Substrate-specific modulation of a multi-substrate proteinase. C-terminal processing of fibrillar procollagens is the only bmp-1 dependent activity to be enhanced by PCPE-1

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Substrate-specific Modulation of a Multisubstrate Proteinase

C-TERMINAL PROCESSING OF FIBRILLAR PROCOLLAGENS IS THE ONLY BMP-1-DEPENDENT ACTIVITY TO BE ENHANCED BY PCPE-1*

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Members of the bone morphogenetic protein-1/tolloid (BMP-1/Tld) family of metalloproteinases, also known as procollagen C-proteinases (PCPs), control multiple biological events (including matrix assembly, cross-linking, cell adhesion/migration and pattern formation) through enzymatic processing of several extracellular substrates. PCP activities on fibrillar procollagens can be stimulated by another family of extracellular proteins, PCP enhancers (PCPE-1, PCPE-2), which lack intrinsic enzymatic activity. While PCPs have multiple substrates, the extent to which PCPEs is involved in the processing of proteins other than fibrillar procollagens is unknown. In the experiments reported here, PCPE-1 was found to have no effect on the *in vitro* BMP-1 processing of procollagen VII, the procollagen V N-propeptide, the laminin 5 γ2 chain, osteoglycin, prolysyl oxidase, or chordin. In contrast, PCPE-1 enhanced C-terminal processing of human fibrillar procollagen III but only when this substrate was in its native, disulfide-bonded conformation. Surprisingly, processing of procollagen III continued to be enhanced when essentially all the triple-helical region was removed. These and previous results (Ricard-Blum, S., Bernocco, S., Font, B., Moali, C., Eichenberger, D., Farjanel, J., Burchardt, E. R., van der Rest, M., Kessler, E., and Hulmes, D. J. S. (2002) *J. Biol. Chem.* 277, 33864–33869; Bernocco, S., Steiglitz, B. M., Svergun, D. I., Petoukhov, M. V., Ruggiero, F., Ricard-Blum, S., Ebel, C., Geourjon, C., Deleage, G., Font, B., Eichenberger, D., Greenspan, D. S., and Hulmes, D. J. S. (2003) *J. Biol. Chem.* 278, 7199–7205) indicate that the mechanism of PCPE-1 action involves recognition sites in both the C-propeptide domain and in the C-telopeptide region of the procollagen molecule. PCPEs therefore define a new class of extracellular adaptor proteins that stimulate proteinase activity in a substrate-specific manner, thereby providing a new target for the selective regulation of PCP activity on fibrillar procollagen substrates.

In recent years, it has become clear that members of the tolloid family of metalloproteinases (BMP-1,[1] mTld, mTLL-1, mTLL-2) are key players in morphogenesis through their ability to control and synchronize the processing of multiple extracellular substrates (1–25). Also called procollagen C-proteinases, tolloid proteinases trigger collagen fibril formation in the extracellular matrix (26) by cleaving the C-propeptide regions from the major fibrillar procollagens (I, II, and III). They are also involved in precursor processing of the minor fibrillar collagens V and XI, necessary for the regulation of heterotypic fibril assembly, in both the N- and C-propeptide regions (8, 15, 17, 19, 23, 27). Tolloid proteinases also cleave precursor forms of small leucine-rich proteoglycans such as biglycan (13) and osteoglycin (24). While some of these proteoglycans have been shown to modulate the kinetics of assembly as well as fibril diameter (28), functional differences between mature and precursor forms are not yet clearly established (24). Biglycan is also important for bone formation (29) and more recently, dentin matrix protein-1, a protein involved in mineralization, has been found to be a substrate for tolloid proteinases (22).

Another important function early attributed to tolloid proteinases is the maturation of the precursor form of lysyl oxidase (LOX) (5, 30), the enzyme responsible for the stabilization of collagen and elastin networks through the formation of covalent cross-links. This tolloid-dependent processing has recently gained in significance with the demonstration that the cleaved propeptide of proLOX accounts for the *ras-recision* gene activity of this protein (31). In addition, four lysyl oxidase-like enzymes are now known, including LOXL, which seems to be essential for elastogenesis (32) and whose precursor has also been shown to be a substrate for BMP-1 *in vitro* (16).

In basement membranes, the γ2-chain of laminin 5 is also subject to processing by tolloid proteinases (12, 20) with implications for the control of epithelial cell adhesion and migration. Another molecule displaying structural homology with the LG domains of laminins is endorepellin, the C-terminal fragment of perlecan. Release of the LG3 domain of endorepellin, which accounts for most of its angiostatic activity, seems to involve tolloid proteinases (25). Also, one of the known ligands of laminin 5 is collagen VII (33), and tolloid proteinases have been shown to cleave the C-propeptide of procollagen VII (18), thereby triggering the formation of bundles of anti-parallel

[1] The abbreviations used are: BMP-1, bone morphogenetic protein-1; mTld, mammalian tolloid; mTLL, mammalian tolloid-like; PCP, procollagen C-proteinase; PCPE, PCP enhancer; LOX, lysyl oxidase; Tsg, twisted gastrulation.

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dimers known as anchoring fibrils (34).

The tolloid proteinase in *Drosophila melanogaster*, after which this family of proteinases is named, has been shown to be essential for dorso-ventral patterning during embryogenesis (11). The corresponding processing event involves short gastrulation (Sog) protein or its human orthologue chordin (3) as substrates, thereby leading to the release of members of the transforming growth factor family from inactive complexes. A similar mechanism governs the control of skeletal muscle growth, except that here the tolloid proteinase target is the transforming growth factor-β-like protein itself, myostatin, where the propeptide blocks activity in a latent complex (21).

Procollagen C-proteinase enhancers (PCPE-1 and -2) are extracellular matrix glycoproteins and potent stimulators of the tolloid-dependent C-terminal processing of procollagens I and II (35–37). These proteins are devoid of intrinsic enzymatic activity and are thought to act via a direct interaction with substrate, reaching a maximum at approximately one molecule of PCPE per molecule of procollagen (36). This mechanism is rather unusual for enzyme activators and raises the question of the specificity of PCPEs, in view of the growing number of functions now attributed to the tolloid proteinases. To address this question, we have characterized the effect of PCPE-1 on a large panel of tolloid proteinase substrates. We demonstrate that C-propeptide cleavage of the fibrillar procollagens is the only BMP-1-dependent activity to be controlled by PCPE-1. In addition, we have probed the structural requirements for the action of PCPE-1 using one of its targets, procollagen III.

EXPERIMENTAL PROCEDURES

Proteins—[^{3}H]Procollagen I was prepared from the medium of freshly isolated chick embryo fibroblasts incubated with [^{3}H]tryptophan as described (38, 39). All other proteins used were recombinant with the exception of laminin 5, which was isolated from the supernatant of confluent SCC25 cells cultured in Dulbecco's modified Eagle's medium/Ham's F-12 nutrient mixture (Sigma) supplemented with 0.4 μg/ml hydrocortisone, 2 mM glutamine, 50 μg/ml gentamycin, and 10 μM BI-1, a specific hydroxamic acid inhibitor of tolloid proteinases (gift from Fibrogen Inc.). The medium was collected every other day for 10 days and precipitated with polyethylene glycol 4000, as described before for procollagen I (39).

Mini-procollagen VII was cloned in frame with the BM40 signal peptide, overexpressed in 293-EBNA cells, and purified by nickel chromatography as described (18). For chordin, the construct was as described (40) and contained a c-Myc tag between the signal peptide and the CR1 domain, starting at residue 40. Myc-tagged human chordin was concentrated from conditioned culture medium by ultrafiltration, then purified on a High S column (Bio-Rad) and dialyzed against 150 mM NaCl, 10 mM HEPES, pH 7.6. The pNcollagen V form of the human proα1(V) homotrimer (with the C-propeptide region removed by furin processing) was obtained from the medium of cells transfected with full-length procollagen V, followed by precipitation at pH 8.6 (41).

Human osteoglycin/mimecan (Image clone 5265880, MRC geneservice, Cambridge, UK) was subcloned in pBluescript KS II (Stratagene) using EcoRI and BamHI. A 215-bp HindIII/BbrPI 5′-fragment was replaced by a PCR product generated as described (42) using the primers: 5′-CCCCAAGCTTGCACCAACCCAGCAGGAC-3′ and 5′-GTA-AGGGTGGTACAGCATCA-3′. The resulting plasmid was cleaved with HindIII/BamHI and the insert ligated in HindIII/BamHI-digested expression vector pCEP4-BM40-hisEK (43). 293-EBNA cells were transfected with the expression vector by electroporation (42), and clones were selected in Dulbecco's modified Eagle's medium, 10% fetal calf serum containing 300 μg/ml hygromycin. Individual clones were picked and tested for osteoglycin expression after labeling in 1 ml of medium containing 20 μCi of [^{35}S]Met and adsorption on 50 μl of nickel-nitrilotriacetic acid-agarose. An osteoglycin producing clone was expanded and the protein purified from 50 ml of supernatant.

Other proteins (BMP-1, PCPE-1, prolysyl oxidase, procollagen III, mini-procollagen III) were produced in 293-EBNA cells after transfection with the pCEP4 plasmid (Invitrogen) bearing the desired gene with its original signal peptide, in the presence of Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24-h posttransfection, hygromycin (300 μg/ml, Calbiochem) was added, and se-

lection was maintained for at least 10 days. Confluent cells in Cellstack culture chambers (Corning) were rinsed twice with phosphate buffer saline buffer and serum-free Dulbecco's modified Eagle's medium (Sigma) containing 50 μg/ml gentamycin was added. Medium was collected every 48 h, centrifuged prior to storage, and replaced by fresh medium. The procedure was repeated between 7 and 20 times depending on the protein produced.

Human BMP-1 encoding cDNA (gift from S. Huet, GlaxoSmithKline) in the pFastBac1 plasmid (Invitrogen) was digested with SalI and HindIII, subcloned in pBluescript-SK$^+$ then inserted into the BamHI and XhoI sites of pCEP4. For expression, culture chambers were precoated with poly-D-lysine (Sigma) to improve adhesion. Purification was as described (44) with the following modifications. The BMP-1-containing fractions were eluted from a Reactive Green 5 (Sigma) column with 20 mM HEPES, pH 7.4, 5 mM CaCl$_2$, 2 M NaCl, and 25% ethylene glycol. Also, fractions from the concanavalin A-Sepharose (Amersham Biosciences) were concentrated by ultrafiltration and loaded onto a 1 ml column of anti-PCPE-Sepharose (prepared by cross-linking the purified polyclonal antibody directed against PCPE-1, described below, to CNBr-activated Sepharose 4B from Amersham Biosciences). The flow-through was then purified to homogeneity over Superdex 200 (Amersham Biosciences) equilibrated with 20 mM HEPES, pH 7.4, 5 mM CaCl$_2$, 1.5 M NaCl, and 0.02% octyl glucoside.

The cDNA for human PCPE-1 (gift from E. Kessler, Tel Aviv, Israel), inserted into pBluescript SK$^+$, was excised with HindIII/BamHI and subcloned into the pCEP4 vector digested with the same enzymes. Production in 293-EBNA cells could be continued for up to 6 weeks with no obvious decrease in PCPE-1 concentration in the culture medium. PCPE-1 was purified as described for the baculovirus-produced protein (36) by Affi-Gel blue (Bio-Rad), HiTrap Heparin HP (Amersham Biosciences), and Superdex 200 chromatography.

The human prolysyl oxidase construct (proLOX inserted in frame with a C-terminal V5-(His)$_6$ tag in the pcDNA3 vector from Invitrogen) was a gift from P. Sommer (Lyon, France). XhoI and HindIII sites were used to introduce the LOX gene into the pCEP4 vector. Addition of the BMP-1 inhibitor (BI-1, Fibrogen Inc.) at a concentration of 10 μM during the production in 293-EBNA cells proved necessary to avoid processing of the proLOX in the culture medium. Nickel chromatography in the presence of 2 M urea was used to purify the protein. Positive fractions were then concentrated and desalted by ultrafiltration.

293-EBNA cells secreting human procollagen III (gift from N. Bulleid, Manchester, UK) were cultured in the presence of 50 μg/ml ascorbic acid during the protein production phase. The collected supernatants were pooled and precipitated with 25% PEG 4000 in 0.15 M NaCl to concentrate the protein, as described (39), and further purified on a DEAE-Sephacel column (Amersham Biosciences) equilibrated with 50 mM Tris, pH 7.4, 10 mM NaCl, 2.5 mM EDTA, and 2 M urea. The protein was eluted with a 10–310 mM NaCl gradient and concentrated by ultrafiltration. Denatured procollagen III was prepared by heating purified procollagen III at 60 °C for 20 min in the presence of 50 mM dithiothreitol, 0.4 M NaCl, and 5.6 M urea. The solution was then allowed to cool for 2 min on ice, and 1/6 volume of 1 M *N*-ethylmaleimide in 50% methanol was added. After 30 min at room temperature, the solution was precipitated with 1/10 volume of trichloroacetic acid for 1 h on ice. The pellet was washed twice with cold acetone and re-suspended in 50 mM Tris, pH 7.4, 0.4 M NaCl, and 0.02% Brij-35.

For mini-procollagen III, the pCEP4 vector containing the procollagen III sequence and a c-Myc tag (EQKLISEEDL) inserted between the signal peptide and the N-propeptide (gift from N. Bulleid) was used as a template for PCR amplifications. To delete the N-propeptide and the major part of the triple helix (except the last 33 Gly-*X*-*Y* triplets), we generated a PCR product starting at position 6 and ending right after the c-Myc tag and another starting at position 3390 and ending at position 4530 (after the STOP codon) in the initial procollagen III sequence (GenBank™ accession number X14420). The two sequences were then simultaneously ligated into the pBluescript SK$^+$ plasmid cut with HindIII and BamHI using HindIII/BsiWI sites inserted in the first product and BsiWI/BamHI sites inserted in the second product. Insertion of the BsiWI site leads to the introduction of an arginine between the c-Myc tag and the triple helix. *Pfx* platinum polymerase (Invitrogen) was used for the PCR reactions and the primers were as follows (sequences used to insert restriction sites are underlined): 5′-AT<u>AAGCT</u>T<u>C</u>GGTGCTGAAGGGCAGG-3′ and 5′-AT<u>CGTACG</u>CAAGTCCTCTTC-AGAAATGA-3′ for the first product and 5′-AT<u>CGTACG</u>GGGTGAACGT-GGAGCTGCT-3′ and 5′-AT<u>GGATCC</u>TTGTTGGGATTTCAGATAGA-G-3′ for the second product. KpnI and BamHI were then used for insertion into the pCEP4 plasmid. The construct was checked by sequencing of the complete coding sequence (Genome Express, Meylan,

France) and transfected into 293-EBNA cells using the standard protocol. For purification, the supernatants were shaken gently for 3 h at 4 °C with heparin-Sepharose CL-6B (Amersham Biosciences) in phosphate-buffered saline buffer, followed by washing with phosphate-buffered saline and elution with a 0.15–1.15 M NaCl gradient. The most concentrated fractions were then immunopurified on an anti-c-Myc column (prepared with the purified c-Myc antibody and CNBr-activated Sepharose 4B). Following elution with 0.1 M glycine HCl, pH 2.3, fractions were quickly neutralized with 1.5 M Tris, pH 8.8, and concentrated by ultrafiltration.

Protein Analysis—Protein concentrations were measured with the ready-to-use Coomassie Blue G-250-based reagent (Pierce) using bovine serum albumin as a standard except for procollagen III and mini-procollagen III for which absorbances at 280 nm were used ($\epsilon = 3 \times 55{,}500$ M^{-1} cm^{-1} for procollagen III and $3 \times 41{,}000$ M^{-1} cm^{-1} for mini-procollagen III). N-terminal sequencing was performed by automated Edman degradation on an Applied Biosystems 473 protein sequencer, and hydroxyproline content was measured after total hydrolysis of procollagen III under acid vapors, followed by high performance liquid chromatography separation and ninhydrin coupling (Protein Analysis Facility, IFR 128, Lyon, France).

Antibodies—The 9E10 anti-c-Myc monoclonal antibody was produced with the hybridoma cells Myc-9E10.2 (ATCC CRL 1729). Procollagen VII was monitored with the NC1-F3 antibody (18), proLOX with anti-V5 from Invitrogen, and laminin 5 with the D4B5 antibody (directed against the γ2 chain) from Chemicon. The polyclonal anti-TSPN antibody was raised in rabbits by injecting the recombinant α1(V) TSPN domain produced in *Escherichia coli*. The polyclonal antibody used to detect PCPE-1 and to prepare the anti-PCPE column was obtained as a rabbit serum after injection of the full-length protein. The polyclonal antibody directed against BMP-1 was a gift from E. Kessler (Tel Aviv, Israel).

Enzymatic Activities—BMP-1 activity was determined as described (38, 45) at 37 °C in the following buffer: 50 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM CaCl$_2$, 0.02% Brij-35. Conversion of [^{3}H]procollagen I to pNcollagen I + C-propeptide trimer was measured for 1.5 h with 1.3 μg of substrate and 11 ng of enzyme in an assay volume of 100 μl. The reaction was stopped with 20 μl of 50 mM Tris, pH 7.4, 0.1 M EDTA, 10 mg/ml fibrinogen (Sigma), and 40 μl of cold ethanol. After 1 h on ice, samples were centrifuged, and 100 μl of supernatant were counted in a scintillation counter with 4 ml of Ultima Gold XR (Packard Bio-Sciences). For other substrates, the same assay buffer was used; reaction times and concentrations were as indicated. Reactions were quenched with 10% trichloroacetic acid (Sigma) and prepared for SDS-PAGE, Western blotting, or Coomassie Blue staining using standard protocols. Collagenase digestion of the mini-procollagen III was carried out at 37 °C for 1 h in the buffer used for BMP-1. One unit of the chromatographically purified collagenase form III (specific activity: 11.3 bovine tendon collagen units/μg) from *Clostridium histolyticum* (Advanced Biofactures, Lynbrook, NY) was used to digest 17 μg of mini-procollagen III.

Electron Microscopy—For rotary shadowing, samples were dialyzed against 0.1 M NH$_4$HCO$_3$, pH 7.8, + 1 mM MgCl$_2$ and diluted to 10–20 mg/ml. After addition of an equal volume of glycerol, the solutions were sprayed onto freshly cleaved mica sheets. The samples were immediately placed on the holder of a MED 010 evaporator (Balzers), and rotary shadowing was carried out under vacuum at an angle of 8°. Observation of replicas was performed with a Philips CM120 microscope at the "Centre Technique des Microstructures" (Université Claude Bernard, Lyon, France).

RESULTS

Recombinant BMP-1, PCPE-1, and PCP Activity—Recombinant human BMP-1 and PCPE-1 were produced in 293-EBNA cells in the absence of any tag and purified to homogeneity (Fig. 1). To remove traces of endogenously produced PCPEs, the BMP-1 purification included an anti-PCPE affinity chromatography step. Yields of purified protein were 20 μg/l for BMP-1 and 1 mg/l for PCPE-1. The native conformation of these proteins was checked by monitoring the conversion of [^{3}H]procollagen I to pNcollagen + C-propeptide trimer. In the presence of 1.6 nM recombinant BMP-1 (11 ng) and 30 nM procollagen I, 2 nM C-propeptide trimer was formed after 1.5 h at 37 °C and 5-fold more in the presence of 1 equivalent of recombinant PCPE-1 (per mol of procollagen). Preliminary results indicate a
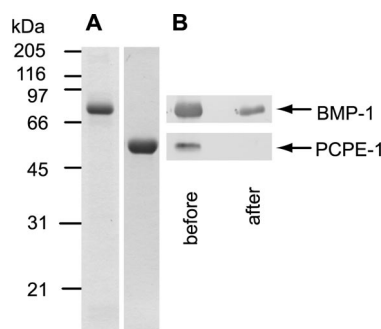

FIG. 1. **Purification of BMP-1 and PCPE-1.** *A*, Coomassie Blue staining of purified proteins after SDS-PAGE (10% acrylamide) under non-reducing conditions. *B*, Western blot analysis of BMP-1 fractions before and after loading onto the anti-PCPE-Sepharose column with both anti-PCPE and anti-BMP-1 antibodies.

k_{cat} value for BMP-1 around 20–30 h^{-1}, to be compared with the published value of 41 h^{-1} for the chick tendon enzyme (45).

Effect of PCPE-1 on Processing of Procollagen III—293-EBNA cells were also used to produce procollagen III. Complete hydrolysis of the protein followed by amino acid analysis revealed a normal level of proline hydroxylation (51%) and electron microscopy after rotary shadowing (Fig. 2) confirmed that the recombinant procollagen III molecules resembled their naturally occurring counterparts. This substrate was then subjected to cleavage by BMP-1 and found to be efficiently cleaved. Fig. 3*A* shows that the reaction rate was strongly increased in the presence of PCPE-1 when a molar ratio of procollagen III:PCPE-1 of 1:1 was used. In a parallel experiment, denatured and alkylated procollagen III (see "Experimental Procedures") was also processed by BMP-1 and gave a product identical to pNcollagen III in terms of electrophoretic migration (Fig. 3*B*). This result strengthens previous reports showing BMP-1 cleavage of heat-denatured procollagen I (45) by demonstrating that cleavage still occurs when disulfide bonds are disrupted. In contrast, when PCPE-1 was included in the reaction, no activation occurred (Fig. 3*B*). This demonstrates that PCPE-1 requires a native conformation of the procollagen molecule whereas BMP-1 is apparently insensitive to the three-dimensional structure of the substrate.

Effect of PCPE-1 on Other Procollagens—Other known procollagen substrates for tolloid proteinases are the minor fibrillar procollagens (V and XI) and procollagen VII, a major component of anchoring fibrils at the dermal-epidermal junction. For procollagens V and XI, in contrast to procollagens I–III, BMP-1 processing can occur in both the N- and C-propeptide regions, and the C-propeptides may also be released by a furin-like enzyme (8, 15, 17). To determine whether PCPE-1 stimulates N-terminal processing of procollagen V, we used the pNcollagen form of the procollagen α1(V) homotrimer, from which the C-propeptide was removed by furin processing. As shown in Fig. 4*A*, no enhancement of BMP-1 cleavage was observed in the presence of PCPE-1 (with a molar ratio of PCPE-1 to pNcollagen V of 5:1), as monitored by the production of the free TSPN domain. In addition, compared with procollagens I and III, BMP-1 cleavage of pNcollagen V was relatively inefficient, requiring overnight incubation with 16 nM BMP-1 instead of 1–2 h with 2–8 nM BMP-1. On a shortened fragment of procollagen V,[2] we also confirmed by N-terminal sequencing after treatment with pyroglutamate aminopeptidase (16) that cleavage occurred at the previously reported SQ site (8), even in the presence of PCPE-1.

Similar results were obtained with mini-procollagen VII,

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[2] M. Berand, S. Ricard-Blum, S. Cogne, C. Bonod-Bidand, and F. Ruggiero, manuscript in preparation.
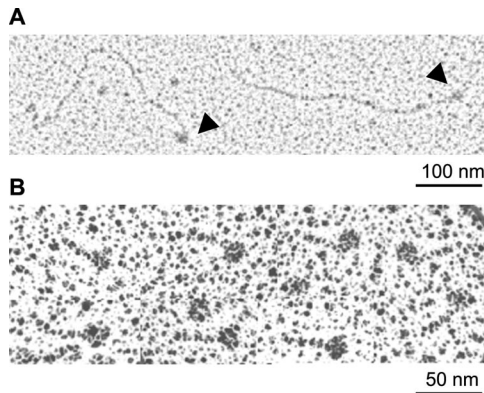
FIG. 2. **Electron microscopy of procollagen III (*A*) and mini-procollagen III (*B*).** Molecules were visualized after rotary shadowing. The globular C-propeptide regions are indicated by *arrowheads* in *A* and are clearly visible in *B*. The rod-like triple-helical region in the full-length molecule (*A*) is ~10× longer than in mini-procollagen III (*B*).
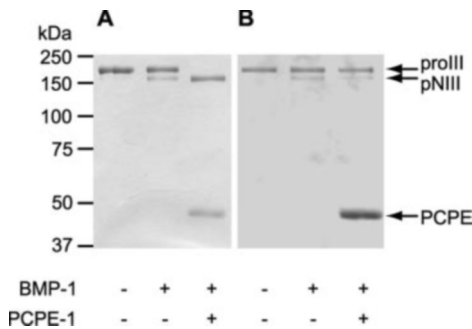

FIG. 3. **Cleavage of procollagen III (*proIII*) to pNcollagen III (*pNIII*) in the presence and absence of PCPE-1.** Samples were analyzed by SDS-PAGE on an 8% acrylamide gel stained with Coomassie Blue, under reducing conditions. *A*, 5.2 pmol of procollagen III (2 µg) were incubated for 2 h at 37 °C in 50 µl of BMP-1 assay buffer (50 mM Tris, pH 7.4, 0.15 M NaCl, 5 mM CaCl$_2$, 0.02% Brij-35) either alone or in the presence of BMP-1 (30 ng) without or with 5.2 pmol PCPE-1. *B*, same as *A* except that the procollagen was denatured and alkylated (see "Experimental Procedures") prior to the cleavage assay. As shown elsewhere (37), PCPEs alone are devoid of proteolytic activity.
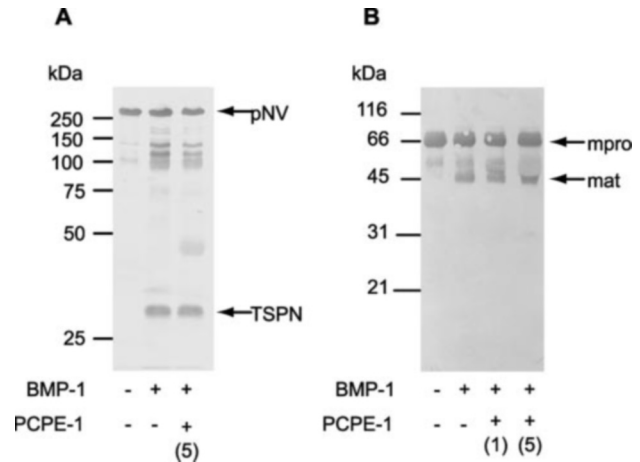

FIG. 4. **Cleavage of procollagen V and mini-procollagen VII in the presence and absence of PCPE-1.** *A*, pNcollagen V (16 pmol) was incubated overnight at 37 °C in 200 µl of assay buffer alone or in the presence of 230 ng of BMP-1 with or without 5 molar equivalents of PCPE-1. Analysis by SDS-PAGE (9% acrylamide reducing conditions) followed by Western blotting with the anti-TSPN antibody to detect both the intact molecule (*pNV*) and the released fragment (*TSPN*) is shown. *B*, mini-procollagen VII (15 pmol) was incubated for 4 h at 37 °C in 100 µl of assay buffer alone or in the presence of BMP-1 (100 ng) with or without 1 or 5 molar equivalents of PCPE-1 as indicated. Analysis by SDS-PAGE (8% acrylamide, reducing conditions) followed by Western blotting using the NC1-F3 antibody to detect both the intact molecule (*mpro*) and the released N-terminal fragment (*mat*) is shown. In the presence of a 5-fold excess of PCPE-1, detection of the N-terminal fragment was hindered because both these proteins have similar electrophoretic mobilities.

which comprises the C-propeptide, a shortened triple helix, and part of the N-propeptide (18). Cleavage by 14 nM BMP-1 resulted in the appearance of an N-terminal fragment of the expected size (Fig. 4*B*). In the presence of PCPE-1, however, no difference in the extent of processing was observed. These results indicate that being a collagenous substrate of BMP-1 is not sufficient for activity enhancement by PCPE-1.

Effect of PCPE-1 on Other Matrix Molecules—Other trimeric extracellular molecules with prominent roles are laminins. Processing of laminin-5 involves multiple sites in at least two of the three chains (α and γ), and the biological significance of these cleavages is a field of intense research (18, 46). BMP-1 and other tolloid enzymes have been shown to cleave the γ2 chain of laminin 5 (20), leading to increased cell migration. For this study, laminin 5 was isolated from the supernatant of SCC25 cells in the presence of a specific tolloid inhibitor, which proved necessary to prevent cleavage by endogenous enzymes. In conditions where the expected cleavage product of approximate molecular mass 105 kDa was obtained after overnight incubation in the presence of 9 nM BMP-1 (γ2′; Fig. 5*A*), the extent of cleavage of the γ2 chain of laminin 5 seemed unaffected by the presence of PCPE-1.

In subsequent experiments, recombinant osteoglycin produced in 293-EBNA cells was subjected to hydrolysis by 7 nM BMP-1 at 37 °C. As shown in Fig. 5*B*, the starting material appeared as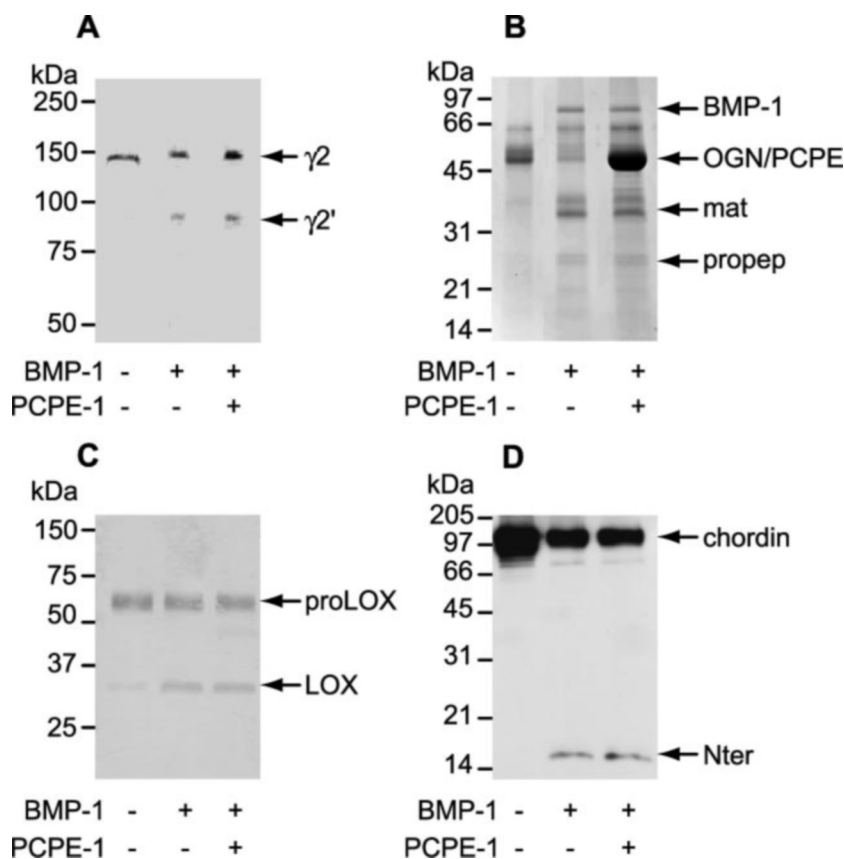 a doublet, probably as a result of heterogenous glycosylation, and had the same electrophoretic mobility as PCPE-1. Nevertheless, the amounts of processed products (as detected by Coomassie Blue staining) clearly showed that the reaction was not modified by the presence of up to 2 equivalents of PCPE-1 per osteoglycin molecule.

The last matrix molecule to be tested was the precursor form of the enzyme lysyl oxidase, processing of which triggers enzymatic activity (5). Unexpectedly, the use of the tolloid proteinase inhibitor also proved essential to avoid processing of 30–50% of the synthesized proLOX during production in 293-EBNA cells. This suggests that tolloid enzymes are expressed by these cells and also that proLOX is more efficiently cleaved than other substrates for which no or little cleavage products were observed in the same cells. This was confirmed during the enzymatic assays by the fact that low concentrations of BMP-1 (<0.5 nM) were sufficient to obtain detectable amounts of mature LOX in 1 h (Fig. 5*C*). However, the reaction rate remained again unaffected by the addition of PCPE-1.

Effect of PCPE-1 on Chordin—Finally, we assayed the ability of PCPE-1 to stimulate BMP-1 processing of chordin. Three main cleavage sites have been described for BMP-1 and mTLL-1, one in the N-terminal part after the CR1 domain, one in the C-terminal part between CR3 and CR4, and one in the central part of the molecule that is only observed in the presence of the twisted gastrulation protein Tsg (3, 47). Three products are thus obtained in the absence of Tsg with molecular masses 13, 15, and 83 kDa. Here, we looked at the BMP-1 cleavage products with the help of a c-Myc (9E10) antibody, which detects full-length chordin and the N-terminal product. As shown in Fig. 5*D*, while the expected cleavage product was produced in the presence of 2.5 nM BMP-1, the extent of processing was not affected by the presence of PCPE-1.

It can be concluded from the above results that the enhancing activity of PCPE-1 is specific to the fibrillar procollagen C-proteinase activity of BMP-1 and that this effect requires a very specific interaction with the procollagen substrate.

Substrate-specific Proteinase Modulation

FIG. 5. **Cleavage of laminin 5, osteo-glycin, prolysyl oxidase, and chordin in the presence and absence of PCPE-1.** *A,* laminin 5 was incubated overnight at 37 °C in 200 µl of assay buffer alone or in the presence of BMP-1 (120 ng) with or without 40 pmol of PCPE-1. Analysis by SDS-PAGE (6% acrylamide, reducing conditions) followed by Western blotting with the D4B5 antibody, which recognizes the intact (γ2) and processed forms (γ2′) of the γ2 chain, is shown. *B,* osteoglycin (*OGN*) was incubated overnight at 37 °C in 200 µl of assay buffer alone or in the presence of 100 ng of BMP-1 with or without 140 pmol of PCPE-1. Analysis by SDS-PAGE (12% acrylamide, reducing conditions) followed by staining with Coomassie Blue allows detection of both the mature form (*mat*) and propeptide (*propep*). *C,* prolysyl oxidase was incubated for 1 h at 37 °C in 100 µl of assay buffer alone or with BMP-1 (3 ng) with or without 4.3 pmol of PCPE-1. SDS-PAGE (10% acrylamide, reducing conditions) followed by Western blotting with the V5 antibody to detect the intact (*proLOX*) and mature (*LOX*) forms of the enzyme. *D,* chordin was incubated for 4.5 h at 37 °C in buffer alone or in the presence of BMP-1 (7 ng) with or without 17 pmol of PCPE-1 in 40 µl of assay buffer. Analysis by SDS-PAGE (12% acrylamide, reducing conditions) followed by Western blotting with the 9E10 c-Myc antibody to detect both the intact molecule and the released N-terminal fragment (*Nter*) is shown.

Interaction between PCPE-1 and Procollagen III—To further investigate the structural requirements for PCPE activity on procollagen substrates, we designed a new substrate derived from procollagen III, called mini-procollagen III, consisting of the C-propeptide, the C-telopeptide, and a very short triple helix made of the last 33 triplets. The length of the remaining triple helix (~30 nm) was chosen to allow interaction with PCPE-1 (length ~15 nm (48)) on both sides of the BMP-1 cleavage site. For purification purposes, a c-Myc tag was inserted between the signal peptide and the triple helix. This substrate was more efficiently produced than the full-length molecule (2 mg/l) and when observed by electron microscopy after rotary shadowing (Fig. 2*B*), it appeared as a bar of length 30 nm and a globule of 15 nm in agreement with the expected dimensions of the triple helical and the C-propeptide domains (49). Moreover, it was efficiently cleaved by BMP-1 (Fig. 6, *lane 2*), at a rate comparable with that observed for full-length procollagen III. Interestingly, the processing of this mini-procollagen III was also enhanced by PCPE-1 (Fig. 6, *lane 3*) apparently to the same extent as with full-length procollagen III. Moreover, N-terminal sequencing of the high molecular mass product revealed that the cleavage site obtained in the presence of PCPE-1 was identical to that reported for procollagen III in the absence of PCPE-1, with the N-terminal sequence DEPMDFKI corresponding to the beginning of the C-propeptide (50). This clearly shows that PCPE-1 does not modify the cleavage specificity of BMP-1. It also demonstrates that the N-propeptide region and the bulk of the triple-helical region of procollagen III are not needed for the enhancing activity of PCPE-1.

To define the interaction sites between PCPE-1 and procollagen III more precisely, we submitted the mini-procollagen III to digestion with highly purified collagenase for 1 h at 37 °C. This led to a unique protein product with a molecular mass of around 100 kDa, as estimated by non-reducing SDS-PAGE

(Fig. 6, *lane 4*). N-terminal sequencing of this product yielded only one sequence (GPPGAPGP*XX*GGV) in agreement with the expected specificity of the bacterial collagenase from *C. histolyticum*, which cleaves *X*-Gly bonds at segments containing the sequence Pro-*X*-Gly-Pro. The resulting product thus lacks most of the triple helix except the last three triplets. Very interestingly, this molecule was also a substrate for BMP-1, and the cleavage was still efficiently enhanced by PCPE-1 (Fig. 6, *lanes 6* and *7*).

DISCUSSION

In this paper, we focus on the conditions required for enhancement of BMP-1 activity by PCPE-1. Previously, we and others (36, 37, 51) have shown that tolloid proteinases and PCPEs act synergistically during C-terminal processing of fibrillar procollagens I and II. Here, we show that this is also the case for procollagen III, at least in the case of BMP-1 and PCPE-1. In contrast, among the other known substrates of BMP-1, we show that cleavage of the pNcollagen α1(V) homotrimer, mini-procollagen VII, the laminin 5 γ2 chain, osteoglycin, prolysyl oxidase, and chordin is not affected by the presence of PCPE-1.

The strong specificity of PCPE-1 gives several insights into the mechanisms by which PCPEs enhance the activities of tolloid proteinases. First, it shows that unlike most enzyme activators, PCPE-1 does not enhance BMP-1 through a direct effect on the enzyme. If this were the case, processing of all substrates would be enhanced by PCPE-1, or BMP-1 would have to act through different mechanisms when processing different substrates, which seems unlikely. Data presented here and elsewhere (35, 52) suggest that a direct substrate-enhancer interaction is a prerequisite for stimulating activity. They do not, however, eliminate the possibility that the enhancer also binds to BMP-1. It should also be noted that there appears to be no link between the catalytic efficiency of BMP-1
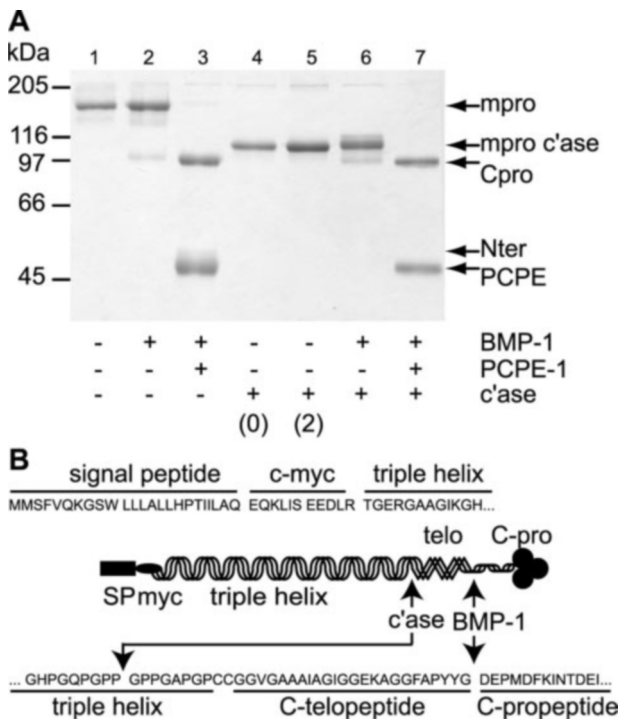
FIG. 6. **Effect of PCPE-1 on BMP-1 cleavage of mini-procollagen III digested or not with collagenase.** *A*: *lanes 1–3*, mini-procollagen III (*mpro*) (16.5 pmol, 2.1 μg) was incubated for 2 h at 37 °C in 50 μl of assay buffer alone or in the presence of BMP-1 (25 ng) with or without 1 molar equivalent of PCPE-1 (16.5 pmol). *Lanes 4–7*, mini-procollagen III (8.5 μg) was first incubated for 1 h at 37 °C with highly purified collagenase (43 ng) in BMP-1 assay buffer. Immediately thereafter, the sample was divided into four aliquots containing or not BMP-1 (25 ng) with or without 1 molar equivalent of PCPE-1 (16.5 pmol) in a final volume of 50 μl. Samples were then further incubated for 2 h at 37 °C. Analysis by SDS-PAGE (8%, non-reducing conditions) followed by staining with Coomassie Blue. Controls without BMP-1 and PCPE-1 showed that no further digestion occurred during the second incubation period, as measured at the end of the collagenase digestion ($t = 0$) and after a subsequent 2 h at 37 °C ($t = 2$). *B*, diagrammatic representation of the mini-procollagen III molecule showing the different structural domains (signal peptide (*SP*), c-Myc tag (*myc*), triple helix, C-telopeptide (*telo*), C-propeptide (*C-pro*)) and the N- and C-terminal amino acid sequences with the collagenase and BMP-1 cleavage sites. *c'ase*, collagenase.

and the enhancing activity of PCPE-1, since qualitatively prolysyl oxidase seemed to be the best BMP-1 substrate, while processing was not enhanced by PCPE-1.

Second, the observation that PCPEs specifically stimulate C-terminal processing of the fibrillar procollagens suggests that these substrates share common PCPE recognition motifs. Our observation that processing of mini-procollagen VII was unaffected by PCPE-1 shows that the presence of a triple-helical region *per se* is not sufficient for enhancing activity. This was confirmed by the observed lack of effect of PCPE-1 on N-terminal processing of the procollagen $\alpha1$(V) homotrimer. We have previously shown (52) that PCPE-1 binds to sites in both the C-propeptide region as well as elsewhere in the procollagen molecule. Our observation that PCPE-1 continued to enhance BMP-1 processing of mini-procollagen III even when all but the last three Gly-*X-Y* triplets were removed by bacterial collagenase suggests that one site is located within the region spanning these triplets and the non-helical C-telopeptide region. To investigate possible sequence motifs involved in PCPE-1 binding, amino acid sequences at the triple helix/C-telopeptide/C-propeptide junction were compared for procollagens I, II, and III (Fig. 7). Several possible PCPE-1 binding sites can be identified. These include the proline-rich region at

the end of the collagen triple helix, the hydrophobic region at the beginning of the telopeptide region (53), the hydrophilic region containing the cross-linking lysine (absent from the pro$\alpha2$(I) chain), and the tyrosine-containing region immediately N-terminal to the BMP-1 cleavage site. Future studies will address the relative contributions of these sequences to PCPE-1 binding.

Third, we demonstrate here that enhancing activity, unlike BMP-1 activity, requires a native three-dimensional structure of the substrate. PCPE-1 therefore recognizes not only a specific region of the amino acid sequence but also structural features in the substrate that are lost on denaturation. Whether these features are contained in individual procollagen chains or imply a trimeric organization is another interesting question. Also, since the C-telopeptide sequence of the pro$\alpha2$(I) chain is shorter than the $\alpha1$ chains of procollagens I-III (Fig. 7), it is possible that not all α chains in heterotrimeric procollagens are equivalent for PCPE action.

Finally, we previously suggested (52) that PCPE-1 induces a conformational change in the substrate molecule which makes it more susceptible to proteolytic cleavage by tolloid proteinases. Another hypothesis has been put forward by Steiglitz and coworkers (37), based on electron microscopy and binding data, whereby PCPEs prevent nonspecific binding of tolloid proteinases to unproductive sites (remote from the cleavage site) within the main triple-helical region of the procollagen molecule. This was based on the observation that PCPEs seem to bind to multiple sites throughout the procollagen molecule and displace tolloid proteinases bound to the collagenous region. Our observation that both mini-procollagen III and collagenase-treated mini-procollagen III behaved like full-length procollagen III, in terms of BMP-1 processing and enhancement by PCPE-1, argues against the latter hypothesis. According to this theory, mini-procollagen III with supposedly fewer unproductive sites than full-length procollagen III would be cleaved at a higher rate by BMP-1 and be little affected by PCPE-1. Also, maximum enhancement activity appears to occur at a PCPE: procollagen molar ratio of 1:1 (35, 52), while the multiple binding site displacement hypothesis (37) would presumably require a large molar excess of PCPE for maximum enhancement.

To our knowledge, the use of a specific enhancer/inhibitor to modulate the substrate choice of a proteinase with broad substrate specificity is rather unique in the field of enzymology. Another example linked to tolloid proteinases is twisted gastrulation (Tsg) that has been shown to enhance activity as well as to modify the pattern of chordin cleavage by BMP-1/mTld (47). While Tsg appears to have a specific role in stabilizing the chordin/growth factor complex, whether it affects cleavage of other tolloid proteinase substrates is unknown. Although we found no evidence that PCPE-1 modifies the site of BMP-1 cleavage in the different substrates tested, the complementary functions of Tsg and PCPEs emphasize the need to adjust tolloid proteinase activity in different morphogenetic processes such as embryonic growth or wound healing. In bacteria, an equivalent but unrelated system has been described for the ClpXP protease that belongs to a family of ATP-dependent proteases related to the 26 S proteasome of eukaryotes (54). In this system, which is designed to prevent the unwanted degradation of cellular proteins, the proteinase selects its substrates through the recognition of a specific amino acid sequence and through adaptor proteins that up- or down-regulate degradation of defined proteins. The use of adaptor proteins to finely tune action of multisubstrate proteinases might thus be more widespread than has been recognized so far.

In conclusion, PCPEs therefore define a new class of extra-

```
                  _____                           ▼                    *    *
proα1(I)    GPPGPP---SAGFDFS-FLPQPPQEKAHDGGRYYRADDAN---VVRDRDLEVDTTLKS
proα1(II)   GPPGPP---GPGIDMSAFAGLGPREKGPDPLQYMRADQAAG--GLRQHDAEVDATLKS
proα1(III)  GPPGAPGPCCGGVGAAAIAGIG-GEKAGG-FAPYYGDEPM---DFKINTDEIMTSLKS

proα2(I)    GPPGPPGVSGGGYDFG-YDGD----------FYRADQPRSAPSLRPKDYEVDATLKS
```

FIG. 7. **Sequence alignment of major human fibrillar procollagen chains at the triple helix/C-telopeptide/C-propeptide junction.** Sequences are aligned starting relative to the final three GXY triplets in the proα1(III) chain. Regions that might be involved in PCPE-1 binding are indicated (see "Discussion"). The BMP-1 cleavage site is indicated by an *arrowhead*. Hydrophobic residues involved in coiled-coil interactions in the C-propeptide region (55) are indicated by *asterisks*.

cellular enzyme adaptors that enhance the activity of a multi-substrate proteinase in a substrate-specific manner. Future work will focus on the elucidation of this unique mechanism of enhancement and on the identification of sites involved in the interaction with major fibrillar procollagens.

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