Inhibition of Bone Morphogenetic Protein 1 by Native and Altered Forms of $\alpha_2$-Macroglobulin*

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The four mammalian bone morphogenetic protein 1 (BMP1)-like proteases act to proteolytically convert procollagens to the major fibrous components of the extracellular matrix. They also activate lysyl oxidase, an enzyme necessary to the covalent cross-linking that gives collagen fibrils much of their tensile strength. Thus, these four proteases are attractive targets for interventions designed to limit the excess formation of fibrous collagenous matrix that characterizes fibrosis. Although it has previously been reported that the serum protein $\alpha_2$-macroglobulin is unable to inhibit the astacin-like proteases meprin $\alpha$ and meprin $\beta$, we herein demonstrate $\alpha_2$-macroglobulin to be a potent inhibitor of the similar BMP1-like proteases. BMP1 is shown to cleave the $\alpha_2$-macroglobulin “bait” region, at a single specific site, which resembles the sites at which BMP1-like proteases cleave the C-propeptides of procollagens I–III. $\alpha_2$-Macroglobulin is an irreversible inhibitor that is shown to bind bone morphogenetic protein 1 in a covalent complex. It is also demonstrated that genetically modified $\alpha_2$-macroglobulin, in which the native bait region is replaced by sequences flanking the probiglycan BMP1 cleavage site, is enhanced ~24-fold in its ability to inhibit BMP1, and is capable of inhibiting the biosynthetic processing of procollagen I by cells. These findings suggest possible therapeutic interventions involving ectopic expression of modified versions of $\alpha_2$-macroglobulin in the treatment of fibrotic conditions.

Bone morphogenetic protein 1 (BMP1)3 is the prototype of a subgroup of structurally similar, secreted metalloproteinases, each member of which has an astacin-like protease domain, complement-uegf-BMP1 protein-protein interaction domains, and epidermal growth factor motifs (1, 2). Mammalian members of this subgroup proteolytically convert a variety of precursor molecules into mature, functional proteins involved in the formation of the extracellular matrix (ECM) (1, 2). Members of this class of proteinases also activate a subset of the transforming growth factor-$\beta$ superfamily of proteins in a broad range of species, through cleavage of extracellular protein antagonists (2). Thus, the four mammalian BMP1-like proteinases are likely to be key regulators and orchestrators of ECM formation and signaling by certain transforming growth factor-$\beta$-like molecules, in morphogenetic events and homeostasis. Surprisingly, studies regarding regulation of the expression and activities of these key proteinases have been limited. Nevertheless, one such study showed that, whereas transcription of the BMP1 gene and secretion of BMP1 are both up-regulated ~8-fold by treatment of cells with transforming growth factor-$\beta$1, induction of detectable procollagen C-proteinase activity is only ~2-fold, suggesting the existence of an endogenous inhibitor(s) (3). In fact, a number of studies have detected unexpectedly low levels of procollagen C-proteinase (pCP) activity in tissues and cell cultures, also leading to suggestions of endogenous inhibitors (4).

$\alpha_2$-Macroglobulin ($\alpha_2$M) is a member of the $\alpha$-macroglobulin family of proteins found in the circulation and egg whites of a broad range of species (5). Human $\alpha_2$M is found at relatively high levels (2–4 mg/ml) in plasma and is produced by hepatocytes, but is also produced by a number of other cell types that include lung fibroblasts, macrophages, astrocytes, and tumor cells (6, 7). Human $\alpha_2$M is a tetramer of four identical 185-kDa subunits, each of which has an exposed 39-amino acid “bait region” that contains cleavage sites for a variety of proteinases (6, 8). Cleavage within the bait region results in exposure of a highly reactive $\alpha_2$M thioester that can covalently bind the cleaving proteinase (6, 8). Cleavage within the bait region also results in a conformational change that entraps the proteinase within the interior of the $\alpha_2$M molecule, thus inhibiting further proteinase activity by steric hindrance (6, 8). The conformational change also gives rise to what can be considered an “activated” form of $\alpha_2$M, with exposed sites for binding of $\alpha_2$M to its cognate cell surface receptor, the low-density lipoprotein receptor-related protein, and for binding to a number of cytokines, including transforming growth factor-$\beta$, platelet-derived growth factor, interleukin-1$\beta$, basic fibroblast growth factor, and nerve growth factor (6). Binding to lipoprotein receptor-related protein results in rapid clearance of $\alpha_2$M-proteinase complexes from the extracellular space and catabolism, although activated $\alpha_2$M appears able to bind cytokines in a reversible manner that allows it to serve as a carrier and targeting protein involved in modulating the biological responses of various cell types (6).

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3 The abbreviations used are: BMP, bone morphogenetic protein; $\alpha_2$M, $\alpha_2$-macroglobulin; ECM, extracellular matrix; EGF, epidermal growth factor; pCP, procollagen C-proteinase; C-propeptide, COOH-terminal propeptide; DMEM, Dulbecco’s modified Eagle’s medium; PBS, phosphate-buffered saline.
BMP1-like proteinases have astacin-like protease domains (1, 2). It has previously been reported that α2M does not inhibit vertebrate proteinases with astacin-like protease domains (7, 9), such as meprin and β meprin (9), although bovine α2M has been shown to have inhibitory activity toward the crayfish digestive protease astacin (10). Here we identify a potential site for cleavage of α2M by BMP1-like proteinases. We demonstrate cleavage at this site by BMP1 and the potent inhibition of BMP1 proteolytic activities via the formation of covalent complexes with cleaved α2M. A genetically altered version of α2M, in which the bait region has been replaced by the BMP1 cleavage site of the small leucine-rich proteoglycan precursor biglycan, is shown to have a greatly enhanced ability to inhibit BMP1, and is able to inhibit procollagen I processing by cells. Implications of the data are discussed.

EXPERIMENTAL PROCEDURES

Cleavage of α2M by BMP1—200 nM FLAG-tagged recombinant BMP1, prepared, and purified as previously described (11), was incubated overnight at 37 °C with 200 nM human α2M (Sigma) in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM CaCl2. 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, separated proteins were transferred to polyvinylidene difluoride membranes and probed with a 1:5000 dilution of anti-FLAG antibody (Sigma). Subsequently, membranes were incubated with a 1:2500 dilution of goat anti-mouse IgG (H+L) antibody.}

Inhibition of BMP1 pCP Activity by α2M—For the dose dependence study, α2M was preincubated with BMP1 for 2 h at 37 °C. The study of time-dependent cleavage of α2M by BMP1 indicates that cleavage of α2M by BMP1 is complete after a 2-h preincubation, under the conditions used. Thus, the rate of procollagen processing is proportional to the amount of active BMP1 remaining uncomplexed to α2M. Relative processing of procollagen was plotted against α2M concentrations and nonlinear regression was performed to obtain IC50 values.

Time-dependent Inhibition of BMP1 Cleavage of Probiglycan by α2M—15 ng of BMP1 (9.4 nM) was preincubated with without 5 times the amount of α2M (47.0 nM) in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM CaCl2, 2 h at 37 °C. Subsequently, 450 ng of biglycan, prepared as previously described (12), was added to a final volume of 20 µl and incubated overnight. Cleavage reactions were quenched by adding 4 µl of chondroitinase ABC (a mixture of 10 µl of 0.01 units/µl protease-free chondroitinase ABC (Seikagaku Corp.), 40 µl of 6× chondroitinase buffer (100 mM Tris-HCl, pH 8.0, 240 mM NaAc, 0.25 mM EDTA), and 10 µl of 500 mM EDTA), followed by incubation at 37 °C for 4 h. Samples were subjected to SDS-PAGE on a 10% gel and Western blot analysis was performed, using anti-probiglycan antibody LF51 (13) (the kind gift of Dr. Larry W. Fisher) at a 1:5000 dilution, and a 1:25000 dilution of goat anti-rabbit IgG alkaline phosphatase conjugate, as the secondary antibody.

Inactivation of α2M by Methylamine—1 mg/ml α2M was incubated with 50 mM Tris-HCl, pH 7.1, in the presence or absence of 20 mM methylamine for 20 h, followed by dialysis against 50 mM Tris-HCl, pH 7.5, 100 mM NaCl. Both methylamine-treated and control α2M samples were tested for the ability to be cleaved by BMP1, and ability to inhibit BMP1 cleavage of biglycan.

Production of Recombinant α2M—Human α2M sequences were PCR amplified from human placenta cDNA in three fragments. Primers used were: fragment 1 (amino acids 24–416), 5′-GCTACGACACTACAAAGACGATGACGA-CAAGTCAGTCTCTGGAAAAACCGCATAT-3′ (forward) and 5′-CATGAAAGAGGAGCTCCATAAC-3′ (reverse); fragment 2 (amino acids 416–1052), 5′-GGTATTGGGTACCTCTCTTACTG3′ (forward) and 5′-ATGTAGGCTGAGTTGAGGC-3′ (reverse); and fragment 3 (amino acids 1052–1474), 5′-GCCAAGCTCGAGCCTACAT-3′ (forward) and 5′-GGGCGCGCTCAAGCATTTTACAGATCTTGGTCTG-3′ (reverse). PCR products were assembled into the full-length α2M sequence in plBlueScript II KS+ (Stratagene), and cloned downstream of (and adjacent via an Nhel site to) BM40/SPARC signal peptide sequences, between the HindIII and NotI sites of the tetracycline-inducible expression vector pcDNA4/TO (Invitrogen). The resulting construct expresses full-length α2M, 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 NH2 terminus of α2M sequences. To generate mutant α2M, an Mfel site was introduced into the 5′-end of α2M bait region sequences, without changing the encoded protein, via two-step PCR. Primers used were: primer 1, 5′-GATATGACAGCTCTCC- TAGAGGA-3′; primer 2, 5′-TGTGGCAATTGTGGACACAGC- TTTGGGTTTACGA-3′; primer 3, 5′-TCACAATTTCG-CACGATGAAATGGTACGAC-3′; and primer 4, 5′-CTCTTCGAGCTCTCGTCTGTA-3′. PCR amplitons of primers 1 and 2, and 3 and 4 were then used as templates, for PCR amplification using primers 1 and 4. The resulting amplon was cloned between BsrGI and BsiWI sites in the α2M sequence. Biglycan sequences were PCR amplified with primers 5′-GAGAGAATTTCTGGGACCTCCCTTGGACA-3′ (forward) and 5′-GAAAGTACCATGGCAGCCTG- TAGGTGTTGT-3′ (reverse). The amplon was then digested with EcoRI and KpnI, and cloned between MfeI and BsiWI sites in the modified α2M sequences.

293 T-Rex cells (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 5 µg/ml blasticidin and 10% fetal bovine serum. Cells at 80% confluence were transfected with 1 µg of expression plasmid/35-mm culture dish using Lipofectamine (Invitrogen). After 48 h, cells were selected in the same type of medium supplemented with 200 µg/ml Zeocin. Production of secreted α2M, upon induction with 1 µg/ml tetracycline, was detected via Western blot.

Confluent cells were washed twice with phosphate-buffered saline (PBS), and incubated 15 min in serum-free DMEM at
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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 after cross-linking was terminated by adding glycine, aspartate, 40 μM 35S-cell labeling mixture (Amersham Biosciences). Conditioned medium using an anti-FLAG M2 column (Sigma), following the manufacturer’s instructions. 40 nM 35S-labeled BMP1 was incubated with 20, 40, 80, 160, and 240 nM plasma αM, or recombinant wild type αM or with 20, 30, 40, 60, and 80 nM b-αM at 37 °C in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 10 mM CaCl2. Reactions were stopped at 10, 30, 60, 90, and 120 min (plasma αM, and recombinant wild type αM), or at 0.5, 1, 2, 3, and 4 h (b-αM) by adding SDS-PAGE sample buffer and boiling 5 min at 95 °C. Protein samples were separated on 7.5% acrylamide SDS-PAGE gels, which were then treated with EN3HANCE (DuPont) and exposed to film. Percentages of BMP1 incorporated into high molecular weight complexes were determined by densitometry and kinetic parameters were determined essentially via the method of Enghild et al. (14).

Inhibition of Procollagen 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 h. Cells were then washed twice with PBS, and incubated 15 min in serum-free DMEM 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 αM, or b-αM, or an equivalent volume of buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl). Conditioned media were harvested after 24 h 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 (13) (a generous gift from Larry Fisher), as described.

RESULTS

BMP1 Cleaves αM—Perusal of the amino acid sequence of the αM bait region found a site comprising residues Ser687, Asp688, and surrounding residues that resembled the majority of known BMP1 cleavage sites (Fig. 1). The resemblance resided primarily in the placement of Phe684 and Tyr685 and 3 and 4 residues, respectively, NH2-terminal to Asp688, because 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-like proteinases (2) (Fig. 1). Moreover, the majority of previously characterized cleavage sites of BMP-like proteinases have residues with small side chains in the P1 positions (Ref. 2 and Fig. 1), such that active site residues of known BMP1 cleavage sites (Fig. 1). The resemblance resided primarily in the placement of Phe684 and Tyr685 and 3 and 4 residues, respectively, NH2-terminal to Asp688, because 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-like proteinases (2) (Fig. 1). Moreover, the majority of previously characterized cleavage sites of BMP-like proteinases have residues with small side chains in the P1 positions (Ref. 2 and Fig. 1), such that the placement of αM Ser687 in relationship to Asp688, Phe684, and Tyr686 is also reminiscent of a BMP1 cleavage site.

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| αM | α2-M | b-αM |
|----|------|------|
| 667| PQLQEYMGPGRLVQFYES...DNVRGCHARLVHVEEPHT shotgun data | 667 |
| 688| POFWDFTLDDGPMNN...DEEASGADTSGVLDPSVTYPYAMV | 688 |

**GDF11**
LDLHDFPQ......DALQPEDF

**Myostatin**
IDQYDVQR......DDSSDSDL

**Procollagens**
α1(I) DGCGRYR......DDANVVRD
α2(I) YDGDPYR......DQPRSAPS
α2(I) DLQYMR......DQAAGGLR
α2(I) GGFAPYGY......DEPDMFFK
α2(V) DLPEPTE......DQAPDDK
α2(VII) RLPSYAAA......DTQAGSHL

**Laminin 5 γ2 chain**
DTGDCYG......DENDPDCIC

**Probiglycan**
DDGPPMN......DEEASGAD

**Chordin**
NH2-terminal site
DPEHRSY.....DRGEPVCYG

**Vertical arrows mark the sites of potential ADAMTS-2 cleavage sites in αM and b-αM.** Aspartate residues conserved at the P1′ positions of the various scissile bonds are in *boldface*, as are aromatic side chains, previously noted NH2-terminal to scissile bonds in many previously identified substrates of BMP1-like proteinases, Phe684, Tyr685, and Asp688 of the αM sequence are similarly presented in *boldface*.
**α₂-Macroglobulin Complexes and Inhibits BMP1**

The 85-kDa band yielded the sequence SVSGKQPQYMV, corresponding to the NH₂-terminus of secreted α₂M. NH₂-terminal amino acid sequencing of the 100-kDa band yielded the sequence DVMGRGHR, thus demonstrating cleavage of α₂M by BMP1 at the predicted site between Ser⁶⁸⁷ and Asp⁶⁸⁸ in the bait region.

_Cleaved α₂M Forms a Complex with BMP1_—Because a common feature in the inhibition of proteinases by α₂M is formation of a covalent complex between α₂M and the proteinase, we next sought to determine the capability of α₂M to covalently bind BMP1. BMP1 is normally detected on SDS-PAGE gels as a ~90-kDa monomer (3, 11), but it can also be detected as a ~270-kDa form on a reduced gel, subsequent to incubation with α₂M (Fig. 2B). Under non-reducing conditions (Fig. 2C), BMP1 can be detected as even higher molecular weight forms. The 85-kDa NH₂-terminal and 100-kDa COOH-terminal fragments of cleaved α₂M can remain covalently bound, via disulfide linkage, and this form can be linked to other 85- and 185-kDa forms via disulfide bonds (8). Thus, the high molecular weight forms observed under non-reducing conditions likely represent BMP1 covalently bound, via reaction with the 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-α₂M antibodies, which show α₂M to co-localize to the same high molecular weight forms as BMP1 under both reducing (Fig. 2D) and non-reducing (Fig. 2E) conditions. The observation here of covalent binding of BMP1 to the 100-kDa α₂M cleavage product is consistent with the mechanism whereby α₂M has been found to covalently bind other proteinases that it inhibits (8).

_α₂M Inhibits the pCP Activity of BMP1_—Because the first identified and best characterized activity of BMP1 is as a pCP (15), we next sought to determine whether this activity is inhibited in the presence of α₂M. Control experiments demonstrated that BMP1 achieves maximum cleavage of plasma α₂M by 2 h (Fig. 3A). Thus, to gauge the effect of α₂M-BMP1 interaction on BMP1 pCP activity, a constant amount of BMP1 (9.4 nM) was preincubated 2 h with increasing concentrations of α₂M (0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM), prior to incubation overnight with ³H-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 α₂M led to potent inhibition of BMP1 pCP activity, with a calculated IC₅₀ of 4.8 nM.

_α₂M Inhibits the Cleavage of Probigglycan by BMP1_—To determine whether α₂M can inhibit BMP1 cleavage of substrates other than procollagens, we tested the ability of α₂M to inhibit BMP1 cleavage of probigglycan. As can be seen (Fig. 4), BMP1 was able to completely convert probigglycan to bigglycan after 30 min under assay conditions, whereas preincubation with a 5-fold molar excess of α₂M for 2 h prior to incubation with probigglycan resulted in inhibition of a majority of bigglycan processing. Thus, α₂M appears to be a general inhibitor of BMP1 activity against various substrates.

_Mechanism of α₂M Inhibition of BMP1_—Binding and inhibition of proteinases by α₂M is thought to follow cleavage of the

To test whether α₂M can be cleaved by BMP1-like proteinases, the two proteins were co-incubated and α₂M examined for processing. Although α₂M incubated alone at 37 °C overnight was stable (Fig. 2A), 185-kDa α₂M co-incubated at 37 °C overnight with BMP1 was cleaved to produce two bands of ~100 and ~85 kDa. NH₂-terminal amino acid sequencing of the

**FIGURE 2.** BMP1 cleaves and forms a complex with α₂M. A, electrophoretic patterns on an SDS-PAGE gel are compared for human α₂M incubated in the absence (α₂M) or presence (α₂M + BMP1) of BMP1. BMP1 alone (BMP1) was electrophoresed in the final lane, to act as a marker for the corresponding band in the α₂M + BMP1 lane. The gel was stained with Coomassie Brilliant Blue R-250. The arrows denote the cleavage products of α₂M. Numbers to the left of the gel correspond to the positions and approximate sizes, in kDa, of molecular mass markers. B–E, Western blots (WB), using anti-FLAG antibodies for the detection of tagged BMP1 (B and C), or anti-α₂M antibodies (D and E) are shown of BMP1 and α₂M incubated alone or together. SDS-PAGE of samples was carried out under reducing (B and D) or non-reducing (C and E) conditions. Numbers to the left of the blots correspond to the positions and approximate sizes, in kDa, of molecular mass markers. Asterisks mark the sites of high molecular weight complexes.
**α₂-Macroglobulin Complexes and Inhibits BMP1**

![Image](https://via.placeholder.com/150)

**FIGURE 3.** Time- and dose-dependent inhibition of BMP1 procollagen C-proteinase activity by α₂M. A, 30 nM BMP1 was incubated with 30 nM α₂M for 0, 10, 30, 60, 90, 120, 240, and 360 min (plasma α₂M and recombinant wild type α₂M) or for 0, 2, 5, 10, 30, 120, 240, and 360 min (b-α₂M) at 37 °C. Cleavage reaction samples were analyzed by SDS-PAGE on 7.5% acrylamide gels, followed by Western blotting with anti-proBGN antibodies, quantification with NIH Image software, and plotting of relative percent cleavage of α₂M versus time in minutes. B, 9.4 nM BMP1 was preincubated with 0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM α₂M for 2 h at 37 °C, followed by incubation overnight with 400 ng of 3H-radiolabeled type I procollagen. Cleavage reaction samples were analyzed by SDS-PAGE on 5% gels, followed by scanning of autoradiograms, quantification of bands, using NIH Image software, and plotting of relative percent cleavage of procollagen versus nanomolar concentration of α₂M. Non-linear regression was performed using SigmaPlot.

**Modified α₂M Has Enhanced Ability to Inhibit BMP1**—We have previously noted wide differences in the efficiency with which different substrates are cleaved by BMP1. One of the substrates processed most efficiently by BMP1 is probiglycan (12). We therefore sought to determine whether we could enhance the ability of α₂M to inhibit BMP1 by replacing the native bait region with sequences surrounding the probiglycan scissile bone (see Fig. 1). It was found that the mutant recombinant α₂M (b-α₂M) forms complexes with and is cleaved by BMP1 more readily than plasma α₂M or recombinant wild type α₂M, prepared under the same conditions as b-α₂M (Fig. 6A). When the pCP inhibitory activities of varying concentrations of recombinant wild type α₂M and b-α₂M were measured (Fig. 6, B and C) (see “Experimental Procedures”), they led to calculated IC₅₀ values of 133 and 1.88 nM, respectively. The IC₅₀ value of 133 nM for recombinant wild type α₂M suggests considerably less effectiveness in BMP1 inhibition than the 4.82 nM value obtained for plasma α₂M (Fig. 3B), whereas the b-α₂M IC₅₀ value of 1.88 is consistent with increased inhibitory effectiveness. In fact, because 9.4 nM BMP1 would be expected to be 50% inhibited by 4.7 nM α₂M under assay conditions, if one molecule of BMP1 is inhibited by one molecule of α₂M, the IC₅₀ value of 1.88 nM suggests that molecules of b-α₂M are capable of inhibiting more than one molecule of BMP1, with an approximate stoichiometry of 2 molecules of BMP1 inhibited by one molecule of b-α₂M. This in turn suggests that b-α₂M is cleaved very rapidly by BMP1, because only in such cases are molecules of α₂M known to inhibit proteases at a greater than 1:1 ratio.

4 Y. Zhang, G. Ge, and D. S. Greenspan, unpublished data.
Although cross-linking experiments demonstrated both recombinant wild type α₂M and b-α₂M to form tetramers (Fig. 6D), some small difference in folding and/or post-translational modification may render the activity of the recombinant wild type protein less stable over the course of the overnight pCP assay than that of the corresponding protein from plasma. Importantly, however, b-α₂M is shown to have markedly improved efficiency in inhibiting BMP1 compared with wild type α₂M prepared under identical conditions, or compared with plasma α₂M. The improved efficiency of interaction of b-α₂M with BMP1, compared with wild type α₂M, is further illustrated by comparing the rapidity with which b-α₂M is cleaved by BMP1 compared with cleavage of wild type recombinant or plasma α₂M (Fig. 3A).

To obtain a quantitative comparison of the rates of interaction of BMP1 with the various wild type and mutant forms of α₂M, we employed the methodology of Enghild et al. (14), which involves quantitation of covalent proteinase-α₂M complex formation, subsequent to incubation of radiolabeled proteinase with α₂M. In Fig. 6E it can be seen that, subsequent to a 2-h co-incubation of 40 nM 35S-radiolabeled BMP1 with 40 nM α₂M, considerably more BMP1 is incorporated into complexes with b-α₂M (42% of sample) than with wild type recombinant (12% of sample) or plasma (28% of sample) α₂M. Time course experiments conducted with a fixed amount of 35S-radiolabeled BMP1 (40 nM) incubated with increasing amounts of each form of α₂M, as in Enghild et al. (14), provided Kᵢ values of 36.4, 39.5, and 25.2 nM for b-α₂M, and wild type recombinant and plasma α₂M, respectively (Fig. 7). Second-order rate constants obtained from the same data showed b-α₂M to be 24-fold more effective in interacting with α₂M than was recombinant α₂M prepared under identical conditions and 16-fold more effective than wild type α₂M from plasma (Table 1).

α₂M Inhibition of Procollagen Processing by Cells—As α₂M is capable of inhibiting the pCP activity of BMP1, we attempted to determine whether it might be able to inhibit the processing of procollagen by cells. Toward this end, MC-3T3-E1 murine osteoblastic cells were incubated either alone or in the presence of recombinant wild type α₂M or b-α₂M, and levels of processing of procollagen and insertion into the cell layer were compared. As can be seen (Fig. 8), MC-3T3 processing of procollagen was inhibited by both wild type and mutant α₂M. However, in the case of media from wild type α₂M-treated cells, most detectable collagens were the form 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 collagens was in the form of unprocessed pro-α1(I) chains (Fig. 8, A and B). These results show efficient inhibition of cellular BMP1-like proteins by b-α₂M, and less efficient inhibition by wild type α₂M. The appearance of pNα1(I) chains in the wild type α₂M-treated sample and 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 (17). b-α₂M may retain the ability to inhibit ADAMTS-2, due to a potential ADAMTS-2 cleavage site at the N terminus of the native bait region that is retained in the b-α₂M sequence (Fig. 1). Collagen was not detected in the media of untreated cells in Fig. 8A, and was only detected upon longer exposure of the Western blot (not shown), 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. 8A). 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. 8A), 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 (3). 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.

DISCUSSION

BMP1-like proteinases are important regulators of ECM deposition in vertebrates. In particular, they are of central importance to the formation of collagen fibrils because 1) they
process types I–III procollagen C-propeptides to yield the major fibrous components of ECM; 2) they cleave a zymogen to produce active lysyl oxidase, the enzyme that catalyzes the formation of covalent cross-links in collagen fibers; and 3) they process the N-propeptides, and in some cases C-propeptides, of procollagen chains of the minor fibrillar collagen types V and XI (2). 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 (18, 19).

Cleavage of procollagen C-propeptides appears to be the essential step that determines whether or not collagen fibrillogenesis will occur, and it has been demonstrated in vitro that, whereas type I collagen monomers that retain N-propeptides are incorporated into growing collagen fibrils as efficiently as mature monomers, monomers which retain C-propeptides are excluded from fibril incorporation (20). Our finding of inclusion of pNα1(I) forms, but not pCα1(I) forms in ECM associated with cell layers (Fig. 8A), is consistent with the previous in vitro fibrillogenesis results. The inhibitory effects of uncleaved C-propeptides on fibrillogenesis may be 2-fold: 1) via steric hindrance by the relatively bulky C-propeptide of the highly ordered packing of monomers necessary for fibrillogenesis, and 2) because C-propeptides confer ~1000-fold increased solubility to collagen monomers, which would be expected to interfere with monomer-to-monomer association (20). Incorporation of monomers with retained N-propeptides results in fibrils with aberrant morphologies and, in vivo, results in the heritable tissue disorder Ehlers-Danlos syndrome type VII (21). In contrast, no genetic disease has yet been associated with inability to cleave C-propeptides, perhaps because such inability would be incompatible with fibrillogenesis, and thus with life itself.

Because BMP1-like proteinases have been demonstrated to provide most, if not all procollagen C-proteinase activity in vivo (15, 22), and because removal of the C-propeptide is essential for collagen fibrillogenesis, the BMP1-like proteinases are attractive targets for therapeutic interventions in situations where inhibition of collagen fibrillogenesis is desirable. Although the formation of collagen fibrils is essential to morphogenesis and to the healing of wounds and bone fractures in the adult, formation of excessive amounts of fibrous collagenous ECM is the cause of much morbidity in the general population. These conditions range from keloids (excessive scarring of the skin), to the formation of surgical adhesions, to deep-seated fibroses of organs such as the lungs, liver, and kidneys. The deep-seated fibroses are particularly

FIGURE 6. Substitution of bait region with probiglycan sequences enhances α2M inhibition of BMP1 ~24-fold. A, a Western blot probed with anti-α2M is shown of plasma α2M, or recombinant wild type α2M (rα2M) or mutant (b-α2M) α2M incubated in the presence (+) or absence (−) of BMP1. B and C, 9.4 nM BMP1 was preincubated with 0, 4.7, 9.4, 18.7, 37.5, 56.2, 75.0, or 93.7 nM wild type α2M (B) or 0, 0.47, 0.94, 1.87, 3.75, 5.62, 9.4, or 18.7 nM b-α2M (C) for 2 h at 37 °C, followed by overnight incubation with 400 ng of 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 substrate versus nanomolar concentration of α2M. Non-linear regression was performed using SigmaPlot. D, a Western blot is shown of recombinant wild type α2M (rα2M) or mutant (b-α2M) α2M cross-linked with glutaraldehyde. E, an autofluorogram shows the SDS-PAGE electrophoretic patterns of 35S-radiolabeled BMP1 incubated alone, or in the presence of plasma α2M (α2M) or recombinant wild type α2M (rα2M) or mutant (b-α2M) α2M. Numbers to the left of the autofluorogram correspond to the positions and approximate sizes, in kDa, of molecular mass markers.
ominous, as the replacement of parenchmal tissue by scar tissue, composed essentially of fibrous collagenous ECM, destroys organ function.

Despite earlier reports that $\alpha_2M$ does not inhibit astacin-like proteinases (7, 9), we herein demonstrate $\alpha_2M$ to be an efficient inhibitor of the BMP1-like proteinases, a subgroup of the astacin-like proteinases (1). Thus, $\alpha_2M$ becomes the second endogenous inhibitor of BMP1-like proteinases to be identified because, as this article was being prepared, De Robertis and colleagues (23) elegantly demonstrated the secreted Xenopus/zebrafish protein sizzled/ogon to be an inhibitor of such proteinases. The demonstration that $\alpha_2M$ is a potent inhibitor of BMP1-like proteinases suggests new approaches toward therapeutic interventions in the fibroses. These could include: 1) topical application in cases of superficial fibroses, such as keloids and corneal scarring; 2) delivery via aerosol for pulmonary fibrosis; and 3) ectopic expression of $\alpha_2M$ via gene therapy.

FIGURE 7. Plots of data for determination of $K_i$ values and second-order rate constants. Plots are shown for percent incorporation of $^{35}S$-radiolabeled BMP1 into complexes with each form of $\alpha_2M$, upon incubation of 40 nM BMP1 with increasing amounts of plasma $\alpha_2M$ (A), wild type recombinant $\alpha_2M$ (B), or b-\(\alpha_2M\) (C).

FIGURE 8. $\alpha_2M$ can inhibit procollagen processing by cells. A, Western blots are shown of media and cell layer samples of MC-3T3 cells incubated in the presence of 20 nM $\alpha_2M$ or b-\(\alpha_2M\), or an equivalent volume of buffer (–). Blots were probed with anti-\(\alpha\)1(I) C-telopeptide antibodies. B, the media sample blot was scanned and quantified, using NIH Image software, providing the graph shown in which the percentage of total signal each for the wild type $\alpha_2M$ lane and for the b-\(\alpha_2M\) lane is given for uncleaved pro-\(\alpha\)1(I) chains (pro), p\(\alpha\)1(I) (pc), and p\(\alpha\)1(I) (pN) processing intermediates, and completely processed mature \(\alpha\)1(I) chains (col \(\alpha\)1(I)).

TABLE 1 Relative BMP1 inhibitory effectiveness of plasma and recombinant $\alpha_2M$ forms

| Form          | $K_i$  | $k_2$  | Second-order rate constant | Relative effectiveness |
|---------------|--------|--------|----------------------------|-----------------------|
| Plasma $\alpha_2M$ | 25.2   | 0.20   | $8.10 \times 10^6$         | 1.51                  |
| Recombinant $\alpha_2M$ | 39.5   | 0.21   | $5.36 \times 10^6$         | 1                     |
| Recombinant b-\(\alpha_2M\) | 36.4   | 4.60   | $1.27 \times 10^6$         | 23.69                 |
Ectopic expression via gene therapy, in addition to targeting delivery of high levels of \( \alpha_2 \)-M to specific tissues, would also result in co-expression of recombinant \( \alpha_2 \)-M with endogenous BMP1-like proteinases in the same cells. The latter result may additionally boost inhibitory effects, as it has been hypothesized that some portion of the cleavages affected by BMP1-like proteinases may occur intracellularly and/or near the cell surface (2, 24), and it has also been previously demonstrated that recombinant \( \alpha_2 \)-M is capable of inhibiting proteolysis both extracellularly and intracellularly, when co-expressed with endogenous proteinases in the same cells (25). Although the above approaches may be undertaken with wild type \( \alpha_2 \)-M sequences, the exciting possibility also exists of engineering the bait region of \( \alpha_2 \)-M, such that the IC\textsubscript{50} for inhibition of BMP1-like proteinases is decreased. The validity of the latter approach has been proven in the current report, in which substitution of the native \( \alpha_2 \)-M bait region with sequences flanking the probrinogen BMP1 cleavage site increased the efficiency about 24-fold (Table 1). The desirability of the latter approach, in terms of inhibiting deposition of a collagenous ECM, is also demonstrated in Fig. 8, where it is shown that the mutant b-\( \alpha_2 \)-M was effective in inhibiting procollagen processing by fibrogenic cells, whereas wild type \( \alpha_2 \)-M was much less effective. Although there is no strict consensus sequence for the cleavage sites of BMP1-like proteinases, we can begin to identify preferred amino acid residues at each position flanking such sites, based on analysis of the sites of known substrates of BMP1-like proteinases (2). In the future, modified versions of \( \alpha_2 \)-M may be further optimized for cleavage by BMP1-like proteinases by substituting preferred residues into positions flanking cleavage sites for BMP1-like proteinases in bait regions. In addition, removal of recognition sites for cleavage of the bait region by other proteinases could make the recombinant \( \alpha_2 \)-M specific for inhibition of BMP1-like proteinases, thereby eliminating the possibility of unwanted secondary effects from inhibition of other proteinases. In regard to the treatment of fibroses, it may be particularly desirable to engineer versions of \( \alpha_2 \)-M that would inhibit BMP1 proteinases, which are involved in forming collagenous ECM while not inhibiting those matrix metalloproteinases involved in the turnover of collagenous ECM. Studies akin to the approaches described above are currently underway.

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