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(71) Applicant: CREATIVE BIOMOLECULES, INC. [US/US]; 45 South Street, Hopkinton, MA 01748 (US).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: RUEGER, David, C.; 19 Downey Street, Hopkinton, MA 01748 (US). JONES, William, K.; 35 Saint Paul Street, Brookline, MA 02146 (US). TUCKER, Robert, F.; 132 Robert Road, Holliston, MA 01746 (US). OPPER-MANN, Hermann; 25 Summer Hill Road, Medway, MA 02053 (US). OZKAYNAK, Engin; 44 Purdue Drive, Milford, MA 01757 (US). SAMPATH, Kuber, T.; 6 Spring Street, Meday, MA 02053 (US).			
(74) Agent: KELLEY, Robin, D.; Patent Dept., Creative BioMolecules, Inc., 45 South Street, Hopkinton, MA 01748 (US).			
(54) Title: METHOD AND COMPOSITIONS FOR RECOMBINANT OSTEOGENIC PROTEIN PRODUCTION			
(57) Abstract Disclosed are novel compositions of osteogenic proteins constituting soluble forms of these proteins, and methods and compositions for distinguishing between soluble and mature forms of these proteins.			

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"METHODS AND COMPOSITIONS
FOR RECOMBINANT OSTEOGENIC PROTEIN PRODUCTION"

Relation to Related Applications

This application is a continuation-in-part of
5 USSN 08/027,070, filed March 4, 1993, which is a
continuation-in-part of USSN 07/841,646, filed February
21, 1992, now US Patent No. 5,266,683, the disclosures
of which are incorporated herein by reference.

10 Field of the Invention

The present invention relates generally to
osteogenic proteins and, more particularly, to methods
and compositions for their production and purification.

15

Background of the Invention

Osteogenic proteins are well known and described in
the art. See, for example, U.S. Pat. Nos. 4, 968,590;
20 5,011,691; 5,018,753 and 5,266,683, as well as various
scholarly articles published in the scientific
literature. See, for example, Ozkaynak et al. (1990)
EMBO J 9:2085-2093; Ozkaynak et al., J.Biol. Chem.
267:13198-13205; Sampath et al. (1993) PNAS 90: 6004-
25 6008; Wozney et al. (1988) Science 242: 1528-1534; Wang
et al. (1988) PNAS 85:9484-9488; Wang et al. (1990)
PNAS 87:2220-2224, and Celeste, et al. (1990) PNAS
87:9843-9847. The art has described how to isolate
osteogenic proteins from bone and how to identify genes

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encoding these protein and express them using recombinant DNA technology.

The proteins that define the class of true
5 osteogenic proteins constitute a group of proteins sharing a number of conserved structural characteristics. Each protein, on its own, can induce endochondral bone formation in a mammal when properly folded, dimerized and disulfide bonded to produce a
10 dimeric species having the appropriate three dimensional conformation, and without requiring the addition of other osteogenic or non-osteogenic proteins. Typically, osteogenic proteins are provided to a site for bone induction in a mammal in association
15 with a suitable matrix having the appropriate conformation to allow the infiltration, proliferation and differentiation of migrating progenitor cells. The construct of osteogenic protein adsorbed to the surfaces of a suitable matrix is generally referred to
20 in the art as an osteogenic device. The proteins can be isolated from bone or, preferably, the gene encoding the protein is produced recombinantly in a suitable host cell. Methods for the production of osteogenic proteins and formulations of osteogenic devices are
25 described in detail in the art. See, for example, U.S.Pat. Nos. 5,011,691, or 5,266,683, the disclosures of which are incorporated hereinabove by reference.

Improved methods for the recombinant expression of
30 osteogenic proteins is an ongoing effort in the art. It is an object of this invention to provide an improvement in the methods for producing and purifying osteogenic proteins having high specific activity, and for formulating osteogenic devices comprising these

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proteins. Still another object is to provide means for distinguishing between the soluble form of the protein and the mature osteogenic protein species typically utilized in formulating osteogenic devices, and to
5 provide polyclonal and monoclonal antibodies capable of distinguishing between these various species. Another object is to provide methods for producing antibodies which can recognize both forms of the protein. Still another object is to provide methods for monitoring
10 each and all these forms of the protein in a fluid, including serum and production media. U.S. Patent No. 4,857,956 and Urist et al. (1984) Proc. Exp. Bio. Med. 176: 472-475, describe a serum assay for detecting a protein purported to have osteogenic activity. The
15 protein is not a member of the family of osteogenic proteins described herein, differing in molecular weight, structural characteristics and solubility from these proteins.

20 Summary of the Invention

It has now been discovered that osteogenic protein as defined herein below, when secreted into cultured medium from mammalian cells contains as a significant
25 fraction of the secreted protein a soluble form of the protein, and that this soluble form comprises the mature dimeric species, including truncated forms thereof, noncovalently associated with at least one, and preferably two pro domains. It further has been
30 discovered that binding partners having specific binding affinity for an epitope on an osteogenic protein or precursor polypeptide chain can be used to discriminate between these two forms of the protein. Preferably the binding partner is a protein. In one

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preferred embodiment the binding protein is an antibody, which may be monoclonal, polyclonal or biosynthetically produced. These binding partners may be used as part of a purification scheme to selectively
5 isolate the mature or the soluble form of the protein, as well as to quantitate the amount of mature and soluble forms produced. The antibodies may be used as part of a production protocol to monitor the pharmacological purity of an osteogenic protein
10 preparation for therapeutic or other clinical applications. Specifically, a method now is provided herein to ensure that only one desired form of a protein is present in a composition. In addition, binding partners can be produced which recognize both
15 protein forms and which may be used to advantage to monitor the quantity of total protein in solution. These binding partners also may be used as part of diagnostic treatments to monitor the concentration of osteogenic protein in solution in a body and to detect
20 fluctuations in the concentration of the proteins in their various forms.

The foregoing and other objects, features and advantages of the present invention will be made more
25 apparent from the following detailed description of the invention.

Brief Description of the Drawings

30 Fig. 1 is a schematic representation of an osteogenic protein polypeptide chain as expressed from a nucleic acid encoding the sequence, wherein the cross-hatched region represents the signal sequence; the stippled region represents the pro domain; the

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hatched region represents the N-terminus ("N-terminal extension") of the mature protein sequence; and the open region represents the C-terminal region of the mature protein sequence defining the conserved seven
5 cysteine domain, the conserved cysteines being indicated by vertical hatched lines;

Fig.2 lists the sequences of the N-terminal extensions of the mature forms of various osteogenic
10 proteins; and

Fig. 3 is a gel filtration column elution profile of a soluble osteogenic protein (OP-1) produced and purified from a mammalian cell culture by IMAC,
15 S-Sepharose and S-200HR chromatography in TBS (Tris-buffered saline), wherein V_0 is the void volume, ADH is alcohol dehydrogenase (MW 150 kDa), BSA is bovine serum albumin (MW 67 kDa), CA is carbonic anhydrase (MW 29kDa) and CytC is cytochrome C (MW 12.5
20 kDa).

Detailed Description

A soluble form of true osteogenic proteins now has
25 been discovered wherein the protein consists essentially of the amino acid sequence of the protein. The soluble form is a non-covalently associated complex comprising the pro domain or a fragment thereof, noncovalently associated or complexed with a dimeric
30 protein species having osteogenic activity, each polypeptide of the dimer having less than 200 amino acids and comprising at least the C-terminal six, and preferably seven cysteine skeleton defined by residues 335-431 and 330-431, respectively, of Seq. ID No. 1.

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Preferably, the polypeptide chains of the dimeric species comprise the mature forms of these sequences, or truncated forms thereof. Preferred truncated forms comprise the intact C-terminal domain and at least 10
5 amino acids of the N- terminal extension sequence e.g., preferably at least the sequence defined by residues 320-330 of Seq. ID 1. The soluble forms of these osteogenic proteins may be isolated from cultured cell medium, a mammalian body fluid, or may be formulated in
10 vitro.

In vivo, under physiological conditions, the pro domain may serve to enhance the transportability of the proteins, and/or to protect the proteins from proteases
15 and scavenger molecules, including antibodies. The pro domains also may aid in targeting the proteins to tissue, e.g., to bone.

The proteins contemplated by the invention and
20 referred to herein as "osteogenic proteins" are true osteogenic proteins capable, on their own, of inducing endochondral bone formation when implanted in a mammal in association with a matrix, without requiring the addition of other osteogenic or non-osteogenic
25 proteins. A detailed description of these proteins appears, for example, in U.S. Pat. Nos. 4968,590, 5,011,691 and USSN 841,646, and includes references to various members of the protein family identified to date. These family members include OP1, OP2, and the
30 proteins referred to in the art as "bone morphogenic proteins": BMP-2, BMP-3, BMP-4, BMP-5, BMP-6 and BMP-9, as well as various known species variants, including Vgr, Vgl, 60A and DPP, and biosynthetic

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osteogenic constructs, including COP1, 3, 5, 7 and 16.

The members of this family of proteins, which are a subclass of the TGF- β super-family of proteins, share characteristic structural features, represented schematically in Fig. 1, as well as substantial amino acid sequence homology in their C-terminal domains, including a conserved seven cysteine structure. As illustrated in the figure, the proteins are translated as a precursor polypeptide sequence 10, having an N-terminal signal peptide sequence 12, (the "pre pro" region, indicated in the figure by cross-hatching), typically less than about 30 residues, followed by a "pro" region 14, indicated in the figure by stippling, and which is cleaved to yield the mature sequence 16. The mature sequence comprises both the conserved C-terminal seven cysteine domain 20, and an N-terminal sequence 18, referred to herein as an N-terminal extension, and which varies significantly in sequence between the various osteogenic proteins. Cysteines are represented in the figure by vertical hatched lines 22. The polypeptide chains dimerize and these dimers typically are stabilized by at least one interchain disulfide bond linking the two polypeptide chain subunits. The mature subunits produced from mammalian cells typically have molecular weights in the range of about 15-23 kD, depending on the degree of glycosylation and N-terminal truncation. The dimeric species then typically have a molecular weight in the range of about 30-40 kD.

The signal peptide is cleaved during or soon after translation, at a cleavage site that can be predicted

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in a given sequence using the method of Von Heijne
((1986) Nucleic Acids Research 14:4683-4691.) The
"pro" form of the protein subunit, 24, in Fig. 1,
includes both the pro domain and the mature domain,
5 peptide bonded together. Typically, this pro form is
cleaved while the protein is still within the cell, and
the pro domain remains noncovalently associated with
the mature form of the subunit to form a soluble
species that appears to be the primary form secreted
10 from cultured mammalian cells. Typically, previous
purification techniques utilized denaturing conditions
that disassociated the complex.

Other soluble forms of osteogenic proteins secreted
15 from mammalian cells include dimers of the pro forms of
these proteins, wherein the pro domain is not cleaved
from the mature domain, and "hemi-dimers", wherein one
subunit comprises a pro form of the polypeptide chain
subunit and the other subunit comprises the cleaved
20 mature form of the polypeptide chain subunit (including
truncated forms thereof), preferably noncovalently
associated with a cleaved pro domain.

The isolated pro domain typically has regions of
25 hydrophobicity, as determined both by analysis of the
sequence and by characterization of its properties in
solution. The isolated pro domains alone typically are
not fully soluble in aqueous solutions. Accordingly,
without being limited to any given theory, the non-
30 covalent association of the cleaved pro domains with
the mature osteogenic protein dimeric species may
involve interaction of a hydrophobic portion of a given
pro domain with a corresponding hydrophobic region on
the dimeric species, the interaction of which

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effectively protects or "hides" an otherwise exposed hydrophobic region of the mature dimer from exposure to aqueous environments, enhancing the affinity of the mature dimer species for aqueous solutions.

5

Like the osteogenic proteins described herein, TGF- β also has a pro domain which associates non-covalently with the mature TGF- β protein form. However, unlike the osteogenic proteins described
10 herein, the TGF- β pro domain contains numerous cysteines and forms disulfide bonds with a specific binding protein. The TGF- β 1 pro domain also is phosphorylated at one or more mannose residues, while the osteogenic protein pro domains typically are not.

15

As described above, the active form of osteogenic protein, exemplified herein below by OP-1, is known to comprise a dimeric species composed of the mature sequence (e.g., amino acids 293-431 of Seq. ID NO. 1)
20 or a truncated form thereof, appropriately disulfide bonded to produce an osteogenic dimeric species. These osteogenic proteins, in their mature forms, are neutral to basic proteins (e.g., pI in the range of about 7 to 8) and are, to varying degrees, relatively insoluble
25 under physiological conditions. It now has been discovered that these proteins are secreted from mammalian cells in a soluble form and that this form comprises the mature dimeric species (also referred to herein as the "purified species") associated
30 noncovalently with one or more copies of the pro domain. This form of the protein likely is the form which is present in the bloodstream. Unlike the latent form of TGF- β , this form of osteogenic protein does not inhibit activity. The soluble form itself may be

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active or, it may dissociate to release the mature dimeric species when the protein has arrived at a target tissue, such as bone tissue.

- 5 Thus antibodies now may be generated which recognize an osteogenic protein of interest, and these antibodies then used to monitor culture medium or endogenous levels of osteogenic protein in a body fluid, such as serum, whole blood or peritoneal fluid.
- 10 Preferably, the antibody has binding specificity for the soluble form. Such antibodies may be generated by using the pro domain or a portion thereof as the antigen, or, preferably, the soluble complex itself. The pro domain may be preferably obtained by isolating
- 15 the soluble complex and then separating the noncovalently associated pro domain from the mature domain using standard procedures, e.g., by separating the complex components by chromatographic means, preferably by ion-exchange chromatography in the
- 20 presence of a denaturant, e.g., 6M urea. Alternatively, the pro protein in its monomeric form may be used as the antigen and the candidate antibodies screened by western blot or other standard immunoassay for those which recognize the pro form or soluble form
- 25 of the protein of interest, but not the mature form. Where antibody capable of identifying both soluble and mature forms of the protein is desired, the complex itself preferably is used as the antigen source. Details of antibody production and exemplary
- 30 immunoassays are provided below. Also provided is an example for detecting soluble osteogenic protein in a body fluid sample.

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The proteins contemplated to be useful in the methods and compositions of the invention include forms having varying glycosylation patterns and varying N-termini. They may be naturally occurring or
5 biosynthetically derived, and may be produced by expression of recombinant DNA in procaryotic or eucaryotic host cells. The proteins are active as a single species (e.g., as homodimers), or combined as a mixed species. Useful sequences and eucaryotic and
10 procaryotic expression systems are well described in the art. See, for example, US Patent Nos. 5,061,911 and 5,266,683 for useful expression systems. Useful sequences are recited in US Pat Nos. 4, 968,590; 5,011,691; 5,018,753 and 5,266,683, and Ozkaynak et al.
15 (1990) EMBO J 9:2085-2093; Ozkaynak et al., J.Biol. Chem. 267:13198-13205; Sampath et al. (1993) PNAS 90: 6004-6008; Wozney et al. (1988) Science 242: 1528-1534; Wang et al. (1988) PNAS 85:9484-9488; Wang et al. (1990) PNAS 87:2220-2224, Celeste, et al. (1990) PNAS
20 87:9843-9847 Weeks et al. (1987) Cell 51:861-867; Padgett, et al. (1987) Nature 325 81-84; Wharton et al. (1991) PNAS 88:9214-9218; Lyons et al. (1989) PNAS 86:4554-4558 and PCT international application WO93/00432 for OP1, OP2, DPP, 60A, Vgl, Vgr-1 and the
25 BMP-2-6, and BMP -9 proteins. Accordingly, these proteins, including allelic, species and other naturally-occurring and biosynthetic sequence variants thereof are contemplated to be useful in the inteant application. Other useful sequences include
30 biosynthetic constructs including, without limitation any of the sequences referred to in U.S. Patent No. 5,011,691 as COP-1, -3, -5, -7, -16; and chimeric constructs created by combining sequences from two or more different osteogenic proteins. As will be

domain has greater than 60% identity, and preferably greater than 65% identity with the amino acid sequence of OPS (e.g., residues 335-431 of Seq. ID No. 1).

15 In another preferred aspect, the invention
contemplates osteogenic proteins comprising species of
polypeptide chains having the generic amino acid
sequence herein referred to as "OPX" which accommodates
the homologies between the various identified species
20 of the osteogenic OP1 and OP2 proteins, and which is
described by the amino acid sequence presented below
and in Sequence ID No. 5.

	Cys	Xaa	Xaa	His	Glu	Leu	Tyr	Val	Ser	Phe	
25	1				5					10	
	Xaa	Asp	Leu	Gly	Trp	Xaa	Asp	Trp	Xaa	Ile	
					15					20	
	Ala	Pro	Xaa	Gly	Tyr	Xaa	Ala	Tyr	Tyr	Cys	
					25					30	
30	Glu	Gly	Glu	Cys	Xaa	Phe	Pro	Leu	Xaa	Ser	
					35					40	
	Xaa	Met	Asn	Ala	Thr	Asn	His	Ala	Ile	Xaa	
					45					50	

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	Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa	
	55	60
	Xaa Val Pro Lys Xaa Cys Cys Ala Pro Thr	
	65	70
5	Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa	
	75	80
	Asp Xaa Ser Xaa Asn Val Ile Leu Xaa Lys	
	85	90
	Xaa Arg Asn Met Val Val Xaa Ala Cys Gly	
10	95	100
	Cys His,	

and wherein Xaa at res. 2 = (Lys or Arg); Xaa at res. 3 = (Lys or Arg); Xaa at res. 11 = (Arg or Gln);

15 Xaa at res. 16 = (Gln or Leu); Xaa at res. 19 = (Ile or Val); Xaa at res. 23 = (Glu or Gln); Xaa at res. 26 = (Ala or Ser); Xaa at res. 35 = (Ala or Ser); Xaa at res. 39 = (Asn or Asp); Xaa at res. 41 = (Tyr or Cys); Xaa at res. 50 = (Val or Leu); Xaa at res. 52 = (Ser or

20 Thr); Xaa at res. 56 = (Phe or Leu); Xaa at res. 57 = (Ile or Met); Xaa at res. 58 = (Asn or Lys); Xaa at res. 60 = (Glu, Asp or Asn); Xaa at res. 61 = (Thr, Ala or Val); Xaa at res. 65 = (Pro or Ala); Xaa at res. 71 = (Gln or Lys); Xaa at res. 73 = (Asn or Ser); Xaa at

25 res. 75 = (Ile or Thr); Xaa at res. 80 = (Phe or Tyr); Xaa at res. 82 = (Asp or Ser); Xaa at res. 84 = (Ser or Asn); Xaa at res. 89 = (Lys or Arg); Xaa at res. 91 = (Tyr or His); and Xaa at res. 97 = (Arg or Lys).

30 In still another preferred aspect, the invention contemplates osteogenic proteins encoded by nucleic acids which hybridize to DNA or RNA sequences encoding the C-terminal seven cysteine domain of OP1 or OP2 under stringent hybridization conditions. As used

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herein, stringent hybridization conditions are defined as hybridization in 40% formamide, 5 X SSPE, 5 X Denhardt's Solution, and 0.1% SDS at 37°C overnight, and washing in 0.1 X SSPE, 0.1% SDS at 50°C. (See, for
5 example, Molecular Cloning: A Laboratory Manual, Maniatis et al., eds. 2d.ed., Cold Spring Harbor Press, Cold Spring Harbor, 1989.)

Useful pro domains include the full length pro
10 domains described below, as well as various truncated forms hereof, particularly truncated forms cleaved at proteolytic Arg-Xaa-Xaa-Arg cleavage sites. For example, in OP-1, possible pro sequences include sequences defined by residues 30-292 (full length
15 form); 48-292; and 158-292. Soluble OP-1 complex stability is enhanced when the pro domain comprises the full length form rather than a truncated form, such as the 48-292 truncated form, in that residues 30-47 show sequence homology to the N-terminal portions of other
20 osteogenic proteins, and are believed to have particular utility in enhancing complex stability for all osteogenic proteins. Accordingly, currently preferred pro sequences are those encoding the full length form of the pro domain for a given morphogen
25 (see below). Other pro sequences contemplated to have utility include biosynthetic pro sequences, particularly those that incorporate a sequence derived from the N-terminal portion of one or more osteogenic protein pro sequences.

30

A brief description of OP-1 is described below, followed by examples disclosing how to isolate soluble forms of these proteins and how to generate and identify antibodies having specificity for the mature

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or "purified" form, and the soluble or "media" form, or for both forms of proteins. In a particularly preferred embodiment, antibodies, or other binding proteins which recognize the soluble form of the

5 protein do not also recognize the precursor form of the pro domain peptide alone. As will be appreciated by those having ordinary skill in the art, with this disclosure it is possible to more accurately identify and/or quantitate the amount of a given, preferred form

10 of a recombinantly produced osteogenic protein present in the culture media, than by previously available methods, e.g., methods which relied on antibody specific for epitopes present only on the mature form or soluble form of the protein. It also now is

15 possible to accurately isolate a desired form of the protein. For example, one can preferentially isolate the soluble complex form by passing the culture media over an affinity column that has bound antibody with binding specificity only for the pro domain form, and

20 then selectively desorbing the bound protein using standard procedures to modify binding conditions, thereby allowing selective isolation of the complex. For example, one can modify binding conditions by using low pH, denaturants, or by competition with a peptide

25 specific for the antibody binding site. It also is anticipated that the antibodies and protocols can be used to identify both forms of the protein in a solution. A particularly useful application of the invention is as part of a protocol for monitoring the

30 pharmacological purity of an osteogenic protein composition to be used for clinical applications.

While the examples demonstrate the utility of the invention by means of an illustrative protein, OP1, it

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is intended to be understood that the methods and compositions taught herein can be extended without undue experimentation to other members of the family of osteogenic proteins. Similarly, while the examples are
5 directed to antibodies as the binding partner having specificity for an epitope on an osteogenic protein, and immunoassays as the detection protocols, any binding partner, particularly any binding protein capable of providing the same discriminatory power as
10 the antibodies described herein are contemplated. Moreover, while only monoclonal and polyclonal antibodies are described in detail, other antibody forms, including biosynthetics such as single chain constructs, referred to in the art as "sFv's" also are
15 contemplated to be within the scope of the invention.

OP1 - Refers generically to the family of osteogenically active proteins produced by expression of part or all of the hOP1 gene. Also referred to in
20 related applications as "OPI" and "OP-1".

hOP1-PP - Amino acid sequence of human OP1 protein (prepro form), Seq. ID No. 1, residues 1-431. Also referred to in related applications as "OP1-PP" and
25 "OPP".

OP1-18Ser - Amino acid sequence of mature human OP1 protein, Seq. ID No. 1, residues 293-431. N-terminal amino acid is serine. Originally identified as
30 migrating at 18 kDa on SDS-PAGE in COS cells. Depending on protein glycosylation pattern in different host cells, also migrates at 23kDa, 19kDa and 17kDa on

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SDS-PAGE. Also referred to in related applications as "OP1-18."

OPS - Amino acid sequence defining the C-terminal six
5 cysteine domain, residues 335-431 of Seq. ID No. 1.

OP7 - Amino acid equence defining the C-terminal seven
cysteine domain, residues 330-431 of Seq. ID No. 1.

10 I. Physical and Antigenic Structure of Soluble OP-1 and Mature, Dimeric OP-1

1a. Solubility of Mature, Dimeric OP-1

15 The solubility properties of purified mature OP-1 dimers (also referred to herein as "pure OP-1" or "purified OP-1" in contrast to OP-1 as found in media, also referred to herein as soluble or "media" OP-1) have been extensively studied. The conclusion of these
20 studies has been that mature OP-1 is soluble typically only under denaturing conditions. In contrast, the recombinantly produced OP-1 initially secreted into mammalian cell (CHO cell) conditioned media remains soluble in the absence of denaturants. Mature OP-1 has
25 been shown to be soluble in low concentrations of detergents including 0.1% SDS and CHAPS, and in mild denaturing conditions such as low ionic strength at low pH, or in the presence of denaturants with non-ionic detergents, e.g., 6 M urea + 0.3% Tween-80, and in the
30 presence of acidified organic solvent like 50% acetonitrile with 0.1% TFA. It has now been discovered that denaturing solvent conditions separate the pro domain from the mature region, and that previously developed purification protocols involving denaturants

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prohibit isolation of the complexed, highly soluble form. As disclosed below, purification of the soluble complexes must be done in the absence of denaturing conditions.

5

1.b Production of Secreted OP-1 by CHO Cells

Mammalian cell produced OP-1 is synthesized and secreted in a soluble dimeric form, as a result of
10 several post-translational modifications, including appropriate folding, dimerization, glycosylation and cleavage at the juncture of the pro domain and mature domain. Some pro-OP-1 also is secreted without being cleaved, resulting in secreted pro OP-1.

15

1.c Identification of Soluble OP-1 (complexed)

Recombinant OP-1 as expressed by CHO (Chinese
20 hamster ovary cells, see U.S. Patent No. 5,266,638 for exemplary protocol) is secreted into serum containing media and exists as in a soluble form. This apparent solubility of OP-1 could be caused by the association of OP-1 with a component of the serum or by the
25 secretion of OP-1 from CHO cells in a more soluble form than its final purified state.

1.d Cleveland Mapping of the 39 kDa Protein

30 Using metabolically labelled protein, a methionine-labeled 39 kDa protein from cultured media co-precipitates in an OP-1-dependent manner with OP-1. Protein was further characterized by Cleveland mapping using standard methodologies (see, for example

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Cleveland, D.W. (1977) J. Biol. Chem. 252:1102). Bands having an apparent molecular weight of 50, 39 and 19/17 kDa-based on comparison with standard molecular weight markers, were isolated from a PAGE gel, the gel slices
5 placed in the wells of a 20% acrylamide gel along with various amounts of endoproteinase lys-C, electrophoresed into the stacking gel and allowed to digest for 30 min. followed by resolving the generated fragments on the 20% gel. The 50 kDa protein was
10 cleaved to yield two fragments. The larger of these was also generated by the 39 kDa protein while the smaller was generated by the 19/17 kDa proteins. This evidence strongly suggested that the 39 kDa protein was the pro domain of OP-1. Further analysis of the
15 secreted form of OP-1 was made possible by the isolation of mature OP-1 as a soluble complex from CHO conditioned media.

20 2. Purification Protocol for Soluble
 Osteogenic Protein

Soluble complexes comprising osteogenic protein can be isolated from conditioned media using a simple, three step chromatographic protocol performed in the
25 absence of denaturants. The protocol involves running the media (or body fluid) over an affinity column, followed by ion exchange and gel filtration chromatographies. The affinity column described below is a Zn-IMAC column. An alternative protocol also
30 envisioned to have utility is an immunoaffinity column, created using standard procedures and, for example, using antibody specific for a given osteogenic protein pro domain (complexed, for example, to a protein A-conjugated Sepharose column.) Protocols for developing

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immunoaffinity columns are well described in the art, (see, for example, Guide to Protein Purification, M. Deutscher, ed., Academic Press, San Diego, 1990, particularly sections VII and XI.)

5

In this experiment OP-1 was expressed in CHO cells as described above. The CHO cell conditioned media containing 0.5% FBS was initially purified using Immobilized Metal-Ion Affinity Chromatography (IMAC).
10 The soluble OP-1 complex from conditioned media binds very selectively to the Zn-IMAC resin, presumably through association with the pro domain, and a high concentration of imidazole (50 mM imidazole, pH 8.0) is required for the effective elution of the bound
15 complex. The Zn-IMAC step separates the soluble OP-1 from the bulk of the contaminating serum proteins that elute in the flow through and 35 mM imidazole wash fractions. The Zn-IMAC purified soluble OP-1 is next applied to an S-Sepharose cation-exchange column
20 equilibrated in 20 mM NaPO_4 (pH 7.0) with 50 mM NaCl. This S-Sepharose step serves to further purify and concentrate the soluble OP-1 complex in preparation for the following gel filtration step. The protein was applied to a Sephacryl S-200HR column equilibrated in
25 TBS. Using substantially the same protocol, soluble osteogenic proteins also may be isolated from one or more body fluids, including serum, cerebro-spinal fluid or peritoneal fluid.

30 IMAC was performed using Chelating-Sepharose (Pharmacia) that had been charged with three column volumes of 0.2 M ZnSO_4 . The conditioned media was titrated to pH 7.0 and applied directly to the ZN-IMAC resin equilibrated in 20 mM HEPES (pH 7.0) with 500 mM

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NaCl. The Zn-IMAC resin was loaded with 80 mL of starting conditioned media per mL of resin. After loading the column was washed with equilibration buffer and most of the contaminating proteins were eluted with
5 35 mM imidazole (pH 7.0) in equilibration buffer. The soluble OP-1 complex is then eluted with 50 mM imidazole (pH 8.0) in 20 mM HEPES and 500 mM NaCl.

The 50 mM imidazole eluate containing the soluble
10 OP-1 complex was diluted with nine volumes of 20 mM NaPO_4 (pH 7.0) and applied to an S-Sepharose (Pharmacia) column equilibrated in 20 mM NaPO_4 (pH 7.0) with 50 mM NaCl. The S-Sepharose resin was loaded with an equivalent of 800 mL of starting conditioned media
15 per mL of resin. After loading the S-Sepharose column was washed with equilibration buffer and eluted with 100 mM NaCl followed by 300 mM and 500 mM NaCl in 20 mM NaPO_4 (pH 7.0). The 300 mM NaCl pool was further purified using gel filtration chromatography. Fifty
20 mls of the 300 mM NaCl eluate was applied to a 5.0 X 90 cm Sephacryl S-200HR (Pharmacia) equilibrated in Tris buffered saline (TBS), 50 mM Tris, 150 mM NaCl (pH 7.4). The column was eluted at a flow rate of 5 mL/minute collecting 10 mL fractions. The apparent
25 molecular of the soluble OP-1 was determined by comparison to protein molecular weight standards (alcohol dehydrogenase (ADH, 150 kDa), bovine serum albumin (BSA, 68 kDa), carbonic anhydrase (CA, 30 kDa) and cytochrome C (cyt C, 12.5 kDa). (see Fig. 3) The
30 purity of the S-200 column fractions was determined by separation on standard 15% polyacrylamide SDS gels stained with coomassie blue. The identity of the mature OP-1 and the pro-domain was determined by N-terminal sequence analysis after separation of the

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mature OP-1 from the pro-domain using standard reverse phase C18 HPLC.

Figure 3 shows the absorbance profile at 280 nm.

5 The soluble OP-1 complex elutes with an apparent molecular weight of 110 kDa. This agrees well with the predicted composition of the soluble OP-1 complex with one mature OP-1 dimer (35-36 kDa) associated with two pro-domains (39 kDa each). Purity of the final complex
10 can be verified by running the appropriate fraction in a reduced 15% polyacrylamide gel.

The complex components can be verified by running the complex-containing fraction from the S-200 or S-
15 200HR columns over a reverse phase C18 HPLC column and eluting in an acetonitrile gradient (in 0.1% TFA), using standard procedures. The complex is dissociated by this step, and the pro domain and mature species elute as separate species. These separate species then
20 can be subjected to N-terminal sequencing using standard procedures (see, for example, Guide to Protein Purification, M. Deutscher, ed., Academic Press, San Diego, 1990, particularly pp. 602-613), and the identity of the isolated 36kD, 39kDa proteins
25 confirmed as mature osteogenic protein and isolated, cleaved pro domain, respectively. N-terminal sequencing of the isolated pro domain from mammalian cell produced OP-1 revealed 2 forms of the pro domain, the predominant form being the intact form (beginning
30 at residue 30 of Seq. ID No. 1) and, as a minor species, a truncated form, (beginning at residue 48 of Seq. ID No. 1.) N-terminal sequencing of the polypeptide subunit of the isolated mature species reveals a range of N-termini for the mature sequence,

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beginning at residues 293, 300, 313, 315, 316, and 318, of Seq. ID No. 1, all of which are active as demonstrated by the standard bone induction assay. (See, for example, US Pat. Nos. 5,011,691 and 5,266,683
5 for descriptions of the standard rat bone induction assay.)

II. Osteogenic Protein Detection

10

As indicated above, the method and compositions of the invention are directed to identifying and/or gauntitating preferred forms of osteogenic protein in a solution, such as a culture medium or body fluid. As
15 will be appreciated by those skilled in the art, any means for specifically identifying and quantifying the protein is contemplated. The current state of the art for identifying proteins in solution is by means of an immunoassay, wherein an antibody capable of binding
20 specifically to the protein of interest is used to identify the protein in solution and the amount of bound complex formed then is determined.

Antibody methodologies are well understood and
25 described in the literature. A more detailed description of their preparation can be found, for example, in Practical Immunology, Butt, W.R., ed., Marcel Dekker, New York, 1984. Broadly, antibodies may be raised against one or more preferred forms of an
30 osteogenic protein by immunizing a suitable animal with an immunogenic preparation under conditions sufficient to induce antibody production in that animal. Monoclonal antibodies then can be obtained by fusing suitable antibody producing cells such as spleen or

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lymph node cells to myeloma cells and screening the fusion products for nuclear reactivity against the immunogen source (e.g., cell line or particular cell type determinant) using standard techniques.

5

The currently preferred method for detecting osteogenic proteins in a solution and/or for quantitating them is by detecting the proteins with osteogenic protein-specific antibodies. The antibodies
10 may be monoclonal or polyclonal in origin, and may be produced by standard methodologies. The osteogenic proteins used as immunogens may be prepared as described above. That is, an intact dimeric species or soluble complex may be used as the antigen (immunogen).
15 Alternatively, a pro domain peptide can be used to advantage, obtained for example, by dissociating a soluble complex and isolating the peptide, or by enzymatic digestion of a precursor form. Alternatively, the entire precursor form may be used as
20 the immunogen.

Antibodies to one or more of these proteins then are raised using standard methods. The antibodies then are exposed to the fluid sample under conditions
25 sufficient to allow specific binding of the antibody to its specific epitope, and the binding partner-osteogenic protein complex formed (here, antibody-osteogenic protein complex) then detected.

30 Immunoassay design considerations include preparation of antibodies (monoclonal or polyclonal) having sufficiently high binding specificity for their antigen that the specifically-bound antibody-antigen complex can be distinguished reliably from nonspecific

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interactions. The higher the antibody binding specificity, the lower the antigen concentration that can be detected. The choice of tagging label for detecting osteogenic protein-antibody complex formation
5 also will depend on the detection limitations desired. Enzyme assays (ELISAs) typically allow detection of a colored product formed by interaction of the enzyme-tagged complex with an enzyme substrate. Alternative labels include radioactive or fluorescent labels. The
10 most sensitive label known to date is a chemiluminescent tag where interaction with a reactant results in the production of light. Useful labels include chemiluminescent molecules such as acridium esters or chemiluminescent enzymes where the reactant
15 is an enzyme substrate. When, for example, acridium esters are reacted with an alkaline peroxide solution, an intense flash of light is emitted, allowing the limit of detection to be increased 100 to 10,000 times over those provided by other labels. In addition, the
20 reaction is rapid. A detailed review of chemiluminescence and immunoassays can be found in Weeks, et al., (1983) Methods in Enzymology 133:366-387. Other considerations include the use of microtiter wells or column immunoassays. Column assays
25 may be particularly advantageous where rapidly reacting labels, such as chemiluminescent labels, are used. The tagged complex can be eluted to a post-column detector which also contains the reactant or enzyme substrate, allowing the subsequent product formed to be detected
30 immediately.

A detailed review of immunological assay design, theory and protocols can be found in numerous texts in the art, including Practical Immunology, Butt, W.R.,

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ed., Marcel Dekker, New York, 1984. Of the various immunoassay formats available, one of the most sensitive is the sandwich technique. In this method, two antibodies capable of binding the analyte of interest are used: one immobilized onto a solid support, and one free in solution, but labeled with some easily detectable chemical compound. As described above, examples of chemical labels that may be used for the second antibody include radioisotopes, fluorescent compounds, and enzymes or other molecules which generate colored or electrochemically active products when exposed to a reactant or enzyme substrate. When samples containing analyte (e.g., osteogenic protein in a given form) are placed in this system, the analyte binds to both the immobilized antibody and the labelled antibody. The result is a "sandwich" immune complex on the support's surface. The analyte is detected by washing away nonbound sample components and excess labeled antibody and measuring the amount of labeled antibody complexed to analyte on the support's surface. The sandwich immunoassay is highly specific and very sensitive, provided that labels with good limits of detection are used.

Another useful form of immunoassay, particularly useful for screening candidates is the Western blot. Here, proteins of interest are dispersed by gel electrophoresis and immobilized on a nitrocellulose membrane. Candidate antibodies then are added, typically complexed with a means for detection, e.g., radioactive label or enzyme as described above, and complex formation is allowed to occur. The membrane then is washed to remove proteins interacting only by non-specific binding interactions, and complexes that

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remain bound are detected. A detailed standard protocol is provided in Molecular Cloning: A Laboratory Manual, Sambrook et al., eds. 2d.ed., Cold Spring Harbor Press, Cold Spring Harbor, 1989. As used
5 herein, typically, osteogenic proteins are electrophoresed under both reducing and oxidizing conditions.

2.a Antibody Production

10

Provided below are standard protocols for polyclonal and monoclonal antibody production. For antibodies which recognize the soluble complex form only, preferably the isolated complex itself is used as
15 the antigen. Alternatively, the antigen may comprise the isolated pro domain or a peptide fragment thereof. Where antibodies specific to the mature protein are desired, the antigen preferably comprises the mature dimeric form (e.g., the "purified" form) or a subunit
20 of the dimer comprising at least the C-terminal domain, or a peptide fragment thereof.

2a. Polyclonal Antibodies

25 Antibodies then are synthesized as described herein below, and tested in vitro for cross-reactivity with the various forms of the protein of interest.

Polyclonal antibody may be prepared as follows.
30 Each rabbit is given a primary immunization of 100 ug/500 μ l of antigen, 500 μ l Complete Freund's Adjuvant. Solubility of a given antigen may be enhanced as needed by combining the antigen in a solubilizing agent, e.g., 0.1% SDS, prior to

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combination with the adjuvant. The antigen is injected subcutaneously at multiple sites on the back and flanks of the animal. The rabbit is boosted after a month in the same manner using incomplete Freund's Adjuvant.

5 Test bleeds are taken from the ear vein seven days later. Two additional boosts and test bleeds are performed at monthly intervals until antibody against the osteogenic protein antigen is detected in the serum using an ELISA assay. Then, the rabbit is boosted

10 monthly with 100 μ g of antigen and bled (15 ml per bleed) at days seven and ten after boosting.

2b. Monoclonal Antibodies.

15 Monoclonal antibody specific for a given osteogenic protein may be prepared as follows. A mouse is given two injections of the osteogenic protein antigen. The protein or protein fragment preferably is recombinantly produced. The first injection contains 100 μ g of

20 antigen in complete Freund's adjuvant and is given subcutaneously. The second injection contains 50 μ g of antigen in incomplete adjuvant and is given intraperitoneally. The mouse then receives a total of 230 μ g of OP-1 in four intraperitoneal injections at

25 various times over an extended period of time (e.g., a one to eight month period.) One week prior to fusion, the mouse is boosted intraperitoneally with antigen (e.g., 100 μ g) and may be additionally boosted with a peptide fragment conjugated to bovine serum albumin

30 with a suitable cross linking agent. This boost can be repeated five days (IP), four days (IP), three days (IP) and one day (IV) prior to fusion. The mouse spleen cells then are fused to commercially available myeloma cells at a ratio of 1:1 using PEG 1500

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(Boehringer Mannheim, Germany), and the fused cells plated and screened for mature or soluble osteogenic protein-specific antibodies using the appropriate portion of the osteogenic protein sequence as antigen.

- 5 The cell fusion and monoclonal screening steps readily are performed according to standard procedures well described in standard texts widely available in the art.

10 2c. Antibody Specificity.

Using these standard procedures, anti-pro domain antisera was prepared from rabbits using the isolated pro domain from OP-1 as the antigen, and monoclonal
15 antibody ("mAb") to the mature domain was produced in mice, using an E. coli-produced truncated form of OP-1 as antigen.

Standard Western blot analysis performed as
20 described herein above under reducing conditions demonstrates that the anti-pro domain antisera ("anti-pro") is specific for the pro domain only, while the mAb to mature OP-1 ("anti-mature OP-1") is specific for the dimer subunits, that the two antibodies do not
25 cross-react, and that the antibodies and can be used to distinguish between soluble and mature protein forms in a sample, e.g., of conditioned media or serum. A tabular representation of the Western blot results is in Table I below, where reactivity of mAb to mature
30 OP-1 is indicated by "yy", and reactivity of the anti-pro antisera is indicated by "xx".

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TABLE I

	Antibody	Purified Sol OP1	Conditioned CHO Cell Media	Isolated Pro Domain	Purified Dimer Subunits
5	"anti-pro"	xx	xx	xx	
10	"anti-mature OP-1"	yy	yy		yy

In a second series, monoclonal antibodies were
 15 raised against each of the following antigens: soluble
 complex and uncomplexed, mature dimeric species.

Clones then were screened for reactivity against
 the various forms of OP-1, in an ELISA assay, as
 20 described above. Here, the various forms of OP1 tested
 were immobilized on a surface, the antibody to be
 screened then was added, and bound antibody detected
 using a goat anti-mouse antibody. Five different
 phenotypes or binding categories were identified and
 25 are described below. In the table, "S" means soluble
 complex; "M" means mature, dimeric species, and "P"
 means isolated pro domain.

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TABLE II

5	<u>Category</u>	Protein Form		
		<u>M</u>	<u>S</u>	<u>P</u>
	1	+	+	
	2		+	+
	3		+	
10	4			+
	5	+		

Antibodies having the binding character of category #1 recognize an epitope present on both the uncomplexed dimeric species and the soluble form.

Antibodies having the binding character of category #2 recognize an epitope present on both the soluble complex and the pro domain. Antibodies having the binding character of category #3 only recognize the soluble complex form, verifying that a conformational change occurs upon complex formation sufficient to create an epitope not present on other forms of the protein. Antibodies having this binding character are particularly useful for verifying the presence of a complex, including the formation of the soluble complex in vitro from its components (e.g., uncomplexed dimer and isolated pro domain peptide.)

Antibodies having the binding character of category #4 only recognize an epitope on the pro domain, verifying that complex formation is sufficient to mask or destroy an epitope present on the soluble complex. Similarly, antibodies having the binding

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character of category #5 only recognize an epitope on the mature, dimeric uncomplexed protein form, but not the soluble complex.

- 5 Of course, individual members within a given category may bind different epitopes. Accordingly, their binding character with respect to the different protein forms may vary depending on the assay conditions. For example, individual members of
- 10 category #1, while still recognizing the mature and soluble forms, show preferentially binding affinity for the soluble form over the mature form under sandwich ELISA conditions where the member of category #1 constituted the capture antibody. Similarly,
- 15 individual members of category #2 demonstrate variable binding for the pro domain under Western blot conditions.

20 III. Immunoassays

- 20 The ability to detect osteogenic proteins in solution and to distinguish between soluble and mature dimeric forms provides a valuable tool for protein production systems. Quality control considerations
- 25 require that means be available for determining both the form of the protein in solution and its quantity. This is particularly true for biological therapeutics where pharmacologically certain forms must be provided for clinical use. The method also provides a useful
- 30 tool for diagnostic assays, allowing one to monitor the level and type of osteogenic protein free in the body, e.g., in serum and other body fluids.

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A currently preferred detection means for evaluating the level of osteogenic protein in a fluid, including culture or a body fluid, comprises an immunoassay utilizing an antibody or other suitable binding protein capable of reacting specifically with an osteogenic protein and being detected as part of a complex with the protein. Immunoassays may be performed using standard techniques known in the art and using antibodies raised against the protein and specific for that protein.

Antibodies which recognize an osteogenic protein form of interest may be generated as described herein and these antibodies then used to monitor the levels of protein in a fluid, including a body fluid, such as serum, whole blood or peritoneal fluid.

To monitor endogenous concentrations of the soluble form of the protein, the antibody chosen preferably has binding specificity for the soluble form. For endogenous proteins, these antibodies may have specificity for the pro domain and/or the soluble complex (e.g., binding categories 1-3, above). Such antibodies may be generated by using the pro domain or a portion thereof as the antigen, or the soluble complex itself, essentially as described herein. A suitable pro domain for use as an antigen may be obtained by isolating the soluble complex and then separating the noncovalently associated pro domain from the mature domain using standard procedures, e.g., by chromatographic means (preferably using an ion-exchange column under denaturing conditions, e.g., 6M urea), as described above or by separation by gel electrophoresis. Alternatively, the pro form of the

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protein in its monomeric form may be used as the antigen and the candidate antibodies screened by Western blot or other standard immunoassay for those which recognize the pro domain of the soluble form of
5 the protein of interest, but not the mature form, also as described above.

Monomeric pro forms can be obtained from cell lysates of CHO produced cells, or from prokaryotic
10 expression of a DNA encoding the pro form, in for example, E.coli, or from a commercially available baculovirus expression system in insect cells. The pro form, which has an apparent molecular weight of about 50 kDa in mammalian cells, can then be isolated
15 as described above.

In order to detect and/or quantitate the amount of osteogenic protein present in a solution, an immunoassay may be performed to detect the osteogenic
20 protein using a polyclonal or monoclonal antibody specific for that protein. Here, soluble and mature forms of the osteogenic protein also may be distinguished by using antibodies that discriminate between the two forms of the proteins as described
25 above. Currently preferred assays include ELISAS and radioimmunoassays, including standard competitor assays useful for quantitating the osteogenic protein in a sample, where an unknown amount of sample protein is allowed to react with anti-osteogenic protein antibody
30 and this interaction is competed with a known amount of labeled antigen. The level of bound or free labeled antigen at equilibrium then is measured to quantitate the amount of unlabeled antigen in solution, the amount of sample antigen being proportional to the amount of

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free labeled antigen. Exemplary protocols for these assays are provided below. However, as will be appreciated by those skilled in the art, variations of these protocols, as well as other immunoassays, including Western blots, are well known in the literature and within the skill of the art. For example, in the ELISA protocol provided below, soluble OP-1 is identified in a sample using biotinylated anti-pro antiserum. Biotinylated antibodies can be visualized in a colormetric assay or in a chemiluminescent assay, as described below. Alternatively, the antibody can be radio-labeled with a suitable molecule, such as ^{125}I . Still another protocol that may be used is a solid phase immunoassay, preferably using an affinity column with anti-osteogenic protein antibody complexed to the matrix surface and over which a serum sample may be passed. A detailed description of useful immunoassays, including protocols and general considerations is provided in, for example, Molecular Cloning: A Laboratory Manual, Sambrook et al., eds. Cold Spring Harbor Press, New York, 1989, particularly Section 18.

For serum assays, the serum preferably first is partially purified to remove some of the excess, contaminating serum proteins, such as serum albumin. Preferably the serum is extracted by precipitation in ammonium sulfate (e.g., 45%) such that the complex is precipitated. Further purification can be achieved using purification strategies that take advantage of the differential solubility of soluble osteogenic protein complex or mature osteogenic proteins relative to that of the other proteins present in serum.

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Further purification also can be achieved by chromatographic techniques well known in the art.

3a. Assays

5

Soluble OP-1 may be detected using a polyclonal or monoclonal antibody in an ELISA, as described below in this experiment, polyclonal antibody specific for the OP-1 pro domain is omalyzedl $\mu\text{g}/100 \mu\text{l}$ of

10 affinity-purified polyclonal rabbit IgG specific for OP-1-pro is added to each well of a 96-well plate and incubated at 37°C for an hour. The wells are washed four times with 0.167M sodium borate buffer with 0.15 M NaCl (BSB), pH 8.2, containing 0.1% Tween 20. To

15 minimize non-specific binding, the wells are blocked by filling completely with 1% bovine serum albumin (BSA) in BSB and incubating for 1 hour at 37°C . The wells are then washed four times with BSB containing 0.1% Tween 20. A $100 \mu\text{l}$ aliquot of an appropriate dilution

20 of each of the test samples of cell culture supernatant or serum sample is added to each well in triplicate and incubated at 37°C for 30 min. After incubation, $100 \mu\text{l}$ biotinylated antibody from rabbit anti-pro anti-serum (stock solution is about 1 mg/ml and diluted 1:400 in

25 BSB containing 1% BSA before use) is added to each well and incubated at 37°C for 30 min. The wells are then washed four times with BSB containing 0.1% Tween 20. $100 \mu\text{l}$ strepavidin-alkaline (Southern Biotechnology Associates, Inc. Birmingham, Alabama, diluted 1:2000 in

30 BSB containing 0.1% Tween 20 before use) is added to each well and incubated at 37°C for 30 min. The plates are washed four times with 0.5M Tris buffered Saline (TBS), pH 7.2. $50 \mu\text{l}$ substrate (ELISA Amplification System Kit, Life Technologies, Inc., Bethesda, MD) is

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added to each well incubated at room temperature for 15 min. Then, 50 μ l amplifier (from the same amplification system kit) is added and incubated for another 15 min at room temperature. The reaction is
5 stopped by the addition of 50 μ l 0.3 M sulphuric acid. The OD at 490 nm of the solution in each well is recorded. To quantitate the level of soluble OP-1 in the sample, a standard curve is performed in parallel with the test samples. In the standard curve, known
10 increasing amounts of purified OP-1-pro is added. Alternatively, using, for example, Lumi-phos 530 (Analytical Luminescence Laboratories) as the substrate and detection at 300-650 nm in a standard luminometer, complexes can be detected by chemiluminescence, which
15 typically provides a more sensitive assay than detection by means of a visible color change.

3b. Plate-based Radioimmunoassay

20 Osteogenic protein (soluble or mature form) may be detected in a standard plated-based radioimmunoassay as follows. Empirically determined limiting levels of anti-osteogenic protein antibody (e.g., anti-OP-1, typically 50-80 ng/well) are bound to wells of a PVC
25 plate e.g., in 50 μ l PBS phosphate buffered saline. After sufficient incubation to allow binding at room temperature, typically one hour, the plate is washed in a borate-buffered saline/Tween 20 solution, ("washing buffer"), and 200 μ l of block (3% BSA, 0.1M lysine in
30 1xBSB) is added to each well and allowed to incubate for 1 hour, after which the wells are washed again in washing buffer. 40 μ l of a sample composed of serially diluted plasma (preferably partially purified as described above) or osteogenic protein standard (e.g.,

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OP-1) is added to wells in triplicate. Samples preferably are diluted in PTH (15 mM KH_2PO_4 , 8 mM Na_2PO_4 , 27 mM KCl, 137 mM NaCl, 0.05% Tween 20, 1 mg/ml HSA, 0.05% NaN_3 , pH 7.2). 10 μl of labelled competitor
5 antigen, preferably 100,000-500,000 cpm/sample is added (e.g., ^{125}I OP-1, radiolabelled using standard procedures), and plates are incubated overnight at 4°C. Plates then are washed in washing buffer, and allowed to dry. Wells are cut apart and bound labelled OP-1
10 counted in a standard gamma counter. The quantities of bound labelled antigen (e.g., ^{125}I OP-1) measured in the presence and absence of sample then are compared, the difference being proportional to the amount of sample antigen (osteogenic protein) present in the
15 sample fluid.

3c. Production Monitoring Considerations

Samples for testing the level of protein production
20 includes culture supernatants or cell lysates, collected periodically and evaluated for OP-1 production by immunoblot analysis (Sambrook et al., eds., 1989, Molecular Cloning, Cold Spring Harbor Press, Cold Spring Harbor, NY), or a portion of the
25 cell culture itself, collected periodically and used to prepare polyA+ RNA for mRNA analysis. To monitor de novo OP-1 synthesis, some cultures are labeled according to conventional procedures with an
 ^{35}S -methionine/ ^{35}S -cysteine mixture for 6-24 hours and
30 then evaluated to OP-1 synthesis by conventional immunoprecipitation methods.

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3.d Diagnostics using Antibodies to Soluble Osteogenic Protein Complex

The antibodies of this invention also may be used
5 to monitor the level of soluble protein in the body.
Fluctuations in osteogenic protein levels present in
the bloodstream or peritoneal fluid then may be used to
evaluate tissue viability. For example, osteogenic
proteins are detected associated with regenerating
10 tissue and/or may be released from dying cells into
surrounding peritoneal fluid.

Serum samples may be obtained by standard
venipuncture and serum prepared by centrifugation at
15 3,000 RPM for ten minutes. Similarly, peritoneal fluid
samples may be obtained by a standard fluid extraction
methodology. The presence of osteogenic protein in the
serum or peritoneal fluid then may be assessed by
standard Western blot (immunoblot), ELISA or RIA
20 procedures. Briefly, for example, with the ELISA,
samples may be diluted in an appropriate buffer, such
as phosphate-buffered saline, and 50 μ l aliquots
allowed to absorb to flat bottomed wells in microtitre
plates pre-coated with soluble osteogenic protein-
25 specific antibody, and allowed to incubate for 18 hours
at 4°C. Plates then may be washed with a standard
buffer and incubated with 50 μ l aliquots of a second
osteogenic protein-specific antibody conjugated with a
detecting agent, e.g., biotin, in an appropriate
30 buffer, for 90 minutes at room temperature. Osteogenic
protein-antibody complexes then may be detected using
standard procedures.

- 40 -

Alternatively, an osteogenic protein-specific affinity column may be created using, for example, soluble osteogenic protein-specific antibodies adsorbed to a column matrix, and passing the fluid sample
5 through the matrix to selectively extract the protein of interest. The protein then is eluted. A suitable elution buffer may be determined empirically by determining appropriate binding and elution conditions first with a control (e.g., purified,
10 recombinantly-produced protein.) Fractions then are tested for the presence of the soluble form protein by standard immunoblot. Protein concentrations in serum or other fluid samples then may be determined using standard protein quantification techniques, including
15 by spectrophotometric absorbance or by quantitation by ELISA or RIA antibody assays. Using this procedure, OP-1 has been identified in serum.

OP-1 was detected in human serum using the
20 following assay. A monoclonal antibody raised against mammalian, recombinantly produced OP-1 using standard immunology techniques well described in the art and described generally herein, was immobilized by passing the antibody over an activated agarose gel (e.g., Affi-
25 GelTM, from Bio-Rad Laboratories, Richmond, CA, prepared following manufacturer's instructions), and used to purify OP-1 from serum. Human serum then was passed over the column and eluted with 3M K-thiocyanate. K-thiocyanate fractions then were
30 dialyzed in 6M urea, 20mM PO₄, pH 7.0, applied to a C8 HPLC column, and eluted with a 20 minute, 25-50% acetonitrile/0.1% TFA gradient. Mature, recombinantly produced OP-1 homodimers elute between 20-22 minutes. Accordingly, these fractions from the affinity-purified

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human serum sample were collected and tested for the presence of OP-1 by standard immunoblot using an OP-1-specific antibody, and the protein identity confirmed by N-terminal sequencing.

5

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiments are therefore to be considered in all respects as
10 illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are therefore intended to be embraced
15 therein.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT:
(A) NAME: CREATIVE BIOMOLECULES, INC.
(B) STREET: 45 SOUTH STREET
10 (C) CITY: HOPKINTON
(D) STATE: MA
(E) COUNTRY: USA
(F) POSTAL CODE (ZIP): 01748
(G) TELEPHONE: 508-435-9001
(H) TELEFAX: 508-435-0992
15 (I) TELEX:
- (ii) TITLE OF INVENTION: METHODS AND COMPOSITIONS
FOR RECOMBINANT OSTEOGENIC PROTEIN PRODUCTION
- 20 (iii) NUMBER OF SEQUENCES: 6
- (iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: TESTA, HURWITZ & THIBEAULT
(B) STREET: 53 STATE STREET
25 (C) CITY: BOSTON
(D) STATE: MASSACHUSETTS
(E) COUNTRY: U.S.A.
(F) ZIP: 02109
- 30 (v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
35 (D) SOFTWARE: Patent In Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: US
(B) FILING DATE: HERewith
40 (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
(A) NAME: PITCHER, EDMUND R.
(B) REGISTRATION NUMBER: 27,829
45 (C) REFERENCE/DOCKET NUMBER: CRP-096PC
- (viii) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617/248-7000
(B) TELEFAX: 617/248-7100

- 43 -

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 1822 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: cDNA

(vi) ORIGINAL SOURCE:

(A) ORGANISM: HOMO SAPIENS
(F) TISSUE TYPE: HIPPOCAMPUS

15 (ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 49..1341
(D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"
/product= "hOP1-PP"
/evidence= EXPERIMENTAL
/standard_name= "hOP1"

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

25 GGTGCGGGCC CGGAGCCCGG AGCCCGGGTA GCGCGTAGAG CCGGCGCG ATG CAC GTG 57
Met His Val
1

30 CGC TCA CTG CGA GCT GCG GCG CCG CAC AGC TTC GTG GCG CTC TGG GCA 105
Arg Ser Leu Arg Ala Ala Ala Pro His Ser Phe Val Ala Leu Trp Ala
5 10 15

35 CCC CTG TTC CTG CTG CGC TCC GCC CTG GCC GAC TTC AGC CTG GAC AAC 153
Pro Leu Phe Leu Leu Arg Ser Ala Leu Ala Asp Phe Ser Leu Asp Asn
20 25 30 35

40 GAG GTG CAC TCG AGC TTC ATC CAC CGG CGC CTC CGC AGC CAG GAG CGG 201
Glu Val His Ser Ser Phe Ile His Arg Arg Leu Arg Ser Gln Glu Arg
40 45 50

CGG GAG ATG CAG CGC GAG ATC CTC TCC ATT TTG GGC TTG CCC CAC CGC 249
Arg Glu Met Gln Arg Glu Ile Leu Ser Ile Leu Gly Leu Pro His Arg
55 60 65

45 CCG CGC CCG CAC CTC CAG GGC AAG CAC AAC TCG GCA CCC ATG TTC ATG 297
Pro Arg Pro His Leu Gln Gly Lys His Asn Ser Ala Pro Met Phe Met
70 75 80

50 CTG GAC CTG TAC AAC GCC ATG GCG GTG GAG GAG GGC GGC GGG CCC GGC 345

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	Leu	Asp	Leu	Tyr	Asn	Ala	Met	Ala	Val	Glu	Glu	Gly	Gly	Gly	Pro	Gly	
	85						90					95					
5	GGC	CAG	GGC	TTC	TCC	TAC	CCC	TAC	AAG	GCC	GTC	TTC	AGT	ACC	CAG	GGC	393
	Gly	Gln	Gly	Phe	Ser	Tyr	Pro	Tyr	Lys	Ala	Val	Phe	Ser	Thr	Gln	Gly	
	100					105					110					115	
10	CCC	CCT	CTG	GCC	AGC	CTG	CAA	GAT	AGC	CAT	TTC	CTC	ACC	GAC	GCC	GAC	441
	Pro	Pro	Leu	Ala	Ser	Leu	Gln	Asp	Ser	His	Phe	Leu	Thr	Asp	Ala	Asp	
					120					125					130		
15	ATG	GTC	ATG	AGC	TTC	GTC	AAC	CTC	GTG	GAA	CAT	GAC	AAG	GAA	TTC	TTC	489
	Met	Val	Met	Ser	Phe	Val	Asn	Leu	Val	Glu	His	Asp	Lys	Glu	Phe	Phe	
				135					140					145			
	CAC	CCA	CGC	TAC	CAC	CAT	CGA	GAG	TTC	CGG	TTT	GAT	CTT	TCC	AAG	ATC	537
	His	Pro	Arg	Tyr	His	His	Arg	Glu	Phe	Arg	Phe	Asp	Leu	Ser	Lys	Ile	
				150				155					160				
20	CCA	GAA	GGG	GAA	GCT	GTC	ACG	GCA	GCC	GAA	TTC	CGG	ATC	TAC	AAG	GAC	585
	Pro	Glu	Gly	Glu	Ala	Val	Thr	Ala	Ala	Glu	Phe	Arg	Ile	Tyr	Lys	Asp	
		165					170					175					
25	TAC	ATC	CGG	GAA	CGC	TTC	GAC	AAT	GAG	ACG	TTC	CGG	ATC	AGC	GTT	TAT	633
	Tyr	Ile	Arg	Glu	Arg	Phe	Asp	Asn	Glu	Thr	Phe	Arg	Ile	Ser	Val	Tyr	
	180					185					190					195	
30	CAG	GTG	CTC	CAG	GAG	CAC	TTG	GGC	AGG	GAA	TCG	GAT	CTC	TTC	CTG	CTC	681
	Gln	Val	Leu	Gln	Glu	His	Leu	Gly	Arg	Glu	Ser	Asp	Leu	Phe	Leu	Leu	
					200					205					210		
35	GAC	AGC	CGT	ACC	CTC	TGG	GCC	TCG	GAG	GAG	GGC	TGG	CTG	GTG	TTT	GAC	729
	Asp	Ser	Arg	Thr	Leu	Trp	Ala	Ser	Glu	Glu	Gly	Trp	Leu	Val	Phe	Asp	
				215					220				225				
	ATC	ACA	GCC	ACC	AGC	AAC	CAC	TGG	GTG	GTC	AAT	CCG	CGG	CAC	AAC	CTG	777
	Ile	Thr	Ala	Thr	Ser	Asn	His	Trp	Val	Val	Asn	Pro	Arg	His	Asn	Leu	
				230				235					240				
40	GGC	CTG	CAG	CTC	TCG	GTG	GAG	ACG	CTG	GAT	GGG	CAG	AGC	ATC	AAC	CCC	825
	Gly	Leu	Gln	Leu	Ser	Val	Glu	Thr	Leu	Asp	Gly	Gln	Ser	Ile	Asn	Pro	
		245					250					255					
45	AAG	TTG	GCG	GGC	CTG	ATT	GGG	CGG	CAC	GGG	CCC	CAG	AAC	AAG	CAG	CCC	873
	Lys	Leu	Ala	Gly	Leu	Ile	Gly	Arg	His	Gly	Pro	Gln	Asn	Lys	Gln	Pro	
	260					265					270					275	
50	TTC	ATG	GTG	GCT	TTC	TTC	AAG	GCC	ACG	GAG	GTC	CAC	TTC	CGC	AGC	ATC	921
	Phe	Met	Val	Ala	Phe	Phe	Lys	Ala	Thr	Glu	Val	His	Phe	Arg	Ser	Ile	
					280					285					290		

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	CGG TCC ACG GGG AGC AAA CAG CGC AGC CAG AAC CGC TCC AAG ACG CCC	969
	Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser Lys Thr Pro	
	295 300 305	
5	AAG AAC CAG GAA GCC CTG CGG ATG GCC AAC GTG GCA GAG AAC AGC AGC	1017
	Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu Asn Ser Ser	
	310 315 320	
10	AGC GAC CAG AGG CAG GCC TGT AAG AAG CAC GAG CTG TAT GTC AGC TTC	1065
	Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr Val Ser Phe	
	325 330 335	
15	CGA GAC CTG GGC TGG CAG GAC TGG ATC ATC GCG CCT GAA GGC TAC GCC	1113
	Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu Gly Tyr Ala	
	340 345 350 355	
20	GCC TAC TAC TGT GAG GGG GAG TGT GCC TTC CCT CTG AAC TCC TAC ATG	1161
	Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn Ser Tyr Met	
	360 365 370	
25	AAC GCC ACC AAC CAC GCC ATC GTG CAG ACG CTG GTC CAC TTC ATC AAC	1209
	Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His Phe Ile Asn	
	375 380 385	
30	CCG GAA ACG GTG CCC AAG CCC TGC TGT GCG CCC ACG CAG CTC AAT GCC	1257
	Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln Leu Asn Ala	
	390 395 400	
35	ATC TCC GTC CTC TAC TTC GAT GAC AGC TCC AAC GTC ATC CTG AAG AAA	1305
	Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile Leu Lys Lys	
	405 410 415	
40	TAC AGA AAC ATG GTG GTC CGG GCC TGT GGC TGC CAC TAGCTCCTCC	1351
	Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His	
	420 425 430	
45	GAGAATTCAG ACCCTTTGGG GCCAAGTTTT TCTGGATCCT CCATTGCTCG CCTTGGCCAG	1411
	GAACCAGCAG ACCAACTGCC TTTTGTGAGA CCTTCCCCTC CCTATCCCCA ACTTTAAAGG	1471
	TGTGAGAGTA TTAGGAAACA TGAGCAGCAT ATGGCTTTTG ATCAGTTTTT CAGTGGCAGC	1531
	ATCCAATGAA CAAGATCCTA CAAGCTGTGC AGGCAAAACC TAGCAGGAAA AAAAAACAAC	1591
50	GCATAAAGAA AAATGGCCGG GCCAGGTCAT TGGCTGGGAA GTCTCAGCCA TGCACGGACT	1651
	CGTTTCCAGA GGTAATTATG AGCGCCTACC AGCCAGGCCA CCCAGCCGTG GGAGGAAGGG	1711
	GGCGTGGCAA GGGGTGGGCA CATTGGTGTC TGTGCGAAAAG GAAAATTGAC CCGGAAGTTC	1771
	CTGTAATAAA TGTCAACAATA AAACGAATGA ATGAAAAAAA AAAAAAAAAA A	1822

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(2) INFORMATION FOR SEQ ID NO:2:

- 5 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 431 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

- 10 (ii) MOLECULE TYPE: protein

- (ix) FEATURE:
 (D) OTHER INFORMATION: /Product="OP1-PP"

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

```

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

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Ser Val Tyr Gln Val Leu Gln Glu His Leu Gly Arg Glu Ser Asp Leu
 195 200 205
 5 Phe Leu Leu Asp Ser Arg Thr Leu Trp Ala Ser Glu Glu Gly Trp Leu
 210 215 220
 Val Phe Asp Ile Thr Ala Thr Ser Asn His Trp Val Val Asn Pro Arg
 225 230 235 240
 10 His Asn Leu Gly Leu Gln Leu Ser Val Glu Thr Leu Asp Gly Gln Ser
 245 250 255
 Ile Asn Pro Lys Leu Ala Gly Leu Ile Gly Arg His Gly Pro Gln Asn
 15 260 265 270
 Lys Gln Pro Phe Met Val Ala Phe Phe Lys Ala Thr Glu Val His Phe
 275 280 285
 20 Arg Ser Ile Arg Ser Thr Gly Ser Lys Gln Arg Ser Gln Asn Arg Ser
 290 295 300
 Lys Thr Pro Lys Asn Gln Glu Ala Leu Arg Met Ala Asn Val Ala Glu
 305 310 315 320
 25 Asn Ser Ser Ser Asp Gln Arg Gln Ala Cys Lys Lys His Glu Leu Tyr
 325 330 335
 Val Ser Phe Arg Asp Leu Gly Trp Gln Asp Trp Ile Ile Ala Pro Glu
 30 340 345 350
 Gly Tyr Ala Ala Tyr Tyr Cys Glu Gly Glu Cys Ala Phe Pro Leu Asn
 355 360 365
 35 Ser Tyr Met Asn Ala Thr Asn His Ala Ile Val Gln Thr Leu Val His
 370 375 380
 Phe Ile Asn Pro Glu Thr Val Pro Lys Pro Cys Cys Ala Pro Thr Gln
 385 390 395 400
 40 Leu Asn Ala Ile Ser Val Leu Tyr Phe Asp Asp Ser Ser Asn Val Ile
 405 410 415
 Leu Lys Lys Tyr Arg Asn Met Val Val Arg Ala Cys Gly Cys His
 45 420 425 430

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 50 (A) LENGTH: 1723 base pairs
 (B) TYPE: nucleic acid

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(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

5

(vi) ORIGINAL SOURCE:

(A) ORGANISM: Homo sapiens

(F) TISSUE TYPE: HIPPOCAMPUS

10

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 490..1696

(D) OTHER INFORMATION: /function= "OSTEOGENIC PROTEIN"

/product= "hOP2-PP"

15

/note= "hOP2 (cDNA)"

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

20	GGCGCCGGCA GAGCAGGAGT GGCTGGAGGA GCTGTGGTTG GAGCAGGAGG TGGCACGGCA	60
	GGGCTGGAGG GCTCCCTATG AGTGGCGGAG ACGGCCAGG AGGCGCTGGA GCAACAGCTC	120
	CCACACCGCA CCAAGCGGTG GCTGCAGGAG CTCGCCATC GCCCCTGCGC TGCTCGGACC	180
25	GCGGCCACAG CCGGACTGGC GGGTACGGCG GCGACAGAGG CATTGGCCGA GAGTCCAGT	240
	CCGCAGAGTA GCCCCGGCCT CGAGGCGGTG GCGTCCCGGT CCTCTCCGTC CAGGAGCCAG	300
30	GACAGGTGTC GCGCGGCGGG GCTCCAGGGA CCGCGCCTGA GGCCGGCTGC CCGCCCGTCC	360
	CGCCCCGCCC CGCCGCCCGC CGCCGCCCGA GCCCAGCCTC CTTGCCGTCG GGGCGTCCCC	420
	AGGCCCTGGG TCGGCCGCGG AGCCGATGCG CGCCCGCTGA GCGCCCCAGC TGAGCGCCCC	480
35	CGGCCTGCC ATG ACC GCG CTC CCC GGC CCG CTC TGG CTC CTG GGC CTG	528
	Met Thr Ala Leu Pro Gly Pro Leu Trp Leu Leu Gly Leu	
	1 5 10	
40	GCG CTA TGC GCG CTG GGC GGG GGC GGC CCC GGC CTG CGA CCC CCG CCC	576
	Ala Leu Cys Ala Leu Gly Gly Gly Gly Pro Gly Leu Arg Pro Pro Pro	
	15 20 25	
	GGC TGT CCC CAG CGA CGT CTG GGC GCG CGC GAG CGC CGG GAC GTG CAG	624
45	Gly Cys Pro Gln Arg Arg Leu Gly Ala Arg Glu Arg Arg Asp Val Gln	
	30 35 40 45	
	CGC GAG ATC CTG GCG GTG CTC GGG CTG CCT GGG CGG CCC CGG CCC CGC	672
50	Arg Glu Ile Leu Ala Val Leu Gly Leu Pro Gly Arg Pro Arg Pro Arg	
	50 55 60	

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	GCG CCA CCC GCC GCC TCC CGG CTG CCC GCG TCC GCG CCG CTC TTC ATG	720
	Ala Pro Pro Ala Ala Ser Arg Leu Pro Ala Ser Ala Pro Leu Phe Met	
	65 70 75	
5	CTG GAC CTG TAC CAC GCC ATG GCC GGC GAC GAC GAC GAG GAC GGC GCG	768
	Leu Asp Leu Tyr His Ala Met Ala Gly Asp Asp Asp Glu Asp Gly Ala	
	80 85 90	
10	CCC GCG GAG CGG CGC CTG GGC CGC GCC GAC CTG GTC ATG AGC TTC GTT	816
	Pro Ala Glu Arg Arg Leu Gly Arg Ala Asp Leu Val Met Ser Phe Val	
	95 100 105	
15	AAC ATG GTG GAG CGA GAC CGT GCC CTG GGC CAC CAG GAG CCC CAT TGG	864
	Asn Met Val Glu Arg Asp Arg Ala Leu Gly His Gln Glu Pro His Trp	
	110 115 120 125	
20	AAG GAG TTC CGC TTT GAC CTG ACC CAG ATC CCG GCT GGG GAG GCG GTC	912
	Lys Glu Phe Arg Phe Asp Leu Thr Gln Ile Pro Ala Gly Glu Ala Val	
	130 135 140	
25	ACA GCT GCG GAG TTC CGG ATT TAC AAG GTG CCC AGC ATC CAC CTG CTC	960
	Thr Ala Ala Glu Phe Arg Ile Tyr Lys Val Pro Ser Ile His Leu Leu	
	145 150 155	
30	AAC AGG ACC CTC CAC GTC AGC ATG TTC CAG GTG GTC CAG GAG CAG TCC	1008
	Asn Arg Thr Leu His Val Ser Met Phe Gln Val Val Gln Glu Gln Ser	
	160 165 170	
35	AAC AGG GAG TCT GAC TTG TTC TTT TTG GAT CTT CAG ACG CTC CGA GCT	1056
	Asn Arg Glu Ser Asp Leu Phe Phe Leu Asp Leu Gln Thr Leu Arg Ala	
	175 180 185	
40	GGA GAC GAG GGC TGG CTG GTG CTG GAT GTC ACA GCA GCC AGT GAC TGC	1104
	Gly Asp Glu Gly Trp Leu Val Leu Asp Val Thr Ala Ala Ser Asp Cys	
	190 195 200 205	
45	TGG TTG CTG AAG CGT CAC AAG GAC CTG GGA CTC CGC CTC TAT GTG GAG	1152
	Trp Leu Leu Lys Arg His Lys Asp Leu Gly Leu Arg Leu Tyr Val Glu	
	210 215 220	
50	ACT GAG GAC GGG CAC AGC GTG GAT CCT GGC CTG GCC GGC CTG CTG GGT	1200
	Thr Glu Asp Gly His Ser Val Asp Pro Gly Leu Ala Gly Leu Leu Gly	
	225 230 235	
55	CAA CGG GCC CCA CGC TCC CAA CAG CCT TTC GTG GTC ACT TTC TTC AGG	1248
	Gln Arg Ala Pro Arg Ser Gln Gln Pro Phe Val Val Thr Phe Phe Arg	
	240 245 250	
60	GCC AGT CCG AGT CCC ATC CGC ACC CCT CGG GCA GTG AGG CCA CTG AGG	1296
	Ala Ser Pro Ser Pro Ile Arg Thr Pro Arg Ala Val Arg Pro Leu Arg	
	255 260 265	

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5 AGG AGG CAG CCG AAG AAA AGC AAC GAG CTG CCG CAG GCC AAC CGA CTC 1344
 Arg Arg Gln Pro Lys Lys Ser Asn Glu Leu Pro Gln Ala Asn Arg Leu
 270 275 280 285
 CCA GGG ATC TTT GAT GAC GTC CAC GGC TCC CAC GGC CGG CAG GTC TGC 1392
 Pro Gly Ile Phe Asp Asp Val His Gly Ser His Gly Arg Gln Val Cys
 290 295 300
 10 CGT CGG CAC GAG CTC TAC GTC AGC TTC CAG GAC CTC GGC TGG CTG GAC 1440
 Arg Arg His Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Leu Asp
 305 310 315
 15 TGG GTC ATC GCT CCC CAA GGC TAC TCG GCC TAT TAC TGT GAG GGG GAG 1488
 Trp Val Ile Ala Pro Gln Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly Glu
 320 325 330
 20 TGC TCC TTC CCA CTG GAC TCC TGC ATG AAT GCC ACC AAC CAC GCC ATC 1536
 Cys Ser Phe Pro Leu Asp Ser Cys Met Asn Ala Thr Asn His Ala Ile
 335 340 345
 25 CTG CAG TCC CTG GTG CAC CTG ATG AAG CCA AAC GCA GTC CCC AAG GCG 1584
 Leu Gln Ser Leu Val His Leu Met Lys Pro Asn Ala Val Pro Lys Ala
 350 355 360 365
 TGC TGT GCA CCC ACC AAG CTG AGC GCC ACC TCT GTG CTC TAC TAT GAC 1632
 Cys Cys Ala Pro Thr Lys Leu Ser Ala Thr Ser Val Leu Tyr Tyr Asp
 370 375 380
 30 AGC AGC AAC AAC GTC ATC CTG CGC AAA GCC CGC AAC ATG GTG GTC AAG 1680
 Ser Ser Asn Asn Val Ile Leu Arg Lys Ala Arg Asn Met Val Val Lys
 385 390 395
 35 GCC TGC GGC TGC CAC T GAGTCAGCCC GCCCAGCCCT ACTGCAG 1723
 Ala Cys Gly Cys His
 400

(2) INFORMATION FOR SEQ ID NO:4:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 402 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: protein

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

50 Met Thr Ala Leu Pro Gly Pro Leu Trp Leu Leu Gly Leu Ala Leu Cys
 1 5 10 15

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

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Phe Asp Asp Val His Gly Ser His Gly Arg Gln Val Cys Arg Arg His
 290 295 300

5 Glu Leu Tyr Val Ser Phe Gln Asp Leu Gly Trp Leu Asp Trp Val Ile
 305 310 315 320

Ala Pro Gln Gly Tyr Ser Ala Tyr Tyr Cys Glu Gly Glu Cys Ser Phe
 325 330 335

10 Pro Leu Asp Ser Cys Met Asn Ala Thr Asn His Ala Ile Leu Gln Ser
 340 345 350

15 Leu Val His Leu Met Lys Pro Asn Ala Val Pro Lys Ala Cys Cys Ala
 355 360 365

Pro Thr Lys Leu Ser Ala Thr Ser Val Leu Tyr Tyr Asp Ser Ser Asn
 370 375 380

20 Asn Val Ile Leu Arg Lys Ala Arg Asn Met Val Val Lys Ala Cys Gly
 385 390 395 400

Cys His

25 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 102 amino acids
 (B) TYPE: amino acid
 30 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

35 (ix) FEATURE:
 (A) NAME/KEY: Protein
 (B) LOCATION: 1..102
 (D) OTHER INFORMATION: /label= OPX
 /note= "WHEREIN EACH XAA IS INDEPENDENTLY SELECTED
 40 FROM A GROUP OF ONE OR MORE SPECIFIED AMINO ACIDS
 AS DEFINED IN THE SPECIFICATION

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

45 Cys Xaa Xaa His Glu Leu Tyr Val Xaa Phe Xaa Asp Leu Gly Trp Xaa
 1 5 10 15

50 Asp Trp Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly
 20 25 30

- 53 -

Glu Cys Xaa Phe Pro Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala
 35 40 45
 5 Ile Xaa Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa Xaa Val Pro Lys
 50 55 60
 Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa
 65 70 75 80
 10 Asp Xaa Ser Xaa Asn Val Xaa Leu Xaa Lys Xaa Arg Asn Met Val Val
 85 90 95
 Xaa Ala Cys Gly Cys His
 100

15

(ix) FEATURE:

(A) NAME/KEY: Protein

(B) LOCATION: 1..102

(D) OTHER INFORMATION: /label= OPX

20

/note= "WHEREIN EACH XAA IS INDEPENDENTLY SELECTED
 FROM A GROUP OF ONE OR MORE SPECIFIED AMINO ACIDS
 AS DEFINED IN THE SPECIFICATION (SECTION II.B.2.)"

25

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

Cys Xaa Xaa His Glu Leu Tyr Val Xaa Phe Xaa Asp Leu Gly Trp Xaa
 1 5 10 15
 30 Asp Trp Xaa Ile Ala Pro Xaa Gly Tyr Xaa Ala Tyr Tyr Cys Glu Gly
 20 25 30
 Glu Cys Xaa Phe Pro Leu Xaa Ser Xaa Met Asn Ala Thr Asn His Ala
 35 40 45
 35 Ile Xaa Gln Xaa Leu Val His Xaa Xaa Xaa Pro Xaa Xaa Val Pro Lys
 50 55 60
 Xaa Cys Cys Ala Pro Thr Xaa Leu Xaa Ala Xaa Ser Val Leu Tyr Xaa
 40 65 70 75 80
 Asp Xaa Ser Xaa Asn Val Xaa Leu Xaa Lys Xaa Arg Asn Met Val Val
 85 90 95
 45 Xaa Ala Cys Gly Cys His
 100

(2) INFORMATION FOR SEQ ID NO:6:

50

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4 amino acids

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(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: peptide

(ix) FEATURE:

(A) NAME/KEY: Cleavage-site

(B) LOCATION: 1..4

10 (D) OTHER INFORMATION: /note= "PROTEOLYTIC CLEAVAGE SITE"

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

15 Arg Xaa Xaa Arg
1

- 55 -

What is claimed is:

- 1 1. An isolated binding partner having specific binding
2 affinity for an epitope on a soluble complex form
3 of an osteogenic protein,
4
5 said soluble complex form being characterized
6 as a dimeric protein comprising a pair of
7 polypeptide chain subunits associated to define a
8 dimeric structure capable of inducing endochondral
9 bone formation in a mammal when implanted in said
10 mammal in association with a matrix, each said
11 subunit having less than about 200 amino acids,
12
13 at least one of said subunits being
14 noncovalently complexed with a peptide comprising a
15 pro domain of a precursor form of an osteogenic
16 protein subunit, or an allelic, species, or
17 sequence variant thereof, to form a complex which
18 is more soluble in aqueous solvents than the
19 uncomplexed pair of subunits.
- 1 2. The isolated binding partner of claim 1 wherein
2 said binding partner is further characterized as
3 having substantially no binding affinity for the
4 mature, dimeric form of said osteogenic protein.
- 1 3. The isolated binding partner of claim 1 wherein
2 said binding partner is further characterized as
3 having substantially no binding affinity for the
4 isolated pro domain of one of said subunits.

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1 4. An isolated binding partner having specific binding
2 affinity for an epitope on a soluble complex form
3 of an osteogenic protein,
4

5 said complex being characterized as a dimeric
6 protein comprising a pair of polypeptide chain
7 subunits associated to define a dimeric structure
8 capable of inducing endochondral bone formation in
9 a mammal when implanted in said mammal in
10 association with a matrix, each said subunit having
11 less than about 200 amino acids,
12

13 at least one of said subunits being
14 noncovalently complexed with a peptide comprising a
15 pro domain of a precursor form of an osteogenic
16 protein subunit, or an allelic, species, or
17 sequence variant thereof, to form a complex which
18 is more soluble in aqueous solvents than the
19 uncomplexed pair of subunits,
20

21 said binding protein being characterized as
22 having affinity for said complex form and a said
23 pro domain of a precursor form, but not said
24 uncomplexed, dimeric protein.

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- 1 5. An isolated binding partner having specific binding
2 affinity for an epitope on a soluble complex form
3 of an osteogenic protein,
4
5 said complex being characterized as a dimeric
6 protein comprising a pair of protein subunits
7 associated to define a dimeric structure capable of
8 inducing endochondral bone formation in a mammal
9 when implanted in said mammal in association with a
10 matrix, each said subunit having less than about
11 200 amino acids,
12
13 at least one of said subunits being
14 noncovalently complexed with a peptide comprising a
15 pro domain of a precursor form of an osteogenic
16 protein subunit, or an allelic, species, or
17 sequence variant thereof, to form a complex which
18 is more soluble in aqueous solvents than the
19 uncomplexed pair of subunits,
20
21 said binding partner being characterized as
22 having binding affinity for said complex form and
23 said uncomplexed, dimeric form, but not a said
24 pro domain of a precursor form of one of said
25 subunits.
- 1 6. An isolated binding partner having specific binding
2 affinity for an epitope on a pro domain of an
3 osteogenic protein precursor form, said binding
4 partner having substantially no binding affinity
5 for the mature, dimeric form or the soluble complex
6 form of said osteogenic protein.

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1 7. An isolated binding partner having specific binding
2 affinity for an epitope on the mature dimeric form
3 of an osteogenic protein, said binding partner
4 having substantially no binding affinity for the
5 soluble complex form or the pro domain of a
6 precursor form of said osteogenic protein.

1 8. The binding partner of claim 1, 4, 5, 6 or 7
2 wherein said binding partner is an antibody.

1 9. The antibody of claim 8 wherein said antibody is a
2 monoclonal antibody.

1 10. The antibody of claim 8 wherein said antibody is a
2 polyclonal antibody.

1 11. The binding partner of claim 1, 4, 5, 6 or 7
2 wherein one said subunit of said dimeric osteogenic
3 protein is an OP-1 polypeptide chain including
4 allelic, species and sequence variants thereof.

1 12. The binding partner of claim 11 wherein the other
2 subunit of said dimeric osteogenic protein is
3 selected from the group consisting of OP1, BMP2,
4 BMP3 or BMP4, including allelic, species and
5 sequence variants thereof.

1 13. The binding partner of claim 11 wherein the other
2 subunit of said dimeric osteogenic protein is
3 selected from the group consisting of OP2, BMP5,
4 BMP6 or BMP9, including allelic, species and
5 sequence variants thereof.

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- 1 14. The binding partner of claim 11 wherein the other
2 subunit of said dimeric osteogenic protein is
3 selected from the group consisting of DPP, 60A,
4 Vgl, Vgr-1, including allelic, species and sequence
5 variants thereof.
- 1 15. The isolated binding partner of claim 1, 4, 5, 6
2 or 7 wherein said peptide comprises at least the
3 first 18 amino acids of an amino acid sequence
4 defining said pro domain.
- 1 16. The isolated binding partner of claim 15 wherein
2 said peptide comprises the full length form of said
3 pro domain.
- 1 17. The binding partner of claim 1, 4, 5, 6 or 7
2 wherein the pro domain peptide non-covalently
3 associated with said dimeric protein species
4 comprises an amino acid sequence selected from the
5 group consisting of OP1, OP2, BMP2, BMP3, BMP4,
6 BMP5, BMP6, DPP, Vgl, Vgr-1, 60A, including
7 allelic, species and sequence variants thereof.
- 1 18. The isolated binding partner of claim 1, 4, 5, 6 or
2 7 wherein said osteogenic protein is a synthetic
3 homolog of an osteogenic protein.
- 1 19. The isolated binding partner of claim 18 wherein
2 said synthetic homolog is selected from the group
3 consisting of COP 1, 3, 5, 7 and 16.
- 1 20. A method for specifically identifying a preselected
2 form of an osteogenic protein in solution, said
3 method comprising the steps of:

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4
5 (a) exposing a solution likely to contain said
6 osteogenic protein to a binding partner having
7 specific binding affinity for a said preselected
8 form of a said osteogenically active protein under
9 conditions to promote specific binding between said
10 preselected protein form and said binding partner
11 to form a complex, and
12
13 (b) detecting the complex formed.

1 21. The method of claim 20 wherein said binding partner
2 is an antibody.

1 22. The method of claim 21 wherein said antibody is a
2 monoclonal antibody.

1 23. The method of claim 21 wherein said antibody is a
2 polyclonal antibody.

1 24. The method of claim 20 wherein said binding partner
2 has means for detection.

1 25. The method of claim 24 wherein said means for
2 detection comprises an enzyme or radioactive atom.

1 26. The method of claim 20 wherein said complex is
2 detected by means of a second binding partner
3 having specificity for said preselected osteogenic
4 protein.

1 27. The method of claim 20 wherein said preselected
2 form is present in admixture with other forms of
3 said protein.

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1 28. The method of claim 20 comprising the additional
2 step of quantitating the amount of complex formed.

1 29. The method of claim 20 wherein said solution in
2 step (a) is exposed to a first immobilized binding
3 partner having specificity for said preselected
4 form under conditions sufficient to promote
5 specific binding interaction between said first
6 binding partner and said preselected osteogenic
7 protein form to form a complex, and
8
9 said step of detecting (b) comprises the step of
10 exposing said complex to a second binding partner
11 having binding specificity for one of the proteins
12 in said complex.

1 30. The method of claim 29 wherein said second binding
2 partner has specific binding affinity for said
3 preselected form of said osteogenic protein.

1 31. The method of claim 20 wherein said osteogenically
2 active protein is a dimeric protein comprising a
3 pair of disulfide bonded subunits, at least one of
4 said subunits comprising an OP-1 specific amino
5 acid sequence, including allelic, species and
6 sequence variants thereof.

1 32. The method of claim 31 wherein the other said
2 subunit comprises an amino acid sequence selected
3 from the group consisting of OP1, BMP2, BMP3 or
4 BMP4, including allelic, species and sequence
5 variants thereof.

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1 33. The method of claim 31 wherein the other said
2 subunit comprises an amino acid sequence selected
3 from the group consisting of OP2, BMP5, BMP6 or
4 BMP9, including allelic, species and sequence
5 variants thereof.

1 34. The method of claim 31 wherein the other said
2 subunit comprises an amino acid sequence selected
3 from the group consisting of DPP, 60A, Vgl, Vgr-1,
4 including allelic, species and sequence variants
5 thereof.

1 35. A kit for detecting a preselected form of an
2 osteogenically active protein in solution, the kit
3 comprising:

4
5 (a) means for capturing a fluid sample comprising
6 osteogenic protein,

7
8 (b) a binding partner having specific binding
9 affinity for a said preselected form of said
10 osteogenic protein, and

11
12 (c) means for detecting said binding partner bound
13 to said preselected osteogenic protein form.

1 36. The kit of claim 35 wherein said binding partner is
2 an antibody.

1 37. The kit of claim 35 wherein said fluid sample is a
2 culture medium.

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1 38. The kit of claim 35 wherein said means for
2 detecting (c) comprises a second binding partner
3 having specific binding affinity for said
4 preselected form of said osteogenic protein.

1 39. The kit according to claim 35 wherein said
2 osteogenically active protein is a dimeric protein
3 comprising a pair of disulfide bonded subunits, at
4 least one of said subunits comprising an OP-1
5 specific amino acid sequence, including allelic,
6 species and sequence variants thereof.

1 40. The kit according to claim 39 wherein said other
2 subunit is selected from the group consisting of
3 OP1, BMP2, BMP3 or BMP4, including allelic, species
4 and sequence variants thereof.

1 41. The kit according to claim 39 wherein said other
2 subunit is selected from the group consisting of
3 DPP, 60A, Vgl, Vgr-1, including allelic, species
4 and sequence variants thereof.

1 42. The kit according to claim 39 wherein said other
2 subunit is selected from the group consisting of
3 OP2, BMP5, BMP6 or BMP9, including allelic, species
4 and sequence variants thereof.

1/2

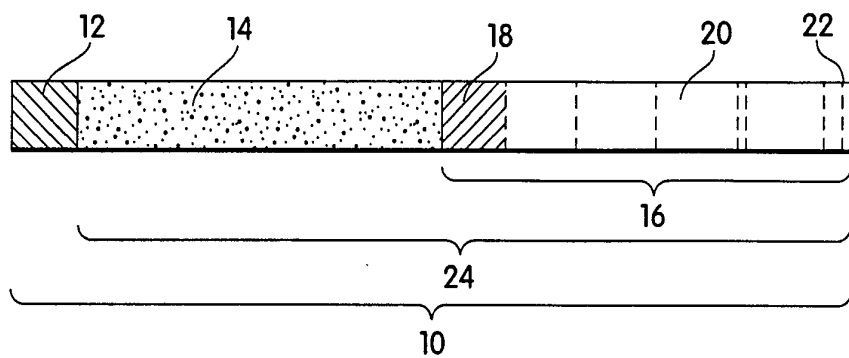


Fig. 1

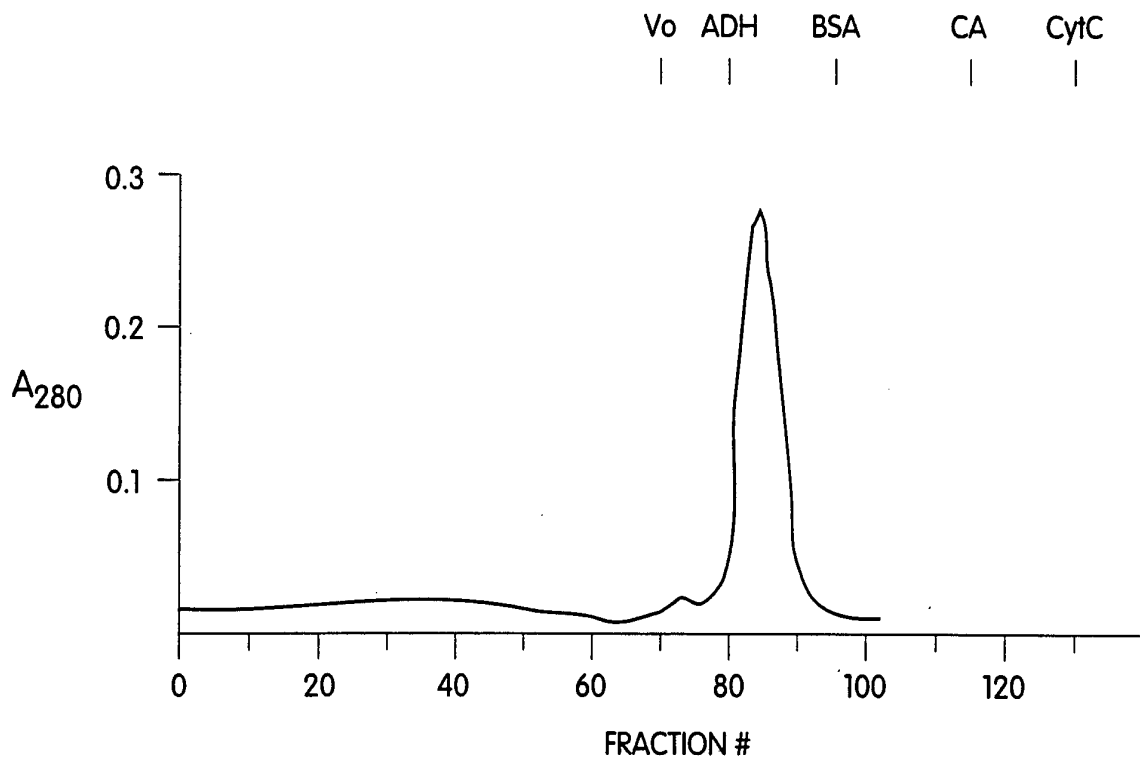


Fig. 3

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OP-2: RAPRSQQPFVVTFFRASPSPIRTTPRAVRPLRRRQPKKSNELPQANRLPGIFDDVHGSHGRQVC
 OP-1: RSIRSTGSKQORSQNRSKTPKNQEALRMANVAENSSSDQRQAC
 Vgr-1: RTTRSASSRRRQQSRNRSTQSDVSRGSGSSDYNGSELKTAC
 BMP-5: RSVRAAANKRKNQNRNKSSSHQDSSSRMSSVGDYNTSEQKQAC
 60A: RSKRSASHPRKRKKSVSPNNVPLLEPMESTRSC
 DPP: RSIRDVSGGEGGKGRNKRHARRPTRRKNHDDTC
 BMP-2: RHVRISRSLHQDEHSWSQIRPLLVTFGHDGKGHPLHK--REKRQAKH--KQRRLKSSC
 BMP-4: RISRSLPQSGNWAQLRPLLVTFGHDGRGHALTRRRRAKRSPKHHSQARAKKNKNC
 Vg-1: RCKRPRRKRSYSKLPFTASNIC
 BMP-3: RKKRSTGVLLPLQ.....KSKNKKKQKGPHRKSQTLQFDEQTLKKARRKQWIEPRNC

Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 94/02335

A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K15/00 C12P21/08 G01N33/577 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K C12P G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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X	DEVELOPMENTAL BIOLOGY vol. 151 , 1992 pages 491 - 505 J. DOCTOR ET AL. 'Sequence, biochemical characterisation and developmental expression of a new member of the TGF-beta superfamily in D. melanogaster' see page 496, column 2 - page 497, column 2, line 11 see page 501 --- -/--	1-18, 20-34

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

12 July 1994

Date of mailing of the international search report

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Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

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Skelly, J

INTERNATIONAL SEARCH REPORT

Inte onal Application No
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