Multiple Bone Morphogenetic Protein 1-related Mammalian Metalloproteinases Process Pro-lysyl Oxidase at the Correct Physiological Site and Control Lysyl Oxidase Activation in Mouse Embryo Fibroblast Cultures

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Lysyl oxidase catalyzes the final enzymatic step required for collagen and elastin cross-linking in extracellular matrix biosynthesis. Pro-lysyl oxidase is processed by procollagen C-proteinase activity, which also removes the C-propeptides of procollagens I–III. The Bmp1 gene encodes two procollagen C-proteinases: bone morphogenetic protein 1 (BMP-1) and mammalian Tolloid (mTLD). Mammalian Tolloid-like (mTLL)-1 and -2 are two genetically distinct BMP-1-related proteinases, and mTLL-1 has been shown to have procollagen C-proteinase activity. The present study is the first to directly compare pro-lysyl oxidase processing by these four related proteinases. In vitro assays with purified recombinant enzymes show that all four proteinases productively cleave pro-lysyl oxidase at the correct physiological site but that BMP-1 is 3-, 15-, and 20-fold more efficient than mTLL-1, mTLL-2, and mTLD, respectively. To more directly assess the roles of BMP-1 and mTLL-1 in lysyl oxidase activation by connective tissue cells, fibroblasts cultured from Bmp1-null, Tll1-null, and Bmp1/Tll1 double null mouse embryos, thus lacking BMP-1/mTLL-1, or all three enzymes, respectively, were assayed for lysyl oxidase enzyme activity and for accumulation of pro-lysyl oxidase and mature 30-kDa lysyl oxidase. Wild type cells or cells singly null for Bmp1 or Tll1 all produced both pro-lysyl oxidase and processed lysyl oxidase at similar levels, indicating apparently normal levels of processing, consistent with enzyme activity data. In contrast, double null Bmp1/Tll1 cells produced predominantly unprocessed 50-kDa pro-lysyl oxidase and had lysyl oxidase enzyme activity diminished by 70% compared with wild type, Bmp1-null, and Tll1-null cells. Thus, the combination of BMP-1/mTLD and mTLL-1 is shown to be responsible for the majority of processing leading to activation of lysyl oxidase by murine embryonic fibroblasts, whereas in vitro studies identify pro-lysyl oxidase as the first known substrate for mTLL-2.

Lysyl oxidase catalyzes the oxidative deamination of peptidyl-lysine in elastin precursors and lysine and hydroxylysine residues in collagen to form peptidyl-α-aminoadipic-δ-semialdehyde and peptidyl-δ-hydroxy-α-aminoadipic-δ-semialdehyde residues, respectively. These aldehydes then undergo nonenzymatic reactions resulting in the cross-linkages known to be critical in the formation of mature functional insoluble elastin and collagens (1, 2). Lysyl oxidase is a copper-dependent enzyme and is specifically inhibited by α-aminopropionitrile. Connective tissue abnormalities known as osteolathyrism result from in vivo inhibition of lysyl oxidase activity by α-aminopropionitrile feeding (3). The abnormalities include malformed and weak bones, as well as increased development of hernias (4). Lysyl oxidase is synthesized as a 50-kDa glycoprotein and is processed extracellularly to produce the ~30-kDa molecular form known to be active (5). The sequence of the proteolytic processing site in pro-lysyl oxidase resembles that of the fibrilar procollagen C-terminal pro-peptide processing sites cleaved by procollagen C-proteinase (PCP)† (6). Moreover, preparations highly enriched in PCP activity have been shown to process pro-lysyl oxidase at the correct physiological site (7).

In mammals, PCP activity is provided by products of the Bmp1 gene (8–10), which encodes alternatively spliced mRNAs for the proteins bone morphogenetic protein 1 (BMP-1) and mammalian Tolloid (mTLD) (11). The mTLD protein product contains a longer C terminus than BMP-1, resulting in a domain structure identical to that of the Drosophila protein Tolloid. The domains in BMP-1, mTLD, and Drosophila Tolloid include the astacin-like proteinase domain and epidermal growth factor-like domains and CUB domains that may mediate binding to other extracellular proteins (12, 13). Drosophila Tolloid plays an important role in pattern formation during embryogenesis by cleaving the secreted protein SOG (short gastrulation), which forms latent complexes with the transforming growth factor β-related protein DPP (decapentaplegic) (14). BMP-1 may play a similar role in mammalian embryonic patterning, because it cleaves the SOG vertebrate homologue Chordin, which binds and inactivates the DPP vertebrate homologues BMP-2/BMP-4 (10, 15). Although mammalian BMP-1 and mTLD both are PCPs, only BMP-1 hydrolyzes Chordin, indicating that Bmp1 gene products have different substrate specificities and some different biological functions (10).

† The abbreviations used are: PCP, procollagen C-proteinase; BMP, bone morphogenetic protein; mTLD, mammalian Tolloid; mTLL, mammalian Tolloid-like; PAGE, polyacrylamide gel electrophoresis.
The mammalian Bmp1 gene is a member of a multigene family, and two genetically distinct mammalian Tolloid-related proteinases, mammalian Tolloid-like 1 and 2 (mTLL-1 and mTLL-2), have recently been described (10). Interestingly, despite highly similar sequences and domain structures, the substrate specificities of BMP-1, mTLD, mTLL-1, and mTLL-2 differ. BMP-1, mTLD, and mTLL-1 all have readily detectable PCP activity. By contrast, mTLL-2 does not appear to process fibrillar procollagens. BMP-1 and mTLL-1 readily cleave Chordin, whereas mTLD and mTLL-2 do not cleave Chordin (10). BMP-1 and mTLL-1 process probiglycan, whereas mTLD-2 does not (16). It has yet to be determined whether differences in specificity occur with other substrates, such as pro-lysyl oxidase. Because lysyl oxidase is critical for production of mature and functional extracellular matrix, it is important to understand whether a single member of the BMP-1-related proteinases predominates in processing pro-lysyl oxidase in connective tissues and cells or whether functional redundancy for this activity exists among BMP-1-related proteins.

Mice homozygous null for the Bmp1 gene have impaired ossification of calvaria and herniation of the gut and do not survive beyond birth but have grossly normal axial and appendicular skeletons (17). In vitro, fibroblasts from Bmp1-null embryos accumulate less insoluble collagen, and collagen fibrils are not normal in appearance. Procollagen processing is diminished, resulting in accumulation of intermediates that retain the C-propeptide and in low amounts of fully processed native collagen monomers (17). These results indicate that in Bmp1-null embryos and fibroblasts, related proteinases are able to partially, but not fully, compensate for the loss of the PCP activity normally provided by BMP-1 and mTLD. Interestingly, the Bmp1-null phenotype that includes gut herniation and abnormalities in calvaria maturation appears similar to lathyrysm (4). As noted above, lathyrysm is caused by a deficiency in lysyl oxidase enzyme activity (1, 3). These observations raise the possibility that lysyl oxidase enzyme activity may be abnormally low in the absence of a functional Bmp1 gene. Tll1-null animals have been recently created, and the homozygous null genotype is embryonic lethal (18). In contrast to Bmp1-null embryos, the Tll1-null embryonic phenotype has abnormalities predominantly in vascular and cardiac tissues. These differences in phenotypes, along with differences in the tissue-specific expression patterns of the different Bmp1-related genes, suggest differences in functions (18), although the absence of more widespread defects in Bmp1-null and Tll1-null embryos suggests some functional overlap between the cognate products of the two genes.

The lysyl oxidase pro-enzyme processing activities of the different BMP-1-related proteinases have not previously been compared, nor has the major enzyme responsible for processing of pro-lysyl oxidase in vivo been identified. The present study compares pro-lysyl oxidase processing in vitro by all four known mammalian BMP-1 proteinase family members (BMP-1, mTLD, mTLL-1, and mTLL-2). In addition, lysyl oxidase enzyme activity and pro-enzyme biosynthetic processing are determined in mouse embryo fibroblasts containing null alleles for Bmp1, for the mTLL-1 gene Tll1, or for both genes. Results from in vitro studies show that BMP-1, mTLD, mTLL-1, and mTLL-2 all process pro-lysyl oxidase at the correct physiological site but that the BMP-1 and mTLL-1 enzymes are the most efficient. Moreover, data from in vitro assays and from cell culture experiments support the important conclusion that the Bmp1 and Tll1 genes together provide the majority of activity for the biosynthetic processing of pro-lysyl oxidase and the generation of lysyl oxidase enzyme activity. The studies also identify pro-lysyl oxidase as the first known substrate for mTLL-2.

**Experimental Procedures**

**Recombinant Enzymes**—Recombinant human BMP-1, mTLD, mTLL-1, and mTLL-2 were produced as C-terminal FLAG-tagged proteins in transfected 293-EBNA cells and purified by affinity chromatography using the same methodology as in Ref. 10. Each enzyme exhibited a single major band on SDS-PAGE gels stained with Coomassie Blue, and purity of each enzyme preparation was estimated to be 90% or greater. The concentration of enzyme solutions was determined by subjecting serial dilutions of enzymes and known amounts of standard proteins to SDS-PAGE and Coomassie Blue staining (10). The relative specific activities of enzymes against known substrates type I procollagen and Chordin were verified prior to initiating experiments with pro-lysyl oxidase (10).

**Substrate and Antibodies**—The maltose-binding protein/lysyl oxidase fusion protein was produced and purified as described previously (19). Rabbit anti-bovine lysyl oxidase antibodies were raised against purified 30-kDa mature bovine aorta lysyl oxidase as described (20) and were provided by Dr. Herbert M. Kagan (Boston University School of Medicine). The antibodies were affinity purified utilizing immobilized lysyl oxidase fusion protein (19). Affinity purification of anti-lysyl oxidase was accomplished utilizing recombinant rat lysyl oxidase fusion protein covalently attached to Sulfolink (Pierce), and the specific antibodies were purified according to the manufacturer’s protocol. The titer of the purified lysyl oxidase antibody was determined by Western blotting against the lysyl oxidase fusion protein. Alkaline phosphatase-coupled goat anti-rabbit antibodies and Western Blue detection reagents were purchased from Promega. Although there is no obvious sequence similarity between lysyl oxidase and the maltose-binding protein, the affinity purified anti-lysyl oxidase antibody recognizes both mature 30-kDa lysyl oxidase itself and the maltose-binding protein (New England BioLabs, Beverly, MA) on Western blots. Thus, by chance, shared epitopes between lysyl oxidase and the maltose-binding protein appear to exist. This provides for recognition of both the lysyl oxidase fusion protein and the maltose-binding protein portions, respectively, of the recombinant fusion protein substrate by this antibody preparation.

**Pro-Lysyl Oxidase Processing Assays**—Maltose-binding protein/lysyl oxidase fusion protein (5 μg) was incubated with 30 ng of recombinant FLAG-tagged BMP-1, mTLD, mTLL-1, or mTLL-2 for either 45 min or 4 h at 37 °C in a final volume of 200 μl of 50 mM Tris, 150 mM NaCl, 5 mM CaCl2, pH 7.5. The reactions all contained 40 μg/ml soybean trypsin inhibitor, 10 μg/ml leupeptin, and 0.4 mM phenylmethylsulfonyl fluoride to inhibit nonspecific proteinases. The reactions were stopped by adding an equal volume of SDS-PAGE sample buffer and boiling for 3 min. Fifty-μl aliquots were subjected to 10% PAGE and Western blotting (21) to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). The blots were blocked with 1% bovine serum albumin in 20 mM Tris, pH 7.5, 150 mM NaCl, 0.05% Tween 20. The blots were incubated with affinity purified anti-lysyl oxidase antibody at a dilution of 1:10,000 at room temperature overnight. The blots were washed and then incubated with alkaline phosphatase goat anti-rabbit IgG according to the manufacturer’s instructions. The bands were detected using stabilized nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate Western Blue reagent (Promega). The relative amount of 86-kDa fusion protein substrate and 30-kDa lysyl oxidase product in each lane was determined by densitometry measurements performed in triplicate utilizing a Bio-Rad Fluor-S MultiImager equipped with Quantityone software (version 4.01), and the percentages of the conversions were determined. The semi-quantitative pro-lysyl oxidase conversion activity/proteinase proteinase was then calculated from reactions in which conversion of 86-kDa fusion protein to 30-kDa product was 50% or less. Previous studies have shown that pro-lysyl oxidase processing activity assays analyzed as described above are linear as a function of time of incubation in reactions in which up to 50% conversion of 86-kDa fusion protein to 30-kDa product is observed (19).

**N-Terminal Amino Acid Sequence Analyses**—For N-terminal sequence analyses of 30-kDa peptides, reactions containing 7 μg of fusion protein, as described above, were incubated at 37 °C for 12 h. The reactions did not contain soybean trypsin inhibitor but did contain all other proteinase inhibitors. The reactions were concentrated by lyophilization, subjected to SDS-PAGE, transferred to polyvinylidene difluoride membranes (Bio-Rad Trans-Blot), and stained with Coomassie Blue. Visualized 30-kDa protein bands were excised and subjected to N-terminal analyses by Edman degradation by Bill Lane and colleagues.
RESULTS

In Vitro Pro-lysyl Oxidase Processing Assays—Recombinant maltose-binding protein/lysyl oxidase fusion proteins were created and used as substrate in assays to assess pro-lysyl oxidase proteolytic processing activity (7, 19). As shown in Fig. 1, the N-terminal end of the intact fusion protein consists of the maltose-binding protein, and the C-terminal end is the rat lysyl oxidase proenzyme. Following proteolytic processing by PCP, two products are generated: 30-kDa mature lysyl oxidase and a 56-kDa maltose-binding protein/lysyl oxidase propeptide product. Immunoblotting of products utilizing anti-30-kDa lysyl oxidase antibodies allowed qualitative and quantitative assessment of lysyl oxidase processing activity (19).

Fusion protein processing by purified recombinant BMP-1, mTLD, mTLL-1, and mTLL-2 was assayed. Thirty ng of each purified enzyme was incubated with 7 µg of fusion protein for 4 h at 37 °C, and products were subjected to SDS-PAGE and Western blotting with lysyl oxidase polyclonal antibodies as described under “Experimental Procedures.” Incubations containing fusion protein and no added proteinase exhibited a single major 86-kDa band and sometimes an additional minor 30-kDa band when probed with the lysyl oxidase antibody (Fig. 2A, lane 1) (19). No 30-kDa product was observed in the absence of added enzymes. By contrast each reaction containing a recombinant enzyme resulted in production of a 30-kDa product recognized by the lysyl oxidase antibody. Under these conditions, BMP-1 and mTLL-1 caused near complete to complete loss of 86-kDa fusion protein and production of large amounts of 30-kDa product, whereas mTLD and mTLL-2 resulted in slight loss of 86-kDa fusion protein and production of small amounts of 30-kDa product. Semi-quantitative densitometric analyses of the relative intensity of 86- and 30-kDa bands in each lane indicate 95–100% conversion of BMP-1 and mTLL-1 and 10–15% conversion by mTLD and mTLL-2, respectively. Similar results were obtained in a total of four experiments performed with two different preparations of enzymes.

Semi-quantitative assessment of lysyl oxidase fusion protein conversion is linear with respect to both the time of incubation and the amount of activity if the percentage of conversion is 50% or less under the experimental conditions utilized in the present study and as previously reported (19). Thus, incubations were carried out for shorter times with the goal of obtaining valid relative semi-quantitative activity estimates for all four enzymes. Incubations carried out for 45 min resulted in lower percentage conversions for all enzymes, as expected (Fig. 2B). BMP-1 converted 50% of the maltose-binding protein/lysyl oxidase fusion protein, and mTLL-1 converted 15%. Conversion activity per hour was then calculated for each enzyme, and the results were expressed as micrograms of fusion protein converted per hour per picomole of enzyme (Table I). The values in Table I are derived from blots shown in Fig. 2A (mTLD and mTLL-2) and Fig. 2B (BMP-1 and mTLL-1). The results clearly show that BMP-1 processes the lysyl oxidase fusion protein with the highest efficiency compared with the other enzymes. As noted above, blots from four independent experiments in which incubations were performed for 2 or 4 h all exhibited

![Diagram showing the structure of the maltose-binding protein/lysyl oxidase fusion protein and products following processing by enzymes with PCP activity.](http://www.jbc.org/Downloaded_from)
higher conversion of 86-kDa fusion protein to 30-kDa lysyl oxidase by BMP-1 compared with the other enzymes, with results similar to the blot shown in Fig. 2A. The data in Table I are derived from assays performed in the linear responsive range of the assay, and relationships identified by these semi-quantitative assay results demonstrate that BMP-1 is the most efficient pro-lysyl oxidase processing enzyme. These results are representative of all of the experiments performed.

N-terminal sequence analyses were performed on the 30-kDa products obtained from lysyl oxidase fusion protein and BMP-1, mTLD, mTLL-1, and mTLL-2, respectively. The expected N terminus of DDPYN corresponding to the known N terminus of the tissue form of mature active 30-kDa lysyl oxidase was obtained with the highest level of confidence from all four reactions. No 30-kDa product was obtained from reactions incubated without added proteinase. Thus, although the rate of processing of the lysyl oxidase fusion protein by the four enzymes differed, the 30-kDa product generated in all cases corresponded to the physiologic cleavage product of pro-lysyl oxidase.

Cell Culture Studies with Bmp1- and Tll1-null Fibroblastic Cells—The data presented above indicate that the two products of the Bmp1 gene, BMP and mTLD, and products from the genetically distinct Tll1 and Tll2 genes encoding mTLL-1 and mTLL-2, respectively, all process pro-lysyl oxidase in vitro. Nevertheless, similarities of the phenotype of Bmp1-null animals to lathyritic suggest that the phenotype might be largely due to diminished lysyl oxidase activity, at least in certain tissues such as calvaria and ventral body wall. The latter consideration, plus the relatively robust pro-lysyl oxidase processing activity of BMP-1, suggest that lysyl oxidase processing and activity in vivo may depend to a large extent on products of the Bmp1 gene and to BMP-1 in particular. To explore this question, levels of lysyl oxidase enzyme activity were first determined from cultures of fibroblasts derived from wild type, Bmp1+/+, Bmp1−/−, Tll1+/+, Tll1−/−, and homozygous double null (Bmp1−/−, Tll1−/−) embryos. The results indicate that lysyl oxidase enzyme activity secreted by the double null embryonic fibroblasts is about 30% of that found in cells from wild type, Bmp1−/−, Bmp1−/−, Tll1−/−, and Tll1−/− cells, respectively (Table II). Thus, only cells homozygous null for both the Bmp1 and Tll1 genes produced a readily detectable reduction in levels of lysyl oxidase enzyme activity. Assessment of accumulating molecular forms of lysyl oxidase was then performed by pulse labeling/immunoprecipitation analyses in wild type, Bmp1−/−, Tll1−/−, and double null cells (Bmp1−/−, Tll1−/−) to determine whether 50-kDa pro-lysyl oxidase processing was diminished only in the double null cells. The cells were cultured to near confluence and then cultured in serum-free medium containing [35S]methionine for 18 h. Media proteins were then immunoprecipitated with anti-lysyl oxidase antibody or preimmune serum and subjected to SDS-PAGE and autoradiography. As shown in Fig. 3, unprocessed 50-kDa lysyl

**TABLE I**

Pro-lysyl oxidase processing activity by recombinant BMP-1, mTLD, mTLL-1, and mTLL-2 enzymes

| Enzyme   | Pro-lysyl oxidase processing activity (μg/ h/ pmol) |
|----------|-----------------------------------------------|
| BMP-1    | 15.0 ± 3.0                                     |
| mTLD     | 0.79 ± 0.10                                    |
| mTLL-1   | 5.38 ± 1.20                                    |
| mTLL-2   | 1.03 ± 0.20                                    |

**TABLE II**

Lysyl oxidase enzyme activity in cultures of wild type, Tll1−/−, and Tll1−/−, Bmp1−/+−, Bmp1−/−−, and double null (Bmp1−/−−, Tll1−/−−) murine embryonic fibroblasts

| Cell culture genotype | Lysyl oxidase activity (dpm/μg DNA) | Fold change vs. wild type |
|-----------------------|-------------------------------------|---------------------------|
| Experiment 1          |                                     |                           |
| Wild type             | 1682 ± 103                          | 1.0                       |
| Tll1−/+−              | 1558 ± 102                          | 0.9                       |
| Tll1−/−−              | 1497 ± 101                          | 0.9                       |
| Bmp1−/+−              | 1696 ± 106                          | 1.0                       |
| Experiment 2          |                                     |                           |
| Wild type             | 968 ± 28                            | 1.0                       |
| Bmp1−/−−              | 1102 ± 54                           | 1.1                       |
| Bmp1−−−, Tll1−/−     | 326 ± 24                            | 0.2                       |

![Image](http://www.jbc.org/content/25/19/22540/F2.large.jpg)
suggest that mTLL-2 also contributes to pro-lysyl oxidase processing and activation. In addition, results indicating that the double null cells accumulated 3.9-, 3-, and metric analyses of the 50-kDa lysyl oxidase pro-enzyme band oxidase proenzyme accumulated at higher levels in media from fibroblasts from mice with different genotypes:

**FIG. 3. Autoradiograms of 10% SDS-PAGE gel of preimmune and lysyl oxidase immunoprecipitates from pulse-labeled embryonic fibroblasts from mice with different genotypes:** Tll1 -/- (T); Bmp1 -/- (B); Tll1 -/-, Bmp1 -/- (T/B); and wild type (W). The markers are in vitro transcription/translation of pro-lysyl oxidase in the presence (+C) or the absence (-C) of canine pancreatic microsomal membranes to produce a mixture of glycosylated 50-kDa and nonglycosylated 45-kDa pro-lysyl oxidase (+C) and only nonglycosylated 45-kDa pro-lysyl oxidase (-C), respectively, to show the mobility of these proteins on SDS-PAGE (5). Lane P shows the mobility of purified mature ~30-kDa bovine aorta lysyl oxidase partially purified as described (38); lane M shows the low molecular weight markers (Bio-Rad). The ~24-kDa protein that co-purifies with lysyl oxidase seen in lane P has previously been identified as tyrosine-rich acidic matrix protein (39, 40). For immunoprecipitation assays, cells were cultured and pulse-labeled with [35S]methionine, and media proteins were isolated and immunoprecipitated with rabbit anti-bovine lysyl oxidase as described under “Experimental Procedures.” 0.6–2.45 × 10⁶ cpm were utilized for immunoprecipitation experiments performed three times with similar results.

oxidase proenzyme accumulated at higher levels in media from double null cells compared with the other cultures. Densitometric analyses of the 50-kDa lysyl oxidase pro-enzyme band indicate that the double null cells accumulated 3.9-, 3-, and 4-fold more 50-kDa lysyl oxidase pro-enzyme than wild type, Bmp1 -/-, and Tll1 -/- cells, respectively. The pro-lysyl oxidase protein immunoprecipitated from cell cultures made from all genotypes is predominantly the 50-kDa N-glycosylated form of this protein but also includes the 45-kDa nonglycosylated proenzyme (Fig. 3), consistent with studies performed in rat smooth muscle cells and murine MC3T3-E1 osteoblastic cells (5, 19). Surprisingly, similar levels of ~30-kDa lysyl oxidase were found in all four cultures, but in double null cells the mobility of this band is faster and more diffuse than in the other three cultures. This suggests that the 30-kDa band in the double null immunoprecipitates may contain abnormally processed lysyl oxidase or a greater proportion of partially degraded and inactive lysyl oxidase. This notion is consistent with lysyl oxidase enzyme activity data that demonstrate diminished lysyl oxidase activity in Bmp1/Tll1 double null cultures (Table II).

Taken together, the data support the conclusion that both Bmp1 and Tll1 genes produce efficient pro-lysyl oxidase processing enzymes, and that these enzymes contribute to pro-lysyl oxidase processing and development of lysyl oxidase enzyme activity in murine embryonic fibroblasts. These data support a previously unrecognized significant role for mTLL-1 in pro-lysyl oxidase processing and activation. In addition, results suggest that mTLL-2 also contributes to pro-lysyl oxidase processing.

**DISCUSSION**

Two recent discoveries lead to the notion that proteinases with PCP activity may be key in initiating and controlling a cascade of extracellular transformations critical for extracellular matrix deposition. The two discoveries are (a) that enzymes with PCP activity have multiple extracellular substrates and (b) that the Bmp1 gene, which is related to genes that regulate morphogenetic patterning in nonmammalian species, encodes BMP-1 and mTLD, two products with PCP activity. In addition to the biosynthetic processing of procollagen types I, II, and III, preparations highly enriched in PCP activity proteolytically process pro-lysyl oxidase (7). Recently BMP-1 has been shown to inactivate Chordin to release growth factors from latent complexes (10, 15) and may biosynthetically process pro-α1(V) collagen (24), laminin-5 (25), and probiglycan (16). The concept of a key proteinase activity that controls a series of subsequent activities leading to a biological phenotype is well known in catabolic cascades, in which extracellular matrix destruction is initiated by collagenases (MMP-1, MMP-8, or MMP-13) or plasmin (26). The existence of anabolic or synthetic extracellular enzymatic cascades now seems likely following characterization of developmentally regulated enzymes such as those with PCP activity.

As with key catabolic proteinases such as the collagenases, a certain degree of redundancy appears to exist for anabolic proteinases, where key enzyme activities may be encoded by different genes in a tissue-specific and developmentally regulated manner. The present study investigates the ability of proteinases encoded by the Bmp1 gene itself and of two highly similar proteinases encoded by Bmp1-related mammalian genes to process pro-lysyl oxidase in vitro. In addition, the effects of the absence of proteinases encoded by the Bmp1 and Tll1 genes, respectively, on lysyl oxidase activity and biosynthesis was studied in fibroblasts cultured from wild type, Bmp1-null, Tll1-null, and Bmp1/Tll1 double null mouse embryos. This experimental approach, utilizing both in vitro assays with recombinant proteins and cultured cells derived from genetically altered animals, provides independent assays to assess the roles of the different procollagen C-proteinases in lysyl oxidase processing. In vitro data were obtained with human recombinant enzymes, all of which were expressed in the same mammalian expression system, all of which contained the same C-terminal epitope tag, and all of which were purified by the same affinity chromatography methodology (10). Moreover, although the maltose-binding protein/pro-lysyl oxidase fusion protein was made using a rat lysyl oxidase cDNA (17), the proteolytic processing site MVG*DDPYN is conserved between rat and human pro-lysyl oxidase (6). Thus, the use of assays investigating relative efficiency of processing of the rat lysyl oxidase substrate by human BMP-1-related proteinases, each of which was prepared in the same manner, represents a valid experimental approach to investigating pro-lysyl oxidase processing activity. The validity of the experimental approach is further supported by the agreement between the in vitro data and data obtained from studies of mouse embryo fibroblasts, all of which support the conclusion that BMP-1 and mTLL-1 together contribute the majority of processing leading to the activation of lysyl oxidase.
The *in vitro* data indicate that BMP-1, mTLD, mTLL-1, and mTLL-2 enzymes all process pro-lysyl oxidase at the physiological site but differ in pro-lysyl oxidase processing efficiency. An important finding is that BMP-1, which has the highest PCP activity, is also the most efficient pro-lysyl oxidase processing enzyme. The high efficiency of BMP-1 processing of both procollagens and pro-lysyl oxidase points toward BMP-1 as a principal activity in the biosynthesis of functional insoluble collagen. Previously reported expression patterns of BMP-1, mTLD, mTLL-1, and mTLL-2 in developing hindlimbs indicate BMP-1 and mTLD to be expressed at high levels throughout limb mesenchyme, whereas mTLL-1 expression appeared more restricted to perichondrium or peristeum, and mTLL-2 expression appeared principally in skeletal muscle (10). The *Bmp1* gene is also expressed at high levels in developing dermis (27).

Thus, patterns of expression of BMP-1 and mTLD in highly collagenous tissues are also consistent with a principal role in lysyl oxidase and mature collagen biosynthesis. The more restricted expression patterns of mTLL-1 and mTLL-2 may indicate tissue-specific roles for these products in the normal development of collagenous extracellular matrices *in vivo*. It is interesting, however, that mTLL-2 is able to process lysyl oxidase slowly but does not appear to process procollagens or biglycan at all (16). Thus, of the procollagen C-proteinase substrates studied so far, lysyl oxidase is unique in that it is processed by mTLL-2. We speculate that because lysyl oxidase-dependent cross-linking is critical for the biosynthesis of mature connective tissues, evolution of greater redundancy providing for lysyl oxidase activation may offer biological advantages.

As noted, two proteins are derived from the *Bmp1* gene by alternative splicing: BMP-1 and mTLD (28). It is of interest that mTLD processes procollagens and pro-lysyl oxidase less efficiently than BMP-1. It is also notable that mTLD does not cleave Chordin, whereas BMP-1 efficiently cleaves Chordin (10). The mTLD protein is longer than the BMP-1 protein and contains two additional CUB domains and an additional epidermal growth factor domain. In addition to modulating proteinase activity, these additional domains may provide binding sites for other extracellular matrix components important for other functions unique to mTLD (29). It is noteworthy that mTLD and BMP-1 proteins are differentially expressed in human and mouse tissues (28).

The *in vitro* finding that the BMP-1 proteinase is a highly efficient pro-lysyl oxidase processing enzyme led to the notion that BMP-1 might be the principal lysyl oxidase processing activity in both cultured fibrogenic cells and *in vivo*. The connective tissue defects in *Bmp1*–/– animals, including gut herniation and delayed development of skull bones, resemble lathyrism and, thus, may be caused in part by below normal lysyl oxidase activity, in at least some tissues in these animals (17). The results from our studies in cultured cells were therefore surprising in that cells derived from *Bmp1*-null embryos processed lysyl oxidase normally and produced similar lysyl oxidase enzyme activity compared with wild type cells. As noted, lysyl oxidase enzyme activity was not diminished, and nearly wild-type levels of 50-kDa pro-lysyl oxidase and of ~30-kDa mature lysyl oxidase accumulated in *Bmp1*–/– cells. These results indicate that other enzymes such as mTLL-1 and/or mTLL-2 might almost fully compensate for the lack of BMP-1 and/or mTLD in these fibroblast cultures, given that mTLL-1 and mTLL-2 mRNAs are expressed in mouse embryo fibroblasts (16). Our data showing that *Bmp1/Tll1* double null fibroblasts produce levels of lysyl oxidase activity diminished by 70% and highly elevated levels of the 50-kDa lysyl oxidase pro-enzyme precursor support the notion that both BMP-1 and mTLL-1 enzymes are together important in processing pro-lysyl oxidase and that mTLL-1 significantly contributes to pro-lysyl oxidase processing in the absence of BMP-1. Future studies closely examining the expression and role of mTLL-2 in processing lysyl oxidase enzyme may account for the residual lysyl oxidase pro-enzyme processing and 30% lysyl oxidase enzyme activity observed in the *Bmp1/Tll1* double null murine embryonic fibroblasts. This information combined with a greater understanding of tissue- and cell-specific expression of different *Bmp1*-related genes will enhance the understanding of the roles of these different gene products in different tissues.

An additional contributing factor to the observed lysyl oxidase activity in *Bmp1/Tll1* double null cells may be that lysyl oxidase enzyme activity could be produced by at least one other gene. In support of this possibility a recent study has shown that some murine osteosarcoma cell clones that produce little or no immunoreactive lysyl oxidase protein do produce lysyl oxidase activity (30). Moreover, lysyl oxidase-like genes have been identified and mapped to human chromosomes 15, 8, and 2, respectively (31–35), whereas the lysyl oxidase gene itself maps to human chromosome 5 (36). The cDNA sequences of all lysyl oxidase relatives predict proenzymezimes that require proteolytic processing. However, only the lysyl oxidase sequence itself contains a G*DD* sequence similar to the A*DD*, A*DQ, and G*DE* sequences present in human types I, II, and III procollagen cleavage sites, respectively (6). These observations raise the possibility that lysyl oxidase activity derived from one or more lysyl oxidase-like genes could develop independently of BMP-1-related enzymes and could partially account for the residual lysyl oxidase enzyme activity observed in cells cultured from *Bmp1/Tll1*–/– animals. It should be noted, however, that the BMP-1/mTLL-1 susceptible sequences in Chordin, A*DG*, and S*DR* differ from the generally more highly anionic procollagen and pro-lysyl oxidase sequences (10). The BMP-1-susceptible site in pro-α1(V) collagen also has unique features and does not contain an Asp in the P position (24).

Thus, the new pro-lysyl oxidase-like precursors may also require processing by BMP-1 and/or BMP-1-related proteinases at a novel site. Studies investigating the biosynthesis and structure/activity relationships of lysyl oxidase and the lysyl oxidase-like proteins are necessary to evaluate relatedness of functional roles. Such studies could potentially involve identification of new pro-lysyl oxidase-like processing enzymes distinct from the BMP-1 family. In addition, a greater understanding of the substrate specificity of BMP-1 itself and of related enzymes will help to further clarify the biological roles of this family of proteinases.

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**REFERENCES**

1. Kagan, H. (1986) in *Biology and Regulation of Extracellular Matrix: Regulation of Matrix Accumulation* (Mecham, R. P., ed) Vol. 1, pp. 321–338, Academic Press, Orlando

2. Kagan, H. M., and Trackman, P. C. (1991) *Am. J. Respir. Cell Mol. Biol.*, 5, 206–210

3. Siegel, R. C. (1979) *Int. Rev. Connect. Tiss. Res.*, 8, 73–118

4. Selye, H. (1957) *Rev. Can. Biol.*, 13, 3–8

5. Trackman, P. C., Bedell-Hogan, D., Tang, J., and Kagan, H. M. (1992) *J. Biol. Chem.*, 267, 8666–8671

6. Cronahaw, A. D., Fothergill-Gilmore, L. A., and Hulmes, D. J. S. (1995) *Biochem. J.*, 306, 279–284

7. Panchenko, M. V., Stelzer-Stevenson, W. G., Trubetskoy, O. V., Gacheru, S. N., Gacheru, S. K., and Kagan, H. M. (1996) *J. Biol. Chem.*, 271, 7113–7119

8. Kessler, E., Takahara, K., Biniaminov, L., Brusel, M., and Greenspan, D. S. (1996) *Science*, 271, 360–362

9. Li, S. W., Sieron, A. L., Fertala, A., Hojima, Y., Arnold, W. V., and Prockop, D. J. (1996) *Proc. Natl. Acad. Sci. U. S. A.*, 93, 5127–5130

10. Scott, I. C., Blitz, I. L., Pappano, W. N., Imamura, Y., Clark, T. G., Steiglitz, B. M., Thomas, C. L., Maas, S. A., Takahara, K., Cho, K. W., and...
11. Takahara, K., Kessler, E., Biniaminov, L., Brusel, M., Eddy, R. L., Jani-Sait, S., Shows, T. B., and Greenspan, D. S. (1994) *J. Biol. Chem.* **269**, 26280–26285

12. Prockop, D. J., Sieron, A. L., and Li, S. W. (1998) *Matrix Biol.* **16**, 399–408

13. Hulmes, D. J., Mould, A. P., and Kessler, E. (1997) *Matrix Biol.* **16**, 41–45

14. Marques, G., Musacchio, M., Shinnell, M. J., Wunnenberg-Stapleton, K., Cho, K. W., and O’Connor, M. B. (1997) *Cell* **91**, 417–426

15. Mullins, M. C. (1999) *Dev. Biol.* **213**, 283–300

16. Uzel, M. I., Shih, S. D., Gross, H., Kessler, E., Gerstenfeld, L. C., and Trackman, P. C. (2000) *J. Bone Miner. Res.* **15**, 1189–1197

17. Kenyon, K., Modi, W. S., Contente, S., and Friedman, R. M. (1993) *J. Biol. Chem.* **268**, 18435–18437

18. Vytasek, R. (1982) *Anal. Biochem.* **120**, 243–248

19. Cronshaw, A. D., MacBeath, J. R., Shackleton, D. R., Collins, J. F., Pothergill-Gilmores, L. A., and Hulmes, D. J. (1993) *Matrix* **13**, 255–266

20. Almassian, B., Trackman, P. C., Iguchi, H., Boak, A., Calvaresi, D., and Kagan, H. M. (1991) *Connect. Tissue Res.* **25**, 197–208

21. Clark, T. G., Conway, S. J., Scott, I. C., Labosky, P. A., Winnier, G., Bundy, J., Hogan, B. L., and Greenspan, D. S. (1999) *Development* **126**, 2631–2642

22. Gerecke, D. R., Keene, D. R., Hudson, D. L., Nishiyama, T., Lee, S., Greenspan, D. S., and Burgeson, R. E. (2000) *J. Biol. Chem.* **275**, 22728–22735

23. Nagase, H., and Woessner, J. F., Jr. (1999) *J. Biol. Chem.* **274**, 21491–21494

24. Cronshaw, A. D., MacBeath, J. R., Shackleton, D. R., Collins, J. F., Pothergill-Gilmores, L. A., and Hulmes, D. J. (1993) *Matrix* **13**, 255–266

25. Liu, G., Nellaiappan, K., and Kagan, H. M. (1997) *J. Biol. Chem.* **272**, 32570–32577
Multiple Bone Morphogenetic Protein 1-related Mammalian Metalloproteinases Process Pro-lysyl Oxidase at the Correct Physiological Site and Control Lysyl Oxidase Activation in Mouse Embryo Fibroblast Cultures

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