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(54) **ANTIBODIES SPECIFIC FOR OSTEOPROTEGERIN BINDING PROTEINS AND METHOD OF USE**

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(57) **ABSTRACT**

A novel polypeptide, osteoprotegerin binding protein, involved in osteoclast maturation has been identified based upon its affinity for osteoprotegerin. Nucleic acid sequences encoding the polypeptide, or a fragment, analog or derivative thereof, vectors and host cells for production, methods of preparing osteoprotegerin binding protein, and binding assays are also described. Compositions and methods for the treatment of bone diseases such as osteoporosis, bone loss due to arthritis or metastasis, hypercalcemia, and Paget's disease are also provided.

GAGCTCGGAT CCACTACTCG ACCCACGCGT CCGGCCAGGA CCTCTGTGAA CCGGTCGGGG	60
CGGGGGCCGC CTGGCCGGGA GTCTGCTCGG CGGTGGGTGG CCGAGGAAGG GAGAGAACGA	120
TCGCGGAGCA GGGCGCCCGA ACTCCGGGCG CCGCGCC ATG CGC CGG GCC AGC CGA	175
Met Arg Arg Ala Ser Arg	
1 5	
GAC TAC GGC AAG TAC CTG CGC AGC TCG GAG GAG ATG GGC AGC GGC CCC	223
Asp Tyr Gly Lys Tyr Leu Arg Ser Ser Glu Glu Met Gly Ser Gly Pro	
10 15 20	
GGC GTC CCA CAC GAG GGT CCG CTG CAC CCC GCG CCT TCT GCA CCG GCT	271
Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro Ala	
25 30 35	

FIG. 1B

CCG GCG CCG CCA CCC GCC GCC TCC CGC TCC ATG TTC CTG GCC CTC CTG	319
Pro Ala Pro Pro Ala Ala Ser Arg Ser Met Phe Leu Ala Leu Leu	
40 45 50	
GGG CTG GGA CTG GGC CAG GTG GTC TGC AGC ATC GCT CTG TTC CTG TAC	367
Gly Leu Gly Leu Gly Gln Val Val Cys Ser Ile Ala Leu Phe Leu Tyr	
55 60 65 70	
TTT CGA GCG CAG ATG GAT CCT AAC AGA ATA TCA GAA GAC AGC ACT CAC	415
Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr His	
75 80 85	
TGC TTT TAT AGA ATC CTG AGA CTC CAT GAA AAC GCA GGT TTG CAG GAC	463
Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Gly Leu Gln Asp	
90 95 100	
TCG ACT CTG GAG AGT GAA GAC ACA CTA CCT GAC TCC TGC AGG AGG ATG	511
Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg Arg Met	
105 110 115	

FIG. 1C

AAA CAA GCC TTT CAG GGG GCC GTG CAG AAG GAA CTG CAA CAC ATT GTG	559
Lys Gln Ala Phe Gln Gln Val Ala Val Gln Lys Glu Leu Gln His Ile Val	
120 125 130	
GGG CCA CAG CGC TTC TCA GGA GCT CCA GCT ATG ATG GAA GGC TCA TGG	607
Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu Gly Ser Trp	
135 140 145 150	
TTG GAT GTG GCC CAG CGA GGC AAG CCT GAG GCC CAG CCA TTT GCA CAC	655
Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro Phe Ala His	
155 160 165	
CTC ACC ATC AAT GCT GCC AGC ATC CCA TCG GGT TCC CAT AAA GTC ACT	703
Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly Ser His Lys Val Thr	
170 175 180	

FIG.1D

CTG	TCC	TCT	TGG	TAC	CAC	GAT	CGA	GGC	TGG	GCC	AAG	ATC	TCT	AAC	ATG	751
Leu	Ser	Ser	Trp	Tyr	His	Asp	Arg	Gly	Trp	Ala	Lys	Ile	Ser	<u>Asn</u>	Met	
		185					190					195				
ACG	TTA	AGC	AAC	GGA	AAA	CTA	AGG	GTT	AAC	CAA	GAT	GGC	TTC	TAT	TAC	799
Thr	Leu	Ser	Asn	Gly	Lys	Leu	Arg	Val	Asn	Gln	Asp	Gly	Phe	Tyr	Tyr	
	200					205					210					
CTG	TAC	GCC	AAC	ATT	TGC	TTT	CGG	CAT	CAT	GAA	ACA	TCG	GGA	AGC	GTA	847
Leu	Tyr	Ala	Asn	Ile	Cys	Phe	Arg	His	His	Glu	Thr	Ser	Gly	Ser	Val	
215					220					225					230	
CCT	ACA	GAC	TAT	CTT	CAG	CTG	ATG	GTG	TAT	GTC	GTT	AAA	ACC	AGC	ATC	895
Pro	Thr	Asp	Tyr	Leu	Gln	Leu	Met	Val	Tyr	Val	Val	Lys	Thr	Ser	Ile	
				235					240						245	

FIG. 1E

AAA ATC CCA AGT TCT CAT AAC CTG ATG AAA GGA GGG AGC ACG AAA AAC	943
Lys Ile Pro Ser 250	
His Ser 255	
Leu Met Lys Gly Ser Thr 260	
Asn 265	
TGG TCG GGC AAT TCT GAA TTC CAC TTT TAT TCC ATA AAT GTT GGG GGA	991
Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly	
265 270 275	
TTT TTC AAG CTC CGA GCT GGT GAA GAA ATT AGC ATT CAG GTG TCC AAC	1039
Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser Asn	
280 285 290	
CCT TCC CTG CTG GAT CCG GAT CAA GAT GCG ACG TAC TTT GGG GCT TTC	1087
Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe	
295 300 305 310	
AAA GTT CAG GAC ATA GAC T GAGACTCATT TCGTGGAACA TTAGCATGGA	1136
Lys Val Gln Asp Ile Asp 315	

FIG. 1F

TGTCCTAGAT	GTTTGGAAC	TTCTTAAAA	ATGGATGATG	TCTATACATG	TGTAAGACTA	1196
CTAAGAGACA	TGGCCCACGG	TGTATGAAAC	TCACAGCCCT	CTCTCTTGAG	CCTGTACAGG	1256
TTGTGTATAT	GTAAAGTCCA	TAGGTGATGT	TAGATTTCATG	GTGATTACAC	AACGGTTTTA	1316
CAATTTTGT	ATGATTTCCT	AGAAATTGAAC	CAGATTGGGA	GAGGTATTCC	GATGCTTATG	1376
AAAAACTTAC	ACGTGAGCTA	TGGAAGGGGG	TCACAGTCTC	TGGGTCTAAC	CCCTGGACAT	1436
GTGCCACTGA	GAACCTTGAA	ATTAAGAGGA	TGCCATGTCA	TTGCAAAGAA	ATGATAGTGT	1496
GAAGGGTTAA	GTTCTTTTGA	ATTGTTACAT	TGCGCTGGGA	CCTGCAAATA	AGTTCCTTTT	1556

FIG.1G

TTCTAATGAG	GAGAGAAAAA	TATATGTATT	TTTATATAAT	GTCTAAAGTT	ATATTTCAGG	1616
TGTAATGTTT	TCTGTGCAAA	GTTTGTAAA	TTATATTTGT	GCTATAGTAT	TTGATTCAAA	1676
ATATTTAAAA	ATGTCTCACT	GTTGACATAT	TTAATGTTTT	AAATGTACAG	ATGTATTTAA	1736
CTGGTGCACT	TTGTAATTCC	CCTGAAGGTA	CTCGTAGCTA	AGGGGGCAGA	ATACTGTTC	1796
TGGTGACCAC	ATGTAGTTTA	TTTCCTTTATT	CTTTTAACT	TAAATAGATC	TTCAGACTTG	1856
TCAAAACTAT	GCAAGCAAAA	TAAATAAATA	AAAATAAAAT	GAATACCTTG	AATAATAAGT	1916
AGGATGTTGG	TCACCAGGTG	CCTTTCAAAT	TTAGAAAGCTA	ATTGACTTTA	GGAGCTGACA	1976
TAGCCAAAAA	GGATACATAA	TAGGCTACTG	AAATCTGTCA	GGAGTATTTA	TGCAATTATT	2036

FIG.1H

GAACAGGTGT	CTTTTTTTAC	AAGAGCTACA	AATGTAAAT	TTTGTTTCTT	TTTTTTCCCA	2096
TAGAAAATGT	ACTATAGTTT	ATCAGCCAAA	AAACAATCCA	CTTTTTAATT	TAGTGAAAGT	2156
TATTTTATTA	TACTGTACAA	TAAAAGCATT	GTCTCTGAAT	GTTAATTTT	TGGTACAAA	2216
AATAAATTG	TACGAAAACC	TGAAAAAAAA	AAAAAAAAA	AAAAAAGGG	CGGCCGCTCT	2276
AGAGGGCCCT	ATTCTATAG					2295

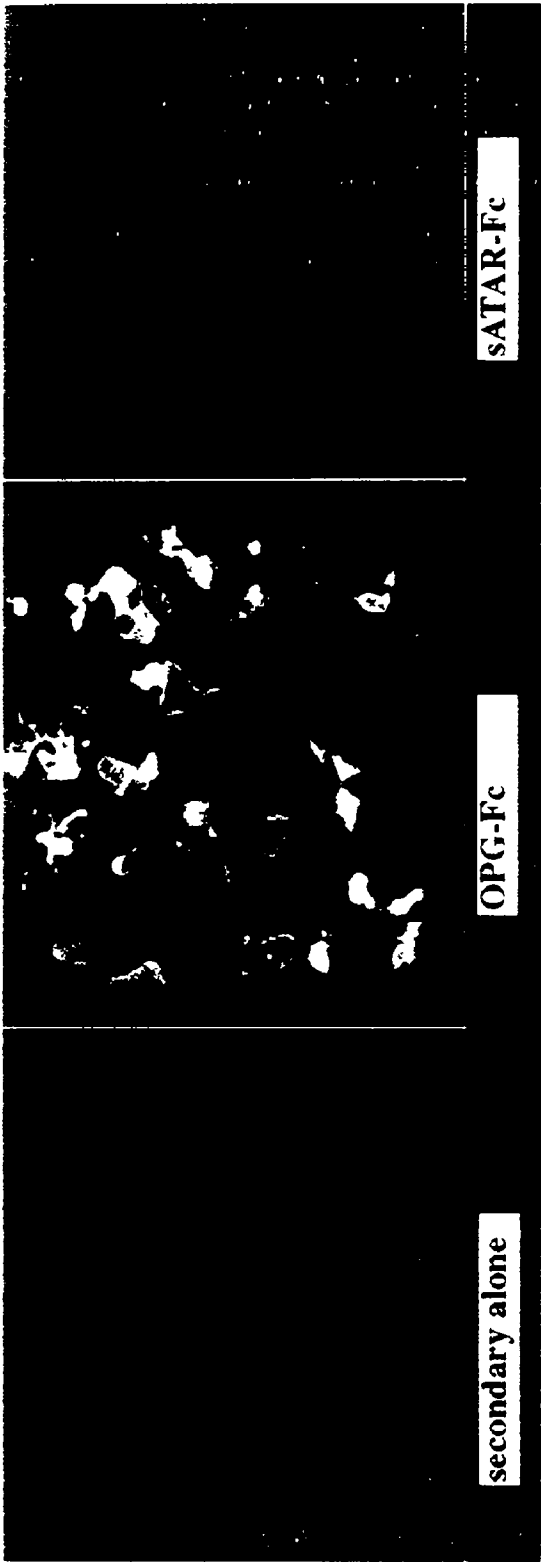


FIG. 2

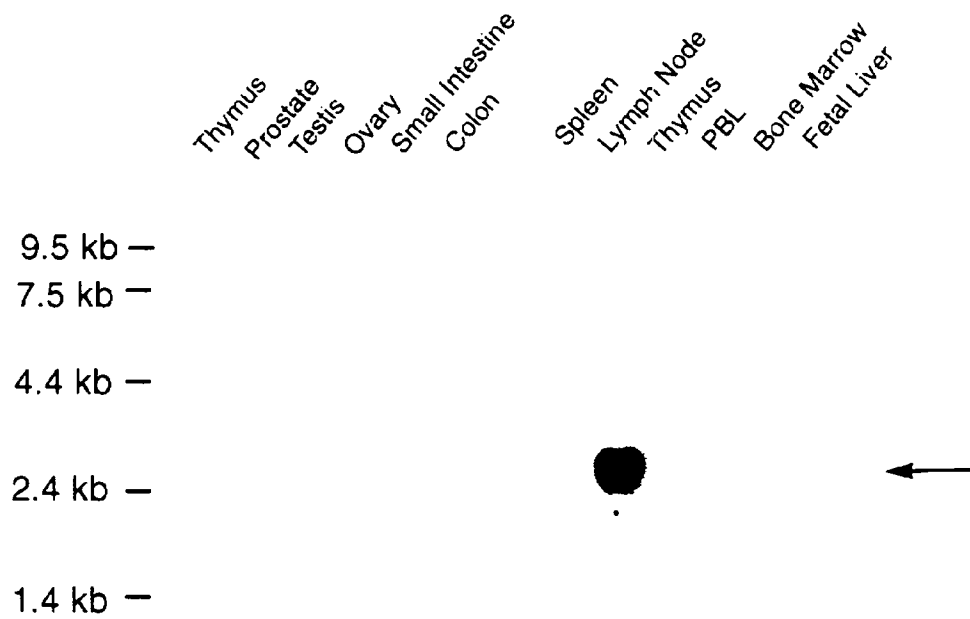


FIG. 3

ANTIBODIES SPECIFIC FOR OSTEOPROTEGERIN BINDING PROTEINS AND METHOD OF USE

FIELD OF THE INVENTION

[0001] The present invention relates to polypeptides which are involved in osteoclast differentiation. More particularly, the invention relates to osteoprotegerin binding proteins, nucleic acids encoding the proteins, expression vectors and host cells for production of the proteins, and binding assays. Compositions and methods for the treatment of bone diseases, such as osteoporosis, bone loss from arthritis, Paget's disease, and hypercalcemia, are also described.

BACKGROUND OF THE INVENTION

[0002] Living bone tissue exhibits a dynamic equilibrium between deposition and resorption of bone. These processes are mediated primarily by two cell types: osteoblasts, which secrete molecules that comprise the organic matrix of bone; and osteoclasts, which promote dissolution of the bone matrix and solubilization of bone salts. In young individuals with growing bone, the rate of bone deposition exceeds the rate of bone resorption, while in older individuals the rate of resorption can exceed deposition. In the latter situation, the increased breakdown of bone leads to reduced bone mass and strength, increased risk of fractures, and slow or incomplete repair of broken bones.

[0003] Osteoclasts are large phagocytic multinucleated cells which are formed from hematopoietic precursor cells in the bone marrow. Although the growth and formation of mature functional osteoclasts is not well understood, it is thought that osteoclasts mature along the monocyte/macrophage cell lineage in response to exposure to various growth-promoting factors. Early development of bone marrow precursor cells to preosteoclasts are believed to mediated by soluble factors such as tumor necrosis factor- α (TNF- α), tumor necrosis factor- β (TNF- β), interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-6 (IL-6), and leukemia inhibitory factor (LIF). In culture, preosteoclasts are formed in the presence of added macrophage colony stimulating factor (M-CSF). These factors act primarily in early steps of osteoclast development. The involvement of polypeptide factors in terminal stages of osteoclast formation has not been extensively reported. It has been reported, however, that parathyroid hormone stimulates the formation and activity of osteoclasts and that calcitonin has the opposite effect, although to a lesser extent.

[0004] Recently, a new polypeptide factor, termed osteoprotegerin (OPG), has been described which negatively regulated formation of osteoclasts in vitro and in vivo (see co-owned and co-pending U.S. Ser. Nos. 08/577,788 filed Dec. 22, 1995, 08/706,945 filed Sep. 3, 1996, and 08/771,777, filed Dec. 20, 1996, hereby incorporated by reference; and PCT Application No. WO96/26271). OPG dramatically increased the bone density in transgenic mice expressing the OPG polypeptide and reduced the extent of bone loss when administered to ovariectomized rats. An analysis of OPG activity in in vitro osteoclast formation revealed that OPG does not interfere with the growth and differentiation of monocyte/macrophage precursors, but more likely blocks the differentiation of osteoclasts from monocyte/macrophage

age precursors. Thus OPG appears to have specificity in regulating the extent of osteoclast formation.

[0005] OPG comprises two polypeptide domains having different structural and functional properties. The amino-terminal domain spanning about residues 22-194 of the full-length polypeptide (the N-terminal methionine is designated residue 1) shows homology to other members of the tumor necrosis factor receptor (TNFR) family, especially TNFR-2, through conservation of cysteine rich domains characteristic of TNFR family members. The carboxy terminal domain spanning residues 194-401 has no significant homology to any known sequences. Unlike a number of other TNFR family members, OPG appears to be exclusively a secreted protein and does not appear to be synthesized as a membrane associated form.

[0006] Based upon its activity as a negative regulator of osteoclast formation, it is postulated that OPG may bind to a polypeptide factor involved in osteoclast differentiation and thereby block one or more terminal steps leading to formation of a mature osteoclast.

[0007] It is therefore an object of the invention to identify polypeptides which interact with OPG. Said polypeptides may play a role in osteoclast maturation and may be useful in the treatment of bone diseases.

SUMMARY OF THE INVENTION

[0008] A novel member of the tumor necrosis factor family has been identified from a murine cDNA library expressed in COS cells screened using a recombinant OPG-Fc fusion protein as an affinity probe. The new polypeptide is a transmembrane OPG binding protein which is predicted to be 316 amino acids in length, and has an amino terminal cytoplasmic domain, a transmembrane domain, and a carboxy terminal extracellular domain. OPG binding proteins of the invention may be membrane-associated or may be in soluble form.

[0009] The invention provides for nucleic acids encoding an OPG binding protein, vectors and host cells expressing the polypeptide, and method for producing recombinant OPG binding protein. Antibodies or fragments thereof which specifically bind OPG binding protein are also provided.

[0010] OPG binding proteins may be used in assays to quantitate OPG levels in biological samples, identify cells and tissues that display OPG binding protein, and identify new OPG and OPG binding protein family members. Methods of identifying compounds which interact with OPG binding protein are also provided. Such compounds include nucleic acids, peptides, proteins, carbohydrates, lipids or small molecular weight organic molecules and may act either as agonists or antagonists of OPG binding protein activity.

[0011] OPG binding proteins are involved in osteoclast differentiation and the level of osteoclast activity in turn modulates bone resorption. OPG binding protein agonists and antagonists modulate osteoclast formation and bone resorption and may be used to treat bone diseases characterized by changes in bone resorption, such as osteoporosis, hypercalcemia, bone loss due to arthritis or metastasis, Paget's disease, osteopetrosis and the like. Pharmaceutical compositions comprising OPG binding proteins and OPG binding protein agonists and antagonists are also encompassed by the invention.

DESCRIPTION OF THE FIGURES

[0012] FIG. 1. Structure and sequence of the 32D-F3 insert encoding OPG binding protein. Predicted transmembrane domain and sites for asparagine-linked carbohydrate chains are underlined.

[0013] FIG. 2. OPG binding protein expression in COS-7 cells transfected with pcDNA/32D-F3. Cells were lipofected with pcDNA/32D-F3 DNA, the assayed for binding to either goat anti-human IgG1 alkaline phosphatase conjugate (secondary alone), human OPG[22-201]-Fc plus secondary (OPG-Fc), or a chimeric ATAR extracellular domain-Fc fusion protein (sATAR-Fc). ATAR is a new member of the TNFR superfamily, and the sATAR-Fc fusion protein serves as a control for both human IgG1 Fc domain binding, and generic TNFR related protein, binding to 32D cell surface molecules.

[0014] FIG. 3. Expression of OPG binding protein in human tissues. Northern blot analysis of human tissue mRNA (Clontech) using a radiolabeled 32D-F3 derived hybridization probe. Relative molecular mass is indicated at the left in kilobase pairs (kb). Arrowhead on right side indicates the migration of an approximately 2.5 kb transcript detected in lymph node mRNA. A very faint band of the same mass is also detected in fetal liver.

DETAILED DESCRIPTION OF THE INVENTION

[0015] The invention provides for a polypeptide referred to as an OPG binding protein, which specifically binds OPG and is involved in osteoclast differentiation. A cDNA clone encoding the murine form of the polypeptide was identified from a library prepared from a mouse myelomonocytic cell line 32-D and transfected into COS cells. Transfectants were screened for their ability to bind to an OPG[22-201]-Fc fusion polypeptide (Example 1). The nucleic acid sequence revealed that OPG binding protein is a novel member of the TNF receptor family and is most closely related to AGP-1, a polypeptide previously described in co-owned and co-pending U.S. Ser. No. 08/660,562, filed Jun. 7, 1996. (A polypeptide identical to AGP-1 and designated TRAIL is described in Wiley et al. *Immunity* 3, 673-682 (1995)). OPG binding protein is predicted to be a type II transmembrane protein having a cytoplasmic domain at the amino terminus, a transmembrane domain, and a carboxy terminal extracellular domain (**FIG. 1**). The amino terminal cytoplasmic domain spans about residues 1-48, the transmembrane domain spans about residues 49-69 and the extracellular domain spans about residues 70-316 as shown in **FIG. 1** (SEQ ID NO: _____). The membrane-associated protein specifically binds OPG (**FIG. 2**). Thus OPG binding protein and OPG share many characteristics of a receptor-ligand pair although it is possible that other naturally-occurring ligands for OPG binding protein exist.

[0016] OPG binding protein refers to a polypeptide having an amino acid sequence of mammalian OPG binding protein, or a fragment, analog, or derivative thereof, and having at least the activity of binding OPG. In preferred embodiments, OPG binding protein is of murine or human origin. In another embodiment, OPG binding protein is a soluble protein having, in one form, an isolated extracellular domain separate from cytoplasmic and transmembrane domains.

OPG binding protein is involved in osteoclast differentiation and in the rate and extent of bone resorption.

[0017] Nucleic Acids

[0018] The invention provides for isolated nucleic acids encoding OPG binding proteins. As used herein, the term nucleic acid comprises cDNA, genomic DNA, wholly or partially synthetic DNA or RNA. The nucleic acids of the invention are selected from the group consisting of:

[0019] a) the nucleic acids as shown in **FIG. 1** (SEQ ID NO: _____);

[0020] b) nucleic acids which hybridize to the polypeptide coding regions of the nucleic acids shown in **FIG. 1** (SEQ ID NO: _____) and remain hybridized to the nucleic acids under high stringency conditions; and

[0021] c) nucleic acids which are degenerate to the nucleic acids of (a) or (b).

[0022] Nucleic acid hybridizations typically involve a multi-step process comprising a first hybridization step to form nucleic acid duplexes from single strands followed by a second hybridization step carried out under more stringent conditions to selectively retain nucleic acid duplexes having the desired homology. The conditions of the first hybridization step are generally not crucial, provided they are not of higher stringency than the second hybridization step. Generally, the second hybridization is carried out under conditions of high stringency, wherein "high stringency" conditions refers to conditions of temperature and salt which are about 12-20° C. below the melting temperature (T_m) of a perfect hybrid of part or all of the complementary strands corresponding to **FIG. 1** (SEQ. ID. NO: _____). In one embodiment, "high stringency" conditions refer to conditions of about 65° C. and not more than about 1M Na⁺. It is understood that salt concentration, temperature and/or length of incubation may be varied in either the first or second hybridization steps such that one obtains the hybridizing nucleic acid molecules according to the invention. Conditions for hybridization of nucleic acids and calculations of T_m for nucleic acid hybrids are described in Sambrook et al. *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Laboratory Press, New York (1989).

[0023] The nucleic acids of the invention may hybridize to part or all of the polypeptide coding regions of OPG binding protein as shown in **FIG. 1** (SEQ ID NO: _____) and therefore may be truncations or extensions of the nucleic acids shown therein. Truncated or extended nucleic acids are encompassed by the invention provided that they retain at least the property of binding OPG. In one embodiment, the nucleic acid will encode a polypeptide of at least about 10 amino acids. In another embodiment, the nucleic acid will encode a polypeptide of at least about 20 amino acids. In yet another embodiment, the nucleic acid will encode a polypeptide of at least about 50 amino acids. The hybridizing nucleic acids may also include noncoding sequences located 5' and/or 3' to the OPG binding protein coding regions. Noncoding sequences include regulatory regions involved in expression of OPG binding protein, such as promoters, enhancer regions, translational initiation sites, transcription termination sites and the like.

[0024] In preferred embodiments, the nucleic acids of the invention encode mouse or human OPG binding protein.

Nucleic acids may encode a membrane bound form of OPG binding protein or soluble forms which lack a functional transmembrane region. The predicted transmembrane region for murine OPG binding protein includes amino acid residues 49-69 inclusive as shown in **FIG. 1** (SEQ. ID. NO: _____). Substitutions which replace hydrophobic amino acid residues in this region with neutral or hydrophilic amino acid residues would be expected to disrupt membrane association and result in soluble OPG binding protein. In addition, deletions of part or all the transmembrane region would also be expected to produce soluble forms of OPG binding protein. Nucleic acids encoding amino acid residues 70-316 as shown in **FIG. 1** (SEQ ID NO: _____), or fragments and analogs thereof, encompass soluble OPG binding protein.

[0025] Nucleic acid sequences of the invention may be used for the detection of sequences encoding OPG binding protein in biological samples. In particular, the sequences may be used to screen cDNA and genomic libraries for related OPG binding protein sequences, especially those from other species. The nucleic acids are also useful for modulating levels of OPG binding protein by anti-sense technology or in vivo gene expression. Development of transgenic animals expressing OPG binding protein is useful for production of the polypeptide and for the study of in vivo biological activity.

[0026] Vectors and Host Cells

[0027] The nucleic acids of the invention will be linked with DNA sequences so as to express biologically active OPG binding protein. Sequences required for expression are known to those skilled in the art and include promoters and enhancer sequences for initiation of RNA synthesis, transcription termination sites, ribosome binding sites for the initiation of protein synthesis, and leader sequences for secretion. Sequences directing expression and secretion of OPG binding protein may be homologous, i.e., the sequences are identical or similar to those sequences in the genome involved in OPG binding protein expression and secretion, or they may be heterologous. A variety of plasmid vectors are available for expressing OPG binding protein in host cells (see, for example, *Methods in Enzymology* v. 185, Goeddel, D. V. ed., Academic Press (1990)). For expression in mammalian host cells, a preferred embodiment is plasmid pDSR α described in PCT Application No. 90/14363. For expression in bacterial host cells, preferred embodiments include plasmids harboring the lux promoter (see co-owned and co-pending U.S. Ser. No. 08/577,778, filed Dec. 22, 1995). In addition, vectors are available for the tissue-specific expression of OPG binding protein in transgenic animals. Retroviral and adenovirus-based gene transfer vectors may also be used for the expression of OPG binding protein in human cells for in vivo therapy (see PCT Application No. 86/00922).

[0028] Prokaryotic and eucaryotic host cells expressing OPG binding protein are also provided by the invention. Host cells include bacterial, yeast, plant, insect or mammalian cells. OPG binding protein may also be produced in transgenic animals such as mice or goats. Plasmids and vectors containing the nucleic acids of the invention are introduced into appropriate host cells using transfection or transformation techniques known to one skilled in the art. Host cells may contain DNA sequences encoding OPG

binding protein as shown in **FIG. 1** or a portion thereof, such as the extracellular domain or the cytoplasmic domain. Nucleic acids encoding OPG binding proteins may be modified by substitution of codons which allow for optimal expression in a given host. At least some of the codons may be so-called preference codons which do not alter the amino acid sequence and are frequently found in genes that are highly expressed. However, it is understood that codon alterations to optimize expression are not restricted to the introduction of preference codons. Examples of preferred mammalian host cells for OPG binding protein expression include, but are not limited to COS, CHod-, 293 and 3T3 cells. A preferred bacterial host cell is *Escherichia coli*.

[0029] Polypeptides

[0030] The invention also provides OPG binding protein as the product of prokaryotic or eucaryotic expression of an exogenous DNA sequence, i.e., OPG binding protein is recombinant OPG binding protein. Exogenous DNA sequences include cDNA, genomic DNA and synthetic DNA sequences. OPG binding protein may be the product of bacterial, yeast, plant, insect or mammalian cells expression, or from cell-free translation systems. OPG binding protein produced in bacterial cells will have an N-terminal methionine residue. The invention also provides for a process of producing OPG binding protein comprising growing prokaryotic or eucaryotic host cells transformed or transfected with nucleic acids encoding OPG binding protein and isolating polypeptide expression products of the nucleic acids.

[0031] Polypeptides which are mammalian OPG binding protein or are fragments, analogs or derivatives thereof are encompassed by the invention. A fragment of OPG binding protein refers to a polypeptide having a deletion of one or more amino acids such that the resulting polypeptide has at least the property of binding OPG. Said fragments will have deletions originating from the amino terminal end, the carboxy terminal end, and internal regions of the polypeptide. Fragments of OPG binding protein are at least about ten amino acids, at least about 20 amino acids, or at least about 50 amino acids in length. In preferred embodiments, OPG binding protein will have a deletion of one or more amino acids from the transmembrane region (amino acid residues 49-69 as shown in **FIG. 1**), or, alternatively, one or more amino acids from the amino-terminus up to and/or including the transmembrane region (amino acid residues 1-49 as shown in **FIG. 1**). In another embodiment, OPG binding protein is a soluble protein comprising, for example, amino acid residues 70-316, or N-terminal or C-terminal truncated forms thereof, which retain OPG binding activity. An analog of an OPG binding protein refers to a polypeptide having a substitution or addition of one or more amino acids such that the resulting polypeptide has at least the property of binding OPG. Said analogs will have substitutions or additions at any place along the polypeptide. Preferred analogs include those of soluble OPG binding proteins. Fragments or analogs may be naturally occurring, such as a polypeptide product of an allelic variant or a mRNA splice variant, or they may be constructed using techniques available to one skilled in the art for manipulating and synthesizing nucleic acids. The polypeptides may or may not have an amino terminal methionine residue.

[0032] Also included in the invention are derivatives of OPG binding protein which are polypeptides that have

undergone post-translational modifications (e.g., addition of N-linked or O-linked carbohydrate chains, processing of N-terminal or C-terminal ends), attachment of chemical moieties to the amino acid backbone, chemical modifications of N-linked or O-linked carbohydrate chains, and addition of an N-terminal methionine residue as a result of prokaryotic host cell expression. In particular, chemically modified derivatives of OPG binding protein which provide additional advantages such as increased stability, longer circulating time, or decreased immunogenicity are contemplated. Of particular use is modification with water soluble polymers, such as polyethylene glycol and derivatives thereof (see for example U.S. Pat. No. 4,179,337). The chemical moieties for derivitization may be selected from water soluble polymers such as polyethylene glycol, ethylene glycol/propylene glycol copolymers, carboxymethylcellulose, dextran, polyvinyl alcohol and the like. The polypeptides may be modified at random positions within the molecule, or at predetermined positions within the molecule and may include one, two, three or more attached chemical moieties. Polypeptides may also be modified at predetermined positions in the polypeptide, such as at the amino terminus, or at a selected lysine or arginine residue within the polypeptide. Other chemical modifications provided include a detectable label, such as an enzymatic, fluorescent, isotopic or affinity label to allow for detection and isolation of the protein.

[0033] OPG binding protein chimeras comprising part or all of an OPG binding protein amino acid sequence fused to a heterologous amino acid sequence are also included. The heterologous sequence may be any sequence which allows the resulting fusion protein to retain the at least the activity of binding OPG. In a preferred embodiment, the carboxy terminal extracellular domain of OPG binding protein is fused to a heterologous sequence. Such sequences include heterologous cytoplasmic domains that allow for alternative intracellular signalling events, sequences which promote oligomerization such as the Fc region of IgG, enzyme sequences which provide a label for the polypeptide, and sequences which provide affinity probes, such as an antigen-antibody recognition.

[0034] The polypeptides of the invention are isolated and purified from tissues and cell lines which express OPG binding protein, either extracted from lysates or from conditioned growth medium, and from transformed host cells expressing OPG binding protein. OPG binding protein may be obtained from murine myelomonocytic cell line 32-D (ATCC accession no. CRL-11346). Human OPG binding protein, or nucleic acids encoding same, may be isolated from human lymph node or fetal liver tissue. Isolated OPG binding protein is free from association with human proteins and other cell constituents.

[0035] A method for the purification of OPG binding protein from natural sources (e.g. tissues and cell lines which normally express OPG binding protein) and from transfected host cells is also encompassed by the invention. The purification process may employ one or more standard protein purification steps in an appropriate order to obtain purified protein. The chromatography steps can include ion exchange, gel filtration, hydrophobic interaction, reverse phase, chromatofocusing, affinity chromatography employing an anti-OPG binding protein antibody or biotin-streptavidin affinity complex and the like.

[0036] Antibodies

[0037] Antibodies specifically binding the polypeptides of the invention are also encompassed by the invention. The antibodies may be produced by immunization with full-length OPG binding protein, soluble forms of OPG binding protein, or a fragment thereof. The antibodies of the invention may be polyclonal or monoclonal, or may be recombinant antibodies, such as chimeric antibodies wherein the murine constant regions on light and heavy chains are replaced by human sequences, or CDR-grafted antibodies wherein only the complementary determining regions are of murine origin. Antibodies of the invention may also be human antibodies prepared, for example, by immunization of transgenic animals capable of producing human antibodies (see, for example, PCT Application No. WO93/12227). The antibodies are useful for detecting OPG binding protein in biological samples, thereby allowing the identification of cells or tissues which produce the protein. In addition, antibodies which bind to OPG binding protein and block interaction with other binding compounds may have therapeutic use in modulating osteoclast differentiation and bone resorption.

[0038] Compositions

[0039] The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the OPG binding protein of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative and/or adjuvant. The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of an OPG binding protein agonist or antagonist. The term "therapeutically effective amount" means an amount which provides a therapeutic effect for a specified condition and route of administration. The composition may be in a liquid or lyophilized form and comprises a diluent (Tris, acetate or phosphate buffers) having various pH values and ionic strengths, solubilizer such as Tween or Polysorbate, carriers such as human serum albumin or gelatin, preservatives such as thimerosal or benzyl alcohol, and antioxidants such as ascorbic acid or sodium metabisulfite. Selection of a particular composition will depend upon a number of factors, including the condition being treated, the route of administration and the pharmacokinetic parameters desired. A more extensive survey of component suitable for pharmaceutical compositions is found in *Remington's Pharmaceutical Sciences*, 18th ed. A. R. Gennaro, ed. Mack, Easton, Pa. (1980).

[0040] In a preferred embodiment, compositions comprising soluble OPG binding proteins are also provided. Also encompassed are compositions comprising soluble OPG binding protein modified with water soluble polymers to increase solubility, stability, plasma half-life and bioavailability. Compositions may also comprise incorporation of soluble OPG binding protein into liposomes, microemulsions, micelles or vesicles for controlled delivery over an extended period of time. Soluble OPG binding protein may be formulated into microparticles suitable for pulmonary administration.

[0041] Compositions of the invention may be administered by injection, either subcutaneous, intravenous or intramuscular, or by oral, nasal, pulmonary or rectal administration. The route of administration eventually chosen will depend upon a number of factors and may be ascertained by one skilled in the art.

[0042] The invention also provides for pharmaceutical compositions comprising a therapeutically effective amount of the nucleic acids of the invention together with a pharmaceutically acceptable adjuvant. Nucleic acid compositions will be suitable for the delivery of part or all of the coding region of OPG binding protein and/or flanking regions to cells and tissues as part of an anti-sense therapy regimen.

[0043] Methods of use

[0044] OPG binding proteins may be used in a variety of assays for detecting OPG and characterizing interactions with OPG. In general, the assay comprises incubating OPG binding protein with a biological sample containing OPG under conditions which permit binding to OPG to OPG binding protein, and measuring the extent of binding. OPG may be purified or present in mixtures, such as in body fluids or culture medium. Assays may be developed which are qualitative or quantitative, with the latter being useful for determining the binding parameters (affinity constants and kinetics) of OPG to OPG binding protein and for quantitating levels of biologically active OPG in mixtures. Assays may also be used to evaluate the binding of OPG to fragments, analogs and derivatives of OPG binding protein and to identify new OPG and OPG binding protein family members.

[0045] Binding of OPG to OPG binding protein may be carried out in several formats, including cell-based binding assays, membrane binding assays, solution-phase assays and immunoassays. In general, trace levels of labeled OPG are incubated with OPG binding protein samples for a specified period of time followed by measurement of bound OPG by filtration, electrochemiluminescent (ECL, ORIGEN system by IGEN), cell-based or immunoassays. Homogeneous assay technologies for radioactivity (SPA; Amersham) and time resolved fluorescence (HTRF, Packard) can also be implemented. Binding is detected by labeling OPG or an anti-OPG antibody with radioactive isotopes (^{125}I , ^{35}S , ^3H), fluorescent dyes (fluorescein), lanthanide (Eu^{3+}) chelates or cryptates, orbipyridyl-ruthenium (Ru^{2+}) complexes. It is understood that the choice of a labeled probe will depend upon the detection system used. Alternatively, OPG may be modified with an unlabeled epitope tag (e.g., biotin, peptides, His_6 , myc) and bound to proteins such as streptavidin, anti-peptide or anti-protein antibodies which have a detectable label as described above.

[0046] In an alternative method, OPG binding protein may be assayed directly using polyclonal or monoclonal antibodies to OPG binding proteins in an immunoassay. Additional forms of OPG binding proteins containing epitope tags as described above may be used in solution and immunoassays.

[0047] Methods for identifying compounds which interact with OPG binding protein are also encompassed by the invention. The method comprises incubating OPG binding protein with a compound under conditions which permit binding of the compound to OPG binding protein, and measuring the extent of binding. The compound may be substantially purified or present in a crude mixture. Binding compounds may be nucleic acids, proteins, peptides, carbohydrates, lipids or small molecular weight organic compounds. The compounds may be further characterized by their ability to increase or decrease OPG binding protein activity in order to determine whether they act as an agonist or an antagonist.

[0048] OPG binding proteins are also useful for identification of intracellular proteins which interact with the cytoplasmic domain by a yeast two-hybrid screening process. As an example, hybrid constructs comprising DNA encoding the N-terminal 50 amino acids of an OPG binding protein fused to a yeast GAL4-DNA binding domain may be used as a two-hybrid bait plasmid. Positive clones emerging from the screening may be characterized further to identify interacting proteins. This information may help elucidate a intracellular signaling mechanism associated with OPG binding protein and provide intracellular targets for new drugs that modulate bone resorption.

[0049] The invention also encompasses modulators (agonists and antagonists) of OPG binding protein and the methods for obtaining them. An OPG binding protein modulator may either increase or decrease at least one activity associated with OPG binding protein, such as ability to bind OPG or some other interacting molecule or to regulate osteoclast maturation. Typically, an agonist or antagonist may be a co-factor, such as a protein, peptide, carbohydrate, lipid or small molecular weight molecule, which interacts with OPG binding protein to regulate its activity. Potential polypeptide antagonists include antibodies which react with either soluble or membrane-associated forms of OPG binding protein, and soluble forms of OPG binding protein which comprise part or all of the extracellular domain of OPG binding protein. Molecules which regulate OPG binding protein expression typically include nucleic acids which are complementary to nucleic acids encoding OPG binding protein and which act as anti-sense regulators of expression.

[0050] OPG binding protein is involved in controlling formation of mature osteoclasts, the primary cell type implicated in bone resorption. An increase in the rate of bone resorption (over that of bone formation) can lead to various bone disorders collectively referred to as osteopenias, and include osteoporosis, osteomyelitis, hypercalcemia, osteopenia brought on by surgery or steroid administration, Paget's disease, osteonecrosis, bone loss due to rheumatoid arthritis, periodontal bone loss, and osteolytic metastasis. Conversely, a decrease in the rate of bone resorption can lead to osteopetrosis, a condition marked by excessive bone density. Agonists and antagonists of OPG binding protein modulate osteoclast formation and may be administered to patients suffering from bone disorders. Agonists and antagonists of OPG binding protein used for the treatment of osteopenias may be administered alone or in combination with a therapeutically effective amount of a bone growth promoting agent including bone morphogenic factors designated BMP-1 to BMP-12, transforming growth factor- β and TGF- β family members, interleukin-1 inhibitors, TNF α inhibitors, parathyroid hormone, E series prostaglandins, bisphosphonates and bone-enhancing minerals such as fluoride and calcium.

[0051] The following examples are offered to more fully illustrate the invention, but are not construed as limiting the scope thereof.

EXAMPLE 1

Identification of a Cell Line Source for an OPG Binding Protein

[0052] Osteoprotegerin (OPG) negatively regulates osteoclastogenesis in vitro and in vivo. Since OPG is a TNFR-

related protein, it is likely to interact with a TNF-related family member while mediating its effects. With one exception, all known members of the TNF superfamily are type II transmembrane proteins expressed on the cell surface. To identify a source of an OPG binding protein, recombinant OPG-Fc fusion proteins were used as immunoprobe to screen for OPG binding proteins located on the surface of various cell lines and primary hematopoietic cells.

[0053] Cell lines that grew as adherent cultures in vitro were treated using the following methods: Cells were plated into 24 well tissue culture plates (Falcon), then allowed to grow to approximately 80% confluency. The growth media was then removed, and the adherent cultures were washed with phosphate buffered saline (PBS) (Gibco) containing 1% fetal calf serum (FCS). Recombinant mouse OPG [22-194]-Fc and human OPG [22-201]-Fc fusion proteins (see U.S. Ser. No. 08/706,945 filed Sep. 3, 1996) were individually diluted to 5 ug/ml in PBS containing 1L % FCS, then added to the cultures and allowed to incubate for 45 min at 0° C. The OPG-Fc fusion protein solution was discarded, and the cells were washed in PBS-FCS solution as described above. The cultures were then exposed to phycoerythrin-conjugated goat F(ab') anti-human IgG secondary antibody (Southern Biotechnology Associates Cat. # 2043-09) diluted into PBS-FCS. After a 30-45 min incubation at 0° C., the solution was discarded, and the cultures were washed as described above. The cells were then analysed by immunofluorescent microscopy to detect cell lines which express a cell surface OPG binding protein.

[0054] Suspension cell cultures were analysed in a similar manner with the following modifications: The diluent and wash buffer consisted of calcium- and magnesium-free phosphate buffered saline containing 1% FCS. Cells were harvested from exponentially replicating cultures in growth media, pelleted by centrifugation, then resuspended at 1×10^7 cells/ml in a 96 well microtiter tissue culture plate (Falcon). Cells were sequentially exposed to recombinant OPG-Fc fusion proteins, then secondary antibody as described above, and the cells were washed by centrifugation between each step. The cells were then analysed by fluorescence activated cell sorting (FACS) using a Becton Dickinson FACScan.

[0055] Using this approach, the murine myelomonocytic cell line 32D (ATCC accession no. CRL-11346) was found to express a surface molecule which could be detected with both the mouse OPG[22-194]-Fc and the human OPG[22-201]-Fc fusion proteins. Secondary antibody alone did not bind to the surface of 32D cells nor did purified human IgG1 Fc, indicating that binding of the OPG-Fc fusion proteins was due to the OPG moiety. This binding could be competed in a dose dependent manner by the addition of recombinant murine or human OPG[22-401] protein. Thus the OPG region required for its biological activity is capable of specifically binding to a 32D-derived surface molecule.

EXAMPLE 2

Expression Cloning of a Murine OPG Binding Protein

[0056] A cDNA library was prepared from 32D mRNA, and ligated into the mammalian expression vector pcDNA3.1(+) (Invitrogen, San Diego, Calif.). Exponentially

growing 32D cells maintained in the presence of recombinant interleukin-3 were harvested, and total cell RNA was purified by acid guanidinium thiocyanate-phenol-chloroform extraction (Chomczynski and Sacchi. Anal. Biochem. 162, 156-159, (1987)). The poly (A+) mRNA fraction was obtained from the total RNA preparation by adsorption to, and elution from, Dynabeads Oligo (dT)25 (DynaL Corp) using the manufacturer's recommended procedures. A directional, oligo-dT primed cDNA library was prepared using the Superscript Plasmid System (Gibco BRL, Gaithersburg, Md.) using the manufacturer's recommended procedures. The resulting cDNA was digested to completion with Sal I and Not I restriction endonuclease, then fractionated by size exclusion gel chromatography. The highest molecular weight fractions were selected, and then ligated into the polylinker region of the plasmid vector pcDNA3.1(+) (Invitrogen, San Diego, Calif.). This vector contains the CMV promoter upstream of multiple cloning site, and directs high level expression in eukaryotic cells. The library was then electroporated into competent *E. coli* (ElectroMAX DH10B, Gibco, N.Y.), and titered on LB agar containing 100 ug/ml ampicillin. The library was then arrayed into segregated pools containing approximately 1000 clones/pool, and 1.0 ml cultures of each pool were grown for 16-20 hr at 37° C. Plasmid DNA from each culture was prepared using the Qiagen QiaWell 96 Ultra Plasmid Kit (catalog #16191) following manufacturer's recommended procedures.

[0057] Arrayed pools of 32D cDNA expression library were individually lipofected into COS-7 cultures, then assayed for the acquisition of a cell surface OPG binding protein. To do this, COS-7 cells were plated at a density of 1×10^6 per ml in six-well tissue culture plates (Costar), then cultured overnight in DMEM (Gibco) containing 10% FCS. Approximately 2 μ g of plasmid DNA from each pool was diluted into 0.5 ml of serum-free DMEM, then sterilized by centrifugation through a 0.2 μ m Spin-X column (Costar). Simultaneously, 10 μ l of Lipofectamine (Life Technologies Cat # 18324-012) was added to a separate tube containing 0.5 ml of serum-free DMEM. The DNA and Lipofectamine solutions were mixed, and allowed to incubate at RT for 30 min. The COS-7 cell cultures were then washed with serum-free DMEM, and the DNA-lipofectamine complexes were exposed to the cultures for 2-5 hr at 37° C. After this period, the media was removed, and replaced with DMEM containing 10%FCS. The cells were then cultured for 48 hr at 37° C.

[0058] To detect cultures that express an OPG binding protein, the growth media was removed, and the cells were washed with PBS-FCS solution. A 1.0 ml volume of PBS-FCS containing 5 μ g/ml of human OPG[22-201]-Fc fusion protein was added to each well and incubated at RT for 1 hr. The cells were washed three times with PBS-FCS solution, and then fixed in PBS containing 2% paraformaldehyde and 0.2% glutaraldehyde in PBS at RT for 5 min. The cultures were washed once with PBS-FCS, then incubated for 1 hr at 65° C. while immersed in PBS-FCS solution. The cultures were allowed to cool, and the PBS-FCS solution was aspirated. The cultures were then incubated with an alkaline-phosphatase conjugated goat anti-human IgG (Fc specific) antibody (SIGMA Product # A-9544) at Rt for 30 min, then washed three-times with 20 mM Tris-Cl (pH 7.6), and 137 mM NaCl. Immune complexes that formed during these steps were detected by assaying for alkaline phosphatase

activity using the Fast Red TR/AS-MX Substrate Kit (Pierce, Cat. # 34034) following the manufacturer's recommended procedures.

[0059] Using this approach, a total of approximately 300,000 independent 32D cDNA clones were screened, represented by 300 transfected pools of 1000 clones each. A single well was identified that contained cells which acquired the ability to be specifically decorated by the OPG-Fc fusion protein. This pool was subdivided by sequential rounds of sib selection, yielding a single plasmid clone 32D-F3 (**FIG. 1**). 32D-F3 plasmid DNA was then transfected into COS-7 cells, which were immunostained with either FITC-conjugated goat anti-human IgG secondary antibody alone, human OPG[22-201]-Fc fusion protein plus secondary, or with ATAR-Fc fusion protein (ATAR also known as HVEM; Montgomery et al. Cell 87, 427-436 (1996)) (**FIG. 2**). The secondary antibody alone did not bind to COS-7/32D-F3 cells, nor did the ATAR-Fc fusion protein. Only the OPG Fc fusion protein bound to the COS-7/32D-F3 cells, indicating that 32D-F3 encoded an OPG binding protein displayed on the surface of expressing cells.

EXAMPLE 3

OPG Binding Protein Sequence

[0060] The 32D-F3 clone isolated above contained an approximately 2.3 kb cDNA insert (**FIG. 1**), which was sequenced in both directions on an Applied Biosystems 373A automated DNA sequencer using primer-driven Taq dye-terminator reactions (Applied Biosystems) following the manufacturer's recommended procedures. The resulting nucleotide sequence obtained was compared to the DNA sequence database using the FASTA program (GCG, University of Wisconsin), and analysed for the presence of long open reading frames (LORF's) using the "Six-way open reading frame" application (Frames) (GCG, University of Wisconsin). A LORF of 316 amino acid (aa) residues beginning at methionine was detected in the appropriate orientation, and was preceded by a 5' untranslated region of about 150 bp. The 5' untranslated region contained an in-frame stop codon upstream of the predicted start codon. This indicates that the structure of the 32D-F3 plasmid is consistent with its ability to utilize the CMV promoter region to direct expression of a 316 aa gene product in mammalian cells.

[0061] The predicted OPG binding protein sequence was then compared to the existing database of known protein sequences using a modified version of the FASTA program (Pearson, Meth. Enzymol. 183, 63-98 (1990)). The amino acid sequence was also analysed for the presence of specific motifs conserved in all known members of the tumor necrosis factor (TNF) superfamily using the sequence profile method of (Gribskov et al. Proc. Natl. Acad. Sci. USA 83, 4355-4359 (1987)), as modified by Lüthy et al. Protein Sci. 3, 139-146 (1994)). There appeared to be significant homology throughout the OPG binding protein to several members of the TNF superfamily. The mouse OPG binding protein appear to be most closely related to the mouse and human homologs of both TRAIL and CD40. Further analysis of the OPG binding protein sequence indicated a strong match to the TNF superfamily, with a highly significant Z score of 19.46.

[0062] The OPG binding protein aa sequence contains a probable hydrophobic transmembrane domain that begins at

a M49 and extends to L69. Based on this configuration relative to the methionine start codon, the OPG binding protein is predicted to be a type II transmembrane protein, with a short N-terminal intracellular domain, and a longer C-terminal extracellular domain (**FIG. 4**). This would be similar to all known TNF family members, with the exception of lymphotoxin alpha (Nagata and Golstein, Science 267, 1449-1456 (1995)).

EXAMPLE 4

Expression of Human OPG Binding Protein mRNA

[0063] Multiple human tissue northern blots (Clontech, Palo Alto, Calif.) were probed with a ³²P-dCTP labelled 32D-F3 restriction fragment to detect the size of the human transcript and to determine patterns of expression. Northern blots were prehybridized in 5×SSPE, 50% formamide, 5× Denhardt's solution, 0.5% SDS, and 100 μg/ml denatured salmon sperm DNA for 2-4 hr at 42° C. The blots were then hybridized in 5×SSPE, 50% formamide, 2× Denhardt's solution, 0.1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5 ng/ml labelled probe for 18-24 hr at 42° C. The blots were then washed in 2×SSC for 10 min at RT, 1×SSC for 10 min at 50° C., then in 0.5×SSC for 10-15 min.

[0064] Using a probe derived from the mouse cDNA and hybridization under stringent conditions, a predominant mRNA species with a relative molecular mass of about 2.5 kb was detected in lymph nodes (**FIG. 3**). A faint signal was also detected at the same relative molecular mass in fetal liver mRNA. No OPG binding protein transcripts were detected in the other tissues examined. The data suggest that expression of OPG binding protein mRNA was extremely restricted in human tissues. The data also indicate that the cDNA clone isolated is very close to the size of the native transcript, suggesting 32D-F3 is a full length clone.

EXAMPLE 5

Molecular Cloning of the Human OPG Binding Protein

[0065] The human homolog of the OPG binding protein is expressed as an approximately 2.5 kb mRNA in human peripheral lymph nodes and is detected by hybridization with a mouse cDNA probe under stringent hybridization conditions. DNA encoding human OPG binding protein is obtained by screening a human lymph node cDNA library by either recombinant bacteriophage plaque, or transformed bacterial colony, hybridization methods (Sambrook et al. *Molecular Cloning: A Laboratory Manual* Cold Spring Harbor Press, New York (1989)). To this the phage or plasmid cDNA library are screened using radioactively-labeled probes derived from the murine OPG binding protein clone 32D-F3. The probes are used to screen nitrocellulose filter lifted from a plated library. These filters are prehybridized and then hybridized using conditions specified in Example 4, ultimately giving rise to purified clones of the human OPG binding protein cDNA. Inserts obtained from any human OPG binding protein clones would be sequenced and analysed as described in Example 3.

EXAMPLE 6

Cloning and Bacterial Expression of OPG Binding Protein

[0066] PCR amplification employing the primer pairs and templates described below are used to generate various

forms of human OPG binding proteins. One primer of each pair introduces a TAA stop codon and a unique SacII site following the carboxy terminus of the gene. The other primer of each pair introduces a unique NdeI site, a N-terminal methionine, and optimized codons for the amino terminal portion of the gene. PCR and thermocycling is performed using standard recombinant DNA methodology. The PCR products are purified, restriction digested, and inserted into the unique NdeI and SacII sites of vector pAMG21 (ATCC accession no. 98113) and transformed into the prototrophic *E. coli* 393. Other commonly used *E. coli* expression vectors and host cells are also suitable for expression. After transformation, the clones are selected, plasmid DNA is isolated and the sequence of the OPG binding protein insert is confirmed.

[0067] pAMG21-Murine OPG Binding Protein [75-316]

[0068] This construct is engineered to be 242 amino acids in length and have the following N-terminal and C-terminal residues, NH₂-Met(75)-Asp-Pro-Asn-Arg-Gln-Asp-Ile-Asp(316)-COOH. The template to be used for PCR is pcDNA/32D-F3 and oligonucleotides #1581-72 and #1581-76 will be the primer pair to be used for PCR and cloning this gene construct.

[0069] 1581-72:

[0070] 5'-GTTCTCCTCATATGGATCCAAACCG-TATTTCTGAAGACAGCACTCACTGCTT-3' (SEQ ID NO: _____)

[0071] 1581-76:

[0072] 5'-TACGCACTCCGCGGTTAGTCTATGTC-CTGAACCTTGA-3' (SEQ ID NO: _____)

[0073] pAMG21-Murine OPG Binding Protein r158-316]

[0074] This construct is engineered to be 160 amino acids in length and have the following N-terminal and C-terminal residues, NH₂-Met-Lys(158)-Pro-Glu-Ala-Gln-Asp-Ile-Asp(316)-COOH. The template to be used for PCR is pcDNA/32D-F3 and oligonucleotides #1581-73 and #1581-76 will be the primer pair to be used for PCR and cloning this gene construct.

[0075] 1581-73:

[0076] 5'-GTTCTCCTCATATGAAACCTGAAGCT-CAACCATTTGCACACCTCACCATCAAT-3' (SEQ ID NO: _____)

[0077] 1581-76:

[0078] 5'-TACGCACTCCGCGGTTAGTCTATGTC-CTGAACCTTGA-3' (SEQ ID NO: _____)

[0079] pAMG21-Murine OPG Binding Protein [166-316]

[0080] This construct is engineered to be 152 amino acids in length and have the following N-terminal and C-terminal residues, NH₂-Met-His(166)-Leu-Thr-Ile-Gln-Asp-Ile-Asp(316)-COOH. The template to be used for PCR is pcDNA/32D-F3 and oligonucleotides #1581-75 and #1581-76 will be the primer pair to be used for PCR and cloning this gene construct.

[0081] 1581-75:

[0082] 5'-GTTCTCCTCATATGCATTTAACTAT-TAACGCTGCATCTATCCATCGGGTTC-CCATAAAGTCACT-3' (SEQ ID NO: _____)

[0083] 1581-76:

[0084] 5'-TACGCACTCCGCGGTTAGTCTATGTC-CTGAACCTTGA-3' (SEQ ID NO: _____)

[0085] pAMG21-Murine OPG binding protein [168-316]

[0086] This construct is engineered to be 150 amino acids in length and have the following N-terminal and C-terminal residues, NH₂-Met-Thr(168)-Ile-Asn-Ala-Gln-Asp-Ile-Asp(316)-COOH. The template to be used for PCR is pcDNA/32D-F3 and oligonucleotides #1581-74 and #1581-76 will be the primer pair to be used for PCR and cloning.

[0087] 1581-74:

[0088] 5'-GTTCTCCTCATATGACTATTAAACGCTG-CATCTATCCCATCGGGTTCCCATAAAGTCACT-3' (SEQ ID NO: _____)

[0089] 1581-76:

[0090] 5'-TACGCACTCCGCGGTTAGTCTATGTC-CTGAACCTTGA-3' (SEQ ID NO: _____)

[0091] It is understood that the above constructs are examples and one skilled in the art may readily obtain other forms of OPG binding protein using the general methodology presented her.

[0092] Growth of transfected *E. coli* 393, induction of OPG binding protein expression and isolation of inclusion bodies containing OPG binding protein is done according to procedures described in U.S. Ser. No. 08/577,788 filed Dec. 22, 1995. Subsequent purification of OPG binding proteins expressed in *E. coli* requires solubilization of bacteria inclusion bodies and renaturing of OPG binding protein using procedures available to one skilled in the art.

[0093] While the present invention has been described in terms of the preferred embodiments, it is understood that variations and modifications will occur to those skilled in the art. Therefore, it is intended that the appended claims cover all such equivalent variations which come within the scope of the invention as claimed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(iii) NUMBER OF SEQUENCES: 7

(2) INFORMATION FOR SEQ ID NO:1:

-continued

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 52 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTTCTCCTCA TATGGATCCA AACCGTATTT CTGAAGACAG CACTCACTGC TT 52

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 37 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

TACGCACTCC GCGGTTAGTC TATGTCCTGA ACTTTGA 37

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 53 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTTCTCCTCA TATGAAACCT GAAGCTCAAC CATTGACACA CCTCACCATC AAT 53

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 45 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTTCTCCTCA TATGCATTTA ACTATTAACG CTGCATCTAT CCCAT 45

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 59 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTTCTCCTCA TATGACTATT AACGCTGCAT CTATCCCATC GGGTTCCCAT AAAGTCACT 59

(2) INFORMATION FOR SEQ ID NO:6:

-continued

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2295 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 158..1105

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GAGCTCGGAT CCACTACTCG ACCCACGCGT CCGGCCAGGA CCTCTGTGAA CCGGTCGGGG	60
CGGGGGCCGC CTGGCCGGGA GTCTGTCTCG CCGTGGGTGG CCGAGGAAGG GAGAGAACGA	120
TCGCGGAGCA GGGCGCCCGA ACTCCGGGCG CCGCGCC ATG CGC CGG GCC AGC CGA	175
Met Arg Arg Ala Ser Arg	
1 5	
GAC TAC GGC AAG TAC CTG CGC AGC TCG GAG GAG ATG GGC AGC GGC CCC	223
Asp Tyr Gly Lys Tyr Leu Arg Ser Ser Glu Glu Met Gly Ser Gly Pro	
10 15 20	
GGC GTC CCA CAC GAG GGT CCG CTG CAC CCC GCG CCT TCT GCA CCG GCT	271
Gly Val Pro His Glu Gly Pro Leu His Pro Ala Pro Ser Ala Pro Ala	
25 30 35	
CCG GCG CCG CCA CCC GCC GCC TCC CGC TCC ATG TTC CTG GCC CTC CTG	319
Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser Met Phe Leu Ala Leu Leu	
40 45 50	
GGG CTG GGA CTG GGC CAG GTG GTC TGC AGC ATC GCT CTG TTC CTG TAC	367
Gly Leu Gly Leu Gly Gln Val Val Cys Ser Ile Ala Leu Phe Leu Tyr	
55 60 65 70	
TTT CGA GCG CAG ATG GAT CCT AAC AGA ATA TCA GAA GAC AGC ACT CAC	415
Phe Arg Ala Gln Met Asp Pro Asn Arg Ile Ser Glu Asp Ser Thr His	
75 80 85	
TGC TTT TAT AGA ATC CTG AGA CTC CAT GAA AAC GCA GGT TTG CAG GAC	463
Cys Phe Tyr Arg Ile Leu Arg Leu His Glu Asn Ala Gly Leu Gln Asp	
90 95 100	
TCG ACT CTG GAG AGT GAA GAC ACA CTA CCT GAC TCC TGC AGG AGG ATG	511
Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro Asp Ser Cys Arg Arg Met	
105 110 115	
AAA CAA GCC TTT CAG GGG GCC GTG CAG AAG GAA CTG CAA CAC ATT GTG	559
Lys Gln Ala Phe Gln Gly Ala Val Gln Lys Glu Leu Gln His Ile Val	
120 125 130	
GGG CCA CAG CGC TTC TCA GGA GCT CCA GCT ATG ATG GAA GGC TCA TGG	607
Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala Met Met Glu Gly Ser Trp	
135 140 145 150	
TTG GAT GTG GCC CAG CGA GGC AAG CCT GAG GCC CAG CCA TTT GCA CAC	655
Leu Asp Val Ala Gln Arg Gly Lys Pro Glu Ala Gln Pro Phe Ala His	
155 160 165	
CTC ACC ATC AAT GCT GCC AGC ATC CCA TCG GGT TCC CAT AAA GTC ACT	703
Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser Gly Ser His Lys Val Thr	
170 175 180	
CTG TCC TCT TGG TAC CAC GAT CGA GGC TGG GCC AAG ATC TCT AAC ATG	751
Leu Ser Ser Trp Tyr His Asp Arg Gly Trp Ala Lys Ile Ser Asn Met	
185 190 195	
ACG TTA AGC AAC GGA AAA CTA AGG GTT AAC CAA GAT GGC TTC TAT TAC	799
Thr Leu Ser Asn Gly Lys Leu Arg Val Asn Gln Asp Gly Phe Tyr Tyr	
200 205 210	
CTG TAC GCC AAC ATT TGC TTT CGG CAT CAT GAA ACA TCG GGA AGC GTA	847
Leu Tyr Ala Asn Ile Cys Phe Arg His His Glu Thr Ser Gly Ser Val	

-continued

215	220	225	230	
CCT ACA GAC TAT CTT CAG CTG ATG GTG TAT GTC GTT AAA ACC AGC ATC				895
Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr Val Val Lys Thr Ser Ile				
	235	240	245	
AAA ATC CCA AGT TCT CAT AAC CTG ATG AAA GGA GGG AGC ACG AAA AAC				943
Lys Ile Pro Ser Ser His Asn Leu Met Lys Gly Gly Ser Thr Lys Asn				
	250	255	260	
TGG TCG GGC AAT TCT GAA TTC CAC TTT TAT TCC ATA AAT GTT GGG GGA				991
Trp Ser Gly Asn Ser Glu Phe His Phe Tyr Ser Ile Asn Val Gly Gly				
	265	270	275	
TTT TTC AAG CTC CGA GCT GGT GAA GAA ATT AGC ATT CAG GTG TCC AAC				1039
Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile Ser Ile Gln Val Ser Asn				
	280	285	290	
CCT TCC CTG CTG GAT CCG GAT CAA GAT GCG ACG TAC TTT GGG GCT TTC				1087
Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala Thr Tyr Phe Gly Ala Phe				
	295	300	305	310
AAA GTT CAG GAC ATA GAC TGAGACTCAT TTCGTGGAAC ATTAGCATGG				1135
Lys Val Gln Asp Ile Asp				
	315			
ATGTCTTAGA TGTTTGAAA CTCTTAAAA AATGGATGAT GTCTATACAT GTGTAAGACT				1195
ACTAAGAGAC ATGGCCACG GTGTATGAAA CTCACAGCCC TCTCTCTGA GCCTGTACAG				1255
GTGTGTATA TGTAAGTCC ATAGGTGATG TTAGATTCAT GGTGATTACA CAACGTTTTT				1315
ACAATTTTGT AATGATTTC TAGAATTGAA CCAGATTGGG AGAGGTATTC CGATGCTTAT				1375
GAAAACTTA CACGTGAGCT ATGGAAGGGG GTCACAGTCT CTGGGTCTAA CCCCTGGACA				1435
TGTGCCACTG AGAACCTTGA AATTAAGAGG ATGCCATGTC ATTGCAAAGA AATGATAGTG				1495
TGAAGGGTTA AGTCTTTTG AATTGTTACA TTGCGCTGGG ACCTGCAAAAT AAGTTCTTTT				1555
TTTCTAATGA GGAGAGAAAA ATATATGTAT TTTTATATAA TGTCTAAAGT TATATTTTCTAG				1615
GTGTAATGTT TTCTGTGCAA AGTTTGTGTA ATTATATTTG TGCTATAGTA TTTGATTCAA				1675
AATATTTTAA AATGTCTCAC TGTTGACATA TTTAATGTTT TAAATGTACA GATGTATTTA				1735
ACTGGTGCAC TTTGTAATTC CCCTGAAGGT ACTCGTAGCT AAGGGGGCAG AATACTGTTT				1795
CTGGTGACCA CATGTAGTTT ATTTCTTTAT TCTTTTAAAC TTAATAGAGT CTTCAGACTT				1855
GTCAAACTA TGCAAGCAAA ATAAATAAAT AAAAATAAAA TGAATACCTT GAATAATAAG				1915
TAGGATGTTG GTCACCAGGT GCCTTTCAAA TTTAGAAGCT AATTGACTTT AGGAGCTGAC				1975
ATAGCCAAAA AGGATACATA ATAGGCTACT GAAATCTGTC AGGAGTATTT ATGCAATTAT				2035
TGAACAGGTG TCTTTTTTTA CAAGAGCTAC AAATTGTAAA TTTTGTCTCT TTTTTTTCCC				2095
ATAGAAAATG TACTATAGTT TATCAGCCAA AAAACAATCC ACTTTTTAAT TTAGTGAAAG				2155
TTATTTTATT ATACTGTACA ATAAAAGCAT TGTCTCTGAA TGTTAATTTT TTGGTACAAA				2215
AAATAAATTT GTACGAAAC CTGAAAAAAA AAAAAAAAAA AAAAAAAGG GCGCCGCTC				2275
TAGAGGGCCC TATTCTATAG				2295

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 316 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

- (ii) MOLECULE TYPE: protein

-continued

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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Met Arg Arg Ala Ser Arg Asp Tyr Gly Lys Tyr Leu Arg Ser Ser Glu
 1          5          10          15
Glu Met Gly Ser Gly Pro Gly Val Pro His Glu Gly Pro Leu His Pro
          20          25          30
Ala Pro Ser Ala Pro Ala Pro Ala Pro Pro Pro Ala Ala Ser Arg Ser
          35          40          45
Met Phe Leu Ala Leu Leu Gly Leu Gly Leu Gly Gln Val Val Cys Ser
 50          55          60
Ile Ala Leu Phe Leu Tyr Phe Arg Ala Gln Met Asp Pro Asn Arg Ile
 65          70          75          80
Ser Glu Asp Ser Thr His Cys Phe Tyr Arg Ile Leu Arg Leu His Glu
          85          90          95
Asn Ala Gly Leu Gln Asp Ser Thr Leu Glu Ser Glu Asp Thr Leu Pro
          100          105          110
Asp Ser Cys Arg Arg Met Lys Gln Ala Phe Gln Gly Ala Val Gln Lys
          115          120          125
Glu Leu Gln His Ile Val Gly Pro Gln Arg Phe Ser Gly Ala Pro Ala
          130          135          140
Met Met Glu Gly Ser Trp Leu Asp Val Ala Gln Arg Gly Lys Pro Glu
          145          150          155          160
Ala Gln Pro Phe Ala His Leu Thr Ile Asn Ala Ala Ser Ile Pro Ser
          165          170          175
Gly Ser His Lys Val Thr Leu Ser Ser Trp Tyr His Asp Arg Gly Trp
          180          185          190
Ala Lys Ile Ser Asn Met Thr Leu Ser Asn Gly Lys Leu Arg Val Asn
          195          200          205
Gln Asp Gly Phe Tyr Tyr Leu Tyr Ala Asn Ile Cys Phe Arg His His
          210          215          220
Glu Thr Ser Gly Ser Val Pro Thr Asp Tyr Leu Gln Leu Met Val Tyr
          225          230          235          240
Val Val Lys Thr Ser Ile Lys Ile Pro Ser Ser His Asn Leu Met Lys
          245          250          255
Gly Gly Ser Thr Lys Asn Trp Ser Gly Asn Ser Glu Phe His Phe Tyr
          260          265          270
Ser Ile Asn Val Gly Gly Phe Phe Lys Leu Arg Ala Gly Glu Glu Ile
          275          280          285
Ser Ile Gln Val Ser Asn Pro Ser Leu Leu Asp Pro Asp Gln Asp Ala
          290          295          300
Thr Tyr Phe Gly Ala Phe Lys Val Gln Asp Ile Asp
          305          310          315

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What is claimed is:

1. An isolated nucleic acid encoding an osteoprotegerin binding protein selected from the group consisting of:

- a) the nucleic acid sequence as in **FIG. 1** (SEQ ID NO: _____);
- b) nucleic acids which hybridize to the polypeptide coding regions as shown in **FIG. 1** (SEQ ID NO: _____) and remain hybridized under high stringency conditions; and

c) nucleic acids which are degenerate to the nucleic acids of (a) or (b).

2. The nucleic acid of claim 1 which is cDNA, genomic DNA, synthetic DNA or RNA.

3. A polypeptide encoded by the nucleic acid of claim 1.

4. The nucleic acid of claim 1 including one or more codons preferred for *Escherichia coli* expression.

5. The nucleic acid of claim 1 having a detectable label attached thereto.

6. The nucleic acid of claim 1 comprising the polypeptide-encoding region of residues 1-316 as shown in **FIG. 1** (SEQ ID NO: _____).

7. A nucleic acid encoding a polypeptide having the amino acid sequence of residues 1-316 or residues 70-316 as shown in **FIG. 1** (SEQ ID NO: _____).

8. An expression vector comprising the nucleic acid of claim 1.

9. The expression vector of claim 8 wherein the nucleic acid comprises the polypeptide-encoding region as shown in **FIG. 1** (SEQ ID NO: _____).

10. A host cell transformed or transfected with the expression vector of claim 8.

11. The host cell of claim 10 which is a eucaryotic or prokaryotic cell.

12. The host cell of claim 11 which is *Escherichia coli*.

13. A process for the production of an osteoprotegerin binding protein comprising:

growing under suitable nutrient conditions host cells transformed or transfected with the nucleic acid of claim 1; and

isolating the polypeptide product of the expression of the nucleic acid.

14. A polypeptide produced by the process of claim 13.

15. A purified and isolated osteoprotegerin binding protein, or fragment, analog, or derivative thereof.

16. The protein of claim 15 which is a human osteoprotegerin.

17. The protein of claim 15 having the amino acid sequence as shown in **FIG. 1** (SEQ ID NO: _____).

18. The protein of claim 15 which has been covalently modified with a water-soluble polymer.

19. The protein of claim 18 wherein the polymer is polyethylene glycol.

20. The protein of claim 15 which is a soluble osteoprotegerin binding protein.

21. The protein of claim 20 having the amino acid sequence from residues 70-316 inclusive as shown in **FIG. 1** (SEQ ID NO: _____), or a fragment, analog, or derivative thereof.

22. An antibody or fragment thereof which specifically binds an osteoprotegerin binding protein.

23. The antibody of claim 22 which is a monoclonal antibody.

24. A method for detecting the presence of an osteoprotegerin binding protein in a biological sample comprising:

incubating the sample with the antibody of claim 22 under conditions that allow binding of the antibody to the osteoprotegerin binding protein; and

detecting the bound antibody.

25. A method for detecting the presence of osteoprotegerin in a biological sample comprising:

incubating the sample with an osteoprotegerin binding protein under conditions that allow binding of the protein to osteoprotegerin; and

measuring the bound osteoprotegerin binding protein.

26. A method to assess the ability of a candidate compound to bind to an osteoprotegerin binding protein comprising:

incubating the osteoprotegerin binding protein with the candidate compound under conditions that allow binding; and

measuring the bound compound.

27. The method of claim 26 wherein the compound is an agonist or an antagonist of an osteoprotegerin binding protein.

28. A method of regulating expression of an osteoprotegerin binding protein in an animal comprising administering to the animal a nucleic acid complementary to the nucleic acids as shown in **FIG. 1** (SEQ ID NO: _____).

29. A pharmaceutical composition comprising a therapeutically effective amount of an osteoprotegerin binding protein in a pharmaceutically acceptable carrier, adjuvant, solubilizer, stabilizer and/or anti-oxidant.

30. The composition of claim 29 wherein the osteoprotegerin binding protein is a human osteoprotegerin binding protein.

31. A method of treating bone disease in a mammal comprising administering a therapeutically effective amount of a modulator of an osteoprotegerin binding protein.

32. The method of claim 31 wherein the modulator is a soluble form of an osteoprotegerin binding protein.

33. The method of claim 32 wherein the modulator is an antibody, or fragment thereof, which specifically binds an osteoprotegerin binding protein.

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