Identification of amino acid residues in bone morphogenetic protein (BMP)-1 important for procollagen C-proteinase activity

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Running title: Procollagen C-proteinase activity of BMP-1

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SUMMARY

Bone morphogenetic protein (BMP)-1, which belongs to the tolloid sub-group of astacin-like zinc metalloproteinases, cleaves the C-propeptides of procollagen at the physiologic site and is, therefore, a procollagen C-proteinase (PCP). Cleavage occurs between a specific alanine or glycine residue (depending on the procollagen chain) and an invariant aspartic acid residue in each of the three chains of procollagen. To learn more about how BMP-1 exhibits PCP activity we mapped the primary structure of BMP-1 onto the X-ray crystal structure of astacin and identified residues in the metalloproteinase domain of BMP-1 for subsequent site-directed mutagenesis studies. Recombinant wild-type and mutant BMP-1 were expressed in COS-7 cells and assayed for PCP activity using type I procollagen as the substrate. We showed that substitution of alanine for E94, which occurs in the HEXXH zinc-binding motif of BMP-1, abolishes PCP activity. Furthermore, mutation of residues K87 and K176, which are located in the S1'-pocket of the enzyme and therefore adjacent to the P1' residue in the substrate, reduced the proteolytic activity of BMP-1 by ≈50%. A surprising observation was that mutation of C66 reduced the activity to 20%, suggesting that this residue is crucial for activity. Further experiments showed that C66 and C63, which are located in the tolloid-specific sequence C63G64C65C66 in the active site, most likely form a disulfide bridge.
INTRODUCTION

Bone morphogenetic protein1 (BMP)-1 is a zinc metalloproteinase whose metalloproteinase domain shares 39% sequence identity with that of astacin, the digestive proteinase from crayfish (1). BMP-1, also known as procollagen C-proteinase (PCP) (2), is fundamental to the synthesis of the extracellular matrix because it cleaves the C-propeptides of type I, II and III procollagen (3,4) and presumably the C-propeptides of the other major fibrillar collagen precursors, type V and XI procollagen. The importance of BMP-1 in tissue assembly is exemplified in the BMP-1 knock-out mouse, which dies soon after birth from failure of ventral body wall closure associated with abnormal collagen fibrillogenesis (5). The occurrence of abnormal fibrils in affected tissues of this mouse is consistent with the persistence of partially-processed procollagen molecules. In addition to its roles in cleaving procollagen, BMP-1 cleaves other ECM macromolecules including prolyl oxidase (6), probiglycan (7) and prolammin-5 (8). BMP-1 and its larger splice variant mammalian tolloid (mTLD) are important in development (9) as well as embryo patterning in drosophila (10).

1 Abbreviations used: BMP-1, bone morphogenetic protein-1; rBMP-1, recombinant BMP-1; BMP-1myc, BMP-1 containing the c-myc peptide at its C-terminus; PCR, polymerase chain reaction; PCP, procollagen C-proteinase; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; EDTA, ethylene diamine tetraacetic acid; SDS, sodium dodecyl sulfate; pNcollagen, a normal intermediate in the conversion of procollagen to collagen containing the N-propeptides but not the C-propeptides; proα chain, a polypeptide chain of procollagen; pNα chain, a polypeptide chain of pNcollagen; P1, P2, P3, and P1’, P2’, P3’ designate substrate/inhibitor residues amino terminal (“non-primed side”) and carboxyl terminal (“primed-side”) to the scissile bond, respectively; S1, S2, S3, and S1’, S2’, S3’ represent the corresponding subsites of the proteinase.
xenopus (11) and sea urchin (12,13). For example, in vertebrates, BMP-1 cleaves chordin (an antagonist of BMP-4) during normal dorsal-ventral patterning (for review see (14)). Therefore, understanding the catalytic mechanism of this enzyme is relevant to studies of animal development and tissue organisation. Furthermore, since collagen is expressed in soft tissues such as liver and lung during progressive fibro-proliferative diseases (e.g. cirrhosis, pulmonary fibrosis and scleroderma) that are characterised by excessive deposition of connective tissue (for review see15)), structure-function studies of BMP-1 are a step towards the rational design of anti-fibrotic drugs.

In all the known substrates of BMP-1 the scissile bond resides between a small side-chained residue and an aspartic acid. For example, the P1 residue in chordin is serine or alanine (16), in procollagen it is alanine or glycine (2) and in prolyl oxidase it is glycine (6). Furthermore, the P1’ residue in these substrates is always aspartic acid. Presumably, the shape and size of the active site of BMP-1 accommodates only small residue side chains in the P1 position and a large acidic side chain in the P1’ position. An understanding of the mechanism of cleavage by BMP-1 requires a detailed knowledge of how the P1 and P1’ residues dock into the active site. In the absence of structural information of the metalloproteinase domain of BMP-1 we reasoned that a valid approach was to examine the function of individual residues by site-directed mutagenesis. This approach is made possible by the fact that the metalloproteinase domain of BMP-1 shares high sequence homology with astacin whose X-ray crystal structure is known (17).

The metalloproteinase domain of astacin is kidney-shaped and has two domains (at the amino and carboxyl-termini) that are separated by an active site cleft (18). A zinc ion sits at the bottom of the cleft and is co-ordinated in a trigonal-bipyramidal geometry by three
histidine residues, a tyrosine residue (Y149) and a water molecule, which is also bound to the
carboxylic acid side chain of E93. It has been shown for astacin that E93 and Y149 are
essential for catalytic activity (19). Although the metalloproteinase domains of astacin and
BMP-1 are homologous and presumably have a similar tertiary structure, it is not obvious
why BMP-1 cleaves scissile bonds between a small side chained residue and an aspartic acid.

In this study we used information from the three-dimension structure of astacin, the X-
ray crystal structure of astacin in complex with a transition state inhibitor (20), and the
primary structures of different members of the astacin family, to identify residues in BMP-1
that might account for the procollagen C-proteinase activity of BMP-1. We subsequently used
site-directed mutagenesis and assay of recombinant enzyme to identify specific lysyl and
cysteine residues in the active site of BMP-1 that are important for PCP activity.
EXPERIMENTAL PROCEDURES

Source of materials

PCR products were purified with Qiaquick kit (Qiagen). Plasmids were extracted with Qiaprep spin miniprep kit (Qiagen). Kaleidoscope protein molecular weight standards (high range) were from BioRad. A full-length cDNA for BMP-1 (accession number P13497) was cloned from a human placental cDNA library. The cDNA was inserted at the KpnI/XbaI sites of the expression vector pcDNA3 (Invitrogen), thereby placing it under the transcriptional control of a CMV promoter. The c-myc amino acid sequence (EQKLISEEDL), which is recognized by the 9E10 monoclonal antibody (21), was introduced immediately 5’ of the stop codon of the BMP-1 clone. The polyclonal 1210 antibody was raised in rabbits using conventional procedures, by Sigma-Genosys. In brief, a peptide corresponding to the 10 N-terminal residues of the mature BMP-1 protein (after removal of the prodomain) was conjugated to keyhole limpet hemocyanin and subsequently used to immunise two separate rabbits. Non-immune serum was collected prior to injections.

Site-directed mutagenesis of BMP-1

Mutants were generated by site-directed mutagenesis of the XcmI/BlpI fragment of the BMP-1 cDNA clone. The XcmI site is located at nucleotide 383. The BlpI site is located at nucleotide position 913. The XcmI/BlpI fragment corresponds to residue numbers S124 to G308 in the BMP-1 protein. The mutations were made by standard procedures using the strand overlap polymerase chain reaction (22), and using Pwo polymerase (Roche), a forward primer containing a XcmI site (underlined) (5’-GTCCCGACCGAGCGTGTGGCC-3’, XcmForward primer) and a reverse primer containing a BlpI site (underlined) (5’-CCCTTGCTGAGCGTGCTCCCT-3’, BlpReverse primer) and oligonucleotides in both
orientations containing the desired modification (in bold): E94A: 5’-ATTGTGGTCACGCTCTGGGGCCACG-3’; K87A: 5’-AACGTGACGCTCCGGCATT-3’; K176A: 5’-GAACGGGCTGGCACCTCCCATTG-3’; C63G: 5’-CCTATCGACCTGGCGCTGCTGCTC-3’; C65A: 5’-GACCTTGCGGCGCTCCTACGT-3’; C66G: 5’-TGCGGGTGCCTACGTG-3’; C85A: 5’-GGCAAGAACGCTGACAAGTT-3’. Pwo DNA polymerase was used to minimize base misincorporation during the polymerase chain reactions. Briefly, a DNA fragment was amplified using the Xcm Forward primer and the antisense mutant primer and an overlapping fragment was amplified using the sense mutant primer and the downstream Blp Reverse primer. Both fragments were gel-purified (Macherey-Nagel) mixed and re-amplified with the Pwo enzyme with the Xcm Forward and Blp Reverse primers. The product was digested using appropriate restriction enzymes, gel-purified and introduced in place of the corresponding wild-type fragment in BMP-1myc. DNA sequencing was used to verify the mutations and to ensure that the cDNA clones were error free.

**Cell culture**

COS-7 cells (ECACC number 87021302) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% foetal calf serum (GIBCO) (complete DMEM) in a 37°C incubator with 5% CO₂. The recombinant wild-type and mutant BMP-1 proteins were expressed in transiently-transfected COS-7 cells. Transfections were made with Fugene 6 reagent (Roche) and 10 µg of plasmid/100 mm tissue culture plate. Cells were grown to approximately 80% confluency by overnight incubation in complete DMEM. After 3 rinses with PBS (GIBCO), cells were transfected in serum-free DMEM/Iscove/HamF12 (1:1:1) (GIBCO) following the manufacturer’s instructions and returned to the incubator. Media were removed after 48 hrs and replaced by DMEM without serum and conditioned for a further 24
hrs. The tissue culture media were collected at 72 hrs post-transfection and cleared of cell debris by centrifugation at 1600 x g for 10 min, and concentrated to 100 µl using Centriprep-30 and Microcon-10 concentrators (Amicon, Inc.). The samples were used immediately or stored at -80°C.

**Assay of procollagen C-proteinase**

Recombinant BMP-1 was assayed for procollagen C-proteinase activity using human $^{14}$C-labeled type I procollagen substrate and analysis of the cleavage products on SDS-gels, as described (3). A minor change was that laser densitometry of film exposed to dried gels was replaced by image plate quantitation of the $^{14}$C-labeled proteins. In brief, $^{14}$C-labeled type I procollagen was obtained from the medium of human skin fibroblasts that had been cultured in DMEM supplemented with ascorbic acid (25 µg/ml), L-glutamine and a mixture of uniformly labeled $^{14}$C-L-amino acids (1 µCi/ml). The procollagen was purified by ammonium sulfate precipitation (176 mg/ml) and ion exchange chromatography, and then concentrated by ultrafiltration on Amicon YM100 membranes. The procollagen content was determined by the sensitive hydroxyproline assay method of Terlink (23). BMP-1 was purified from the culture medium of MG63 cells by liquid chromatography on sequential columns of matrix Green A, Con A sepharose, Heparin-Sepharose and Superose S-300 and S-200 as described (24).

**Electrophoresis and immunoblotting**

Cells were rinsed once with PBS and incubated on ice for 15 min with 500 µl of RIPA buffer (150 mM NaCl, 1% deoxycholate, 0.1% SDS, 10 mM Tris pH 7.6) containing 10 mM EDTA, and protease inhibitor cocktail (Roche). Cells in RIPA buffer were scraped on ice and sonicated. Lysates were subjected to a 5 min centrifugation at 14,000 x g at 4°C. Supernatants
were retained and stored at –80°C for further analysis. Proteins were concentrated on Centriprep YM30 membranes (Amicon) and separated by discontinuous SDS-PAGE. BMP-1myc was examined by Western blot analysis in which the primary antibody was either the mouse monoclonal anti c-myc peptide antibody, 9E10, or the rabbit 1210 neoeptitope polyclonal antibody. Secondary antibodies were either horseradish peroxidase conjugated to anti-mouse or anti-rabbit IgG and were detected by the enhanced chemiluminescence (ECL) method (Supersignal West Dura Extended Duration, Pierce). The levels of BMP-1myc were quantitated by laser densitometry of ECL flurograms exposed to pre-flashed film.
RESULTS

Sequence analysis

In preliminary studies we performed a multiple sequence alignment of 31 members of the astacin family of metalloproteinases using MultAlin (25) (data not shown). This included astacin, BMP-1, mTLD, mammalian tolloid like (mTLL)-1, mTLL-2 and meprins. Fig. 1 contains a trimmed-down version of this alignment in which only the sequences of astacin and human BMP-1 are shown. The high degree of sequence homology between the metalloproteinase domains of astacin and BMP-1, and the fact that the metalloproteinase domains are similar in size, suggests that the structure of the metalloproteinase domain of BMP-1 is similar to that of astacin (26). With this information, we identified the following residues for site-directed mutagenesis studies.

E94 - In astacin the active site zinc is penta-coordinated by three histidines, a unique tyrosine residue (in the Met-turn) and a water molecule. The zinc-bound water is thought to be polarized for nucleophilic attack of the scissile bond by the glutamic acid residue in the consensus sequence HEXXH. In BMP-1, this glutamic acid is at amino acid position 94. To test the hypothesis that E94 is important for the PCP activity of BMP-1, this residue was replaced by alanine.

K87 and K176 - It has been suggested that K87 and the K176-containing S1’ loop define the S1’ pocket of BMP-1 (26). Furthermore, the K176-containing S1’ loop of BMP-1 is absent from astacin and non-tolloid members of the astacin family. It was an attractive idea that the positively charged side chains of these lysyl residues might bind to the acidic side chain of aspartic acid in the P1’ position of the procollagen chains.
**CXCC motif** - Of particular interest was the anti-parallel β-strand IV (edge strand), which forms the upper edge of the active site cleft. Noteworthy, when astacin binds its substrate, the residues in the substrate N terminal to the scissile bound (i.e. the non-primed side) align with the β-strand IV of the enzyme, forming two or three hydrogen bonds (27). It has been pointed out that the β-strand IV in BMP-1 contains three cysteine residues. Two of these, C63 and C66, might form a disulfide bond with each other (26). These cysteine residues straddle C65, which is conserved across the astacin family. In astacin, the equivalent cysteine (C64) forms a vital disulfide bond with a non-active site cysteine (C84). It has been suggested that the equivalent disulfide bond in BMP-1 is formed between C65 and C85 (26). The strategic position of C63, C65 and C66 in the active site β-strand IV of BMP-1 and the proposed disulfide bond between C65 and C85 made these residues good targets for site-directed mutagenesis studies of the structure and function of BMP-1.

**Recombinant latent BMP-1myc is correctly processed and secreted by COS-7 cells.**

The pcDNA3 vector containing the cDNA for BMP-1myc was transfected into COS-7 cells and the conditioned culture medium and the cell lysate were analysed by Western blotting using the 9E10 antibody (which detects the c-myc tag at the C-terminus of the molecule) and the 1210 neoeptope antibody (which was raised to a peptide corresponding to the 10 residues at the N-terminus of mature BMP-1). Fig. 2 shows that the 9E10 antibody detected the latent BMP-1myc in cell lysates and the mature BMP-1myc in the culture medium. The 1210 neoeptope anti-peptide antibody detected only the mature enzyme, which occurred in the culture medium and not in the cell lysate. Furthermore, the culture medium from COS-7 cells transfected with the empty vector contained no immunoreactive proteins, which shows that
the endogenous levels of BMP-1 were very low. Furthermore, Western blots using the preimmune rabbit serum were blank (data not shown).

**BMP-1myc expressed in COS-7 cells exhibits procollagen C-proteinase activity**

Previous studies had shown that the addition of the Flag peptide at the C-terminus of BMP-1 has negligible effect on the ability of the molecule to cleave procollagen (16). Therefore, we anticipated that the c-myc was unlikely to influence the PCP activity of BMP-1. Nevertheless, we assayed c-myc tagged BMP-1 for PCP activity and compared it to that of native BMP-1. Figs. 3 and 4 show that type I procollagen is readily cleaved by BMP-1myc. We wanted to know if BMP-1myc cleaved procollagen at the physiological site. We used a neoepitope antibody that recognised the N-terminal 10 residues of the \( \alpha_1(1) \) chain C-propeptide. Western blot analysis showed that the antibody recognised the C-propeptides after cleavage of procollagen with recombinant BMP-1 and BMP-1myc (data not shown).

**The E94, K87, K176 and K87A/K176A mutants were secreted efficiently**

COS-7 cells were transfected with pcDNA3 containing cDNAs encoding for BMP-1myc and E94, K87, K176 and K87A/K176A mutants. The culture media of the cells were examined by Western blot analysis using the 9E10 monoclonal antibody. Fig. 5 shows that BMP-1myc and the mutants were secreted as mature enzymes. In some experiments small levels of latent BMP-1 were detectable in the culture medium, which showed that cleavage of the prodomain of BMP-1 was not a prerequisite for secretion. Medium harvested from COS-7 cells transfected with the empty vector contained no immunoreactive proteins.

**C85 but not C63, C65 and C66 is essential for stability of BMP-1**
Constructs encoding BMP-1myc and the mutants C63G, C65A, C66G and C85A were transfected into COS-7 cells and the conditioned culture media were subjected to Western blot analysis using the 9E10 antibody. A typical result is shown in Fig. 6. The results showed that BMP-1myc was secreted into the culture medium mostly as the mature enzyme. The C63 and C65 mutants were efficiently secreted into the culture medium and as both mature and latent enzymes. C66G was also secreted efficiently into the culture medium. In contrast to the other mutants studied, the C85A mutant was poorly secreted and could only be detected in the culture medium when the gel was overloaded. Cell lysates contained only the latent form of BMP-1.

**Evidence for C63-C66 and C65-C85 disulfide bonds in BMP-1**

The low level of secretion of the C85A mutant suggested to us that C85 is crucial for the stability of the protein, presumably because it participates in the formation of an important structural disulfide bond with C65 in the β-strand IV. This was in agreement with the suggestion that C85 bonds with C65. However, the fact that the C65A mutant was well secreted raised the possibility that another cysteine residue could substitute for C65 in bonding to C85. The only candidates were C63 and C66. We examined the migration of the C63G and C65A mutants in SDS-gels under non-reducing conditions. Fig. 7 shows that wild-type BMP-1 migrated mostly as a single band (M-SS). In comparison, the C63 and C65 mutants migrated as a slower band (M-SH). This indicated that the C63 and C65 mutant molecules have a more open conformation than the wild-type molecule.

**Identification of residues important for PCP activity: E94 and C66 are crucial.**

Culture media from COS-7 cells transfected with cDNAs encoding BMP-1myc and the mutants described above was concentrated on YM30 membranes and the levels of BMP-1myc
determined by Western blot analysis using the 9E10 antibody. The concentration of BMP-1 molecules was normalised. The preparations were then examined in assays of procollagen C-proteinase. The results are summarised in Fig. 8. All the mutants exhibited reduced PCP activity. Most notably, the E94A mutant exhibited no PCP activity. This showed that E94 is essential for PCP activity of BMP-1. Furthermore, this result showed that endogenous levels of BMP-1 in the culture medium of COS-7 cells were below the detection limit of the assay and that non-specific proteinases that might occur in the preparations did not interfere with the PCP assays. The K87 and K176 mutants had reduced PCP activity and the PCP activity of the double lysine mutant was 48% of control values. The results also showed that the C63 and C65 mutants exhibited a marked reduction in PCP activity (38 and 33%, respectively). A surprising result was that the C66G mutant exhibited very low levels of PCP activity (~20% of control).
DISCUSSION

In this study we have used site-directed mutagenesis to identify residues in the metalloproteinase domain of BMP-1 that are important for its PCP activity. It was first necessary to establish and validate a system capable of expressing recombinant BMP-1 that exhibited PCP activity. We found that COS-7 cells efficiently expressed a cDNA clone encoding the full-length latent BMP-1 (driven by a CMV promoter) to produce active procollagen C-proteinase. Furthermore, if these cells synthesised endogenous BMP-1, it was undetectable in assays of procollagen C-proteinase and in Western blotting analyses using a neopeptide antibody that recognises the N-terminus of mature BMP-1. The amino acid sequence within the N-terminal 10 residues of mature BMP-1 that is recognised by the 1210 antibody has not been characterised. However, since the 1210 antibody does not recognise latent BMP-1 and only recognises the mature BMP-1, it is likely that COS-7 cells convert latent BMP-1 to mature BMP-1 at, or close to, the physiologic site. The study also showed that the introduction of the c-myc tag at the C-terminus of BMP-1 did not prohibit subsequent assays of PCP activity.

The results show that mature BMP-1 (lacking the prodomain) was not observed in lysates of transfected COS-7 cells. This is consistent with processing of latent BMP-1 by COS-7 cells occurring after, or during, secretion from the cells. Latent BMP-1 contains the dibasic RSRR peptide at the junction of the prodomain and the mature protein. Furin and furin-like subtilisin-related proprotein convertases are known to process a variety of protein precursors at dibasic sequences including growth factors, receptors and matrix metalloproteinases (28,29), and most probably BMP-1/mTLD-like proteases (17). In some experiments we noticed that latent BMP-1 was detected in the culture medium. This might have occurred
when the rate of synthesis of latent BMP-1 exceeded the capacity of the BMP-1 convertase to convert all the latent BMP-1 to mature BMP-1. Nevertheless, these experiments demonstrated that, in COS-7 cells, removal of the prodomain is not a prerequisite for secretion of the protein.

One of our targets in site-directed mutagenesis experiments was E94, which occurs in the zinc-binding consensus motif HExxHxxGxxH. We showed that substitution of alanine for E94 eliminated the PCP activity of BMP-1 and confirmed that this glutamic acid residue is essential for catalytic activity of this enzyme. The observation that the culture medium of cells transfected with the E94A mutant did not contain detectable PCP activity also validated the use of COS-7 cells as a good model cell system in which to express recombinant BMP-1.

By mapping the primary structure of BMP-1 onto the secondary structure of astacin, Stocker and colleagues showed that one side of the S1’ pocket of BMP-1 is formed from an S1’ loop containing residues 170-184 (30). This loop contains K176. When this residue was mutated to alanine the PCP activity of BMP-1 was reduced by 25%, possibly because the positive charge of its side chain stabilises the carboxylic side chain of the aspartic acid in P1’ position of procollagen. The S1’ pocket of BMP-1 is supposedly bigger than the corresponding pocket in astacin, which explains how BMP-1 can accommodate the large polar side chain of aspartic acid in procollagen. Of special interest, the S1’ pocket of meprin A is thought to be larger than that in astacin (17) and accounts for how bulky and charged side chains in the P1’ position of substrates can be accommodated in the S1’ pocket (31).

We noted that BMP-1 has a lysine residue at position 87 that occurs in all tolloid but is a tyrosine residue in astacin (W86). Interestingly, neither astacin nor meprin A, which also
exhibits a tyrosine in this position (32), can accept an acidic side chain in P1'. In contrast, meprin B has an arginine in place of K87 (an equivalent basic amino acid) and prefers an acidic side chain in P1' (33). Taken together, these observations led us to hypothesise that K87 might be important for PCP activity. To test this hypothesis we mutated K87 to alanine and assayed the mutant enzyme. The results showed that the PCP activity of the K87 mutant was 64% of control and was, therefore, less effective than the K176 mutant. When both K87 and K176 were mutated, the PCP activity of BMP-1 was reduced to ≈50%. These results indicated that K87 and K176 act together to stabilise enzyme-substrate interactions involving the primed side of the substrate. As these two residues contribute to only 50% of the PCP activity of BMP-1, it was of interest to determine which other residues might be important for PCP activity. The most conspicuous difference between BMP-1 and other non-PCP astacins was the occurrence of two unique cysteine residues at the active site of the enzyme.

The X-ray crystal structure of astacin shows a disulfide bond between a cysteine in the upper edge of the active site cleft and a cysteine buried in the body of the metalloproteinase domain (34). These cysteine residues are C64 and C84, respectively, and are invariant in all astacin family members (26). As this disulfide bridge clamps the β-strand IV to the loop between strand V and helix B, it is likely to be crucial for stabilising the structure of the metalloproteinase domain. The equivalent cysteine residues in BMP-1 are C65 and C85. The importance of C85 in stabilising the structure of the metalloproteinase domain of BMP-1 was confirmed when C85A was found to be poorly secreted. Surprisingly, however, the C65 mutant was secreted efficiently. Also, BMP-1 molecules in which the other two cysteine residues on the active site edge strand, i.e. C63 and C66, had been mutated were also well secreted. This raised the possibility that C85 could disulfide bond with a cysteine other than C65. The most likely candidate was C63 because, based on structure comparison with astacin
in complex with an inhibitor, its side chain is oriented towards the C85 residue (20). This suggestion is supported by the observation that the C63G and C65A mutants were equivalent to each other both in PCP assays and migration position in non-reducing SDS-gels.

In Fig. 9 we propose a model for the PCP activity of BMP-1 which explains the results obtained in this study and takes into account published studies on the structure of astacin. We propose that the disulfide-bonded side chain of C66 is fundamental to the PCP activity of BMP-1 because it backs onto the P1 residue in procollagen. This is a direct analogy with what happens in astacin when W65 (the equivalent of C66 in BMP-1) backs onto the P1 position of the astacin substrate. The absence of the C63-C66 disulfide bond in: (1) the C66 mutant, (2) the C63 mutant (because C66 has a free thiol group in this mutant), and (3) the C65 mutant (because C85 has bonded with C63 thus leaving a free thiol group on C66), alters the chemical structure of the S1 site and decreases PCP activity.

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Figure 1. The sequence alignment of astacin and BMP-1. The alignment of the amino acid sequences of human BMP-1 (BMP-1_hu) and astacin was based on the work of Stöcker et al. (26) and with the secondary structures identified by boxes. Residue numbers are labeled with an asterisk, in blocks of 10 residues, and specific for BMP-1 and astacin. Amino acid positions are numbered from the start of the metalloproteinase domain of BMP-1, in which the first alanine residue of the domain is residue number 1. The numbers of the metalloproteinase domain can be converted to the positions in the BMP-1 sequence by adding 120. Green indicates identical residues. Bold indicates homologous substitutions. Red indicates residues that are different between BMP-1 and astacin and which also occur at functionally-significant sites in the proteins. Closed triangles indicate the residues in BMP-1 that were chosen for site-directed mutagenesis.

Figure 2. Expression and characterization of BMP-1myc in COS-7 cells. Wild-type BMP-1myc was transiently expressed in cultured COS-7 cells. After 72 hours post-transfection the cells were lysed and the proteins in the culture medium were concentrated on YM membranes. Proteins from the cell medium (M) and lysates (L) were separated by discontinuous SDS-PAGE (10% separating gel) in the presence of β-mercaptoethanol. BMP-1myc and BMP-1 were detected by Western blot analysis using anti-c-myc monoclonal antibody (9E10) and the neoepitope 1210 antibody (1210), respectively. In control samples (pcDNA3 empty vector) COS-7 cells were transfected with the empty vector.

Figure 3. Cleavage of type I procollagen by recombinant BMP-1myc (analysed under non-reducing conditions). Lane 1, 14C-labeled type I procollagen (0.3 µg) was incubated at
37°C for 24 hours with unconcentrated medium (90 µl) from COS-7 cells transfected with the empty vector (Lane 1) or from the vector encoding BMP-1myc (Lane 3). Lane 2, ¹⁴C-labeled procollagen (0.3 µg) incubated with BMP-1 purified from MG63 cells. The proteins were separated in non-reducing SDS-gels (10% separating) and the ¹⁴C-labeled proteins were detected using a phosphorimager. In samples containing wild-type BMP-1 and recombinant BMP-1myc the procollagen was converted to pNcollagen. Undigested procollagen (P, containing disulfide-bonded chain) migrates near the top of the SDS-gel. The cleaved C-propeptides (CP) migrate as a single disulfide-bonded trimer.

Figure 4. Cleavage of type I procollagen by recombinant BMP-1myc (analysed under reducing conditions). Human type I procollagen (0.3 µg) was incubated at 37°C for 24 hrs with and without EDTA: Lane 1, without enzyme; Lane 2, purified MG63-derived BMP-1; Lane 3, concentrated medium (4 µl) from COS-7 cells transfected with BMP-1myc. Products of the reactions were separated on a 7% SDS-gel under reducing conditions. Arrows indicate the position of the proα1(I) and proα2(I) chains of procollagen and the pNα1(I) and pNα2(I) chains of pNcollagen. The proα2(I) and pNα1(I) chains migrated as a single band.

Figure 5. Expression of wild-type and E94A, K87A, K176 and K87A/K176A mutant BMP-1myc in COS-7 cells. Vectors containing cDNA encoding BMP-1myc and BMP-1myc containing the mutations E94A, K87 and K176A as well as the double lysine mutant K87A/K176A were transiently expressed in COS-7 cells and the culture media were concentrated. Protein samples (4 µl) were separated by SDS-PAGE (10%) in the presence of β-mercaptoethanol and detected by Western blot analysis using the 9E10 anti-c-myc monoclonal antibody. The proteins were secreted mostly as mature BMP-1. Small quantities
of latent BMP-1myc were detected in some samples. pcDNA3, cells transfected with the empty vector.

Figure 6. Expression of wild-type and C63G, C65A, C66G and C85A mutant BMP-1myc in COS-7 cells. Wild-type and mutant BMP-1myc were transiently expressed in COS-7 cells. Proteins in the concentrated culture media (4 µl) (M) and cell lysates (L) were separated on SDS-gels (10%) in the presence of β-mercaptoethanol and the BMP-1 molecules detected by Western blotting using the 9E10 antibody. Latent denotes the latent form of BMP-1. Mature denotes the mature form of BMP-1. The C85A mutant was only detectable in samples in which a minimum of 100 µl of sample was loaded on the gel (indicated by the asterisk).

Figure 7. Analysis of C63G and C65A BMP-1myc by non-reducing SDS-PAGE. The migration positions of C63G and C65A mutant BMP-1myc were examined by SDS-PAGE under non-reducing conditions. BMP-1myc migrated as a fast migrating oxidized form (M-SS) and a slower migrating reduced form (M-SH) in which the oxidised form was the most abundant. The C63G and C65A mutants migrated exclusively as the slower migrating reduced form. The far right lane contains latent BMP-1myc, which migrates at a slower position than the oxidised (M-SS) form of BMP-1.

Figure 8. BMP-1myc mutants exhibit decreased PCP activity. COS-7 cells were transfected with vectors encoding BMP-1myc and mutant BMP-1myc, as indicated. The proteins in the culture medium were concentrated by ultrafiltration and the levels of BMP-1myc quantitated by Western blot analysis, using the 9E10 antibody. The PCP activity of the BMP-1myc mutants, normalised for BMP-1myc concentration, was assayed by cleavage of 14C-labeled type I procollagen. Percentage of procollagen is shown from 5 separate
experiments (3 in the case of C65A and C66G) (+ S.E.). The results were normalised to the PCP activity of BMP-1myc lacking mutations.

Figure 9. Schematic representation of the presumed binding interactions between BMP-1 β-strand IV and the non-primed side of the substrate (type I procollagen) (adapted from 35). The substrate (in red) runs antiparallel to the edge strand (β-strand IV) in black. Dashed lines, hydrogen bonds. Green lines, putative disulfide bonds. Subsites are shown as arched lines.
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Identification of amino acid residues in bone morphogenetic protein (BMP)-1 important for procollagen C-proteinase activity
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