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(54) **COMPOSITION FOR TREATING A FIBROTIC DISORDER COMPRISING AN INHIBITOR OF A BMP-1-LIKE PROTEIN**

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(51) **Int. Cl.**
A61K 38/00	(2006.01)
A61K 38/55	(2006.01)
A61K 38/16	(2006.01)
C07K 14/00	(2006.01)
C07K 14/81	(2006.01)
C12N 15/62	(2006.01)

(52) **U.S. Cl.** **514/1.1**; 514/9.4; 514/20.1; 514/21.2; 530/350; 435/69.7

(58) **Field of Classification Search** .... None
See application file for complete search history.

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(74) *Attorney, Agent, or Firm*—Quarles & Brady LLP

(57) **ABSTRACT**

α₂-macroglobulin-related agents for treating or preventing a fibrotic disorder associated with fibrillogenesis are disclosed along with methods for using the agents, as well as methods for producing agents suited for use in the disclosed methods for treating or preventing a fibrotic disorder.

9 Claims, 10 Drawing Sheets

α_2M		^{667}PQLQQYEMHGPEGLRVGFYES	......	DVMGRGHARLVHVEEPHT^{705}	SEQ ID NO:1
b-α_2M		PQFWDFTLDDGPFMMN	......	DEEASGADTSGFLDPDSVTPTYSAMV	SEQ ID NO:2
GDF11		LDLHDFQG	......	DALQPEDF	SEQ ID NO:3
Myostatin		IDQYDVQR	......	DDSSDGSL	SEQ ID NO:4
Procollagens	α1 (I)	DGGRYYRA	......	DDANVVRD	SEQ ID NO:5
	α2 (I)	YDGDFYRA	......	DQPRSAPS	SEQ ID NO:6
	α1 (II)	DPLQYMRA	......	DQAAGGLR	SEQ ID NO:7
	α1 (III)	GGFAPYYG	......	DEPMDFKI	SEQ ID NO:8
	α2 (V)	DPLPEFTE	......	DQAAPDDK	SEQ ID NO:9
	α1 (VII)	RPLPSYAA	......	DTAGSQLH	SEQ ID NO:10
Laminin 5 γ2 chain		DTGDCYSC	......	DENPDIEC	SEQ ID NO:11
Probiglycan		DDGPFMMN	......	DEEASGAD	SEQ ID NO:12
Chordin					
N-terminal site		DPEHRSYS	......	DRGEPGVG	SEQ ID NO:13

687 688

FIG. 1

FIG. 2A

FIG. 2B   **FIG. 2C**

WB: α FLAG
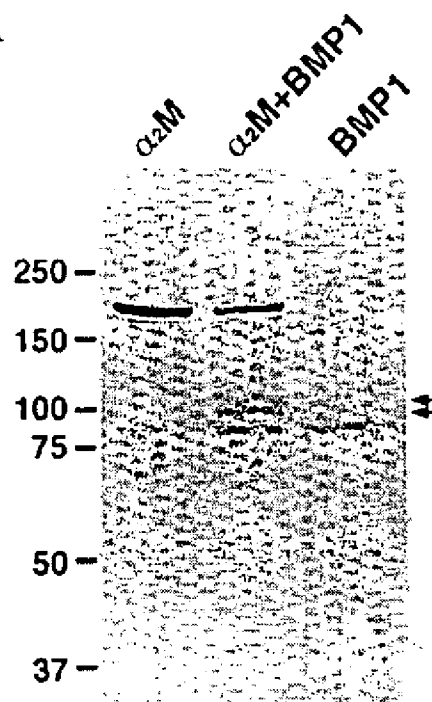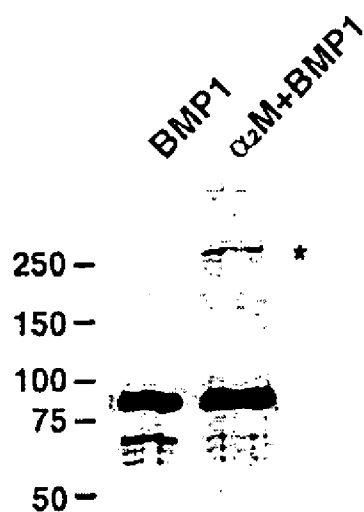
FIG. 2D

FIG. 2E
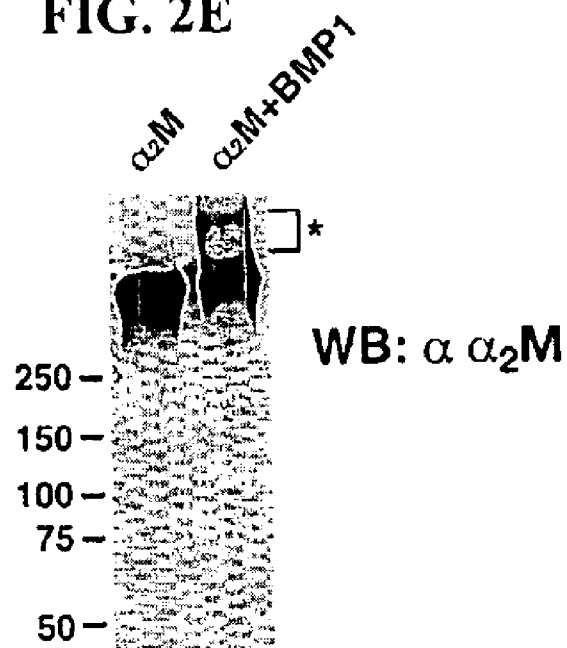
FIG. 3A
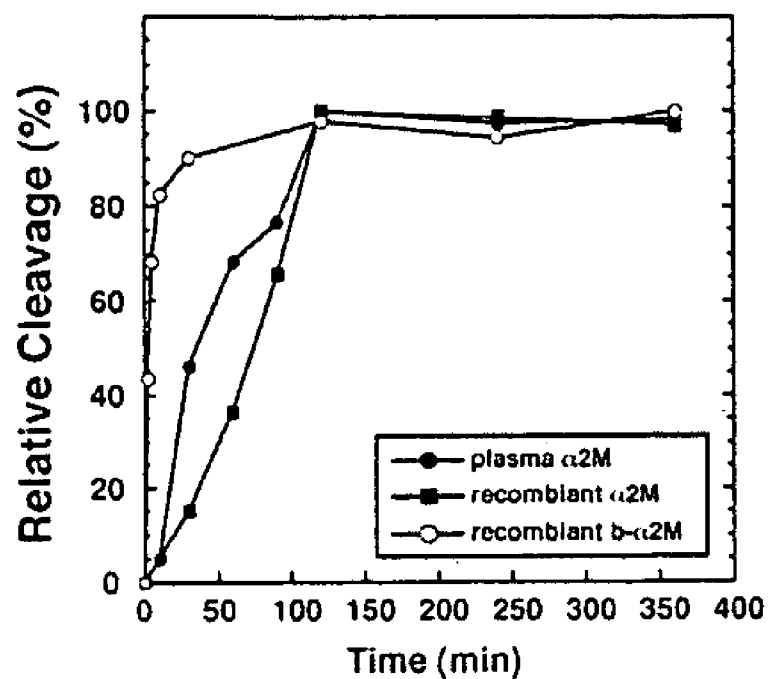
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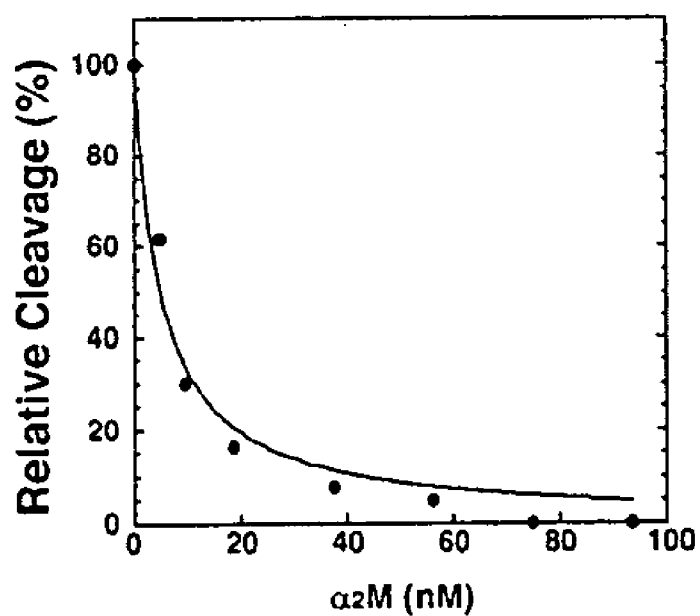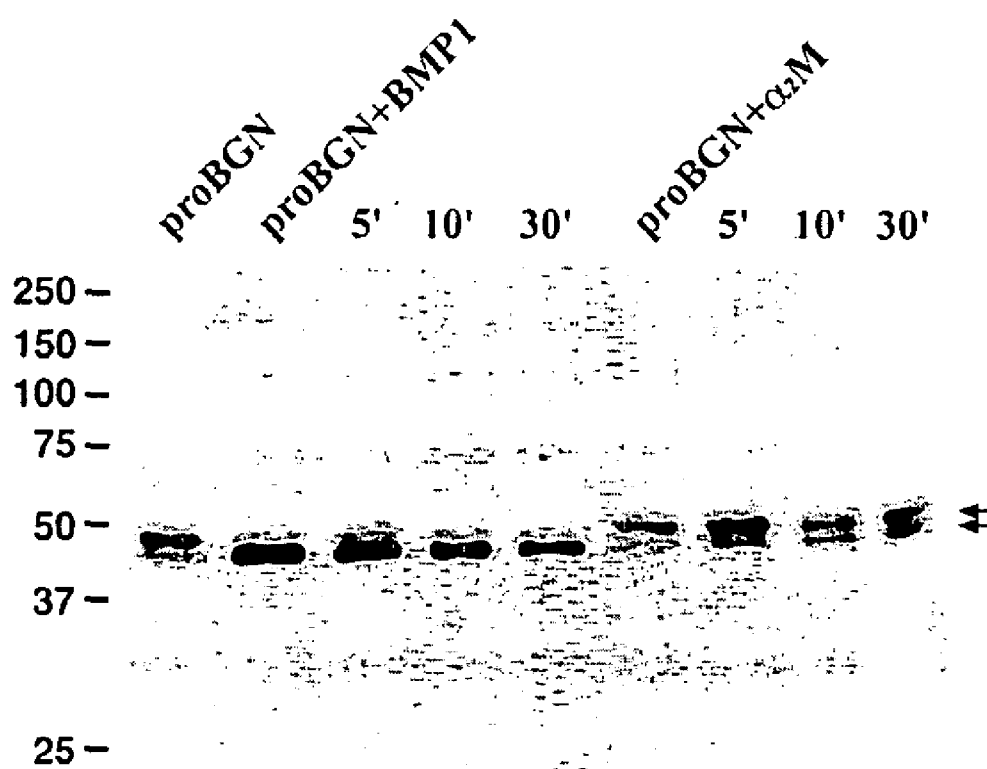
FIG. 4

FIG. 5A
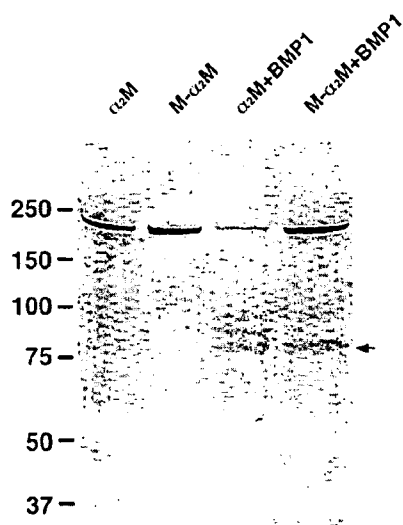
FIG. 5B
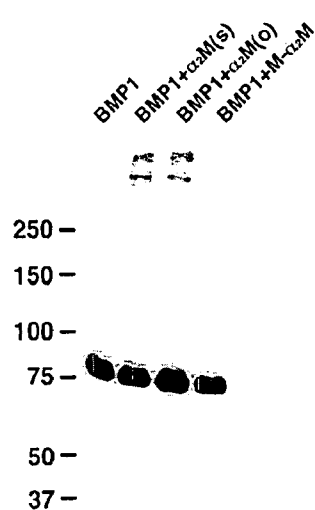
FIG. 5C
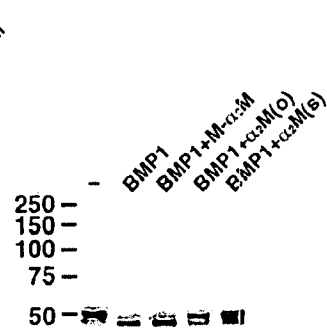
FIG. 6A
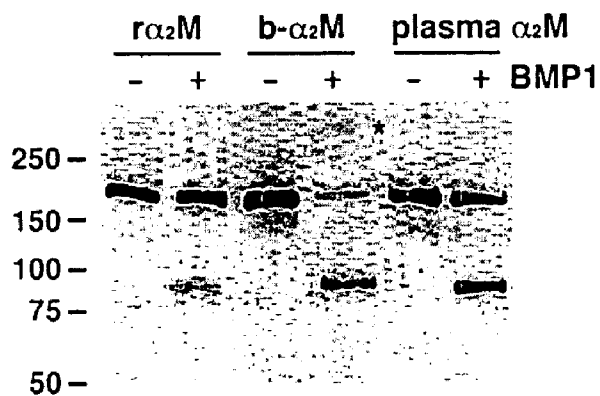
FIG. 6D
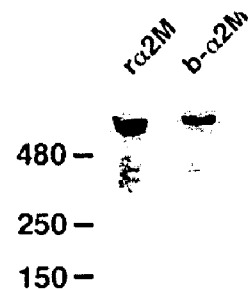
FIG. 6B
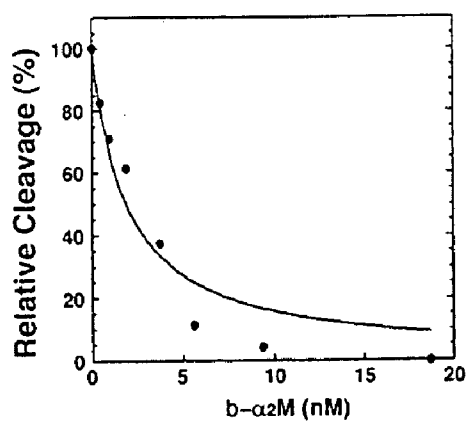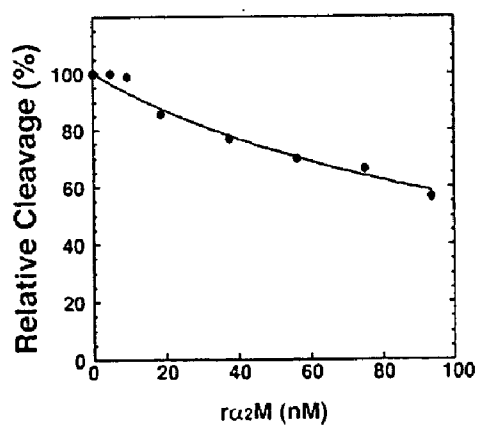
FIG. 6E
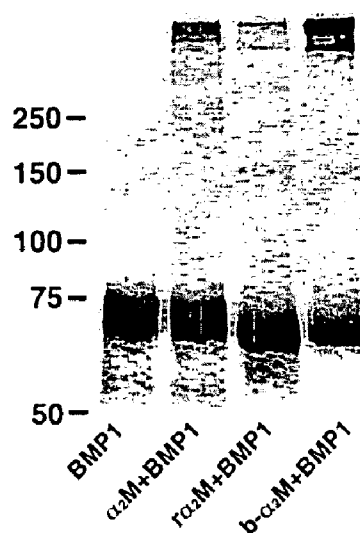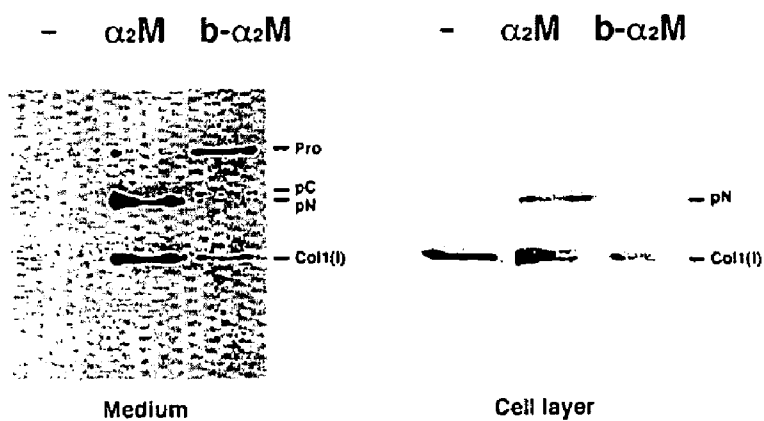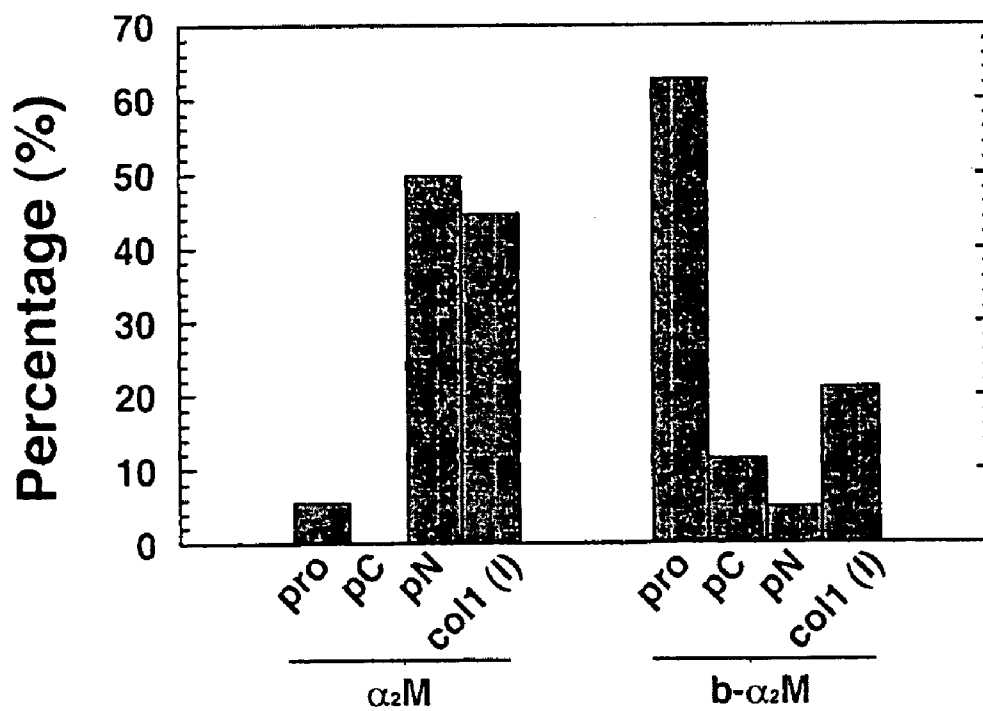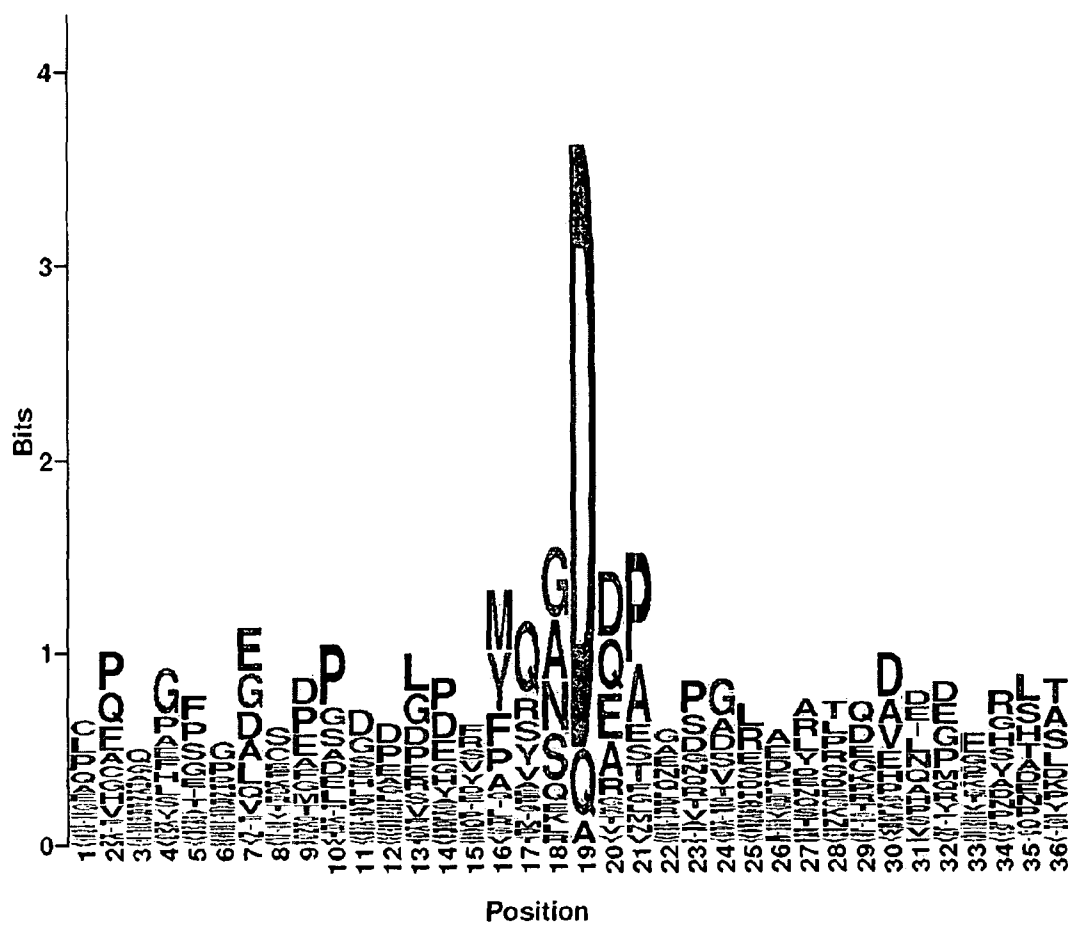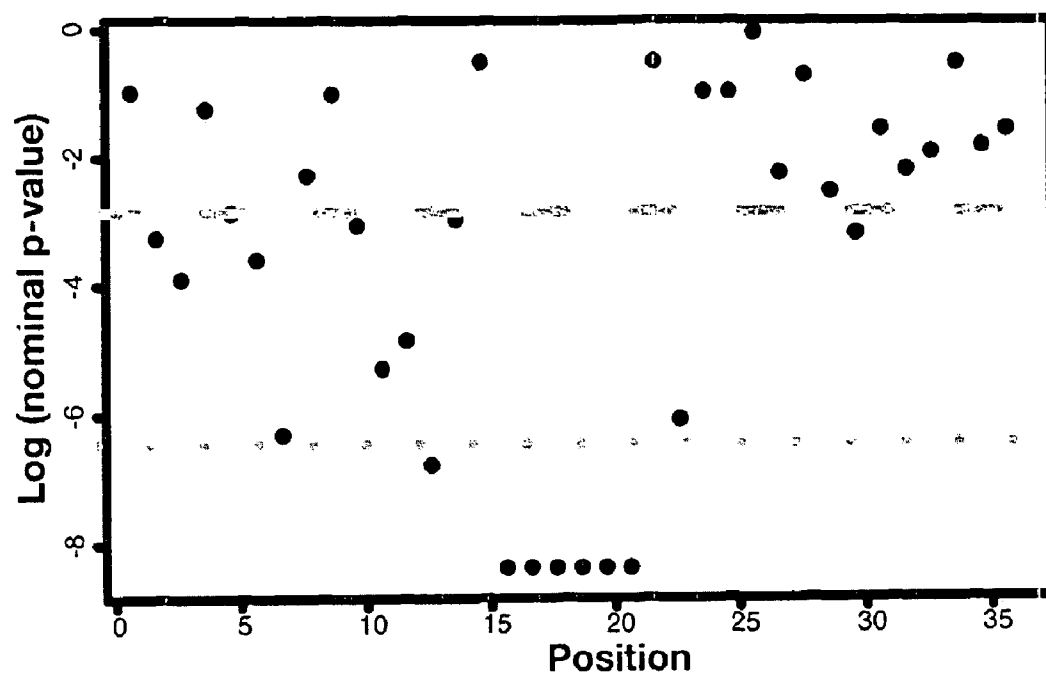
FIG. 6C

FIG. 7A

Medium   Cell layer

FIG. 7B

FIG. 8A

FIG. 8B

COMPOSITION FOR TREATING A FIBROTIC DISORDER COMPRISING AN INHIBITOR OF A BMP-1-LIKE PROTEIN

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Patent Application No. 60/839,019, filed Aug. 21, 2006, incorporated herein by reference as if set forth in its entirety.
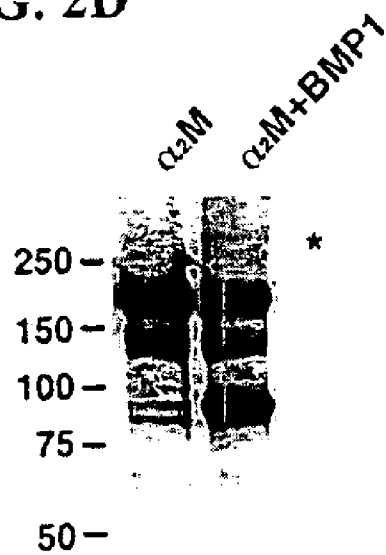
STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States government support awarded by the following agencies: NIH GM063471, GM071679 and AR047746. The United States government has certain rights in this invention.

BACKGROUND

α_2-macroglobulin (α_2M) is a member of an α-macroglobulin family of proteins found in plasma and egg whites of a broad range of animal species. α_2M is present in human plasma at relatively high levels (i.e., 2-4 mg/ml) and is produced by several cell types, such as hepatocytes, lung fibroblasts, macrophages, astrocytes and tumor cells. Borth W, "Alpha 2-macroglobulin. A multifunctional binding and targeting protein with possible roles in immunity and autoimmunity," Ann. N.Y. Acad. Sci. 737:267-272 (1994), incorporated herein by reference as if set forth in its entirety.

α_2M was initially thought to function in plasma and tissue as a humoral defense barrier that binds host or foreign peptides and particles via exposed 39 amino acid "bait regions" present on each of four identical 185 kDa subunits. Each bait region contains sites at which various proteinases, or other nucleophiles, can cleave the subunits, thereby activating α_2M by changing the subunit conformation, and exposing a highly reactive internal thioester. Borth W, "Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics," FASEB J. 6:3345-3353 (1992); and Sottrup-Jensen L, "Alpha-macroglobulins: structure, shape, and mechanism of proteinase complex formation," J. Biol. Chem. 264:11539-11542 (1989). The thioester covalently binds and entraps the proteinase or nucleophile, inhibiting further activity by steric hindrance. For example, α_2M inhibits metalloproteinases belonging to a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family, see Tortorella M, et al., "Alpha 2-macroglobulin is a novel substrate for ADAMTS-4 and ADAMTS-5 and represents an endogenous inhibitor of these enzymes," J. Biol. Chem. 279:17554-17561 (2004), but was previously reported that it does not inhibit proteinases with astacin-like protease domains, see Baker A, et al., "Metalloproteinase inhibitors: biological actions and therapeutic opportunities," J. Cell Sci. 115:3719-3727 (2002).

Activated α_2M is also a targeting carrier for cytokines or growth factors (e.g., TGF-β, PDGF; IL-1β, basic FGF and NGF) involved in modulating biological responses of various cell types. The cytokine or growth factor dissociates either on the cell surface of a cell expressing the low-density lipoprotein receptor-related protein (LPR) or in an endocytic compartment within the cell expressing the LPR. Activated α_2M binds to LRP, which results in rapid clearance of α_2M-proteinase complexes from the plasma or extracellular space for subsequent catabolism. In addition, α_2M functions as a protective factor against many pathogens by binding to certain peptides (e.g. toxins or cell surface proteins) of some parasites, bacteria and viruses.

Bone morphogenetic protein-1 (BMP-1) is a prototype of a subgroup of structurally similar, secreted metalloproteinases having an astacin-like protease domain, CUB protein-protein interaction domains and EGF motifs. Bond J & Benyon R, "The astacin family of metalloendopeptidases," Protein Sci. 4:1247-1261 (1995). In mammals, members of this subgroup proteolytically cleave precursors into mature, extracellular matrix-(ECM) forming proteins. Members of this subgroup also activate some members of the TGF-β superfamily in a broad range of species by cleaving extracellular protein antagonists. Thus, mammalian BMP-1-like proteinases (i.e., BMP-1, mammalian Tolloid (mTLD) and mammalian Tolloid-like 1 and 2 (mTLL-1 and mTLL-2, respectively)) are likely to be involved in forming ECM and in signaling by certain TGF-β-like molecules in morphogenetic events and homeostasis.

BMP-1 and Tolloid-like proteinases have a distinct protein domain, structure that includes (starting at the N-terminus) a signal peptide (for secretion), a prodomain (that must be cleaved to activate the proteinase), a conserved protease domain found in the astacin M12A family of metzincin metalloproteases, and then a number of CUB and EGF-like protein-protein interaction domains. See Ge G & Greenspan D, "Developmental roles of the BMP1/TLD metalloproteinases," Birth Defects Res. (Part C) 78:47-68 (2006); and Hopkins D, et al., "The bone morphogenetic protein 1/Tolloid-like metalloproteinases," Matrix Biol. (Epub ahead of print, May 18, 2007), each of which is incorporated herein by reference as if set forth in its entirety. These proteinases play diverse roles in morphogenetic events, via biosynthetic cleavage of precursors into mature functional proteins involved in formation of the ECM, and via activation of certain members of the TGFβ superfamily of growth factors. Greenspan D, "Biosynthetic processing of collagen molecules," Top. Curr. Chem. 247:149-183 (2005).

In particular, BMP-1-like proteinases (1) process type I-III procollagen C-propeptides to yield the major fibrous components of ECM; (2) cleave a zymogen to produce active lysyl oxidase enzyme that catalyzes covalent cross-linking in collagen fibers; and (3) process procollagen N-propeptides, and in some cases C-propeptides, of the minor fibrillar collagen types V and XI. Ge G, et al., "Bone morphogenetic protein-1/tolloid-related metalloproteinases process osteoglycin and enhance its ability to regulate collagen fibrillogenesis," J. Biol. Chem. 279:41626-41633 (2004). The latter are incorporated into growing fibrils of collagen types I and II, respectively, and appear to control the geometries of the resulting heterotypic fibrils.

Because BMP-1-like proteinases provide most, if not all procollagen C-proteinase (pCP) activity in vivo, and because C-propeptide removal is essential for collagen fibrillogenesis, the BMP-1-like proteinases are attractive targets for therapeutic interventions where inhibition of collagen fibrillogenesis is desirable. Although the formation of collagen fibrils is essential to morphogenesis and to healing of wounds and bone fractures in the adult, excessive formation of fibrous collagenous ECM causes much morbidity in the general population. These conditions include keloids (excessive skin scarring), surgical adhesions, and deep-seated fibroses of organs including lungs, liver and kidneys. The deep-seated fibroses are particularly ominous, as the replacement of parenchymal tissue by scar tissue composed essentially of fibrous, collagenous ECM destroys organ function.

Accordingly, there is a need for new methods and compositions for treating fibrotic disorders, particularly those that inhibit the activities of BMP-1-like proteinases.

BRIEF SUMMARY

In a first aspect, the present invention is summarized as a method of inhibiting a BMP-1-like proteinase in a human or non-human animal experiencing or susceptible to a fibrotic disorder caused by the BMP-1-like proteinase that includes administering to the animal an amount of an inhibitor of a BMP-1-like proteinase effective to reduce BMP-1-like proteinase activity in the animal, where the reduction is characterized by a reduction in severity or occurrence of the fibrotic disorder.

In some embodiments of the first aspect, the administered inhibitor is natural or modified α_2M, and in related embodiments the α_2M is modified-relative to natural α_2M in that in place of a native bait region, a bait region of probiglycan, a small leucine-rich proteoglycan precursor, is present.

In a second aspect, the present invention is summarized as a method for engineering an inhibitor of a BMP-1-like proteinase from a naturally-occurring α_2M protein having a bait region that includes substituting the bait region with a bait region from a protein other than α_2M protein that is cleaved by BMP-1 to produce an engineered α_2M protein. In a related embodiment, the other protein can be a probiglycan, and can be a probiglycan from a human. The source of the naturally occurring α_2M protein is not critical, as long as the engineered protein contains a bait region that can be cleaved by a BMP-1-like proteinase.

These and other features, aspects and advantages of the present invention will become better understood from the description that follows. In the description, reference is made to the accompanying drawings, which form a part hereof and in which there is shown by way of illustration, not limitation, embodiments of the invention. The description of preferred embodiments is not intended to limit the invention but rather to cover all modifications, equivalents and alternatives. Reference should therefore be made to the claims recited herein for interpreting the scope of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

The embodiments described herein will be better understood and features, aspects and advantages other than those set forth above will become apparent when consideration is given to the following detailed description thereof. Such detailed description makes reference to the following drawings, wherein:

FIG. 1 shows α_2M as a candidate substrate for cleavage by BMP-1-like proteinases. The bait region from human α_2M (residues 667-705) is aligned with the cleavage sites of a number of protein substrates of BMP-1-like proteinases, including growth differentiation factor 11 (GDF-11), myostatin, procollagens I-III and the pro-$\alpha2$(V) and pro-$\alpha1$ (VII) procollagen chains, a $\gamma2$ chain of laminin 5, probiglycan and an N-terminal cleavage site of chordin immediately beneath the α_2M sequence is the probiglycan sequence that substitutes for the native bait region in a mutant α_2M (b-α_2M). Vertical arrows mark the sites of potential ADAMTS-2 cleavage sites in α_2M and b-α_2M. Aspartate residues conserved at the P1' positions of the various scissile bonds are in boldface, as are aromatic side chains, previously noted NH_2-terminal to scissile bonds in many previously identified substrates of BMP-1-like proteinases. Phe^{684}, Tyr^{685} and Asp^{688} of the α_2M sequence are similarly presented in boldface;

FIG. 2 shows that BMP-1 cleaves and forms a complex with α_2M. (FIG. 2A) Electrophoretic patterns on an SDS-PAGE gel are compared for human α_2M incubated in the absence (α_2M) or presence (α_2M+BMP-1) of BMP-1. BMP-1 alone (BMP-1) was electrophoresed in the final lane, to act as a marker for the corresponding band in the α_2M+BMP-1 lane. The gel was stained with Coomassie Brilliant Blue R-250. The arrows denote the cleavage products of α_2M. Numbers to the left of the gel correspond to the positions and approximate sizes, in kDa, of molecular mass markers. (FIGS. 2B and 2C) Western blots, using anti-Flag antibodies for the detection of tagged BMP-1, of α_2M incubated alone (α_2M) or with BMP-1 (α_2M+BMP-1). BMP-1 starting material [BMP-1(s)] or BMP-1 incubated in the absence of α_2M [BMP-1(0)], were co-electrophoresed as controls. SDS-PAGE of samples was carried out under reducing (FIG. 2B) or non-reducing (FIG. 2C) conditions. Numbers to the left of the blots correspond to the positions and approximate sizes, in kDa, of molecular mass markers. Western blots probed with anti-α_2M antibodies show α_2M to co-localize to the same high molecular weight forms as BMP-1 under both reducing (FIG. 2D) and non-reducing (FIG. 2E) conditions;

FIG. 3 shows the time- and dose-dependent inhibition of BMP-1 procollagen C-proteinase activity by native and recombinant forms of α_2M. (FIG. 3A) 30 nM BMP-1 was incubated with 30 nM α_2M for 0, 10, 30, 60, 90, 120, 240, and 360 min (plasma α_2M and recombinant wild type α_2M) or for 0, 2, 5, 10, 30, 120, 240, and 360 min (b-α_2M) at 37° C. Cleavage reaction samples were analyzed by SDS-PAGE on 7.5% acrylamide gels, followed by Western blotting with anti-α_2M antibodies, quantification with NIH Image software, and plotting of relative percentage cleavage of α_2M vs time in minutes. (FIG. 3B) 9.4 nM BMP- was preincubated with 0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM α_2M for 2 hours at 37° C., followed by incubation with 400 ng ^3H-radiolabeled type I procollagen. Cleavage reaction samples were analyzed by SDS-PAGE on 5% gels, followed by scanning of autofluorograms, quantification of bands, using NIH Image software, and plotting of relative percent cleavage of procollagen vs nM concentration of α_2M. Non-linear regression was performed using SigmaPlot;

FIG. 4 shows that α_2M inhibits probiglycan cleavage by BMP-1. Western blot analysis, using anti-human biglycan antibody LF-51 (available from National Institutes of Health) raised against amino acids 27-40 of SEQ ID NO:2 (GVLD-PDSVTPTYSA)—the mature region of biglycan—was employed to monitor the processing of probiglycan to biglycan by incubation for 5, 10 or 30 minutes with BMP-1 (proBGN+BMP-1) or with BMP-1 preincubated with α_2M (proBGN+α_2M). The upper and lower arrows denote probiglycan and mature biglycan, respectively. Numbers to the left of the blot correspond to the positions and approximate sizes, in kDa, of molecular mass markers;

FIG. 5 shows that methylamine-treated α_2M is neither cleaved nor complexed by BMP-1 and does not inhibit cleavage of probiglycan by BMP-1. (FIG. 5A) Electrophoretic patterns on an SDS-PAGE gel are compared for human α_2M preincubated either alone (α_2M) or with methylamine (M-α_2M) and then incubation of BMP-1 with either the untreated (α_2M+BMP-1) or methylamine-treated (M-α_2M+BMP-1) α_2M samples. The gel was stained with Coomassie Brilliant Blue R-250. The arrow denotes BMP-1. Numbers to the left of the blot and gel correspond to the positions and approximate sizes, in kDa, of molecular mass markers. (FIG. 5B) A western blot, using anti-Flag antibodies to detect tagged BMP-1, of BMP-1 alone (BMP-1) or BMP-1 incubated with untreated α_2M [BMP-1+α_2M(s)] or with α_2M

preincubated in the absence [BMP-1+α_2M(o)] or presence [BMP-1+M-α_2M] of methylamine. (FIG. **5**C) Western blot analysis using LF-51 antibody was employed to monitor the processing of probiglycan to biglycan by incubation alone (−), with BMP-1 (BMP-1), or with BMP-1 preincubated with α_2M [BMP-1+α_2M(s)] or with BMP-1 preincubated with α_2M that had itself been preincubated either in the presence (BMP-1+M-α_2M) or absence [BMP-1+α_2M(0)] of methylamine. Numbers to the left of the blot correspond to the positions and approximate sizes, in kDa, of molecular mass markers;

FIG. **6** shows that substituting the probiglycan bait region sequences in place of the native α_2M bait region enhances α_2M inhibition of BMP-1~25-fold. (FIG. **6**A) A Western blot probed with anti-α_2M is shown of plasma α_2M, or recombinant wild type α_2M (rα_2M) or mutant (b-α_2M) α_2M incubated in the presence (+) or absence (−) of BMP-1. (FIGS. **6**B and **6**C) 9.4 nM BMP-1 was preincubated with 0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM wild type α_2M (FIG. **6**B) or 0, 0.47, 0.94, 1.87, 3.75, 5.62, 9.4, or 18.7 nM b-α_2M (FIG. **6**C) for 2 hours at 37° C., followed by incubation with 400 ng ^{3}H-radiolabeled type I procollagen. Cleavage reaction samples were analyzed by SDS-PAGE on 5% gels, followed by scanning of autofluorograms, quantification of bands, using NIH Image software, and plotting of relative percent cleavage of substrate vs nM concentration of α_2M. Non-linear regression was performed using SigmaPlot. (FIG. **6**D) A Western blot is shown of recombinant wild type (rα_2M) or mutant (b-α_2M) α_2M cross-linked with glutaraldehyde. (FIG. **6**E) An autofluorogram shows the SDS-PAGE electrophoretic patterns of ^{35}S-radiolabeled BMP-1 incubated alone, or in the presence of plasma α_2M (α_2M) or recombinant wild type α_2M (rα_2M) or mutant (b-α_2M) α_2M. Numbers to the left of the autofluorogram correspond to the positions and approximate sizes, in kDa, of molecular mass markers;

FIG. **7** shows that α_2M can inhibit procollagen processing by cells. (FIG. **7**A) Western blots of media and cell layer samples of MC-3T3 cells incubated in the presence of 20 nM α_2M or b-α_2M, or an equivalent volume of buffer (−). Blots were probed with anti-α1(I) C-telopeptide antibodies. (FIG. **7**B) The media sample blot was scanned and quantified using NIH Image software to provide the graft shown in which the percentage of total signal each for the wild-type α_2M lane and for the b-α_2M lane is given for uncleaved pro-α1(I) chains (pro), pCα1(I) (pC) and pNα1(I) (pN) processing intermediates, and completely processed mature α1(I) chains [col1 (I)]; and

FIG. **8** shows the relative conservation of amino acid residues flanking BMP-1 cleavage sites. (FIG. **8**A) Sequence logo of the thirty-six positions around the cleavage site. The sequence logo was produced by the WebLogo application of Crooks et al. for sequences flanking the BMP-1 cleavage sites of twenty-six published and unpublished substrates. Crooks et al., "WebLogo: a sequence logo generator," Genome Res. 14:1188-1190 (2004). Height of each letter is proportional to the corresponding amino acid and the overall height of each stack is proportional to the sequence conservation at that position. Sequence conservation, measured in bits, has a maximum theoretical value of 4.32. The cleavage site resides between positions eighteen and nineteen. (FIG. **8**B) Logged nominal p-values of the goodness of fit tests across the positions around the cleavage site. For each position, a chi-square goodness-of-fit test is employed for testing the null hypothesis that all amino acids have equal probability of occurrence at that site. Plotted are nominal p-values of the tests in the log 10 scale. Horizontal dashed gray lines correspond to p-value

cut-offs of 0.05 and 0.05/36, respectively. The second cut-off is the Bonferroni adjusted multiple testing cut-off for controlling the overall Type-I error rate at the nominal level of 0.05.

DESCRIPTION OF PREFERRED EMBODIMENTS

Disclosed herein is a site for cleavage of α_2M by some astacin-like proteinases, particularly BMP-1. See also, Zhang Y, et al., "Inhibition of bone morphogenetic protein 1 by native and altered forms of alpha2-macroglobulin," J. Biol. Chem. 281:39096-39104 (2006), incorporated herein by reference as if set forth in its entirety. Cleavage at this site by BMP-1 results in formation of covalent complexes with cleaved α_2M and potent inhibition of BMP-1 proteolytic activities. Also, a modified α_2M, containing the probiglycan BMP-1 cleavage site in place of the native bait region, has enhanced ability to inhibit BMP-1 and to inhibit procollagen I processing by cells.

One inhibits a BMP-1-like proteinase such as BMP-1, mTLD, mTLL-1 or mTLL-2 in a human or non-human animal by administering an amount of an inhibitor of a BMP-1-like proteinase effective to reduce BMP-1-like proteinase activity in the animal. BMP-1-like proteinase activity is inhibited relative to pre-administration activity by at least 60%, alternatively by at least 75%, alternatively by at least 80%, alternatively by at least 85% and alternatively by at least 90%. The route of delivery varies depending upon the type of disorder being treated. That is, the inhibitor is administered topically for disorders such as keloids or for preventing excess scarring after cornea surgery or after other surgeries as well. Other routes of delivery include inhalation for pulmonary fibrosis. Furthermore, systemic delivery is contemplated for a surgical adhesion or for deep-seated fibroses of an organ. Preferred inhibitor delivery routes are topical and intravenous. The skilled artisan will appreciate the desirability of non-invasive administration of the agent. For topical delivery, creams, lotions or ointments comprising the inhibitor are a preferred delivery vehicle. For intravenous administration, the agent is provided in a pharmaceutically acceptable carrier, such as saline.

The inhibitor is natural or modified α_2M. As described further below, the modified α_2M is modified relative to natural α_2M in that in place of the native bait region, a bait region of probiglycan, a small leucine-rich proteoglycan precursor, is present. The number of doses a human or non-human animal receives, the time allowed between doses and the length of time the human or non-human animal receives a natural or modified α_2M can depend on the severity of the fibrotic disorder. Dosage level and the time between doses can be modified in accord with clinical assessment of the human or non-human animal.

It is contemplated that the methods described herein can be used with any fibrotic disorder, especially those disorders involving deposition of collagen I. Examples of fibrotic disorders include, but are not limited to, a keloid or other abnormal wound healing, a surgical adhesion or deep-seated fibroses of an organ.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the embodiments, the preferred methods and materials are now described.

As used herein, an "effective amount" refers to that concentration of α_2M or of a modified α_2M that is effective in attenuating fibrillogenesis and, hence, a fibrotic disorder.

As used herein, a "BMP-1-like proteinase" refers to a proteinase including, without limitation, BMP-1, mTLD, mTLL-1 and mTLL-2 that possesses an astacin-like protease domain, CUB protein-protein interaction domains and EGF motifs.

The invention embraced by the claims recited below will be more fully understood upon consideration of the following non-limiting Examples.

EXAMPLES

Example 1

Inhibition of BMP-1 by α_2M

Methods:

Cleavage of α_2M by BMP-1: 200 nM Flag-tagged recombinant BMP-1, prepared and purified as previously described, was incubated overnight at 37° C. with 200 nM human α_2M (Sigma-Aldrich; St. Louis, Mo.) in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$. Scott I, et al., "Mammalian BMP-1/Tolloid-related metalloproteinases, including novel family member-mammalian Tolloid-like 2, have differential enzymatic activities and distributions of expression relevant to patterning and skeletogenesis," Dev. Biol. 213:283-300 (1999). Subsequently, reaction proteins were analyzed by SDS-PAGE under reducing conditions on a 7.5% gel and staining with Coomassie Brilliant Blue R-250. For Western blot analysis, 320 nM BMP-1 was incubated with 200 nM α_2M at 37° C. overnight, and the samples were loaded on a 7.5% gels and separated by SDS-PAGE, under both nonreducing and reducing conditions. Separated proteins were transferred to polyvinylidene difluoride membranes and probed with a 1:5,000 dilution of anti-FLAG antibody (Sigma-Aldrich). Subsequently, membranes were incubated with a 1:25,000 dilution of goat anti-mouse IgG HRP conjugate as the secondary antibody.

For a dose-dependence study, α_2M was pre-incubated with BMP-1 for 2 hours at 37° C. The study of time-dependent inhibition of BMP-1 by α_2M indicates that complex formation between α_2M and BMP-1 is complete (reaches equilibrium) after 2 hours pre-incubation, under the conditions used. Thus, the rate of procollagen processing is proportional to the amount of active BMP-1 remaining uncomplexed to α_2M.

Time-Dependent Inhibition of BMP-1 Cleavage of Probiglycan by α_2M: 15 ng BMP-1 (9.4 nM) was preincubated with or without 5 times the amount of α_2M (47.0 nM) in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM $CaCl_2$ for 2 hours at 37° C. Subsequently, 450 ng probiglycan, prepared as previously described in Scott et al., was added to a final volume of 20 μl and incubated overnight. Scott I, et al., "Bone morphogenetic protein-1 processes probiglycan," J. Biol. Chem. 275:30504-30511 (2000). Cleavage reactions were quenched by adding 4 μl chondroitinase ABC (a mixture of 10 μl 0.01 U/μl protease-free chondroitinase ABC (Seikaguku Corp.; Tokyo, Japan), 40 μl 6× chondroitinase buffer (100 mM Tris-HCl, pH 8.0, 240 mM NaAc, 0.25 mM EDTA) and 10 μl 500 mM EDTA), followed by incubation at 37° C. for 4 hours. Samples were subjected to SDS-PAGE on a 10% gel and Western blot analysis was performed, using anti-probiglycan antibody LF51 at a 1:5,000 dilution, and a 1:25,000 dilution of goat-anti-rabbit IgG horse radish peroxidase (HRP) conjugate, as the secondary antibody.

Inactivation of α_2M by Methylamine: 1 mg/ml α_2M was incubated with 50 mM Tris-HCl, pH 7.1 in the presence or absence of 20 mM methylamine 20 hours, followed by dialysis against 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. Both methylamine-treated and control α_2M samples were tested for ability to be cleaved by BMP-1 and for ability to inhibit BMP-1 cleavage of probiglycan.

Production of recombinant α_2M: Human α_2M (NM_000014; SEQ ID NO:14 and SEQ ID NO:15) sequences were PCR-amplified from human placenta cDNA in three fragments. The following primers were used: fragment 1 (amino acids 24-416 of SEQ ID NO:15), 5'-GCTAGCAGACTA-CAAAGACGATGACGACAAGT-CAGTCTCTGGAAAACCGCAGTAT-3' (SEQ ID NO:16; forward) and 5'-CAGTAAGAGAGGTACCCATAAC-3' (SEQ ID NO:17; reverse); fragment 2 (amino acids 416-1052 of SEQ ID NO:15), 5'-GTTATGGGTACCTCTCGTTACTG-3' (SEQ ID NO:18; forward) and 5'-ATGTAGGCTCGAGCT-TGGGC-3' (SEQ ID NO:19; reverse); fragment 3 (amino acids 1052-1474 of SEQ ID NO:15), 5'-GCCCAAGCTC-GAGCCTACAT-3' (SEQ ID NO:20; forward) and 5-GCG-GCCGCTCAAGCATTTrCCAAGATCTTTGCTG-3' (SEQ ID NO: 21; reverse).

PCR products were assembled into the full-length α_2M sequence into pBlueScript II KS+ (Stratagene), and cloned downstream of (and adjoined via an NheI site to) BM40/SPARC signal peptide sequences, between the HindIII and NotI of the tetracycline-inducible expression vector pcDNA4/TO (Invitrogen Corp.; Carlsbad, Calif.). The resulting construct expresses full-length α_2M, except that the native signal peptide is replaced by the BM40 signal peptide, for optimization of secretion. In addition, upon cleavage of the signal peptide, a FLAG epitope remains at the NH_2-terminus of α_2M sequences.

To generate mutant α_2M, an MfeI site was introduced into the 5'-end of α_2M bait region sequences, without changing the encoded protein, via two-step PCR. Primers used were: primer 1: 5'-GATATGTACAGCTTCCTAGAGGA-3' (SEQ ID NO:22); primer 2: 5'-GTTGCAATTGTGGACA-CATTTTGGGTTTACGA-3' (SEQ ID NO:23); primer 3: 5'-TCCACAATTGCAACAGTATGAAATGCATGGACCT-3' (SEQ ID NO:24); and primer 4: 5'-CTTTCGTACG-GTCTCCGTGTGA-3' (SEQ ID NO:25). PCR amplicons of primer 1 and 2, 3 and 4 were then used as templates for PCR amplification using primers 1 and 4. The resulting amplicon was cloned between BsrG I and BsiW I sites in the α_2M sequence.

Biglycan sequences were PCR amplified with primers 5'-GAGAGAATTCTGGGACTTCACCCTGGACGA-3' (SEQ ID NO:26; forward) and 5'-GAAAGGTACCATG-GCGCTGTAGGTGGGTGT-3' (SEQ ID NO:27; reverse). The amplicon was then digested with EcoRI and KpnI, and cloned between MfeI and BsiWI sites in the modified α_2M sequences. In the resulting mutant α_2M (b-α_2M) the sequences flanking the human probiglycan BMP-1 cleavage site substitute for the bait region (see FIG. 1).

293 T-REx cells (Invitrogen Corp.) were maintained in Dulbecco's Modified Eagle's medium (DMEM), with 5 μg/ml blasticidin and 10% fetal bovine serum (FBS). Cells at 80% confluence were transfected with 1 μg expression plasmid/35 mm culture dish using Lipofectamine (Invitrogen Corp.). After 48 hours, cells were selected in the same type of medium supplemented with 200 μg/ml Zeocin. Production of secreted α_2M, upon induction with 1 μg/ml tetracycline, was detected via Western blot.

Confluent cells were washed twice with phosphate buffered saline (PBS) and incubated in serum-free DMEM 15

minutes at 37° C. Cells were then washed once with PBS, followed by addition of serum-free DMEM containing 1 µg/ml tetracycline, to induce protein expression, and 40 µg/ml soybean trypsin inhibitor. Conditioned medium was harvested every 24 hours, and protease inhibitors were added to final concentrations of 5 mM EDTA, 1 mM phenylmethyl-sulfonyl fluoride, 1 mM N-ethylmaleimide, and 1 mM p-aminobenzoic acid. Conditioned medium was centrifuged to remove debris and supernatants were stored at −70° C. FLAG-tagged α_2M and b-α_2M were affinity-purified from conditioned medium using an anti-FLAG M2 column (Sigma-Aldrich), following manufacturer's instructions.

Cross-linking: 6.9 nM recombinant α_2M or b-α_2M were incubated 5 minutes with 80 mM glutaraldehyde (Sigma) in PBS at room temperature. Cross-linking was terminated by adding glycine to a final concentration of 0.2 M. Samples were separated by SDS-PAGE on 4-15% acrylamide gradient gels and were subjected to immunoblotting.

Determination of K_i Values and second-order Rate Constants for BMP-1-α_2M Interactions: Confluent 293 T-Rex cells producing recombinant BMP-1 were washed twice with PBS, and incubated 15 minutes in scrum-free, Cys/Met-free DMEM at 37° C. Cells were then washed once with PBS, followed by addition of serum-free DMEM containing 1 µg/ml tetracycline, to induce protein expression, 40 µg/ml soybean trypsin inhibitor and 60 µCi/ml Pro-Mix ^{35}S cell labeling mix (Amersham). Conditioned medium was harvested every 24 hours, and protease inhibitors were added to final concentrations of 1 mM phenylmethylsulfonyl fluoride, 1 mM N-ethylmaleimide, and 1 mM p-aminobenzoic acid. Metabolically ^{35}S-radiolabeled BMP-1 was affinity-purified from conditioned medium using an anti-FLAG M2 column (Sigma), following manufacturer's instructions.

40 nM ^{35}S-labeled BMP-1 was incubated with 20, 40, 80, 160, and 240 nM plasma α_2M, or recombinant wild type α_2M or with 5, 10, 20, 40, and 80 nM b-α_2M at 37° C. in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl and 10 mM CaCl$_2$. Reactions were stopped at 10, 30, 60, 90 and 120 minutes (plasma α_2M and recombinant wild type α_2M), or at 2, 5, 10, 20 and 30 minutes (b-α_2M) by adding. SDS-PAGE sample buffer and boiling 5 minutes at 95° C. Protein samples were separated on 7.5% acrylamide SDS-PAGE gels, which were then treated with EN^{3}HANCE (DuPont) and exposed to film. Percentages of BMP-1 incorporated into high molecular weight complexes were determined by densitometry and kinetic parameters were determined essentially via the method of Enghild J, et al, "Interaction of human rheumatoid synovial collagenase (matrix metalloproteinase 1) and stromelysin (matrix metalloproteinase 3) with human alpha 2-macroglobulin and chicken ovostatin. Binding kinetics and identification of matrix metalloproteinase cleavage sites," J. Biol. Chem. 264:8779-8785 (1989).

Inhibition of Collagen Processing by Cells: 2×10^5 MC-3T3-E1 cells were plated in a 24 well plate, allowed to attach overnight, and then treated with 50 µg/ml ascorbate in DMEM 10% FBS for 24 hours. Cells were washed twice with PBS, and incubated in serum-free DMEM 15 minutes at 37° C. Cells were then washed once with PBS, followed by addition of serum-free DMEM containing 50 µg/ml ascorbate, 40 µg/ml soybean trypsin inhibitor, and 20 nM α_2M or b-α_2M, or an equivalent volume of buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). Conditioned medium was harvested after 24 hours as described above. Cell layers were washed twice with ice-cold PBS, and scraped into hot SDS-sample buffer. Medium and cell layer samples were subjected to SDS-PAGE on acrylamide gels, transferred to nitrocellulose membranes

and probed with anti-collagen α1(I) C-telopeptide polyclonal antibody LF67 (a generous gift from Larry Fisher), as described.

Amino Acid Specificity Analysis of Identified Cleavage Sites of BMP-1-like Proteinases: We investigated the amino acid sequence specificity of the cleavage sites of twenty-six substrates of BMP-1-like proteinases by considering a thirty-six amino acid region around these sites. The sequence logo of the sequences is given in FIG. 8A, in which the cleavage site is between positions 18 and 19. It is easily observed that positions around the cleavage site exhibit bias towards certain amino acids. We further tested, for each of the thirty-six positions, the null hypothesis that the amino acid composition of the site follows a random amino acid distribution, which assigns an equal occurrence probability to each amino acid. The nominal p-values from the chi-square goodness-of-fit test of each position are plotted in FIG. 8B on the log 10 scale. P-value cut-offs of 0.05 and 0.05/36 are marked as gray dashed horizontal lines on this plot. The second cut-off is the Bonferroni adjusted multiple testing cut-off that provides the control of the overall Type-I error rate at level 0.05. We observe that positions 13, 16, 17, 18, 19, 20 and 21 exhibit strong evidence against the null hypothesis of equal amino acid frequency. Furthermore, although positions 7 and 23 do not pass the Bonferroni adjusted p-value cut-off, they have quite small p-values (0.0016 and 0.002, respectively).

Results:

BMP-1 Cleaves α_2M: Perusal of the amino acid sequence of the α_2M bait region found a site comprising residues Ser^{687}, Asp^{688} and surrounding residues that resembled the majority of known BMP-1 cleavage sites (FIG. 1). The resemblance resided primarily in the placement of Phe^{684} and Tyr^{686} 3 and 4 residues, respectively, NH$_2$-terminal to Asp^{688}, since residues with aromatic side chains are frequently found in positions P2-P5, and an Asp is almost always found in the P1' position of previously identified substrates of BMP-1-like proteinases (FIG. 1). Moreover, the majority of previously characterized cleavage sites of BMP-1-like proteinases have residues with small side chains in the P1 positions (FIG. 1), such that the placement of α_2M Ser^{687} in relationship to Asp^{688}, Phe^{684} and Tyr^{686} is also reminiscent of a BMP-1 cleavage site.

As can be seen (FIG. 2A), α_2M incubated alone at 37° C. overnight was stable. In contrast, 185-kDa α_2M incubated at 37° C. overnight in the presence of BMP-1 was cleaved to produce two bands of ~100- and ~85-kDa. NH$_2$-terminal amino acid sequencing of the 85-kDa band yielded the sequence SVSGKPQYMV (SEQ ID NO:28), which corresponds to the NH$_2$-terminus of secreted α_2M. NH$_2$-terminal amino acid sequencing of the 100-kDa band yielded the sequence DVMGRGHXR (SEQ ID NO:29, wherein X represents an unidentified residue), thus demonstrating that BMP-1 cleaves α_2M at the predicted site between Ser^{687} and Asp^{688} within the bait region.

Cleaved α_2M Forms a Complex with BMP-1: As can be seen (FIG. 2B), in the presence of α_2M, BMP-1, which is normally detected on SDS-PAGE gels as a ~90-kDa monomer can be detected as a ~270-kDa form on a reduced gel. This result is consistent with the interpretation that a single cleaved 100-kDa C-terminal portion of α_2M forms a ~190-kDa covalent complex with a single molecule of BMP-1. Under non-reducing conditions (FIG. 2C), BMP-1 can be detected as even higher molecular weight forms. The 85-kDa NH$_2$-terminal and the 100-kDa COOH-terminal fragments of cleaved α_2M can remain covalently bound, via disulfide linkage, and this form can be linked to other 85-kDa and 185-kDa forms via disulfide bonds. Thus, the high molecular weight

forms observed under non-reducing conditions likely represent BMP-1 covalently bound, via thioester, to 185-, 270- and 375-kDa disulfide bonded oligomers, although the exact identity of each band on the non-reducing-gel remains somewhat speculative. The above interpretations are consistent with Western blots probed with anti-α_2M antibodies, which show α_2M to co-localize to the same high molecular weight forms as BMP-1 under both reducing (FIG. 2D) and non-reducing (FIG. 2E) conditions. The observation here of covalent binding of BMP-1 to the 100-kDa α_2M cleavage product is consistent with the mechanism whereby α_2M has been found to covalently bind other proteinases that it inhibits.

α_2M Inhibits the pCP Activity of BMP-1: Since the first identified, and best characterized activity of BMP-1 is as a pCP, Kessler E, et al., "Bone morphogenetic protein-1: the type I procollagen C-proteinase," Science 271:360-362 (1996), we next sought to determine whether this activity is inhibited in the presence of α_2M. Control experiments demonstrated that BMP-1 achieves maximum cleavage of plasma α_2M by 2 hours (FIG. 3A). Thus, to gauge the effect of α_2M-BMP-1 interaction on BMP-1 pCP activity, a constant amount of BMP-1 (9.4 nM) was preincubated 2 hours with increasing concentrations of α_2M (0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM), prior to incubation with ^{3}H-radiolabeled type I procollagen. Reaction mixtures were subjected to SDS-PAGE and cleavage of procollagen was measured by densitometric analysis of autofluorograms. As can be seen (FIG. 3B), prior incubation with α_2M led to potent inhibition of BMP-1 pCP activity, with a calculated IC_{50} of 4.8 nM.

α_2M Inhibits the Cleavage of Probiglycan by BMP-1: As can be seen (FIG. 4), whereas BMP-1 was able to completely convert probiglycan to biglycan after 30 minutes under assay conditions, preincubation with a five-fold molar excess of α_2M for 2 hours prior to incubation with probiglycan resulted in inhibition of a majority of probiglycan processing. Thus, α_2M appears to be a general inhibitor of BMP-1 activity against various substrates.

Mechanism of α_2M Inhibition of BMP-1: As can be seen, BMP-1 was unable to cleave methylamine-treated α_2M (FIG. 5A). In addition, α_2M pretreated with methylamine did not form complexes with BMP-1 (FIG. 5B). Furthermore, α_2M pretreated with methylamine was unable to inhibit the processing of probiglycan to biglycan by BMP-1 (FIG. 5C). Inability of BMP-1 to cleave methylamine-treated α_2M was probably the consequence of the conformational change induced in α_2M by interaction of methylamine with the α_2M thioester. Subsequent to this conformational change, the bait region is presumably not available to proteinases for cleavage. Together, these results bolster the conclusion that inhibition and complexing of BMP-1 by α_2M involves formation of a thioester bond between BMP-1 and α_2M, and a consequent α_2M conformational change.

Example 2

Efficiency of b-α_2M

Methods and Results:

b-α_2M has enhanced ability to inhibit BMP-1: We previously noted wide differences in the efficiency (e.g., percent cleavage and rate of cleavage) with which different substrates art cleaved by BMP-1 (unpublished data). One of the substrates processed most efficiently by BMP-1 is probiglycan. We therefore sought to determine whether we could enhance the ability of α_2M to inhibit BMP-1 by replacing the native bait region with sequences surrounding the probiglycan scissile bond (see FIG. 1).

As can be seen (FIG. 6A), b-α_2M is cleaved by BMP-1 more readily than recombinant wild-type α_2M (rα_2M). When the pCP inhibitory activities of varying concentrations of rα_2M and b-α_2M were measured (FIGS. 6B and 6C), they led to IC_{50} values of 133 nM and 1.88 nM, respectively. The IC_{50} value of 133 nm for rα_2M suggests considerably less effectiveness in BMP-1 inhibition than the 4.82 value obtained for serum α_2M (FIG. 3B, Table I); whereas the b-α_2M IC_{50} value of 1.88 is consistent with increased inhibitory effectiveness. Although cross-linking experiments demonstrated both recombinant wild type α_2M and b-α_2M to form tetramers (FIG. 6D), some small difference in folding and/or post-translational modification appears to render the recombinant wild type protein somewhat less effective at BMP-1 inhibition than the corresponding protein from plasma. Importantly however, b-α_2M is shown to have markedly improved efficiency in inhibiting BMP-1 compared to wild type α_2M prepared under identical conditions, or compared to plasma α_2M. The improved efficiency of interaction of b-α_2M with BMP-1, compared to wild type α_2M, is further illustrated by comparing the rapidity with which b-α_2M is cleaved by BMP-1 compared to cleavage of wild type recombinant or plasma α_2M (FIG. 3A).

To obtain a quantitative comparison of the rates of interaction of BMP-1 with the various wild type and mutant forms of α_2M, we employed the methodology of Enghild et al., supra, which involves quantification of covalent proteinase-α_2M complex formation, subsequent to incubation of radiolabeled proteinase with α_2M. FIG. 6E shows that subsequent to 2 hour co-incubation of 40 nM ^{35}S-radiolabeled BMP-1 with 40 nM α_2M, considerably more BMP-1 is incorporated into complexes with b-α_2M (42% of sample), than with wild type recombinant (12% of sample) or plasma (28% of sample) α_2M. Time course experiments conducted with a fixed amount of ^{35}S-radiolabeled BMP-1 (40 nM) incubated with increasing amounts of each form of α_2M, as in Enghild et al., supra, provided K_i values of 25.2, 39.5, and 36.4 nM for b-α_2M, and wild type recombinant and plasma α_2M, respectively. Second order rate constants obtained from the same data showed b-α_2M to be 23.69-fold more effective in interacting with BMP-1 than was recombinant α_2M prepared under identical conditions and 15.69-fold more effective than wild type α_2M from plasma (Table I).

TABLE I

	Relative BMP-1 inhibitory effectiveness of plasma and recombinant α_2M forms			
	K_i (nM)	k_2 (s^{-1})	2nd-order rate constant ($M^{-1}s^{-1}$)	Relative effectiveness
Plasma α_2M	25.2	0.20	8.10×10^6	1.51
Recombinant α_2M	39.5	0.21	5.36×10^6	1
Recombinant b-α_2M	36.4	4.60	1.07×10^8	23.69

α_2M Inhibition of Procollagen Processing by Cells: As α_2M is capable of inhibiting the pCP activity of BMP-1, we determined whether it might be able to inhibit the processing of procollagen by cells. Towards this end, MC-3T3-E1 murine osteoblastic cells were incubated either alone or in the presence of rα_2M or b-α_2M. The levels of processing of procollagen and insertion into the cell layer were compared. As can be seen (FIG. 7), MC-3T3 processing of procollagen was inhibited by both recombinant wild type (r) α_2M and mutant (b)-α_2M. However, in the case of media from rα_2M-treated cells, most detectable collagenous material was the in the form of processing intermediate pNα1(I) (in which the

N-, but not the C-propeptide is retained), or mature α1(I) chains; whereas, in media from b-α₂M-treated cells, most detectable collagenous material was in the form of unprocessed pro-α1(I) chains (FIGS. 7A and 7B). These results show efficient inhibition of cellular BMP-1-like proteins by b-α₂M, and less efficient inhibition by wild-type α₂M.

The appearances of pNα1(I) chains in the wild type α₂M-treated sample and of procollagen in the b-α₂M-treated sample indicate that both forms of α₂M are able to inhibit N-propeptide cleavage in cell culture by the proteinase ADAMTS-2. This is consistent with a previous report that α₂M is capable of inhibiting ADAMTS-2 in vitro. Colige A, et al., "Domains and maturation processes that regulate the activity of ADAMTS-2, a metalloproteinase cleaving the aminopropeptide of fibrillar procollagens types I-III and V," J. Biol. Chem. 280:34397-34408 (2005). b-α₂M may retain the ability to inhibit ADAMTS-2, as a potential ADAMTS-2 cleavage site at the N-terminus of the native bait region is retained in the b-α₂M sequence (FIG. 1).

Collagen was not detected in the media of untreated cells (FIG. 7A), presumably because of efficient processing and insertion of mature collagen into the cell layer. Untreated cell layers contained only fully processed mature α1(I) chains, whereas cell layers of cultures treated with either wild type α₂M or b-α₂M contained both mature α1(I) chains and pNα1 (I) forms (FIG. 7A). pCα1(I) forms (in which the C-, but not the N-propeptide is retained) were detected only in the media of b-α₂M-treated cultures (FIG. 7A), and were not found in the cell layers of any of the cultures, presumably because pCα1(I) chains are not inserted into ECM under normal circumstances. Treatment of MC-3T3-E1 cells with plasma α₂M (data not shown), yielded effects on procollagen processing similar to those obtained from treatment of cells with recombinant wild type α₂M.

Example 3

Prophetic

Treatment of Fibrotic Disorders

A subject experiencing or at risk for a fibrotic disorder, such as lung fibrosis, kidney fibrosis or keloids is adminis-

tered a an inhalant, injection or topical lotion (e.g., cream or ointment), respectively, comprising α₂M or a modified α₂M in an amount effective to reduce occurrence or severity of the fibrotic disorder. For example, a subject predisposed to keloids applies a topical cream, lotion or ointment comprising α₂M or a modified α₂M at least once a day. After treatment, fibrosis is prevented or reduced.

The invention can be tested by ascertaining anti-fibrotic effects in a model system in which pulmonary fibrosis is induced in mice via intratracheal (IT) instillation of bleomycin (2 U/kg). At 3 and 5 days following instillation with bleomycin, mice can be given IT installations of 40 nM wild type or altered α₂M (30 µl/mouse), or PBS, as a negative control. Two weeks after initial bleomycin instillation, lung tissue can be isolated and subjected to histological (via hematoxylin and eosin staining and light microscopy) and ultrastructural (electron microscopic) analyses to test for septal thickening and reduction of alveolar space, typical of pulmonary fibrosis. Similarly, Masson trichrome-staining of paraffin sections and a Sircol assay, can be performed to determine the extent of deposition of collagenous matrix in lung (a strong indicator of fibrosis). These and additional tests of fibrotic outcome can be performed essentially as described previously. See, e.g., Giri S, et al., "Antifibrotic effect of Decorin in a bleomycin hamster model of lung fibrosis," Biochem. Pharmacol. 54:1205-1216 (1997); and Avivi-Green C, et al., "Discoidin domain receptor 1-deficient mice are resistant to bleomycin-induced lung fibrosis," Am. J. Respir. Crit. Care Med. 174:420-427 (2006), each of which is incorporated herein by reference as if set forth in its entirety.

The invention has been described in connection with what are presently considered to be the most practical and preferred embodiments. However, the present invention has been presented by way of illustration and is not intended to be limited to the disclosed embodiments. Accordingly, those skilled in the art will realize that the invention is intended to encompass all modifications and alternative arrangements within the spirit and scope of the invention as set forth in the appended claims.

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<210> SEQ ID NO 7
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 7

Asp Pro Leu Gln Tyr Met Arg Ala Asp Gln Ala Ala Gly Gly Leu Arg
1               5                   10                  15


<210> SEQ ID NO 8
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 8
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Gly Gly Phe Ala Pro Tyr Tyr Gly Asp Glu Pro Met Asp Phe Lys Ile
1               5                   10                  15


<210> SEQ ID NO 9
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 9

Asp Pro Leu Pro Glu Phe Thr Glu Asp Gln Ala Ala Pro Asp Asp Lys
1               5                   10                  15


<210> SEQ ID NO 10
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 10

Arg Pro Leu Pro Ser Tyr Ala Ala Asp Thr Ala Gly Ser Gln Leu His
1               5                   10                  15


<210> SEQ ID NO 11
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 11

Asp Thr Gly Asp Cys Tyr Ser Gly Asp Glu Asn Pro Asp Ile Glu Cys
1               5                   10                  15


<210> SEQ ID NO 12
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 12

Asp Asp Gly Pro Phe Met Met Asn Asp Glu Glu Ala Ser Gly Ala Asp
1               5                   10                  15


<210> SEQ ID NO 13
<211> LENGTH: 16
<212> TYPE: PRT
<213> ORGANISM: Mus musculus

<400> SEQUENCE: 13

Asp Pro Glu His Arg Ser Tyr Ser Asp Arg Gly Glu Pro Gly Val Gly
1               5                   10                  15


<210> SEQ ID NO 14
<211> LENGTH: 4678
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (114)..(4535)

<400> SEQUENCE: 14

gcacacagag cagcataaag cccagttgct ttgggaagtg tttgggacca gatggattgt      60

agggagtagg gtacaataca gtctgttctc ctccagctcc ttctttctgc aac atg       116
                                                            Met
                                                             1

ggg aag aac aaa ctc ctt cat cca agt ctg gtt ctt ctc ctc ttg gtc     164
Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu Leu Val
         5                   10                  15
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ctc ctg ccc aca gac gcc tca gtc tct gga aaa ccg cag tat atg gtt        212
Leu Leu Pro Thr Asp Ala Ser Val Ser Gly Lys Pro Gln Tyr Met Val
        20              25                  30

ctg gtc ccc tcc ctg ctc cac act gag acc act gag aag ggc tgt gtc        260
Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys Gly Cys Val
    35              40                  45

ctt ctg agc tac ctg aat gag aca gtg act gta agt gct tcc ttg gag        308
Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu Glu
50              55                  60                  65

tct gtc agg gga aac agg agc ctc ttc act gac ctg gag gcg gag aat        356
Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu Asn
                70                  75                  80

gac gta ctc cac tgt gtc gcc ttc gct gtc cca aag tct tca tcc aat        404
Asp Val Leu His Cys Val Ala Phe Ala Val Pro Lys Ser Ser Ser Asn
                85                  90                  95

gag gag gta atg ttc ctc act gtc caa gtg aaa gga cca acc caa gaa        452
Glu Glu Val Met Phe Leu Thr Val Gln Val Lys Gly Pro Thr Gln Glu
            100                 105                 110

ttt aag aag cgg acc aca gtg atg gtt aag aac gag gac agt ctg gtc        500
Phe Lys Lys Arg Thr Thr Val Met Val Lys Asn Glu Asp Ser Leu Val
        115                 120                 125

ttt gtc cag aca gac aaa tca atc tac aaa cca ggg cag aca gtg aaa        548
Phe Val Gln Thr Asp Lys Ser Ile Tyr Lys Pro Gly Gln Thr Val Lys
130                 135                 140                 145

ttt cgt gtt gtc tcc atg gat gaa aac ttt cac ccc ctg aat gag ttg        596
Phe Arg Val Val Ser Met Asp Glu Asn Phe His Pro Leu Asn Glu Leu
                150                 155                 160

att cca cta gta tac att cag gat ccc aaa gga aat cgc atc gca caa        644
Ile Pro Leu Val Tyr Ile Gln Asp Pro Lys Gly Asn Arg Ile Ala Gln
                165                 170                 175

tgg cag agt ttc cag tta gag ggt ggc ctc aag caa ttt tct ttt ccc        692
Trp Gln Ser Phe Gln Leu Glu Gly Gly Leu Lys Gln Phe Ser Phe Pro
            180                 185                 190

ctc tca tca gag ccc ttc cag ggc tcc tac aag gtg gtg gta cag aag        740
Leu Ser Ser Glu Pro Phe Gln Gly Ser Tyr Lys Val Val Val Gln Lys
        195                 200                 205

aaa tca ggt gga agg aca gag cac cct ttc acc gtg gag gaa ttt gtt        788
Lys Ser Gly Gly Arg Thr Glu His Pro Phe Thr Val Glu Glu Phe Val
210                 215                 220                 225

ctt ccc aag ttt gaa gta caa gta aca gtg cca aag ata atc acc atc        836
Leu Pro Lys Phe Glu Val Gln Val Thr Val Pro Lys Ile Ile Thr Ile
                230                 235                 240

ttg gaa gaa gag atg aat gta tca gtg tgt ggc cta tac aca tat ggg        884
Leu Glu Glu Glu Met Asn Val Ser Val Cys Gly Leu Tyr Thr Tyr Gly
            245                 250                 255

aag cct gtc cct gga cat gtg act gtg agc att tgc aga aag tat agt        932
Lys Pro Val Pro Gly His Val Thr Val Ser Ile Cys Arg Lys Tyr Ser
        260                 265                 270

gac gct tcc gac tgc cac ggt gaa gat tca cag gct ttc tgt gag aaa        980
Asp Ala Ser Asp Cys His Gly Glu Asp Ser Gln Ala Phe Cys Glu Lys
        275                 280                 285

ttc agt gga cag cta aac agc cat ggc tgc ttc tat cag caa gta aaa      1028
Phe Ser Gly Gln Leu Asn Ser His Gly Cys Phe Tyr Gln Gln Val Lys
290                 295                 300                 305

acc aag gtc ttc cag ctg aag agg aag gag tat gaa atg aaa ctt cac      1076
Thr Lys Val Phe Gln Leu Lys Arg Lys Glu Tyr Glu Met Lys Leu His
                310                 315                 320

act gag gcc cag atc caa gaa gaa gga aca gtg gtg gaa ttg act gga      1124
Thr Glu Ala Gln Ile Gln Glu Glu Gly Thr Val Val Glu Leu Thr Gly
            325                 330                 335
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agg cag tcc agt gaa atc aca aga acc ata acc aaa ctc tca ttt gtg     1172
Arg Gln Ser Ser Glu Ile Thr Arg Thr Ile Thr Lys Leu Ser Phe Val
        340                 345                 350

aaa gtg gac tca cac ttt cga cag gga att ccc ttc ttt ggg cag gtg     1220
Lys Val Asp Ser His Phe Arg Gln Gly Ile Pro Phe Phe Gly Gln Val
        355                 360                 365

cgc cta gta gat ggg aaa ggc gtc cct ata cca aat aaa gtc ata ttc     1268
Arg Leu Val Asp Gly Lys Gly Val Pro Ile Pro Asn Lys Val Ile Phe
370                 375                 380                 385

atc aga gga aat gaa gca aac tat tac tcc aat gct acc acg gat gag     1316
Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp Glu
                390                 395                 400

cat ggc ctt gta cag ttc tct atc aac acc acc aat gtt atg ggt acc     1364
His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly Thr
                405                 410                 415

tct ctt act gtt agg gtc aat tac aag gat cgt agt ccc tgt tac ggc     1412
Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr Gly
        420                 425                 430

tac cag tgg gtg tca gaa gaa cac gaa gag gca cat cac act gct tat     1460
Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala Tyr
        435                 440                 445

ctt gtg ttc tcc cca agc aag agc ttt gtc cac ctt gag ccc atg tct     1508
Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met Ser
450                 455                 460                 465

cat gaa cta ccc tgt ggc cat act cag aca gtc cag gca cat tat att     1556
His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr Ile
                470                 475                 480

ctg aat gga ggc acc ctg ctg ggg ctg aag aag ctc tcc ttc tat tat     1604
Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr Tyr
                485                 490                 495

ctg ata atg gca aag gga ggc att gtc cga act ggg act cat gga ctg     1652
Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly Leu
        500                 505                 510

ctt gtg aag cag gaa gac atg aag ggc cat ttt tcc atc tca atc cct     1700
Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile Pro
        515                 520                 525

gtg aag tca gac att gct cct gtc gct cgg ttg ctc atc tat gct gtt     1748
Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala Val
530                 535                 540                 545

tta cct acc ggg gac gtg att ggg gat tct gca aaa tat gat gtt gaa     1796
Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val Glu
                550                 555                 560

aat tgt ctg gcc aac aag gtg gat ttg agc ttc agc cca tca caa agt     1844
Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln Ser
                565                 570                 575

ctc cca gct tca cac gcc cac ctg cga gtc aca gcg gct cct cag tcc     1892
Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln Ser
        580                 585                 590

gtc tgc gcc ctc cgt gct gtg gac caa agc gtg ctg ctc atg aag cct     1940
Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys Pro
        595                 600                 605

gat gct gag ctc tcg gcg tcc tcg gtt tac aac ctg cta cca gaa aag     1988
Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu Lys
610                 615                 620                 625

gac ctc act ggc ttc cct ggg cct ttg aat gac cag gac gat gaa gac     2036
Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu Asp
                630                 635                 640

tgc atc aat cgt cat aat gtc tat att aat gga atc aca tat act cca     2084
Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr Pro
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            645              650              655

gta tca agt aca aat gaa aag gat atg tac agc ttc cta gag gac atg   2132
Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp Met
        660              665              670

ggc tta aag gca ttc acc aac tca aag att cgt aaa ccc aaa atg tgt   2180
Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met Cys
    675              680              685

cca cag ctt caa cag tat gaa atg cat gga cct gaa ggt cta cgt gta   2228
Pro Gln Leu Gln Gln Tyr Glu Met His Gly Pro Glu Gly Leu Arg Val
690              695              700              705

ggt ttt tat gag tca gat gta atg gga aga ggc cat gca cgc ctg gtg   2276
Gly Phe Tyr Glu Ser Asp Val Met Gly Arg Gly His Ala Arg Leu Val
                710              715              720

cat gtt gaa gag cct cac acg gag acc gta cga aag tac ttc cct gag   2324
His Val Glu Glu Pro His Thr Glu Thr Val Arg Lys Tyr Phe Pro Glu
                725              730              735

aca tgg atc tgg gat ttg gtg gtg gta aac tca gca ggt gtg gct gag   2372
Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly Val Ala Glu
        740              745              750

gta gga gta aca gtc cct gac acc atc acc gag tgg aag gca ggg gcc   2420
Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly Ala
    755              760              765

ttc tgc ctg tct gaa gat gct gga ctt ggt atc tct tcc act gcc tct   2468
Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala Ser
770              775              780              785

ctc cga gcc ttc cag ccc ttc ttt gtg gag ctc aca atg cct tac tct   2516
Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met Pro Tyr Ser
                790              795              800

gtg att cgt gga gag gcc ttc aca ctc aag gcc acg gtc cta aac tac   2564
Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val Leu Asn Tyr
                805              810              815

ctt ccc aaa tgc atc cgg gtc agt gtg cag ctg gaa gcc tct ccc gcc   2612
Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala Ser Pro Ala
        820              825              830

ttc cta gct gtc cca gtg gag aag gaa caa gcg cct cac tgc atc tgt   2660
Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His Cys Ile Cys
    835              840              845

gca aac ggg cgg caa act gtg tcc tgg gca gta acc cca aag tca tta   2708
Ala Asn Gly Arg Gln Thr Val Ser Trp Ala Val Thr Pro Lys Ser Leu
850              855              860              865

gga aat gtg aat ttc act gtg agc gca gag gca cta gag tct caa gag   2756
Gly Asn Val Asn Phe Thr Val Ser Ala Glu Ala Leu Glu Ser Gln Glu
                870              875              880

ctg tgt ggg act gag gtg cct tca gtt cct gaa cac gga agg aaa gac   2804
Leu Cys Gly Thr Glu Val Pro Ser Val Pro Glu His Gly Arg Lys Asp
                885              890              895

aca gtc atc aag cct ctg ttg gtt gaa cct gaa gga cta gag aag gaa   2852
Thr Val Ile Lys Pro Leu Leu Val Glu Pro Glu Gly Leu Glu Lys Glu
        900              905              910

aca aca ttc aac tcc cta ctt tgt cca tca ggt ggt gag gtt tct gaa   2900
Thr Thr Phe Asn Ser Leu Leu Cys Pro Ser Gly Gly Glu Val Ser Glu
        915              920              925

gaa tta tcc ctg aaa ctg cca cca aat gtg gta gaa gaa tct gcc cga   2948
Glu Leu Ser Leu Lys Leu Pro Pro Asn Val Val Glu Glu Ser Ala Arg
930              935              940              945

gct tct gtc tca gtt ttg gga gac ata tta ggc tct gcc atg caa aac   2996
Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala Met Gln Asn
                950              955              960

aca caa aat ctt ctc cag atg ccc tat ggc tgt gga gag cag aat atg   3044
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Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Cys Gly Glu Gln Asn Met
        965                 970                 975

gtc ctc ttt gct cct aac atc tat gta ctg gat tat cta aat gaa aca    3092
Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu Thr
        980                 985                 990

cag cag ctt act cca gag atc  aag tcc aag gcc att  ggc tat ctc aac   3140
Gln Gln Leu Thr Pro Glu Ile  Lys Ser Lys Ala Ile  Gly Tyr Leu Asn
        995             1000                 1005

act  ggt tac cag aga cag  ttg aac tac aaa cac  tat gat ggc tcc      3185
Thr  Gly Tyr Gln Arg Gln  Leu Asn Tyr Lys His  Tyr Asp Gly Ser
1010             1015                 1020

tac  agc acc ttt ggg gag  cga tat ggc agg aac  cag ggc aac acc      3230
Tyr  Ser Thr Phe Gly Glu  Arg Tyr Gly Arg Asn  Gln Gly Asn Thr
1025             1030                 1035

tgg  ctc aca gcc ttt gtt  ctg aag act ttt gcc  caa gct cga gcc      3275
Trp  Leu Thr Ala Phe Val  Leu Lys Thr Phe Ala  Gln Ala Arg Ala
1040             1045                 1050

tac  atc ttc atc gat gaa  gca cac att acc caa  gcc ctc ata tgg      3320
Tyr  Ile Phe Ile Asp Glu  Ala His Ile Thr Gln  Ala Leu Ile Trp
1055             1060                 1065

ctc  tcc cag agg cag aag  gac aat ggc tgt ttc  agg agc tct ggg      3365
Leu  Ser Gln Arg Gln Lys  Asp Asn Gly Cys Phe  Arg Ser Ser Gly
1070             1075                 1080

tca  ctg ctc aac aat gcc  ata aag gga gga gta  gaa gat gaa gtg      3410
Ser  Leu Leu Asn Asn Ala  Ile Lys Gly Gly Val  Glu Asp Glu Val
1085             1090                 1095

acc  ctc tcc gcc tat atc  acc atc gcc ctt ctg  gag att cct ctc      3455
Thr  Leu Ser Ala Tyr Ile  Thr Ile Ala Leu Leu  Glu Ile Pro Leu
1100             1105                 1110

aca  gtc act cac cct gtt  gtc cgc aat gcc ctg  ttt tgc ctg gag      3500
Thr  Val Thr His Pro Val  Val Arg Asn Ala Leu  Phe Cys Leu Glu
1115             1120                 1125

tca  gcc tgg aag aca gca  caa gaa ggg gac cat  ggc agc cat gta      3545
Ser  Ala Trp Lys Thr Ala  Gln Glu Gly Asp His  Gly Ser His Val
1130             1135                 1140

tat  acc aaa gca ctg ctg  gcc tat gct ttt gcc  ctg gca ggt aac      3590
Tyr  Thr Lys Ala Leu Leu  Ala Tyr Ala Phe Ala  Leu Ala Gly Asn
1145             1150                 1155

cag  gac aag agg aag gaa  gta ctc aag tca ctt  aat gag gaa gct      3635
Gln  Asp Lys Arg Lys Glu  Val Leu Lys Ser Leu  Asn Glu Glu Ala
1160             1165                 1170

gtg  aag aaa gac aac tct  gtc cat tgg gag cgc  cct cag aaa ccc      3680
Val  Lys Lys Asp Asn Ser  Val His Trp Glu Arg  Pro Gln Lys Pro
1175             1180                 1185

aag  gca cca gtg ggg cat  ttt tac gaa ccc cag  gct ccc tct gct      3725
Lys  Ala Pro Val Gly His  Phe Tyr Glu Pro Gln  Ala Pro Ser Ala
1190             1195                 1200

gag  gtg gag atg aca tcc  tat gtg ctc ctc gct  tat ctc acg gcc      3770
Glu  Val Glu Met Thr Ser  Tyr Val Leu Leu Ala  Tyr Leu Thr Ala
1205             1210                 1215

cag  cca gcc cca acc tcg  gag gac ctg acc tct  gca acc aac atc      3815
Gln  Pro Ala Pro Thr Ser  Glu Asp Leu Thr Ser  Ala Thr Asn Ile
1220             1225                 1230

gtg  aag tgg atc acg aag  cag cag aat gcc cag  ggc ggt ttc tcc      3860
Val  Lys Trp Ile Thr Lys  Gln Gln Asn Ala Gln  Gly Gly Phe Ser
1235             1240                 1245

tcc  acc cag gac aca gtg  gtg gct ctc cat gct  ctg tcc aaa tat      3905
Ser  Thr Gln Asp Thr Val  Val Ala Leu His Ala  Leu Ser Lys Tyr
1250             1255                 1260
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gga gca gcc aca ttt acc agg act ggg aag gct gca cag gtg act		3950
Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val Thr		
1265 1270 1275		

atc cag tct tca ggg aca ttt tcc agc aaa ttc caa gtg gac aac   3995
Ile Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp Asn
1280   1285   1290

aac aac cgc ctg tta ctg cag cag gtc tca ttg cca gag ctg cct   4040
Asn Asn Arg Leu Leu Leu Gln Gln Val Ser Leu Pro Glu Leu Pro
1295   1300   1305

ggg gaa tac agc atg aaa gtg aca gga gaa gga tgt gtc tac ctc   4085
Gly Glu Tyr Ser Met Lys Val Thr Gly Glu Gly Cys Val Tyr Leu
1310   1315   1320

cag aca tcc ttg aaa tac aat att ctc cca gaa aag gaa gag ttc   4130
Gln Thr Ser Leu Lys Tyr Asn Ile Leu Pro Glu Lys Glu Glu Phe
1325   1330   1335

ccc ttt gct tta gga gtg cag act ctg cct caa act tgt gat gaa   4175
Pro Phe Ala Leu Gly Val Gln Thr Leu Pro Gln Thr Cys Asp Glu
1340   1345   1350

ccc aaa gcc cac acc agc ttc caa atc tcc cta agt gtc agt tac   4220
Pro Lys Ala His Thr Ser Phe Gln Ile Ser Leu Ser Val Ser Tyr
1355   1360   1365

aca ggg agc cgc tct gcc tcc aac atg gcg atc gtt gat gtg aag   4265
Thr Gly Ser Arg Ser Ala Ser Asn Met Ala Ile Val Asp Val Lys
1370   1375   1380

atg gtc tct ggc ttc att ccc ctg aag cca aca gtg aaa atg ctt   4310
Met Val Ser Gly Phe Ile Pro Leu Lys Pro Thr Val Lys Met Leu
1385   1390   1395

gaa aga tct aac cat gtg agc cgg aca gaa gtc agc agc aac cat   4355
Glu Arg Ser Asn His Val Ser Arg Thr Glu Val Ser Ser Asn His
1400   1405   1410

gtc ttg att tac ctt gat aag gtg tca aat cag aca ctg agc ttg   4400
Val Leu Ile Tyr Leu Asp Lys Val Ser Asn Gln Thr Leu Ser Leu
1415   1420   1425

ttc ttc acg gtt ctg caa gat gtc cca gta aga gat ctg aaa cca   4445
Phe Phe Thr Val Leu Gln Asp Val Pro Val Arg Asp Leu Lys Pro
1430   1435   1440

gcc ata gtg aaa gtc tat gat tac tac gag acg gat gag ttt gca   4490
Ala Ile Val Lys Val Tyr Asp Tyr Tyr Glu Thr Asp Glu Phe Ala
1445   1450   1455

att gct gag tac aat gct cct tgc agc aaa gat ctt gga aat gct   4535
Ile Ala Glu Tyr Asn Ala Pro Cys Ser Lys Asp Leu Gly Asn Ala
1460   1465   1470

tgaagaccac aaggctgaaa agtgctttgc tggagtcctg ttctcagagc tccacagaag   4595

acacgtgttt ttgtatcttt aaagacttga tgaataaaca cttttctgg tcaatgtcaa   4655

aaaaaaaaaa aaaaaaaaaa aaa   4678

<210> SEQ ID NO 15
<211> LENGTH: 1474
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 15

Met Gly Lys Asn Lys Leu Leu His Pro Ser Leu Val Leu Leu Leu
1   5   10   15

Val Leu Leu Pro Thr Asp Ala Ser Val Ser Gly Lys Pro Gln Tyr Met
            20   25   30

Val Leu Val Pro Ser Leu Leu His Thr Glu Thr Thr Glu Lys Gly Cys
        35   40   45

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Val Leu Leu Ser Tyr Leu Asn Glu Thr Val Thr Val Ser Ala Ser Leu
    50              55              60

Glu Ser Val Arg Gly Asn Arg Ser Leu Phe Thr Asp Leu Glu Ala Glu
65              70              75              80

Asn Asp Val Leu His Cys Val Ala Phe Ala Val Pro Lys Ser Ser Ser
            85              90              95

Asn Glu Glu Val Met Phe Leu Thr Val Gln Val Lys Gly Pro Thr Gln
            100             105             110

Glu Phe Lys Lys Arg Thr Thr Val Met Val Lys Asn Glu Asp Ser Leu
    115             120             125

Val Phe Val Gln Thr Asp Lys Ser Ile Tyr Lys Pro Gly Gln Thr Val
    130             135             140

Lys Phe Arg Val Val Ser Met Asp Glu Asn Phe His Pro Leu Asn Glu
145             150             155             160

Leu Ile Pro Leu Val Tyr Ile Gln Asp Pro Lys Gly Asn Arg Ile Ala
            165             170             175

Gln Trp Gln Ser Phe Gln Leu Glu Gly Gly Leu Lys Gln Phe Ser Phe
            180             185             190

Pro Leu Ser Ser Glu Pro Phe Gln Gly Ser Tyr Lys Val Val Val Gln
            195             200             205

Lys Lys Ser Gly Gly Arg Thr Glu His Pro Phe Thr Val Glu Glu Phe
    210             215             220

Val Leu Pro Lys Phe Glu Val Gln Val Thr Val Pro Lys Ile Ile Thr
225             230             235             240

Ile Leu Glu Glu Glu Met Asn Val Ser Val Cys Gly Leu Tyr Thr Tyr
            245             250             255

Gly Lys Pro Val Pro Gly His Val Thr Val Ser Ile Cys Arg Lys Tyr
            260             265             270

Ser Asp Ala Ser Asp Cys His Gly Glu Asp Ser Gln Ala Phe Cys Glu
    275             280             285

Lys Phe Ser Gly Gln Leu Asn Ser His Gly Cys Phe Tyr Gln Gln Val
    290             295             300

Lys Thr Lys Val Phe Gln Leu Lys Arg Lys Glu Tyr Glu Met Lys Leu
305             310             315             320

His Thr Glu Ala Gln Ile Gln Glu Glu Gly Thr Val Val Glu Leu Thr
            325             330             335

Gly Arg Gln Ser Ser Glu Ile Thr Arg Thr Ile Thr Lys Leu Ser Phe
            340             345             350

Val Lys Val Asp Ser His Phe Arg Gln Gly Ile Pro Phe Phe Gly Gln
    355             360             365

Val Arg Leu Val Asp Gly Lys Gly Val Pro Ile Pro Asn Lys Val Ile
    370             375             380

Phe Ile Arg Gly Asn Glu Ala Asn Tyr Tyr Ser Asn Ala Thr Thr Asp
385             390             395             400

Glu His Gly Leu Val Gln Phe Ser Ile Asn Thr Thr Asn Val Met Gly
            405             410             415

Thr Ser Leu Thr Val Arg Val Asn Tyr Lys Asp Arg Ser Pro Cys Tyr
            420             425             430

Gly Tyr Gln Trp Val Ser Glu Glu His Glu Glu Ala His His Thr Ala
            435             440             445

Tyr Leu Val Phe Ser Pro Ser Lys Ser Phe Val His Leu Glu Pro Met
    450             455             460

Ser His Glu Leu Pro Cys Gly His Thr Gln Thr Val Gln Ala His Tyr
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465                   470                   475                   480

Ile Leu Asn Gly Gly Thr Leu Leu Gly Leu Lys Lys Leu Ser Phe Tyr
                485                   490                   495

Tyr Leu Ile Met Ala Lys Gly Gly Ile Val Arg Thr Gly Thr His Gly
            500                   505                   510

Leu Leu Val Lys Gln Glu Asp Met Lys Gly His Phe Ser Ile Ser Ile
            515                   520                   525

Pro Val Lys Ser Asp Ile Ala Pro Val Ala Arg Leu Leu Ile Tyr Ala
        530                   535                   540

Val Leu Pro Thr Gly Asp Val Ile Gly Asp Ser Ala Lys Tyr Asp Val
545                   550                   555                   560

Glu Asn Cys Leu Ala Asn Lys Val Asp Leu Ser Phe Ser Pro Ser Gln
                565                   570                   575

Ser Leu Pro Ala Ser His Ala His Leu Arg Val Thr Ala Ala Pro Gln
            580                   585                   590

Ser Val Cys Ala Leu Arg Ala Val Asp Gln Ser Val Leu Leu Met Lys
            595                   600                   605

Pro Asp Ala Glu Leu Ser Ala Ser Ser Val Tyr Asn Leu Leu Pro Glu
        610                   615                   620

Lys Asp Leu Thr Gly Phe Pro Gly Pro Leu Asn Asp Gln Asp Asp Glu
625                   630                   635                   640

Asp Cys Ile Asn Arg His Asn Val Tyr Ile Asn Gly Ile Thr Tyr Thr
                645                   650                   655

Pro Val Ser Ser Thr Asn Glu Lys Asp Met Tyr Ser Phe Leu Glu Asp
            660                   665                   670

Met Gly Leu Lys Ala Phe Thr Asn Ser Lys Ile Arg Lys Pro Lys Met
        675                   680                   685

Cys Pro Gln Leu Gln Gln Tyr Glu Met His Gly Pro Glu Gly Leu Arg
        690                   695                   700

Val Gly Phe Tyr Glu Ser Asp Val Met Gly Arg Gly His Ala Arg Leu
705                   710                   715                   720

Val His Val Glu Glu Pro His Thr Glu Thr Val Arg Lys Tyr Phe Pro
                725                   730                   735

Glu Thr Trp Ile Trp Asp Leu Val Val Val Asn Ser Ala Gly Val Ala
            740                   745                   750

Glu Val Gly Val Thr Val Pro Asp Thr Ile Thr Glu Trp Lys Ala Gly
            755                   760                   765

Ala Phe Cys Leu Ser Glu Asp Ala Gly Leu Gly Ile Ser Ser Thr Ala
        770                   775                   780

Ser Leu Arg Ala Phe Gln Pro Phe Phe Val Glu Leu Thr Met Pro Tyr
785                   790                   795                   800

Ser Val Ile Arg Gly Glu Ala Phe Thr Leu Lys Ala Thr Val Leu Asn
                805                   810                   815

Tyr Leu Pro Lys Cys Ile Arg Val Ser Val Gln Leu Glu Ala Ser Pro
            820                   825                   830

Ala Phe Leu Ala Val Pro Val Glu Lys Glu Gln Ala Pro His Cys Ile
            835                   840                   845

Cys Ala Asn Gly Arg Gln Thr Val Ser Trp Ala Val Thr Pro Lys Ser
        850                   855                   860

Leu Gly Asn Val Asn Phe Thr Val Ser Ala Glu Ala Leu Glu Ser Gln
865                   870                   875                   880

Glu Leu Cys Gly Thr Glu Val Pro Ser Val Pro Glu His Gly Arg Lys
                885                   890                   895
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Asp Thr Val Ile Lys Pro Leu Leu Val Glu Pro Glu Gly Leu Glu Lys
            900   905   910

Glu Thr Thr Phe Asn Ser Leu Leu Cys Pro Ser Gly Gly Glu Val Ser
            915   920   925

Glu Glu Leu Ser Leu Lys Leu Pro Pro Asn Val Val Glu Glu Ser Ala
        930   935   940

Arg Ala Ser Val Ser Val Leu Gly Asp Ile Leu Gly Ser Ala Met Gln
945   950   955   960

Asn Thr Gln Asn Leu Leu Gln Met Pro Tyr Gly Cys Gly Glu Gln Asn
                965   970   975

Met Val Leu Phe Ala Pro Asn Ile Tyr Val Leu Asp Tyr Leu Asn Glu
            980   985   990

Thr Gln Gln Leu Thr Pro Glu Ile Lys Ser Lys Ala Ile Gly Tyr Leu
            995   1000   1005

Asn Thr Gly Tyr Gln Arg Gln Leu Asn Tyr Lys His Tyr Asp Gly
        1010   1015   1020

Ser Tyr Ser Thr Phe Gly Glu Arg Tyr Gly Arg Asn Gln Gly Asn
        1025   1030   1035

Thr Trp Leu Thr Ala Phe Val Leu Lys Thr Phe Ala Gln Ala Arg
        1040   1045   1050

Ala Tyr Ile Phe Ile Asp Glu Ala His Ile Thr Gln Ala Leu Ile
        1055   1060   1065

Trp Leu Ser Gln Arg Gln Lys Asp Asn Gly Cys Phe Arg Ser Ser
        1070   1075   1080

Gly Ser Leu Leu Asn Asn Ala Ile Lys Gly Gly Val Glu Asp Glu
        1085   1090   1095

Val Thr Leu Ser Ala Tyr Ile Thr Ile Ala Leu Leu Glu Ile Pro
        1100   1105   1110

Leu Thr Val Thr His Pro Val Val Arg Asn Ala Leu Phe Cys Leu
        1115   1120   1125

Glu Ser Ala Trp Lys Thr Ala Gln Glu Gly Asp His Gly Ser His
        1130   1135   1140

Val Tyr Thr Lys Ala Leu Leu Ala Tyr Ala Phe Ala Leu Ala Gly
        1145   1150   1155

Asn Gln Asp Lys Arg Lys Glu Val Leu Lys Ser Leu Asn Glu Glu
        1160   1165   1170

Ala Val Lys Lys Asp Asn Ser Val His Trp Glu Arg Pro Gln Lys
        1175   1180   1185

Pro Lys Ala Pro Val Gly His Phe Tyr Glu Pro Gln Ala Pro Ser
        1190   1195   1200

Ala Glu Val Glu Met Thr Ser Tyr Val Leu Leu Ala Tyr Leu Thr
        1205   1210   1215

Ala Gln Pro Ala Pro Thr Ser Glu Asp Leu Thr Ser Ala Thr Asn
        1220   1225   1230

Ile Val Lys Trp Ile Thr Lys Gln Gln Asn Ala Gln Gly Gly Phe
        1235   1240   1245

Ser Ser Thr Gln Asp Thr Val Val Ala Leu His Ala Leu Ser Lys
        1250   1255   1260

Tyr Gly Ala Ala Thr Phe Thr Arg Thr Gly Lys Ala Ala Gln Val
        1265   1270   1275

Thr Ile Gln Ser Ser Gly Thr Phe Ser Ser Lys Phe Gln Val Asp
        1280   1285   1290

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Asn Asn  Asn Arg Leu Leu Leu  Gln Gln Val Ser Leu  Pro Glu Leu
    1295             1300             1305

Pro Gly  Glu Tyr Ser Met Lys  Val Thr Gly Glu Gly  Cys Val Tyr
    1310             1315             1320

Leu Gln  Thr Ser Leu Lys Tyr  Asn Ile Leu Pro Glu  Lys Glu Glu
    1325             1330             1335

Phe Pro  Phe Ala Leu Gly Val  Gln Thr Leu Pro Gln  Thr Cys Asp
    1340             1345             1350

Glu Pro  Lys Ala His Thr Ser  Phe Gln Ile Ser Leu  Ser Val Ser
    1355             1360             1365

Tyr Thr  Gly Ser Arg Ser Ala  Ser Asn Met Ala Ile  Val Asp Val
    1370             1375             1380

Lys Met  Val Ser Gly Phe Ile  Pro Leu Lys Pro Thr  Val Lys Met
    1385             1390             1395

Leu Glu  Arg Ser Asn His Val  Ser Arg Thr Glu Val  Ser Ser Asn
    1400             1405             1410

His Val  Leu Ile Tyr Leu Asp  Lys Val Ser Asn Gln  Thr Leu Ser
    1415             1420             1425

Leu Phe  Phe Thr Val Leu Gln  Asp Val Pro Val Arg  Asp Leu Lys
    1430             1435             1440

Pro Ala  Ile Val Lys Val Tyr  Asp Tyr Tyr Glu Thr  Asp Glu Phe
    1445             1450             1455

Ala Ile  Ala Glu Tyr Asn Ala  Pro Cys Ser Lys Asp  Leu Gly Asn
    1460             1465             1470

Ala
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<211> LENGTH: 55
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polynucleotide

<400> SEQUENCE: 16

gctagcagac tacaaagacg atgacgacaa gtcagtctct ggaaaaccgc agtat          55


<210> SEQ ID NO 17
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 17

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<210> SEQ ID NO 18
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<400> SEQUENCE: 18

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<400> SEQUENCE: 19

atgtaggctc gagcttgggc                                                    20


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gcccaagctc gagcctacat                                                    20


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<400> SEQUENCE: 21

gcggccgctc aagcatttcc aagatctttg ctg                                     33


<210> SEQ ID NO 22
<211> LENGTH: 23
<212> TYPE: DNA
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<220> FEATURE:
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<400> SEQUENCE: 22

gatatgtaca gcttcctaga gga                                                23


<210> SEQ ID NO 23
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<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 23

gttgcaattg tggacacatt ttgggtttac ga                                      32


<210> SEQ ID NO 24
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
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<400> SEQUENCE: 24

tccacaattg caacagtatg aaatgcatgg acct                                    34


<210> SEQ ID NO 25
<211> LENGTH: 22
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polynucleotide

<400> SEQUENCE: 25

ctttcgtacg gtctccgtgt ga                                                 22
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<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polynucleotide


<400> SEQUENCE: 26

gagagaattc tgggacttca ccctggacga                                    30


<210> SEQ ID NO 27
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polynucleotide


<400> SEQUENCE: 27

gaaaggtacc atggcgctgt aggtgggtgt                                    30


<210> SEQ ID NO 28
<211> LENGTH: 10
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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polypeptide


<400> SEQUENCE: 28

Ser Val Ser Gly Lys Pro Gln Tyr Met Val
1               5                   10


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<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Synthetic polypeptide
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<221> NAME/KEY: UNSURE
<222> LOCATION: (8)..(8)
<223> OTHER INFORMATION: X is an unidentified residue.


<400> SEQUENCE: 29

Asp Val Met Gly Arg Gly His Xaa Arg
1               5
```

The invention claimed is:

1. An engineered α_2-macroglobulin protein comprising an engineered bait region, the engineered bait region being a bone morphogenetic protein-1 bait region from a protein other than α_2-macroglobulin that is cleaved by BMP-1, wherein the engineered α_2-macroglobulin protein is characterized by an at least about 25-fold enhanced BMP-1 inhibitory effectiveness compared to a wild type α_2-macroglobulin protein, and wherein the protein other than $\alpha2$-macroglobulin is probiglycan.

2. The protein of claim 1, wherein the protein other than α_2-macroglobulin is probiglycan from a human.

3. The protein of claim 1, wherein the bait region of the protein other than α_2-macroglobulin consists of an amino acid sequence encoded by a nucleic acid sequence amplifi-able from genomic DNA from a human using SEQ ID NO:26 and SEQ ID NO:27 as amplification primers.

4. The protein of claim 1, wherein the engineered bait region comprises the amino acid sequence of SEQ ID NO: 2.

5. A method of inhibiting a bone morphogenetic protein-1 (BMP-1)-like proteinase in a human or non-human animal experiencing or susceptible to a fibrotic disorder caused by the BMP-1-like proteinase, the method comprising the step of: administering to the animal an amount of an inhibitor of the BMP-1-like proteinase effective to reduce BMP-1-like proteinase activity in the animal, where the reduction is characterized by a reduction in severity or occurrence of the fibrotic disorder, and wherein the inhibitor of the BMP-1-like proteinase is an engineered α_2-macroglobulin protein according to claim 1.

6. The method of claim **5**, wherein the bait region of the protein other than α_2-macroglobulin consists of an amino acid sequence encoded by a nucleic acid sequence amplifiable from genomic DNA from a human using SEQ ID NO:26 and SEQ ID NO:27 as amplification primers.

7. The method of claim **5**, wherein the bait region comprises the amino acid sequence of SEQ ID NO:2.

8. The method of claim **5**, wherein the bone morphogenetic protein-1-like proteinase is selected from the group consist-

ing of bone morphogenetic protein-1, mammalian Tolloid, mammalian Tolloid-like 1 and mammalian Tolloid-like 2.

9. The method of claim **5**, wherein the fibrotic disorder is selected from the group consisting of a keloid or other abnormal wound healing, a surgical adhesion and a deep-seated fibrosis of an organ.

* * * * *