Bone morphogenetic protein (BMP)-1 is a zinc-dependent metalloproteinase that cleaves a variety of extracellular matrix substrates, including type I procollagen. Little is known about the site of action of BMP-1, although the extracellular matrix seems likely to be it. BMP-1 is synthesized with an N-terminal prodomain. The removal of the prodomain presumably activates the proteinase. In this study we show that the prodomain is cleaved in the trans-Golgi network (TGN) and by furin-like/paired basic proprotein convertases. Inhibitors of furin resulted in the secretion of pro-BMP-1, which could not cleave procollagen. Recombinant furin cleaved the prodomain from pro-BMP-1. Site-directed mutagenesis of the prodomain cleavage site (RSRR) to RSAA resulted in efficient secretion of pro-BMP-1. Therefore, prodomain cleavage was not required for secretion. Using peptide N-glycosidase and neuraminidase digestion to determine the post-translational status of pro-BMP-1 during its conversion to BMP-1, we showed that BMP-1 first appears in the TGN during sialylation of the molecule. Furthermore, immunofluorescence studies using an antibody to the nascent N terminus of BMP-1 showed localization to the TGN and plasma membrane. The observation that BMP-1 occurs inside the cell raises the possibility that BMP-1 might begin to cleave its substrates prior to secretion to the extracellular matrix.

Bone morphogenetic protein (BMP)-1 is a zinc-dependent metalloproteinase (1, 2) that is fundamental to dorsal-ventral patterning and tissue morphogenesis of vertebrates (3, 4). During early embryogenesis, BMP-1 fragments chordin, a BMP antagonist, thereby allowing BMPs to bind to cognate receptors. During tissue morphogenesis, BMP-1 cleaves the latent peptides of extracellular matrix (ECM) macromolecules, including type I–III procollagen (5, 6), biglycan (7), prolyl oxidase (8), and type VII procollagen (9) as well as chains of laminin-5 (10, 11). Effort has been focused on identifying substrates of BMP-1, but little is known about its activation and in particular where, in relation to the cell, it cleaves its substrates. BMP-1 is synthesized as an inactive proenzyme (for review see Ref. 12), and the site of removal of the prodomain is expected to determine the site of action of BMP-1. The active proteinase can be isolated from the medium of cultured human cells and tendon organ cultures. However, this does not exclude the possibility that the prodomain of BMP-1 can be cleaved within the cell, with secretion of the active molecule.

Pro-BMP-1 comprises a signal peptide, a prodomain, a catalytic domain, three CUB domains, and an epidermal growth factor-like domain (12). The function of the prodomain is unknown, although latency is a likely role. Expression of recombinant BMP-1 lacking a prodomain showed that its absence does not abrogate secretion (13), but this does not rule out a function for the prodomain in the secretion of BMP-1 and the regulation of its site of action. A major question is whether the prodomain maintains BMP-1 in an inactive form until it is transported to its site of action, whereupon removal of the prodomain leads to the release of active BMP-1.

Evidence from expression studies in bacteria suggests that cleavage of the prodomain is required for BMP-1 activity (5, 6). Removal of the prodomain is predicted to occur after the dibasic motif RSRR (14, 15), which is a consensus sequence for proprotein convertases (PCs). Furin, PACE4, PC5/6, and PC7 are the four members that recognize the general RX(K/R)R motif (15, 16). The cellular site where this maturation occurs is not well defined, despite extensive studies of the localization of PCs. For example, furin has a transmembrane domain that targets the protein to the trans-Golgi network (TGN) and is known to cycle between the TGN and the plasma membrane (PM) via the endosome retrieval pathway (17). There is also evidence to suggest that furin can be cleaved at the PM and released as a secreted form into the ECM (18). Furin is involved in the processing of a wide variety of molecules, for example, notch (19), fibrillin (20), type V procollagen (21), MT1-MMP (22), and MT3-MMP (23). Furin also autoactivates with cleavage of its prodomain being required for exit from the endoplasmic reticulum (ER). After cleavage, the prodomain remains associated with the mature molecule and inhibits the furin. Upon reaching the acidic environment of the TGN, the prodomain dissociates and furin becomes active (24).

**MATERIALS AND METHODS**

**Cell Culture and Reagents—**BMP-1 antibody (N-ter antibody) was from Oncogene, and the anti-FLAG M2 antibody was from Sigma. All PCR products and plasmid purifications were carried out using Qiagen kits. Preparative protein markers were obtained from Bio-Rad. Decanoyl-RVKR-chloromethyl ketone was obtained from Bachem. A full-length cDNA for BMP-1 (P13497) was cloned from a human placental...
cDNA library and inserted at the KpnI/XcmI site of pcDNA3 (Invitrogen). HT-1080 human fibrosarcoma cells (ATCC CCL-121) were maintained in DMEM (Invitrogen), supplemented with 100 mM L-glutamine and 10% fetal bovine serum (FBS) (complete DMEM), at 37 °C with 5% CO2.

The mutant BMP-1 proteins were stably expressed in HT-1080 cells. Transfections were carried out using Lipofectin reagent (Invitrogen) with 10 μg of plasmid/100-mm tissue culture dish, according to the manufacturer’s instructions. Pools were selected and maintained with 250 μg/ml Geneticin. The culture media was collected and cleared of cell debris by centrifugation at 1600 × g for 10 min and concentrated using Centriprep-30 (Amicon, Inc.). The samples were immediately or stored at −80 °C.

Plasmid Constructs and Transformations—The FLAG epitope (DYKDDDDK) was added to the C terminus of the BMP-1 construct (5′ of the stop codon) by PCR modification of the wild-type construct. A forward primer (−5′-CTGTTGAAGAATCAGCCGAGGGA-3′) and a reverse primer (5′-CCCCCTCTCGAGTCCTGTGCATGGTCGCTTGAACCGT-CGGGGGTCCGGTTTCTTTTCTGCACTCGGAATTTGAGC-3′) with a XhoI site (boldface) and the FLAG epitope (underlined) were used to amplify a fragment of −1500 bp. An internal BamHI site and the XhoI were used to generate a fragment that was introduced in place of the wild-type fragment. The 117RSRR20 → 117RSAAA20 (pro-BMP-1AA) mutant was generated by site-directed mutagenesis of the KpnI/XcmI fragment of the BMP-1 FLAG construct. KpnI is located prior to the start codon, and XcmI is located at nucleotide 383. The mutations were made by PCR and procedures using standard PCR (25) using Pwo DNA polymerase (Roche Applied Science). A forward primer containing a KpnI site (boldface) (−5′-GGGTTGGAACATCCAGCCAGGGCA-3′, seq8), and oligonucleotides in both directions containing the desired mutation (underlined) (−5′-AGGGTCTCTCCACAGGCTGGGCAC-3′; furF; 5′-GATCCCCGATACCAGGGGCGCC-3′, furR; 5′-GATCCCCGTTAGGGCGCCGAGGGCGCC-3′, furP) were used. Pwo DNA polymerase was used to minimize base misincorporation during the polymerase chain reactions. Briefly, a DNA fragment was amplified using the KpnI primer and the antisense mutant primer (furR), and an overlapping fragment was amplified using the sense mutant primer (furF) and the downstream seq8 primer. Both fragments were gel-purified (see Fig. 1), mixed, and re-amplified with the KpnIII and seq8 primers. The product was digested using appropriate restriction enzymes (KpnI and XcmI), gel-purified, and introduced in place of the corresponding wild-type fragment in BMP-1 FLAG. DNA sequencing (Big Dye, ABI Biosystems) was used to verify the mutations and to ensure that the cDNA clones were error-free.

Western Blotting—Nonidet P-40 extracts were prepared as follows. Cells were rinsed three times with phosphate-buffered saline and incubated on ice for 15 min with 500 μl of Nonidet P-40 buffer (1% Nonidet P-40, 50 mM Tris, pH 7.6) containing 10 mM EDTA, protease inhibitors mixture (Roche Applied Science). Cells in Nonidet P-40 buffer were scraped on ice, and lysates were subjected to a 5-min centrifugation at 14,000 × g at 4 °C. Supernatants were retained and stored at −80 °C for further analysis. Secreted proteins were concentrated on Centriprep-30 membranes and separated by discontinuous SDS-PAGE (4/20% or 4/7%). Most samples were grown for 5–24 h prior to harvest in DMEM with 0% FBS. For BFA and MON treatment, cells were grown for 4–5 h in DMEM with 0% FBS in the presence of 3.5 μM inhibitor. FI was used at 20 and 40 μM in 0% DMEM for 4–5 h. Pro-BMP-1 and pro-BMP-1AA were examined by Western blot analysis in which the primary antibody was either the mouse monoclonal FLAG antibody or the rabbit polyclonal N-ter antibody. Secondary antibodies were either horseradish peroxidase-conjugated to anti-mouse or anti-FLAG IgG and were detected by the enhanced chemiluminescence method (SuperSignal West Dura Extended Duration, Pierce).

Assay of Procollagen C-proteinase—Recombinant BMP-1 was assayed for procollagen C-proteinase activity using human 14C-labeled type I procollagen substrate and analysis of the cleavage products on SDS gels as described (38). In brief, 14C-labeled type I procollagen was obtained from the medium of human skin fibroblasts that had been cultured in DMEM supplemented with ascorbic acid (25 μM) for 24 h. The medium was clarified and concentrated by ultrafiltration on Centriprep-100 membranes. The samples were used immediately or stored at −80 °C.

In Vitro Furin Digest and Deglycosylation—For furin digests, 1–2 μg of protein was incubated overnight at 37 °C in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl2, 1 mM β-mercaptoethanol, protease inhibitor, with or without 5 units of recombinant furin (Affinity Bioreagents). PNGase and neuraminidase (New England BioLabs) were used to digest protein samples per the manufacturer’s instructions.

Immunofluorescence—HT-1080 cells were grown on coverslips in complete DMEM. The tetrabasic motif at 117RSRR20 was the presumed C-terminal end of the prodomain, with activation being carried out by a member of the furin-like proprotein convertase (PC) family. Previous work had established that pro-BMP-1 is cleaved following an Arg-Arg sequence, based on the reactivity of the antiserum raised to the N terminus of the cleaved product (25). In this study, we wanted to know where the prodomain was removed, in particular whether this occurred intracellularly, at the PM or extracellularly. In preliminary experiments we engineered a pro-BMP-1 molecule containing a FLAG epitope at the C terminus. Previous work had established that the FLAG epitope did not interfere with expression or proteolytic activity of BMP-1 (3). The potential PC cleavage site in pro-BMP-1 was changed from 117RSRR20 to 117RSAAA20 by site-directed mutagenesis of pro-BMP-1AA (Fig. 1). The potential PC cleavage site in pro-BMP-1 was changed from 117RSRR20 to 117RSAAA20 by site-directed mutagenesis of pro-BMP-1AA (Fig. 1).

HT-1080 cells were transfected separately with cDNAs encoding pro-BMP-1 and pro-BMP-1AA, and the proteins in the cell lysate and culture medium were examined by Western blot analysis using the anti-prodomain antibody (N-ter antibody) and the anti-FLAG M2 antibody (Fig. 2). The N-ter antibody recognized a protein of ~95 kDa in Nonidet P-40 extracts of cells transfected with pro-BMP-1 and pro-BMP-1AA. A protein of ~105 kDa was detected in the medium of cells transfected with cDNA for pro-BMP-1AA but not pro-BMP-1. The anti-FLAG antibody reacted with the ~95 kDa protein in the cell extract and a protein of ~85 kDa in the medium of cells expressing pro-BMP-1 as well as the ~105 kDa protein in the medium of cells expressing pro-BMP-1AA. Taken together, these results showed that the ~85 kDa protein in the medium corresponded to BMP-1 (which lacked the prodomain), the ~95 kDa protein in the Nonidet P-40 extract corresponded to pro-BMP-1 (retaining the prodomain), and the ~105 kDa pro-BMP-1AA (retaining the prodomain).
Western blots of the Nonidet P-40 extracts had been extracted, and these demonstrated that: (i) the majority of protein detected on the proteins were examined in Nonidet P-40 extracts of the cell layer (C) or medium (M) using either the N-ter-antibody (A) or the anti-FLAG antibody (B). Samples were separated by 4/10% discontinuous SDS-PAGE. A, pro-BMP-1 and pro-BMP-1AA were detected in the cell layer (C), and pro-BMP-1AA was detected in the culture medium (M). Pro-BMP-1AA was detected in the medium but at a noticeably higher molecular weight. The N-ter antibody did not react with BMP-1 in the medium or with the vector control (not shown). B, BMP-1 was observed in the culture medium. Pro-BMP-1AA was detected in the cell layer and in the medium. Anti-FLAG Western blots had a common background band observed in the vector control and all Nonidet P-40 extracts (asterisk).

From these results we drew several conclusions. For example, the processing of pro-BMP-1 occurs only at the tetrabasic motif; removal of the P1 and P2 arginine residues blocked cleavage of the prodomain. Furthermore, no alternate cleavage site was used when the prodomain cleavage site was removed. The results also showed that removal of the prodomain is not required for secretion of BMP-1. The medium of cells expressing pro-BMP-1 contained a protein of ~15 kDa that was recognized by the N-ter antibody (data not shown). This was most likely to be the cleaved prodomain of pro-BMP-1. However, pro-BMP-1 was only detected in the cell extracts and not in the culture medium.

Pro-BMP-1AA migrated slower (~105 kDa) than intracellular pro-BMP-1 (~95 kDa). To determine the reason for this, the glycosylation state of the protein in cell extracts and culture medium was examined by treatment with PNGase (which removes all N-linked glycosylation) or neuraminidase (which removes sialic acid residues). As shown in Fig. 3, digestion of intracellular pro-BMP-1 with PNGase resulted in a marked decrease in molecular weight, which was consistent with the removal of the five N-linked glycans that occur on pro-BMP-1 (26). Digestion of pro-BMP-1AA with PNGase resulted in the protein migrating to the same position as deglycosylated pro-BMP-1. Digestion of secreted pro-BMP-1AA with neuraminidase resulted in a decrease in molecular weight, which was indicative of sialylation of the secreted protein. Of particular relevance to this study, the absence of the ~105 kDa form of pro-BMP-1AA in the cell extracts and the presence of TGN-mediated addition of sialic acid residues of this protein showed that the rate of transit of pro-BMP-1AA through the TGN was rapid. The deglycosylation studies showed that the secreted and intracellular forms of pro-BMP-1AA differed only in the state of glycosylation. The clearly detectable difference in migration of secreted pro-BMP-1AA before or after treatment with neuraminidase and the identical migration of the intracellular pro-BMP-1AA before and after neuraminidase treatment demonstrated that: (i) the majority of protein detected on Western blots of the Nonidet P-40 extracts had been extracted from the ER or in transit to the Golgi and (ii) cleavage of the prodomain and sialylation of the pro-BMP-1 molecule occurs at a late stage in the secretory pathway. The absence of pro-BMP-1 in the medium, coupled with the rapid transit of pro-BMP-1 through the late secretory pathway, was consistent with complete cleavage of the prodomain in the late secretory pathway.

Pro-BMP-1 Does Not Exhibit Procollagen C-proteinase Activity—To determine if the prodomain inhibited proteolytic activity of BMP-1, pro-BMP-1AA and BMP-1 were assayed for pro-collagen C-proteinase activity using type I [U-14C]procollagen as substrate (25). The results showed that BMP-1 is an effective C-proteinase, which is consistent with previous observations of the recombinant protein (3, 5). However, pro-BMP-1AA was inactive (Fig. 4). The C-proteinase activity of BMP-1 was inhibited by EDTA. In control experiments, medium from cells transfected with empty vector lacked C-proteinase activity (Fig. 4).

Furin-like/Proprotein Convertase Activity Was Responsible for Prodomain Cleavage of Pro-BMP-1—Of the PC family of proteinases, furin, PACE4, PC5/6, and PC7 cleave target molecules in the constitutive secretory pathway (16) utilizing the tetrabasic motif of RX(K/R)R. The arginine residues at P1 and P4 are critical for specific cleavage, whereas additional arginine residues at positions P6 and P8 contribute to enhance cleavage (18), with P6 controlling the pH dependence of the cleavage (17). BMP-1 has arginine residues at the potential P1, P2, P4, P6, and P8 positions (11RGRSRRR120), which strongly suggests that pro-BMP-1 is a substrate for a furin-like PC. Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (FI) is a potent inhibitor of furin-like proprotein convertases (27), leading to inhibition of cleavage of PC substrates. To investigate if these specific PCs are responsible for the maturation of pro-BMP-1, FI was added to HT-1080 cells expressing pro-BMP-1 (Fig. 5). In control samples, pro-BMP-1 occurred in the cell lysate and BMP-1 occurred in the medium (see Figs. 2, 5A, and 5B). Pro-BMP-1 was not observed in the culture medium in the
of L-[U-14C]procollagen. Proteins were separated in SDS gels, and the medium, but pro-BMP-1 was the predominant species. At 40°C, treatment.

Concentrated media from overnight cultures of stable transfectants grown in DMEM without FCS were incubated with 1 μg of 1-[U-14C]procollagen. Proteins were separated in SDS gels, and the radiolabeled proteins were detected by 14C-phosphorimaging. BMP-1 converted pro-α1(I) and pro-α2(I) chains to pNα1(I) and pNα2(I), respectively, by removal of the C-propeptides. EDTA (E) inhibited the procollagen C-proteinase activity of BMP-1. Pro-BMP-1AA did not cleave the procollagen chains. Far right two lanes are empty vector and water control samples, respectively. All samples were incubated at 37°C for 4 h.

FIG. 4. Pro-BMP-1 was inactive in assays of procollagen C-proteinase. Concentrated media from overnight cultures of stable transfectants grown in DMEM without FCS were incubated with 1 μg of 1-[U-14C]procollagen. Proteins were separated in SDS gels, and the radiolabeled proteins were detected by 14C-phosphorimaging. BMP-1 converted pro-α1(I) and pro-α2(I) chains to pNα1(I) and pNα2(I), respectively, by removal of the C-propeptides. EDTA (E) inhibited the procollagen C-proteinase activity of BMP-1. Pro-BMP-1AA did not cleave the procollagen chains. Far right two lanes are empty vector and water control samples, respectively. All samples were incubated at 37°C for 4 h.

absence of FI. However, treatment of the cells with 20 μM FI resulted in the secretion of the ~105-kDa form of pro-BMP-1 that we had observed in the culture medium of cells expressing pro-BMP-1AA. Of particular interest, the levels of pro-BMP-1AA, together with those on the effects of FI, suggested that the prodomain of pro-BMP-1 could be cleaved by furin (or a similar PC) within the cell or at the cell-ECM boundary. Interestingly, furin contains a transmembrane domain, which localizes the protein to the TGN, although active cycling between the TGN and the PM occurs (17). Additional evidence suggests that the catalytic region of furin can be cleaved to produce a secreted enzyme (18). In the next series of experiments, we used brefeldin A (BFA) and monensin (MON) to test the possibility that the prodomain of pro-BMP-1 is cleaved within the ER and Golgi, respectively. BFA depolymerizes the Golgi apparatus and causes it to fuse with the ER. This effectively blocks protein transport from ER to Golgi (29, 30). MON is a Na+/H+ ionophore, which interferes with transport to the late Golgi, effectively blocking protein transport from Golgi to TGN (31). Additionally, MON alters the pH of the TGN, which may inhibit the activity of TGN-resident proteins, for example furin (31).

Western blot analysis of cells treated separately with BFA and MON showed that BMP-1 was not secreted (Fig. 7A). This confirmed that BFA and MON had stopped protein secretion. The results also showed that neither pro-BMP-1 nor pro-BMP-1AA was secreted (Fig. 7A). Analysis of BFA- and MON-treated cells with the anti-FLAG antibody (Fig. 7B) showed identical results. Importantly, treatment of the pro-BMP-1- or pro-BMP-1AA-transfected cells with either BFA or MON showed no accumulation of BMP-1. This indicated that removal of the prodomain occurs post-Golgi.

Pro-BMP-1 Is Converted to BMP-1 during Sialylation of the Molecule—A consistent observation was that pro-BMP-1 was never detected in the culture medium (unless the cells were treated with FI). Furthermore, we had not detected BMP-1 in cell lysates. Therefore, pro-BMP-1 was either cleaved in the medium by a mechanism that was highly efficient so that pro-BMP-1 occurred transiently, or it was cleaved inside the cell in the late secretory pathway or at the plasma membrane. If efficient cleavage occurred in the culture medium, then we would expect to detect small amounts of pro-BMP-1 in highly concentrated solutions. Likewise, if efficient cleavage occurred

FIG. 5. Decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (FI), a specific furin inhibitor, blocks the cleavage of pro-BMP-1 in a dose-dependent manner. A, stable transfectants were treated with 0, 20, or 40 μM FI, and proteins in the Nonidet P-40 extracts (C) of the cells and concentrated media (M) were analyzed by Western blot analysis using the N-ter antibody. Pro-BMP-1 was secreted in the presence of FI. No differences were observed in the Nonidet P-40 extracts upon FI treatment. B, same as A, but the anti-FLAG antibody was used to detect the proteins. BMP-1 was observed in the medium (M) of untreated cells. At 20 μM FI, both pro-BMP-1 and BMP-1 were observed in the medium, but pro-BMP-1 was the predominant species. At 40 μM FI, the levels of pro-BMP-1 in the medium were much reduced compared with 20 μM FI. The asterisk indicates a background band in all samples (including empty vector controls, data not shown).
Within cells, we would expect to see low levels of BMP-1 in concentrated solutions of cell extracts. In the next series of experiments, we examined highly concentrated solutions of the culture medium and Nonidet P-40 cell extracts of cells taken from 90–95% confluent 100-mm dishes. Care was taken to cool the extracts rapidly and prepare them for 4/7% discontinuous SDS-PAGE in the presence of protease inhibitors to avoid nonspecific cleavage of the proteins. Cells expressing pro-BMP-1 were used in additional control experiments. The results showed the absence of pro-BMP-1 in the culture medium, except in medium of cells expressing pro-BMP-1 (see Fig. 8A). In contrast, BMP-1 was detected in the Nonidet P-40 extract and culture medium (see Fig. 8B). Intracellular BMP-1 was detected with the anti-FLAG antibody and not with the N-ter antibody, consistent with the proposal that cleavage of pro-BMP-1 to BMP-1 begins in the TGN. This suggested to us that a proportion of the intracellular BMP-1 was post-translationally immature. To test this hypothesis, we digested the BMP-1 with PNGase and neuraminidase.

Treatment of intracellular pro-BMP-1 with PNGase (Fig. 9A) showed that the protein was N-linked glycosylated. Furthermore, treatment of the secreted form of BMP-1 showed that this protein was also N-linked glycosylated (see right-hand panel of Fig. 9B). Treatment of the secreted BMP-1 with neuraminidase (see right-hand panel of Fig. 9B) resulted in a marked sharpening and downward shift of BMP-1, which showed that this protein was sialylated. Results from the anti-FLAG antibody (Fig. 9, B and C) showed that intracellular pro-BMP-1 had no sialic acid modification (as shown by its insensitivity to treatment, see Fig. 9C), whereas both intracellular and secreted BMP-1 molecules were sialylated. Upon treatment with neuraminidase, secreted BMP-1 and intracellular mature BMP-1 migrated to the same position in SDS gels. The presence of sialic acid modification on intracellular BMP-1, whereas intracellular pro-BMP-1 lacks sialic acid, implies that sialic acid modification is required prior to removal of the prodomain. Additionally, the observation that secreted BMP-1 had more sialylation compared with intracellular mature BMP-1 indicates that removal of the prodomain and sialylation occurs in the same compartment. These results provide evidence that cleavage of the prodomain occurs in the trans-Golgi network.

In further experiments we carried out temperature drop experiments in which the pro-BMP-1-transfected cells were incubated at 20 °C to stop vesicular transport from the TGN (32), and extracts of the cells were examined by western blotting. The results showed a small amount of mature BMP-1 in the Nonidet P-40 extract and none in the culture medium of cells treated at 20 °C (data not shown). These results are consistent with the proposal that cleavage of pro-BMP-1 to BMP-1 begins in the TGN.

**Immunolocalization of BMP-1 to the trans-Golgi Network and Plasma Membrane—**To pinpoint the localization of BMP-1 inside cells, we carried out immunofluorescence studies using a neoeptope antibody (1210) that recognizes the N terminus of mature BMP-1 but not pro-BMP-1 (25). The results showed TGN and plasma membrane localization of BMP-1 (Fig. 10A). For comparison, Fig. 10B shows labeling of TGN46, which is a TGN marker (33). Localization of mature BMP-1 to the PM was similar to that obtained with Na⁺/K⁺ ATPase (data not shown).

**DISCUSSION**

In this study we showed that the prodomain of pro-BMP-1 can be removed inside the cell by a furin-like (paired basic) proprotein convertase. This removal is required for procollagen...
C-proteinase activity of BMP-1 but is not essential for its secretion. Lee et al. (34) examined secretion of BMP-1 in several cell types and showed that TGF-$\beta 1$ induced the secretion of BMP-1 from fibroblasts. Both pro-BMP-1 and BMP-1 were secreted, supporting the results reported here that removal of the prodomain is not required for secretion. Interestingly, TGF-$\beta 1$ is activated by furin (15, 35), which implies that secretion of pro-BMP-1 under TGF-$\beta 1$ induction involves a complex feedback system between BMP-1, TGF-$\beta 1$, furin, or a related PC.

Decanoyl-RVKR-chloromethyl ketone, the intracellularly active furin inhibitor, blocked the removal of the prodomain of pro-BMP-1. The inhibitor functions by either binding active furin or by inhibiting autoactivation (18). To show that furin could remove the prodomain of pro-BMP-1, we incubated pro-BMP-1 with recombinant furin in vitro. The results showed that furin was able to cleave pro-BMP-1. The scissile bond was predicted between P1 Arg$^{120}$ and P1’ Ala$^{121}$. Site-directed mutagenesis of the P1 and P2 arginine residues resulted in accumulation of pro-BMP-1$^{AA}$ in cell culture medium and failure of recombinant furin to remove the prodomain in vitro.

The identity of the PC or PCs responsible for the removal of the prodomain of pro-BMP-1 were not identified, and it is likely that more than one PC is capable of carrying out this function. The fact that FI was an effective inhibitor of the removal of the prodomain indicated that furin is a good candidate for the PC responsible for cleavage of pro-BMP-1. PACE4, PC5/6, and PC7 are additional candidates, because they are resident within the constitutive secretory pathway, although only furin and PC7 (like BMP-1 (14)), have ubiquitous tissue distribution (16, 18).

The secretion profile of pro-BMP-1$^{AA}$ was identical to that of the pro-BMP-1 in FI-treated cells. Moreover, the majority of intracellular pro-BMP-1 ($\sim 95 \text{kDa}$) detected on SDS gels was located in the ER and early Golgi compartments. Furthermore, TGN modifications were identified on the secreted form of BMP-1 $\sim 105 \text{kDa}$) but not the intracellular form (compare Figs. 3B and 9B). This showed that secretion rates from the ER were fast, and, that TGN- and post-TGN-located BMP-1 occurred transiently within the cell boundary. The rapid conversion of pro-BMP-1 inside cells presumably maximizes the efficiency of BMP-1 in ECM assembly, which might not be possible if pro-BMP-1 were secreted into the ECM. This explained the practical difficulties of detecting BMP-1 within the cell boundary. The fact that no pro-BMP-1 was identified outside the cell shows that there was efficient removal of the prodomain within the cell boundary. In some experiments we attempted to use immunofluorescence to co-localize BMP-1 and furin within the cell. However, the inherent difficulties associated with detecting extremely low levels of furin in most cultured cell lines precluded this approach (36). Single immunofluorescence was
Intracellular Cleavage of Pro-BMP-1

possible using an antibody raised to a peptide corresponding to the neoepitope of BMP-1 (25). The results from these experiments showed that mature BMP-1 (i.e., lacking the prodomain) occurs in the TGN and at the plasma membrane.

Numerous ECM molecules, other than BMP-1, require PCs for their maturation. Fibrillin, a major component of microfibrils in the ECM, requires removal of its C terminus by furin for incorporation into the matrix (20), although the cellular site of this maturation was not determined. Type V procollagen is a component of the heterotypic collagen fibrils in the cornea and has been implicated in regulating the size of the narrow-diameter fibrils in the cornea (37). The α1(V) N-propeptides and α2(V) C-propeptides of type V procollagen are removed by BMP-1, whereas the α1(I) C-propeptides are removed by furin (21).

Collectively, therefore, the concerted actions of furin and related PCs together with BMP-1 initiate the critical steps in the assembly of the extracellular matrix. It is intriguing to speculate why cells evolved to use BMP-1 as a convertase for extracellular matrix macromolecules when furin-like PCs would appear to have all the attributes of effective ECM convertases, including being located in the late secretory pathway. Presumably, the use of two convertases, perhaps in different post-TGN compartments, provides a further level of control with which to assemble an ordered and stable ECM.

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