Mammalian Tolloid-like 1 Binds Procollagen C-proteinase Enhancer Protein 1 and Differs from Bone Morphogenetic Protein 1 in the Functional Roles of Homologous Protein Domains

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Bone morphogenetic protein 1 (BMP1) is the prototype of a sub-group of metalloproteinases with manifold roles in morphogenesis. Four mammalian subgroup members exist, including BMP1 and mammalian Tolloid-like 1 (mTLL1). Subgroup members have a conserved protein domain structure: an NH2-terminal astacin-like protease domain, followed by a fixed order of CUB and epidermal growth factor-like protein-protein interaction motifs. Previous structure/function studies have documented those BMP1 protein domains necessary for secretion, and activity against various substrates. Here we demonstrate that, in contradiction to previous reports, the most NH2-terminal CUB domain (CUB1) is not required for BMP1 secretion nor is the next CUB domain (CUB2) required for enzymatic activity. The same is true for mTLL1. In fact, secreted protease domains of BMP1 and mTLL1, devoid of CUB or epidermal growth factor-like domains, have procollagen C-proteinase (pCP) activity and activity for biosynthetic processing of biglycan, the latter with kinetics superior to those of the full-length proteins. Structure-function analyses herein also suggest differences in the functional roles played by some of the homologous domains in BMP1 and mTLL1. Surprisingly, although BMP1 has long been known to be Ca2+ dependent, a property previously assumed to apply to all members of the subgroup, mTLL1 is demonstrated to be independent of Ca2 levels in its ability to cleave some, but not all, substrates. We also show that pCP activities of only versions of BMP1 and mTLL1 with intact COOH termini are enhanced by the procollagen C-proteinase enhancer protein 1 (PCOLCE1) and that mTLL1 binds PCOLCE1, thus suggesting reappraisal of the accepted paradigm for how PCOLCE1 enhances pCP activities.

BMP12-like proteinases mediate morphogenetic events by biosynthetically processing precursors into a variety of mature functional protein components of the extracellular matrix (ECM). Such components include collagen types I–III, V, VII, and XI; small leucine-rich proteoglycans biglycan and osteoglycin; the ECM cross-linking enzyme lysyl oxidase; the basement membrane protein laminin 5; and dentin matrix protein 1, which is thought to be involved in initiating mineralization of ECM in hard connective tissues (1). In addition, the BMP1-like proteinases act in morphogenesis by activating certain members of the transforming growth factor-β superfamily of growth factors. This includes activation of BMPs 2 and 4 via cleavage of the extracellular antagonist chordin and activation of growth and differentiation factors (GDFs) 8 (also known as myostatin) and 11 (also known as BMP11) via cleavage within prodomain sequences to release mature GDF8 and 11 from non-covalent, latent complexes (1, 2). Thus, BMP1-like proteinases not only regulate formation of the ECM and signaling by a subset of transforming growth factor-β-like factors but may orchestrate the two in morphogenetic events. There are four mammalian BMP1-like proteinases: BMP1, mammalian Tolloid (mTLD), mammalian Tolloid-like 1 (mTLL1), and mammalian Tolloid-like 2 (mTLL2) (1, 3–5). BMP1 and mTLL1 are encoded by alternatively spliced mRNAs of the same gene (3), whereas mTLL1 and mTLL2 are genetically distinct (4, 5).

The BMP1-like proteinases share a common protein domain structure comprising an NH2-terminal prodomain that must be cleaved by furin-like proprotein convertases to yield the mature proteinase. This is followed by a protease domain common to the astacin M12A family of the M12 subclade of metalloproteinases (6) and then by EGF-like motifs and CUB (complement-Uegf-BMP1) (6) domains, thought to be involved in protein-protein interactions (1, 7, 8). BMP1 differs from the other three mammalian members of this family in lacking the most COOH-terminal EGF motif and two most COOH-terminal CUB domains found in the other three proteinases (1). The four mammalian BMP1-like proteinases differ in their distributions of expression and in the phenotypes resulting from ablation of their cognate genes (1, 5, 9, 10). In regard to the latter difference, ablation of the Bmp1 gene, which encodes both BMP1 and mTLD, results in perinatal lethality and defects of ventral wall closure, probably reflecting defects in ECM formation and dorsal-ventral patterning (9); whereas ablation of the mTLL1 gene results in embryonic lethality and defects in positioning and septation of the heart, probably reflecting defects in the regulation of BMP signaling (10).

In previous studies (11, 12), Kadler and colleagues have analyzed the structure-function relationships of various protein domains of BMP1. They have reported that the most NH2-terminal CUB domain (CUB1) is required for secretion and that the next most NH2-terminal CUB domain (CUB2) is required for procollagen C-proteinase (pCP) activity (11), the activity involved in cleaving the C-propeptides from procollagens I–III and from the pro-α2 chain of type V procollagen (1). They have also reported that retention of the most COOH-terminal BMP1 CUB domain (CUB3) is required for full enhancement of BMP1 pCP activity by the PCOLCE1. The latter has previously been thought to
enhance cleavage of procollagen C-propeptides solely by binding to procollagens, thus inducing conformational changes that make them fitter substrates for BMP1-like proteinases (13, 14). In the present report, we demonstrate that CUB1 is not necessary for secretion of either BMP1 or mTLL1 and that secreted BMP1 and mTLL1 protease domains devoid of CUB or EGF-like domains retain pCP activity and have enhanced activity, compared with full-length BMP1 or mTLL1, for the biosynthetic processing of biglycan at the physiologically appropriate site. We also demonstrate that PCOLCE1 only enhances full-length mTLL1 and BMP1 and not truncated versions missing the most COOH-terminal EGF and CUB domains and that full-length mTLL1, but not truncated versions of mTLL1, binds PCOLCE1. Finally, mTLL1 is shown to be independent of Ca$^{2+}$ levels in the ability to cleave some, but not all, substrates. This is in contrast to the long known Ca$^{2+}$ dependence of BMP1 proteolytic activities (13, 15, 16). Possible implications of the data are discussed.

EXPERIMENTAL PROCEDURES

Production of Recombinant Proteins—A cDNA encoding full-length human pro-MTLL1 (5) was ligated between the HindIII and NotI sites of the tetracycline-inducible vector pCDNA4/TO (Invitrogen). To generate a form of MTLL1 truncated after the CUB3 domain (TLL1-CUB3), oligonucleotides 5′-AATGAGCCCTCAGAATGCTATGATATCAGATACAGC-3′ and 5′-GGCCGCTCTAGACTACTTGTCACTGTCGTCCTTGACTCTAGTTGTAAGATATCCAGATAC-3′ were annealed to create a sequence encoding the COOH-terminal portion of the CUB3 domain fused to a thrombin cleavage site and a COOH-terminal FLAG epitope. The annealed oligonucleotides were cloned between an EcoRI site in the TLL1 sequence and an NotI site in pCDNA4/TO. Expression vectors for the other truncated forms of mTLL1 were constructed via PCR amplification and cloning of amplimers between a BsU36I site in mTLL1 coding sequences and a SacII site in the thrombin cleavage site sequence. A common forward primer used for all such amplification was 5′-AATGAGCCCTCAGGCAATCTCTATC-3′, whereas reverse primers were 5′-TCCCCGCGCAGCTACTAATGTTAAGATATCCAGATACACCAAGGGGGACATTGCCCAAGCCCGCAAGCTTTACAAGTG-3′ (for TLL1-protease): 5′-TCCCCGCGGAGCTAGATAAGACGCCTGCAAGGCTCTTCCC-3′ (TLL1-CUB1) and 5′-TCCCCCGCGGCAC-TAGTGTGTGTCCCCCTGACAGTACGAAAAC-3′ (for TLL1-CUB2). To generate truncated forms that retained the mTLL1 COOH-terminal domain, oligonucleotides 5′-CTATGCTTATAGTATCCAGATACACCAACATACACCAAGGGGGACATTGCCCAAGCCCGCAAGCTTTACAAGTG-3′ (sense) and 5′-TTTTTTGGTATGTTGGAATCTGTTACTTTTACACCAAGGGGGACATTGCCCAAGCCCGCAAGCTTTACAAGTG-3′ (antisense) were annealed to each other, thus creating the sequences encoding the COOH-terminal domain. The annealed oligomers were cloned between SpeI and SacII sites in the thrombin cleavage site sequences in the expression vectors for truncated forms described above.

To produce BMP1 and truncated forms, a cDNA encoding full-length human pro-BMP1 (5) was ligated between the HindIII and NotI sites of pCDNA4/TO. To generate a form of BMP1 truncated after the CUB2 domain (BMP1-CUB2), PCR was performed using forward primer, 5′-TGCCGATCCAGGCTTGCTAGGCTCTTT-3′, and reverse primer, 5′-GATCCGGCGGCTACTTGTGCATCGCTGCTCTTGATATC-GATTGGTATAGGACCCGTCAAGACAGCA-3′. The PCR amplifier was cloned between a BamHI site in the BMP1 sequence and a NotI site in pCDNA4/TO. The resulting construct contains a COOH-terminal FLAG epitope, separated from BMP1 sequences by a Clal site. To generate a form of BMP1 truncated after the CUB1 domain (BMP1-CUB1), oligonucleotides 5′-AATTCGCGACGCGACGACAAAGGTCCTTGGAGCTCTA-3′ (sense) and 5′-CTGAGACTGCAAGGAGCCCTTCAAGCTGGCTGCTGGCGG-3′ (antisense) were annealed and cloned between an EcoRI site in BMP1 sequences and a Clal site in the CUB3 construct. To generate a form of BMP1 truncated after the protease domain (BMP1-protease), oligonucleotides 5′-TCAAGAATGACATCGCCACGACCCCGAAGCTTTTACATGATCGGACAGTTTTCACAGTTCGTCAGAAAC-3′ (sense) and 5′-CGCCTTTGTAAGCTGGGCTTGGGCAATGCGATTGATACCTTGGC-3′ (antisense) were annealed, and cloned between a Bpl site in BMP1 sequences and a Clal site in the CUB3 construct.

293 T-REx cells (Invitrogen) were maintained in Dulbecco’s modified Eagle’s medium, 5 μg/ml blasticidin, and 10% fetal bovine serum. Cells at 80% confluence were transfected with 1 μg of expression plasmid/35-mm culture dish using Lipofectamine (Invitrogen). After 48 h, cells were selected in the same type of medium containing 200 μg/ml Zeocin. Resistant clones were isolated by ring cloning, and clones producing high levels of secreted full-length or truncated BMP1 or BMP1 upon induction with 1 μg/ml tetracycline were identified by Western blot.

For the production of recombinant proteinases for enzymatic assays, confluent cells were washed twice with phosphate-buffered saline and incubated in serum-free Dulbecco’s modified Eagle’s medium 15 min at 37 °C. Cells were then washed once with phosphate-buffered saline, followed by addition of serum-free Dulbecco’s modified Eagle’s medium containing 1 μg/ml tetracycline, to induce protein expression, and 40 μg/ml soybean trypsin inhibitor. Conditioned medium was harvested every 24 h, and protease inhibitors were added to final concentrations of 1 mM phenylmethylsulfonfluride, 1 mM N-ethylmaleimide, and 1 mM P-amino benzoic acid. Conditioned medium was centrifuged to remove debris, and supernatants were stored at −70 °C. Proteins were affinity-purified from medium using an anti-FLAG M2 affinity column (Sigma), as described (5), except that for some experiments proteinases were purified in the absence of Ca$^{2+}$. For the Western blot analyses of Fig. 2, cell layers were washed once with ice-cold phosphate-buffered saline, after the second harvest of media, and then scraped into hot SDS-sample buffer. 20 μl of second harvest conditioned media and equivalent amounts of cell layer samples were then separated on 4–15% SDS-PAGE gels, transferred to nitrocellulose membranes, and probed with anti-FLAG antibody (Sigma).

In Vitro Enzyme Assays—2 μg of full-length MTLL1 was incubated at 37 °C with 15 ng of trypsin or chymotrypsin (Promega) in 50 mM NH$_4$HCO$_3$, pH 7.8, or 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, respectively, for the indicated times. Reactions were stopped by adding SDS-sample buffer and boiling 5 min at 100 °C. Proteins were separated on 4–15% acrylamide SDS-PAGE gradient gels and visualized by Coomassie Blue R-250 staining.

Collagen processing was performed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$, 400 ng of $^3$H-labeled type I procollagen, prepared as previously described (5), was incubated 30 min at 37 °C with or without 50 ng of procollagen PCOLCE1, prepared as described (17). Full-length or truncated mTLL1 or BMP1 was then added to a 12 nM final concentration (except that for experiments in which collagen processing in the absence or presence of Ca$^{2+}$ was compared, 24 nM of isolated BMP1 and mTLL1 protease domains was used), and the reaction was stopped after 16 h at 37 °C by adding SDS-sample buffer. Proteinase were separated on a 5% SDS-PAGE gel and followed by treatment of the gel with ENHANCE (DuPont) and autoradiography.

500 ng of biglycan, prepared as described (18), was incubated with 3 nM full-length or truncated mTLL1 or BMP1 in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl$_2$ at 37 °C for indicated times. Subsequently, 0.01 unit of chondroitinase ABC (Seikagaku Corp.)
and 6× chondroitinase buffer (100 mM Tris-HCl, pH 8.0, 240 mM sodium acetate, 100 mM EDTA) were added to the reaction, and samples were incubated an additional 4 h at 37 °C. Reactions were stopped by adding SDS-sample buffer, and proteins were separated on a 7.5% acrylamide SDS-PAGE gel. For studies on Ca2+-dependence of probiglycan cleavage, assays were performed essentially as above, except that 500 ng of probiglycan was incubated 16 h with 1 nM full-length or truncated BMP1 or mTLL1 in the presence or absence of 5 mM CaCl2.

Recombinant chordin was prepared, and processing reactions were performed essentially as previously described (5, 19). 5 nM of each proteinase or isolated protease domain was incubated with 20 nM chordin for 16 h at 37 °C. Western blotting was with a previously described antibody specific for the chordin NH2-terminus (20).

Amino Acid Sequence Analysis—The products of cleavage assays, performed as described above, were resolved by SDS-PAGE and electrotransferred to Sequi-Blot polyvinylidene difluoride membranes (Bio-Rad). Proteins were revealed with 0.025% Coomassie Brilliant Blue R-250, and NH2-terminal amino acid sequences were determined by automated Edman degradation at the Harvard University Microchemistry Facility.

Far Western Blotting—100 ng of full-length mTLL1 and equal molar amounts of truncated forms of mTLL1 were separated on a 4–15% acrylamide SDS-PAGE gel under non-reducing conditions and were electrotransferred to a nitrocellulose membrane. The membrane was blocked in 5% nonfat dry milk in 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, and 5 mM CaCl2 (TBS-T/Ca2+) 1 h at room temperature. The membrane was then incubated with or without 200 ng/ml PCOLCE1 in 0.5% nonfat dry milk/TBS-T/Ca2+ overnight at 4 °C. After extensive washing with TBS-T/Ca2+ buffer, the membrane was incubated with anti-PCOLCE1 antibodies and washed as previously described for PCOLCE1 Western blots (17).

Co-immunoprecipitation—200 ng of PCOLCE1 was preincubated with or without 100 ng of mTLL1 or equivalent molar amounts of truncated mTLL1 forms in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM CaCl2 for 1 h at 4 °C. Anti-FLAG M2-agarose (Sigma) was then added, and the mixture was allowed to rotate 3 h at 4 °C. Agarose was then washed with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100 5 times at 20 min and divided into two parts. Agarose beads were centrifuged, and samples were eluted with SDS sample buffer and separated on 4–15% acrylamide SDS-PAGE gels (Bio-Rad). Samples were then transferred to nitrocellulose membranes and blotted with polyclonal anti-PCOLCE1 antisera (17) or polyclonal antibody 531 against the protease domain of mTLL1 (21).

RESULTS

Characterization of mTLL1 Domain Structure by Limited Proteolysis—Limited digestions were performed to define boundaries of folded protein domains in mature, active 120-kDa mTLL1. Digestion with trypsin produced major protein fragments of ~60, 45, and 38 kDa (Fig. 1, A and C), whereas digestion of mTLL1 with α-chymotrypsin produced ~92, 65, 60, 45, 39, and 17 kDa protein fragments (Fig. 1, B and C). Subsequent NH2-terminal amino acid sequencing showed the 60- and 38-kDa trypsin products to correspond to COOH-terminal fragments resulting from cleavage between mTLL1 residues 564KA565 and 724KA725, respectively, in the published human pro-mTLL1 sequence (Ref. 4, GenBankTM/EBI accession number U91963). NH2-terminal sequences of the 45-kDa fragment corresponded to the NH2-terminus of mature mTLL1, beginning with the first amino acid after the predicted furin cleavage site for removal of the prodomain (5).

Thus, based on SDS-PAGE mobility and NH2-terminal sequence, the 45-kDa band is predicted to represent the NH2-terminal fragment resulting from cleavage at the 564KA565 site (Fig. 1C). A somewhat heterogeneous ~65-kDa band (Fig. 1A) may correspond to the NH2-terminal product of cleavage at the 564KA565 site but was not in sufficient amounts to obtain amino acid sequences.

Edman degradation of chymotrypsin fragments showed the 60- and 39-kDa bands to both correspond to COOH-terminal portions resulting from cleavage between mTLL1 residues Ser558 and Asp559. Thus, based on SDS-PAGE mobility, the 60-kDa fragment is predicted to extend to the COOH terminus of mature mTLL1, whereas the size of the 39-kDa fragment is consistent with a COOH terminus somewhere near the junction of the CUB4 and CUB5 domains (Fig. 1C). NH2-terminal sequences of the 92-, 65-, 45-, and 17-kDa fragments all correspond to the NH2-terminus of mature mTLL1. Thus, based on SDS-PAGE mobility and NH2-terminal sequences, the 45-kDa fragment is predicted to represent the NH2-terminal fragment resulting from cleavage at the 558SD559 site, whereas the 92-, 65-, and 17-kDa NH2-terminal fragments are predicted to be truncated at positions illustrated in Fig. 1C. Cleavage by chymotrypsin and trypsin between CUB2 and EGF1 and by trypsin between CUB3 and EGF2 demonstrates these to be linker regions, which lie between individually folded domains and to be relatively exposed to proteases. Bands relatively resistant to protease digestion likely represent tightly folded protein domains. These limited proteolysis results differ in marked ways from results obtained by Sieron et al. (22) for limited proteolysis of mTLD with chymotrypsin. These differences, illustrated in Fig. 1C, suggest that, despite identical domain structures, mTLL1 and mTLD may differ in folding. However, use of pro-mTLD in the earlier study and mature, active mTLL1 here makes direct comparison difficult, because retention of the prodomain may affect folding and protease accessibility of the protease, CUB, and EGF domains.

Structure-Function Analyses of mTLL1 Domains: Secretion—To determine the roles of various mTLL1 protein domains, a number of recombinant truncated versions of mTLL1 were produced for functional comparison to full-length mTLL1. Proteins TLL1-CUB3, TLL1-CUB2, TLL1-CUB1, and TLL1-protease are versions of mTLL1 truncated at, and inclusive of, the CUB3, CUB2, CUB1, and protease domains, respectively (Fig. 2A). The COOH termini of TLL1-CUB2 and TLL1-CUB3 were Lys564 and Lys724, respectively, thus corresponding to COOH termini resulting from limited trypsin proteolysis (see above). This represents an attempt to have ends of these forms correspond to natural boundaries of mTLL1 protein domains, as defined by the limited proteolysis studies described above. Surprisingly, all of these truncated proteins were secreted from transfected 293 T-REx cells as efficiently as full-length, except for the longest truncated protein, TLL1-CUB3 (Fig. 2B), which was secreted less efficiently, because a faint CUB3 band was only visible in the media sample after prolonged exposure of the blot (data not shown). These results were surprising for three reasons: 1) truncated versions shorter than TLL1-CUB3 were efficiently secreted, whereas TLL1-CUB3 was not; 2) TLL1-CUB3 has a domain structure very similar to that of wild-type BMP1, which normally ends at CUB3; and 3) previous studies with truncated versions of BMP1 have shown that retention of CUB1 in its proper position is necessary for secretion of the protease domain to occur (11). For a direct structure-function comparison of mTLL1 and BMP1 protein domains, a series of truncated versions of BMP1, with COOH termini corresponding to those of the truncated mTLL1 proteins (Fig. 2A), were expressed, together with full-length BMP1, under conditions identical to those under which mTLL1 and its
FIGURE 1. Characterization of mTLL1 domain structure by limited proteolysis. 2 μg of full-length mTLL1 was incubated with 15 ng of trypsin (A) or chymotrypsin (B) for the indicated times. Cleavage products were subjected to SDS-PAGE on 4–15% acrylamide gels and visualized by staining with Coomassie Blue R-250. C, beneath a schematic representation of the protein domain structure shared by mTLL1 and mTLD are predicted identities of the various bands (with approximate molecular masses in kDa) produced by cleavage of mTLL1 with trypsin or chymotrypsin. Arrows above the schematic denote cleavage sites of chymotrypsin (C) and trypsin (T). Arrows below the schematic denote mTLD chymotrypsin (C) cleavage sites previously identified by Sieron et al. (22). The domain structure of BMP1 is identical to that of mTLL1 and mTLD, but, unlike the other two proteinases, ends after the CUB3 domain (3).
truncated forms were expressed in Fig. 2B. As can be seen (Fig. 2C), all truncated forms of BMP1 were secreted. Although the form corresponding to isolated BMP1 protease domain (BMP1-protease) was secreted with less efficiency than the other forms, it has previously been reported that isolated BMP1 protease is not secreted at all (11). Thus, CUB1 is not essential for secretion of either mTLL1 or BMP1 protease domains but does enhance secretion in the case of BMP1. Full-length and truncated forms of mTLL1 and BMP1 to be used in enzymatic assays (below) were affinity-purified from media using an anti-FLAG affinity column and subjected to SDS-PAGE and Coomassie Blue staining to confirm purity (Fig. 2, E and F).

Structure-Function Analyses of mTLL1 Domains: Enzymatic Activities—To determine roles of various mTLL1 protein domains in provision of pCP activity, 12 nM full-length mTLL1 and each of the truncated versions of mTLL1 were separately incubated with 50 nM type I procollagen. As can be seen (Fig. 3A), although full-length mTLL1 had the highest levels of pCP activity, some pCP activity was detected for all of the truncated mTLL1 proteins, with the exception of TLL1-CUB2. Of particular note, TLL1-CUB1 had relatively robust pCP activity and even TLL1-protease, which is devoid of CUB and EGF domains, showed some pCP activity. These results were surprising, because it has previously been shown that truncated forms of BMP1 lacking the CUB2, EGF, and CUB3 domains, or lacking just the CUB2 and EGF domains, are devoid of detectable levels of pCP activity (11).

When full-length BMP1 and each of the truncated versions of BMP1 were tested for pCP activity under conditions identical to those used for mTLL1 and its truncated forms, it was found that, whereas BMP1-CUB2 and BMP1-CUB1 had no detectable pCP levels, isolated BMP1 protease domain (BMP1-protease) did have such activity (Fig. 3C). Thus, in contrast to the findings of Hartigan et al. (11), the present results indicate that the
CUB2 domain is not essential for BMP1 pCP activity. In addition, it can be seen that mTLL1 and BMP1 differ, in that mTLL1-CUB1 has pCP activity, whereas BMP1-CUB1 does not (Fig. 3, A and C).

PCOLCE1 Enhances the pCP Activity of Only Full-length mTLL1 and BMP1—PCOLCE1 is a non-protease that can potentiate the pCP activity of proteinases like mTLL1 and BMP1 by ~10-fold (13, 23), appar-

FIGURE 3. Comparison of pCP activity levels in full-length and truncated forms of mTLL1 and BMP1, in the presence/absence of PCOLCE1. A, an autofluorogram is shown of purified human type I procollagen incubated in the absence (No Enzyme) or presence of full-length (FL) mTLL1, or the various mTLL1-truncated forms (without the COOH-terminal domain) and analyzed by SDS-PAGE. B–D, autofluorograms are shown of purified human type I procollagen incubated in the absence (No Enzyme) or presence of full-length or various truncated forms of mTLL1 (B and D) or BMP1 (C), in the presence (+) or absence (−) of PCOLCE1, followed by SDS-PAGE analysis. Truncated forms of mTLL1 either lack (B) or are fused to (D) the unique mTLL1 COOH-terminal domain. S.M., starting material. Graphic representations are shown of the scanned and quantified results of each autofluorogram as the percentage of each type I procollagen sample constituted by pNα1(I).
Structure-Function Analyses of mTLL1

ently by a mechanism that involves binding to type I procollagen C-propeptides and C-telopeptides, thereby inducing a conformational change that renders procollagen a better pCP substrate (13, 14). According to this model, any proteinase with pCP activity should have levels of that activity enhanced in the presence of PCOLCE1. To test this possibility, full-length mTLL1 and the various truncated versions of mTLL1 were separately incubated with type I procollagen in the presence or absence of PCOLCE1, and levels of pCP activity were compared. As can be seen (Fig. 3B), whereas PCOLCE1 markedly enhanced the pCP activity of full-length mTLL1, there was no evidence of enhancement of the pCP activity of any of the truncated forms of mTLL1. This result was surprising in the context of the existing model of how PCOLCE1 is thought to exert its effects on processing of procollagen C-propeptides. We also found that the pCP activity of only full-length BMP1, and of none of the truncated forms, is enhanced by PCOLCE1 (Fig. 3C). The latter results differ somewhat from the results of Petropoulou et al. (12), who found BMP1 forms lacking the CUB3 domain, or both the CUB3 and EGF domains, to be enhanced to some degree by PCOLCE1.

PCOLCE1 Binds mTLL1—The results of Fig. 3 (B and C) suggested the possibility of protein-protein interactions between PCOLCE1 and protein domains present in full-length mTLL1 and BMP1 but absent in the truncated forms. To assay for possible protein-protein interactions between the various forms of mTLL1 and PCOLCE1, far Western blotting was performed. Consistent with the enhancement assays of Fig. 3B, full-length mTLL1, but none of the truncated forms, bound PCOLCE1 (Fig. 4, A–C). Unexpectedly, it was found that, under the non-reducing SDS-PAGE conditions necessitated by the far Western assay, a significant proportion of TLL1-CUB3 and TLL1-CUB2 formed oligomeric aggregates, perhaps reflecting misfolding of some portion of these molecules. Because this might affect the ability of these forms to function as proteases and to bind PCOLCE1, a new generation of truncated mTLL1 forms was generated, members of which retained the small COOH-terminal domain found in full-length mTLL1 (Fig. 2, A and D), because it was thought this might help prevent misfolding. As can be seen (Fig. 4F), the TLL1-CUB3 and TLL1-CUB2 forms fused to the mTLL1 COOH-terminal domain migrated primarily as single bands under non-reducing SDS-PAGE conditions, suggesting correct folding. Nevertheless, full-length mTLL1 was still the only form to bind PCOLCE1 (Fig. 4D). Similarly, full-length mTLL1 was still the only form for which pCP activity was enhanced by PCOLCE1 (Fig. 3D).

Interestingly, the TLL1-CUB3 construct with the COOH-terminal domain showed higher levels of pCP activity than did full-length mTLL1 (in the absence of PCOLCE1) and than did TLL1-CUB3-truncated protein without the COOH-terminal domain (compare Fig. 3, B and D). The latter difference probably reflects the fact that a greater proportion of TLL1-CUB3 with the COOH-terminal domain is correctly folded (Fig. 4, compare C and F). In regard to the observation that TLL1-CUB3-C can have greater activity than full-length mTLL1, it is of potential interest that BMP1, which ends at CUB3, has greater pCP activity than does mTLD (5), which retains EGF2, CUB4, and CUB5 domains (see Fig. 1). Thus, the more COOH-terminal domains can actually limit pCP activity in both mTLL1 and mTLD/BMP1, for as yet obscure reasons.

To independently validate mTLL1-PCOLCE1 binding via a second methodology, PCOLCE1 was incubated either alone, or in the presence of FLAG-tagged full-length mTLL1 or individually with each of the truncated forms, with or without the mTLL1 COOH-terminal domain. As can been seen (Fig. 4G), PCOLCE1 co-immunoprecipitated with, and thus bound, full-length mTLL1. In contrast, PCOLCE1 did not co-immunoprecipitate with any of the truncated mTLL1 forms. Thus, PCOLCE1 binds mTLL1, and the presence of one or more of the mTLL1 COOH-terminal CUB and/or EGF domains is essential for such binding.

Proteinases like mTLL1 and BMP1 are also responsible for the proteolytic removal of an N-propeptide to yield the mature form of the small leucine-rich proteoglycan biglycan (1). To test the roles of the various protein domains on this activity, full-length mTLL1 and the first set of various truncated forms (lacking the small COOH-terminal domain of full-length mTLL1) were separately incubated overnight with biglycan to assay for possible differences in levels of activity. As can be seen (Fig. 5A), full-length mTLL1 and all of the original set of truncated mTLL1 forms, with the exception of TLL1-CUB2, had readily detectable levels of biglycan processing activity. Moreover, NH2-terminal amino acid sequencing of mature biglycan resulting from cleavage by the truncated TLL1-CUB3, TLL1-CUB1, and even TLL1-protease forms showed that in each case cleavage had occurred at the previously reported, physiologically relevant site (18, 24), between residues Asn77 and Asp56. Thus, none of the CUB or EGF domains are necessary, and the proteinase domain is sufficient, for providing the specificity with which mTLL1 processes biglycan to the mature, tissue form of biglycan. To compare levels of biglycan processing activity in full-length mTLL1 and the original TLL1-CUB3, TLL1-CUB1, and TLL1-protease forms (lacking the mTLL1 COOH-terminal domain) in a more quantitative fashion, a time-course study was performed. As can be seen (Fig. 5B), although TLL1-CUB3 appeared to process biglycan with reduced kinetics compared with full-length mTLL1, both TLL1-CUB1 and TLL1-protease showed increased kinetics compared with full-length mTLL1. A graphic presentation of the quantified scanned data (Fig. 5C) demonstrates TLL1-CUB1 to have the highest biglycan-processing kinetics, with somewhat lesser levels found in TLL1-protease.

When full-length BMP1 and each of the truncated versions of BMP1 were tested for biglycan-processing activity under conditions identical to those used for mTLL1 and its truncated forms, it was found that, as for mTLL1, the isolated protease domain had more activity than did the full-length proteinase (Fig. 5, D and E). However, mTLL1 and BMP1 differed in that, whereas TLL1-CUB1 had highest levels of biglycan-processing activity, the corresponding BMP1-truncated protein BMP1-CUB1 had no discernable biglycan activity.

Because TLL1-CUB2 and TLL1-CUB3 without the mTLL1 COOH-terminal domain showed marked levels of oligomerization, suggestive of misfolding (Fig. 4C), the forms of TLL1-CUB2 and TLL1-CUB3 with the COOH-terminal domain, which showed low levels or no oligomerization, respectively (Fig. 4F), were compared with full-length mTLL1 in a time-course assay of biglycan cleavage (Fig. 5F). TLL1-CUB2 again showed no biglycan activity, even with the COOH-terminal domain, whereas TLL1-CUB3 with the COOH-terminal domain showed marked biglycan processing. A graphic presentation of the quantified scanned data of Fig. 5F is shown in Fig. 5G. The absence of pCP or biglycan cleaving activity in TLL1-CUB2, even in the absence of oligomerization, may reflect an inhibitory role for the CUB2 domain in either interfering with the binding of substrate or obscuring the active site in the TLL1-CUB2 constructs.

mTLL1 Is Ca2+-independent—Although we previously predicted that the EGF-like domains of BMP1 might be responsible for the Ca2+-dependence of this proteinase (25), Garrigue-Antar et al. (26) have demonstrated that BMP1 Ca2+-dependence/binding resides instead in its CUB and/or protease domain(s). Because we had successfully produced isolated BMP1 and mTLL1 protease domains, we examined whether the proteolytic activities of these domains were Ca2+-dependent or -independent. As can be seen (Fig. 6A), full-length BMP1 had almost no probi-
glycan-processing activity in the absence of Ca\(^{2+}\), whereas isolated BMP1 protease domain retained ~50% of its activity in the absence of Ca\(^{2+}\), suggesting that some of the Ca\(^{2+}\) dependence/binding of BMP1 resides in its protease domain. Surprisingly, however, neither full-length mTLL1, nor any of its truncated forms, showed evidence of Ca\(^{2+}\) dependence for probiglycan-processing activity. In fact, full-length mTLL1 showed less probi-
FIGURE 5. Comparison of probiglycan-processing activity levels of full-length and truncated forms of mTLL1 and BMP1. A, a Coomassie Blue-stained gel is shown of probiglycan incubated in the absence (No enzyme) or presence of full-length or truncated forms of mTLL1. S.M., starting material. B and D, Coomassie Blue-stained gels are shown of probiglycan incubated with full-length (FL) or truncated forms of mTLL1 (B) or BMP1 (D). F, a Coomassie Blue-stained gel is shown of probiglycan incubated with full-length mTLL1 or truncated forms retaining the COOH-terminal domain. Incubations were for 5, 10, 30, or 60 min. Graphic representations of the scanned and quantified results of B, D, and F are shown in C, E, and G, respectively, as percentages of processing of probiglycan as a function of time.
glycan-processing activity in the presence than in the absence of Ca\textsuperscript{2+}, a result that was reproducible over the course of three independent experiments. To determine whether this seeming Ca\textsuperscript{2+} independence of mTLL1 activity held for other substrates, full-length BMP1 and mTLL, and their respective protease domains were incubated with type I procollagen in the presence or absence of Ca\textsuperscript{2+}, to determine effects on levels of pCP activity.

As can be seen (Fig. 6C), the pCP activity of both BMP1 and BMP1-protease domain showed marked Ca\textsuperscript{2+} dependence. Surprisingly, full-length mTLL1 and the mTLL1-protease domain showed somewhat higher levels of pCP activity in the presence than in the absence of Ca\textsuperscript{2+}, although the difference did not approach that observed for BMP1 and its protease domain.
The full-length proteinases and their respective protease domains were prepared and stored in buffer containing 5 mM Ca\(^{2+}\) (see Ref. 5), such that final concentrations of Ca\(^{2+}\) in cleavage reactions using full-length proteinases or protease domains were 10 nM (mTLL1) and 20 nM (BMP1), respectively, in reactions not raised to a final Ca\(^{2+}\) concentration of 5 mM Ca\(^{2+}\). To ensure that Ca\(^{2+}\) concentrations of 10 nM were not the basis for the ability of mTLL1 and its protease domain to efficiently cleave probiglycan and to partially cleave procollagen in the absence of 5 mM Ca\(^{2+}\), new batches of full-length enzymes and protease domains were prepared in the absence of Ca\(^{2+}\): BMP1

**FIGURE 7.** Ca\(^{2+}\) independence/dependence of mTLL1 and BMP1 and protease domains against probiglycan, procollagen, and chordin. A Coomassie Blue-stained gel of probiglycan (A), an autofluorogram of type I procollagen (B), and a Western blot of chordin (C) incubated with BMP1, mTLL1, or their respective protease domains (all of which were prepared in the absence of Ca\(^{2+}\), in the presence (+) or absence (−) of 5 mM CaCl\(_2\), are shown. To the right of each gel is a graphic representation of the scanned and quantified results, given as percentage of processing of probiglycan (A) or as percentage of a total collagen sample represented by the pN1(I)-processing intermediate (B), or by the ratio of cleavage fragment (produced by cleavage at the COOH-terminal chordin site) to full-length chordin (frag/FL) (C).
showed clear-cut Ca\(^{2+}\) dependence for probiglycan and procollagen processing, whereas mTLL1 and its protease domain showed Ca\(^{2+}\) independence in the ability to cleave probiglycan but slightly improved pCP activity in the presence, than in the absence of Ca\(^{2+}\). A difference between the results of Figs. 6 and 7 was that BMP1 protease domain prepared in the absence of Ca\(^{2+}\) was devoid of activity, even when cleavage assays were run in the presence of 5 mM Ca\(^{2+}\).

It has previously been reported by Ricard-Blum et al. (27) that procollagen C-propeptides bind Ca\(^{2+}\), and that such binding is necessary for certain protein-protein interactions involving these protein domains. Thus, it was possible that the slightly improved pCP activity of mTLL1 and its protease domain in the presence of Ca\(^{2+}\) was due to C-propeptide-Ca\(^{2+}\), rather than mTLL1-Ca\(^{2+}\), interactions. To gain further insight into the degree to which mTLL1 and its protease domain might be Ca\(^{2+}\)-independent, BMP1, mTLL1, and their respective protease domains were all prepared in the absence of Ca\(^{2+}\) and then evaluated for their ability to cleave chordin, another substrate of BMP1-like proteases (5, 20), in the presence or absence of 5 mM Ca\(^{2+}\). Surprisingly, the chordinase activities of mTLL1 and its protease domain, like BMP1 chordinase activity, were greatly enhanced in the presence of 5 mM Ca\(^{2+}\) (Fig. 7C). Thus, mTLL1 and its protease domain seem independent to Ca\(^{2+}\) levels in their ability to cleave probiglycan but dependent on Ca\(^{2+}\) levels in the ability to efficiently cleave chordin.

**DISCUSSION**

The three mammalian genes, two *Drosophila* genes, and multiple genes in other species, such as *Xenopus*, that encode BMP1-like proteinases (4, 5, 7, 28–31) doubtless arose via gene duplication, and the protein domain structure identical to that of *Drosophila* proteases Tolloid (TLD) and Tolloid-related 1 (TLR1) (1, 7). Thus, the complex modular array of domains that characterizes the BMP1-like proteinases has been conserved for the 800 million years that separate humans and *Drosophila* from their last common ancestor. Nevertheless, although the protein domain structure has been retained, sequences constituting the various domains in the different proteinases have diverged (5), and the extent to which functions of these related but different proteinases and their constituent domains have diverged or been conserved has remained, in large part, an open question.

It has previously been reported that the most NH\(_2\)-terminal CUB domain (CUB1) of BMP1 and mTLD, products of alternatively spliced RNAs encoded by the same gene (13), is required for secretion and that the next most NH\(_2\)-terminal CUB domain (CUB2) is required for pCP activity (11). Here we demonstrate that CUB1 is neither necessary for efficient mTLL1 secretion, nor is either CUB1 or CUB2 required for mTLL1 pCP activity, and that the mTLL1 astacin-like protease domain, devoid of CUB or EGF-like domains, is secreted and retains pCP activity. We also demonstrate that, contrary to a previous report (11), CUB1 is not required for BMP1 secretion, although it enhances the efficiency of secretion, nor is the CUB2 domain required for BMP1 pCP activity, because the isolated BMP1 protease domain exhibits pCP activity. It is notable that the isolated BMP1 and mTLL1 astacin-like protease domains cleave the large procollagen I molecule only in the small C-telopeptide region that separates the C-propeptide from the main collagenous domain. In contrast, the prototypic protease astacin, a digestive enzyme composed solely of a protease domain, degrades a broad range of proteins via cleavage at many sites (7). Thus, although it has become increasing clear that the CUB and EGF domains of the BMP1-like proteinases have marked effects upon the specificities of these enzymes (5, 11, 12, 22, 26), data presented here for the first time provide evidence that marked specificity can also reside in the protease domain itself. In fact, we demonstrate that the isolated mTLL1 protease domain has greater probiglycan-cleaving activity than does full-length mTLL1, as does protease domain fused only to the CUB1 domain. It is also demonstrated that isolated mTLL1 protease domain and the truncated TLL1-CUB1 form both cleave probiglycan solely at the physiologically relevant site. Thus, the remainder of full-length mTLL1 is clearly not necessary for either specificity or activity in the processing of probiglycan to the mature, tissue form of biglycan. We also demonstrate that isolated BMP1 protease domain has probiglycan processing activity superior to that of full-length BMP1 but that BMP1-CUB1 has no such activity. The finding here that BMP1-CUB1 has neither pCP nor probiglycan processing activity, plus the previous finding of Hartigan et al. (11) that a construct analogous to BMP1-CUB1 has no pCP activity, may mean that CUB1 can interfere with the BMP1 proteolytic site in the absence of CUB2, rather than the previous suggestion (11) of some intrinsic property of CUB2 that is essential for pCP activity. The pCP and probiglycan-processing activities of TLL1-CUB1 and the lack of both activities in BMP1-CUB1 demonstrates fundamental differences in the attributes of BMP1 and mTLL1 CUB1 domains.

Interestingly, the current study provided the unexpected result that the pCP activity of full-length mTLL1, but of none of the truncated forms, is enhanced by PCOLCE1. This result was unexpected as various data have previously suggested PCOLCE1 to enhance cleavage of procollagen C-propeptides solely by binding to the substrate and thereby inducing conformational changes that render the substrate more readily cleavable by BMP1-like proteinases (13, 14). Previous data, however, have not excluded the possibility of physical interactions between PCOLCE1 and BMP1-like proteinases. The occurrence of such physical interactions between full-length mTLL1 and PCOLCE1 is demonstrated herein, constituting the first report of physical interactions between a PCOLCE protein and a member of the BMP1 subgroup of proteinases. It has been known for some time that PCOLCE1 specifically binds the procollagen I C-propeptide (13). Moreover, Hulmes and colleagues (14) have recently presented evidence that PCOLCE1 binds sites in both the procollagen I C-propeptide and adjacent C-telopeptide regions. However, the finding here of a physical interaction between PCOLCE1 and mTLL1 suggests for the first time that induction of a conformation change in procollagen may not be the only way in which PCOLCE1 can act to enhance the pCP activities of BMP1-like proteinases. The fact that PCOLCE1 can bind both mTLL1 and procollagen invites speculation that it may be involved in a ternary complex with the two other molecules and, in this way, help to join proteinase and substrate in a fashion that positively affects the cleavage event. Formation of such a ternary complex is consistent with evidence that PCOLCE1 enhances the activity of BMP1-like proteinases solely toward procollagens and not toward other substrates (1, 12, 14). Data presented herein suggest that mTLL1 amino acid residues necessary for physical interactions with PCOLCE1 reside in the EGF2, CUB4, and/or CUB5 domains. Petropoulou et al. (12) previously noted that the BMP1 CUB3 domain, which is the most COOH-terminal BMP1 CUB domain (see Fig. 2), is necessary for full enhancement of BMP1 pCP activity by PCOLCE1, although they observed some enhancement with shortened BMP1 forms lacking the CUB3 and adjacent EGF domains (12). They also speculated that such results are consistent with interactions between PCOLCE1 and the BMP1 EGF and CUB3 domains (12). Here we have detected enhancement of only full-length BMP1 by PCOLCE1. Thus, results presented here support a model in which the most COOH-terminal EGF2, CUB4, and/or CUB5 domains of mTLL1 and the COOH-terminal CUB3 domain of BMP1 are necessary for enhancement of pCP activity by PCOLCE1. *Drosophila* members of the BMP1 proteinase family have a protein domain structure identical to that of mammalian proteases mTLL1.
Structure-Function Analyses of mTLL1

mTLL2, and mTLD (5, 7, 28), suggesting the shortened domain structure of BMP1 to be a later evolutionary innovation. Perhaps the BMP1 CUB3 domain has adapted to this innovation by developing the ability to interact with PCOLCE1, an ability found in the more COOH-terminal domains of related proteinases. Such adaptation underscores the different roles that seemingly orthologous domains can play in different members of the BMP1 subgroup of metalloproteinases.

Another surprise of the current study is the finding that mTLL1 is Ca$^{2+}$-independent in its ability to cleave biglycan, and perhaps in terms of its pCP activity, but that it requires Ca$^{2+}$ for efficient cleavage of chordin. This is in stark contrast to the long known Ca$^{2+}$ dependence of BMP1 pCP activity (15, 16) and to our demonstration that BMP1 and any one BMP1-like proteinase are not necessarily applicable to other like proteinases can be Ca$^{2+}$-dependent in some of its activities, 2) that results of structure-function analyses of all of their tested activities. The demonstration, that isolated BMP1 protease domain and, in the case of chordinase activity, mTLL1 protease domain are highly Ca$^{2+}$-dependent in all of their tested activities. The demonstration, that isolated BMP1 protease domain and, in the case of chordinase activity, mTLL1 protease domain are highly Ca$^{2+}$-dependent, indicates that these astacin domains probably bind Ca$^{2+}$. Presumably, differences in the Ca$^{2+}$ dependence of BMP1 and mTLL1 translates into different modes of regulation of these different proteinases in morphogenetic events in vivo. It should be noted that we did not attempt to strip BMP1, mTLL1, or their protease domains with chelators, such as EDTA, because this would not only have removed Ca$^{2+}$ but would also have removed Zn$^{2+}$, a necessary cofactor, from the active site. Thus, we do not exclude the possibility that, even in the absence of external Ca$^{2+}$, mTLL1 and its protease domain retain tightly bound Ca$^{2+}$, obtained during intracellular biosynthesis.

In summary, the present study provides the first structure-function analysis of the developmentally important metalloproteinase mTLL1, and demonstrates 1) that mTLL1 can be independent of external Ca$^{2+}$ in some of its activities, 2) that results of structure-function analyses of any one BMP1-like proteinase are not necessarily applicable to other members of the subgroup, 3) that isolated protease domains of BMP1-like proteinases can be Ca$^{2+}$-dependent, and 4) that physical interactions occur between PCOLCE1 and at least some members of the BMP1 proteinase family. The latter result suggests reappraisal of the current model for how PCOLCEs enhance pCP activity in such proteinases.

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