt
Met Pro Gly Asp Phe Arg Ala Asp Ala Pro Ser Phe Leu Pro Pro Arg
145 150 155

cgc tca ctc ccc atg ttg ttc cgg atg gag tgg gcc tgt gcc ctc aca
Arg Ser Leu Pro Met Leu Phe Arg Met Glu Trp Gly Cys Ala Leu Thr
160 165 170
ggc agc cag tcc gct gtc tgg cca tca tct cct gtc cct gag gaa att
Gly Ser Gln Ser Ala Val Trp Pro Ser Ser Pro Val Pro Glu Glu Ile
175 180 185

ccc aga atg cac cct gga agg aac gga aaa aag cag cca aag aag cct
Pro Arg Met His Pro Gly Arg Asn Gly Lys Lys Gln Gln Arg Lys Pro
190 195 200

c tg cgg gag aag atg aag ccc gag aag agg aag aag aag ccc aag ggc agg ggg
Leu Arg Glu Lys Met Lys Pro Glu Arg Arg Arg Pro Lys Gly Arg Gly
205 210 215 220

aag agg agg aga gag aag cag aag ccc aca gac tgc cac ctg tgc ggc
Lys Arg Arg Arg Glu Lys Gln Arg Pro Thr Asp Cys His Leu Cys Gly
225 230 235

gat gct gtt ccc cgg agg taa cccaccccct tggagagaga gcacccgcac
Asp Ala Val Pro Arg Arg
240

cctgtgctg tatttattc cgtcactc ttcagtgact cctgctggt a cctgccaagg
895

gcaattccga gcacac
911

<210>  8
<211>  242
<212> PRT
<213> Homo sapiens

<400>  8
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly
1  5  10  15

Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly
20  25  30

Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Glu Glu Val Trp Gly
35  40  45

Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu
50  55  60

Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu
65  70  75  80

Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro
Val Glu Thr Ala Asn Val Thr Met Gln Leu Leu Lys Ile Arg Ser Gly
   85     90     95
Asp Arg Pro Ser Tyr Val Glu Leu Thr Phe Ser Gln His Val Arg Cys
  100    105    110
Glu Cys Arg His Ser Pro Gly Arg Arg Gln Ser Pro Asp Met Pro Gly Asp
  115    120    125
  130    135    140
Phe Arg Ala Asp Ala Pro Ser Phe Leu Pro Pro Arg Arg Ser Leu Pro
  145    150    155    160
Met Leu Phe Arg Met Glu Trp Gly Cys Ala Leu Thr Gly Ser Gln Ser
  165    170    175
Ala Val Trp Pro Ser Ser Pro Val Pro Glu Glu Ile Pro Arg Met His
  180    185    190
Pro Gly Arg Asn Gly Lys Lys Gln Gln Arg Lys Pro Leu Arg Glu Lys
  195    200    205
Met Lys Pro Glu Arg Arg Arg Pro Lys Gly Arg Gly Lys Arg Arg Arg
  210    215    220
Glu Lys Gln Arg Pro Thr Asp Cys His Leu Cys Gly Asp Ala Val Pro
  225    230    235    240
Arg Arg

<210>  9
<211>  678
<212>  DNA
<213>  Homo sapiens

<220>
<221>  CDS
<222>  (77) .. (331)
<400>  9
gtggtgcgtgga attcgccctt gggatgagc atgggtggttt tcccctcggag ccccttggtc
60
cgggacgtct gagaag atg ccg gtc atg agg ctc cct gcc ttc ctc cag cag
112
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln
5
ctc ctc gcc ggg ctc cgc ctc cct gcc ccc ccc cag cag tgg gcc
160
Leu Leu Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala
15 20 25
ttg tct gct ggg aac gcc tcg tca gag gtc gaa gga agt gtc ggg ccg
208
Leu Ser Ala Gly Asn Gly Ser Ser Glu Val Glu Gly Ser Val Gly Pro
15 20 25
cag cta ctc ccg ggc gct gga gag gct ggt gga cgt cgt gtc cga gta
256
Gln Leu Leu Leu Pro Gly Ala Gly Leu Ala Gly Arg Arg Val Arg Val
45 50 55 60
ccc cag cga ggt gga gca cat gtt cag ccc atc ctc ctt ctc cct gct
304
Pro Gln Glu Gly Ala His Val Gln Pro Ile Leu Cys Leu Pro Ala
65 70 75
gcg ctc cac ccg ctc ctc ccg cga tga gaatctgac cttggtgccgg
351
Ala Leu His Arg Leu Leu Arg Arg
80
tggagacgcc caatgtcacc atgcagctcc taaagatccg ttctggggac cggcctctctc
411
acgttggagct gacgtttctct cagcaagcttc gctgcaaatg cagccctcttg ccggagaaga
471
tgaagccgga aaggaggaga cccaaagggca ggggaagag gaggagagag aacgagagac
531
ccacacagcta cctgtgcggc gatgctgttcc cccggaggta accacacccct tggagggagac
591
agaccccccga cccggctcgtg gtattttatta ccctcacact cttcagtgcac ttctgtgctgt
651
acctgccaag ggcgaaattct gcagata
678

<210> 10
<211> 84
<212> PRT
<213> Homo sapiens

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly
1 5 10 15
Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly
20 25 30
Asn Gly Ser Ser Glu Val Glu Gly Ser Val Gly Pro Gln Leu Leu Pro  
35 40 45

Gly Ala Gly Glu Ala Gly Gly Arg Arg Arg Val Arg Val Pro Gln Arg Gly  
50 55 60

Gly Ala His Val Gln Pro Ile Leu Cys Leu Pro Ala Ala Leu His Arg  
65 70 75 80

Leu Leu Arg Arg

<210> 11
<211> 702
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (77) .. (664)

<400> 11
gtgtgctgga attcgccctt gggtgatgc atggcttt tccctcg gag cccccctgct 60
cgggacgtct gagaag atg ccg tgc atg agg ctt cct tgt tgt cag  
112
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln

ctc tgt gcc ggg tgt gcc tgt cct gct tgt ccc cag cag tgg gcc  
160
Leu Leu Ala Gly Leu Ala Leu Pro Ala Val Pro Gln Gln Trp Ala

ttg tct gct ggg aac ggc tgg tca gag tgt gaa gtt gta ccc ttc cag  
208
Leu Ser Ala Gly Asp Gly Ser Ser Glu Val Glu Val Val Phe Gln

30 35 40

45 50 55 60

Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Gln Arg Leu Val Asp

50 55 60

Glu Val Ser Glu Tyr Pro Ser Glu Val Val Glu His Met Phe Ser Pro Ser

65 70 75

Val Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu

80 85 90

352
Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu

80 85 90

352

cac tgt gct ggt gag acg gcc aat gtg acc atg cag gag aag gag 400
His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln Glu Arg Glu
95 100 105

aag agt gcc gga aac tgc tgg gcc act ccc caa tcc agg cat cca ctc
Lys Ser Ala Gly Asn Ser Trp Ala Thr Pro Gln Ser Arg His Gln Leu
110 115 120

ttt cct cct tca gct cct aac gat ccg ttc tgg gga ccg gcc ctc cta
Phe Leu Pro Ser Ala Pro Lys Asp Pro Phe Trp Gly Pro Ala Leu Leu
125 130 135 140

agt gct gac gtt ctc tca gca gct tgc tgc tca gta ccg gcc ctc cta
Arg Gly Ala Asp Val Leu Ser Ala Arg Ser Leu Arg Met Pro Ala Ser
145 150 155

gcg gga gaa gat gaa gcc gga aag gtg ccg cga tgc tgt tcc ccg gag
Ala Gly Glu Asp Glu Ala Gly Lys Val Arg Arg Cys Cys Ser Pro Glu
160 165 170

gta acc cac ccc ttg gag gag cag cag ccc gca ccc ggc tgt att
Val Thr His Pro Leu Glu Glu Arg Asp Pro Ala Pro Gly Ser Ile
175 180 185

tat tac cgt cac act ctt cag tga ctctgttgg tacctgccaag gggcgaattc
Tyr Tyr Arg His Thr Leu Gln
190 195

tgcagata

<210> 12
<211> 195
<212> PRT
<213> Homo sapiens

<400> 12

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly
1 5 10 15

Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly
20 25 30

Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly
35 40 45

Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu
50 55 60

Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu
Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro
85 90 95

Val Glu Thr Ala Asn Val Thr Met Gln Glu Arg Glu Lys Ser Ala Gly
100 105 110

Asn Ser Trp Ala Thr Pro Gln Ser Arg His Gln Leu Phe Leu Pro Ser
115 120 125

Ala Pro Lys Asp Pro Phe Trp Gly Pro Ala Leu Leu Arg Gly Ala Asp
130 135 140

Val Leu Ser Ala Arg Ser Leu Arg Met Pro Ala Ser Ala Gly Glu Asp
145 150 155 160

Glu Ala Gly Lys Val Arg Arg Cys Cys Ser Pro Glu Val Thr His Pro
165 170 175

Leu Glu Glu Arg Asp Pro Ala Pro Gly Ser Cys Ile Tyr Tyr Arg His
180 185 190

Thr Leu Gln
195

<210> 13
<211> 762
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (74) .. (724)

<400> 13
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  60
gacgtctgag aag atg ccg gtc atg agg ctg ttc cct tgc ttc ctg cag
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln
  109
  15

ctc ctg gcc ggg ctg gcc ctg cct gct tgt gtc ccc ccc cag cag tgg gcc
Leu Leu Ala Gly Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala
  157

[Sequence information and DNA sequence]
ttg tct gct ggg aac ggc tcg tca gag gtg gaa gtg gta ccc ttc cag
Leu Ser Ala Gly Asn Ser Ser Ser Glu Val Glu Val Val Pro Phe Gln
30 35 40

gaa gtg tgg ggc cgc agc tac tgc cgg gcg ctg gag agg ctg gtg gac
Glu Val Trp Gly Arg Ser Tyr Cys Arg Ala Leu Arg Leu Val Asp
45 50 55 60

gtc gtg tcc gag tac ccc agc gag gtg gag cac atg ttc agc cca tcc
Val Val Ser Glu Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser
65 70 75

tgt gtc tcc ctc ctc cgc tgc acc ggc tgc ggc gat gag aat ctc
Cys Val Ser Leu Leu Arg Cys Thr Gly Cys Gly Asp Glu Asn Leu
80 85 90

cac tgt gtg cg cgc gag acg gcc aat gtc acc atg cag gag aga gag
His Cys Val Pro Val Glu Thr Ala Asn Val Thr Met Gln Glu Arg Glu
95 100 105

aag agt gcc gca aac tgc tgg gcc act ccc caa tcc agg cat caa ctc
Lys Ser Ala Gly Asn Ser Trp Ala Thr Pro Glu Ser Arg His Glu Leu
110 115 120

ttc ctt cct tca gct cct aar gaa cgt tgg gga ccg gcc ctc cta
Phe Leu Pro Ser Ala Pro Lys Asp Pro Phe Try Gly Pro Ala Leu
125 130 135 140

cgt gga gct gac gtt ctc tca gca cgt tgc ctc cga atg ccg gcc ctc
Arg Gly Ala Asp Val Leu Ser Ala Arg Ser Leu Arg Met Pro Ala Ser
145 150 155

gcg gga gaa gat gaa ggc gga aag gag gag acc caa ggg cag ggg gaa
Ala Gly Glu Asp Glu Ala Gly Lys Glu Glu Glu Gly Gln Gly Gly Glu
160 165 170

gag gag gag aga gaa gca gag acc cac aga ctg cca cct gtg ccg cga
Glu Glu Glu Arg Glu Ala Glu Thr His Arg Leu Pro Pro Val Arg Arg
175 180 185

tgc tgt tcc cgc gat ctc cac ccc ctc gta acc cac ccg tgc gag aga gac ccc gca
Cys Cys Ser Pro Glu Val Thr His Pro Leu Glu Arg Asp Pro Ala
190 195 200

ccc gcc tgt tgt att tat tac cgt cac act ctt cag tca ctcctgcctgg
Pro Gly Ser Cys Ile Tyr Tyr Arg His Thr Leu Gln
205 210 215

tacctgcca ga gggca attc tgcagata

<210> 14
<211>  216
<212>  PRT
<213>  Homo sapiens

<400>  14

 Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly
 1      5       10       15

 Leu Ala Leu Pro Ala Val Pro Pro Gln Gln Trp Ala Leu Ser Ala Gly
 20     25       30

 Asn Gly Ser Ser Glu Val Glu Val Val Pro Phe Gln Glu Val Trp Gly
 35     40       45

 Arg Ser Tyr Cys Arg Ala Leu Glu Arg Leu Val Asp Val Val Ser Glu
 50     55       60

 Tyr Pro Ser Glu Val Glu His Met Phe Ser Pro Ser Cys Val Ser Leu
 65     70       75       80

 Leu Arg Cys Thr Gly Cys Cys Gly Asp Glu Asn Leu His Cys Val Pro
 85     90

 Val Glu Thr Ala Asn Val Thr Met Gln Glu Arg Glu Lys Ser Ala Gly
100    105       110

 Asn Ser Trp Ala Thr Pro Gln Ser Arg His Gln Leu Phe Leu Pro Ser
115    120       125

 Ala Pro Lys Asp Pro Phe Trp Gly Pro Ala Leu Leu Arg Gly Ala Asp
130    135       140

 Val Leu Ser Ala Arg Ser Leu Arg Met Pro Ala Ser Ala Gly Glu Asp
145    150       155       160

 Glu Ala Gly Lys Glu Glu Thr Gln Gly Glu Gly Glu Glu Arg
165    170       175

 Glu Ala Glu Thr His Arg Leu Pro Pro Val Arg Arg Cys Cys Ser Pro
180    185       190
-17-

Glu Val Thr His Pro Leu Glu Glu Arg Asp Pro Ala Pro Gly Ser Cys
195  200  205

Ile Tyr Tyr Arg His Thr Leu Gln
210  215

<210> 15
<211> 652
<212> DNA
<213> Homo sapiens

<220>
<221> CDS
<222> (77)...(355)
<400> 15
tatctgcaga attgcctcct gggatgacg atggtgttt tcctcggag ccccctggtct 60
cggagctct gagaag atg ccg tgc ctc atg agg tgt ttc cct ttc ctg cag
Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln 1  5  10
ctc ctg gcc ggg ctg ctg gcc ccc cag tgg tac cct
Leu Leu Ala Gly Leu Ala Ala Val Pro Pro Glu Trp Tyr Pro 15  20  25
tcc agg aag tgt ggg gcc gca gct act gcc ggg gcc tgg aga ggc tgg
Ser Arg Lys Cys Gly Ala Ala Ala Thr Ala Gly Arg Trp Arg Gly Trp 30  35  40
tgg acg tcg tgt ccc aag tgg agg tgg aca tgt tca gcc
Trp Thr Ser Cys Pro Ser Ala Arg Trp Ser Thr Cys Ser Ala 45  50  55  60
cat cct gtc tct ccc tgc tgt cgg gcc gct gct gcc cgg gcc ctg aga
His Pro Val Ser Cys Pro Ala Ala Ala Ala Ala Ala Met Arg 65  70  75
atc tgc act tgt cgg tgt aga cgg cca atg cca ctc tgt cgc
Ile Cys Thr Val Cys Arg Trp Arg Arg Met Ser Pro Cys Ser Ser 80  85  90
taa agatccgttc tggggacagg cccctctcag tggagctgac gttctctcag 405
cacgttcgct gcgaatgccg gctcttgccg gagaagatga agccggagag gaggagaccc 465
aagggcaggg ggaagaggg ggaagagaag gagaagccca cagactgcca cctgtgcggc 525
gatgctgttc cccggaggta accaacccct tggaggagag agaaccggca cccgccgtgct 585
gtatattata cggctcacact ctctcagtgac tcctgctggc acctgccaag gggaaattcc 645
agcacac

<210> 16
<211> 92
<212> PRT
<213> Homo sapiens

<400> 16

Met Pro Val Met Arg Leu Phe Pro Cys Phe Leu Gln Leu Leu Ala Gly
1 5 10 15

Leu Ala Leu Pro Ala Val Pro Pro Gln Trp Tyr Pro Ser Arg Lys Cys
20 25 30

Gly Ala Ala Thr Ala Gly Arg Trp Arg Gly Trp Trp Thr Ser Cys
35 40 45

Pro Ser Thr Pro Ala Arg Thr Ser Thr Cys Ser Ala His Pro Val Ser
50 55 60

Pro Cys Ala Ala Pro Ala Ala Ala Ala Met Arg Ile Cys Thr Val
65 70 75 80

Cys Arg Trp Arg Arg Pro Met Ser Pro Cys Ser Ser
85 90