Bone Morphogenetic Protein-1/Tolloid-like Proteinases Process Dentin Matrix

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SUMMARY

Bone Morphogenetic Protein-1 (BMP-1)/Tolloid-like metalloproteinases play key roles in formation of mammalian extracellular matrix (ECM), through the biosynthetic conversion of precursor proteins into their mature functional forms. These proteinases likely play a further role in formation of bone through activation of transforming growth factor β-like BMP’s. Dentin matrix protein-1 (DMP1), deposited into the ECM during assembly and involved in initiating mineralization of bones and teeth, is thought to undergo proteolysis in vivo to generate functional cleavage fragments found in extracts of mineralized tissues. Here, we have generated recombinant DMP1 and demonstrate that it is cleaved, to varying extents, by all four mammalian BMP-1/Tolloid-like proteinases, to generate fragments similar in size to those previously isolated from bone. Consistent with possible roles for the BMP-1/Tolloid-like proteinases in the physiological processing of DMP1, NH₂-terminal sequences of products generated by BMP-1 cleavage of DMP1 match those predicted from processing at the predicted DMP1 site that shows greatest cross-species conservation of sequences. Moreover, fibroblasts derived from mouse embryos homozygous null for genes encoding three of the four mammalian BMP-1/Tolloid-like proteinases appear to be deficient in processing of DMP1. Thus, a further role for BMP-1-Tolloid-like proteinases in formation of mineralized tissues is indicated, via proteolytic processing of DMP1.
Bone morphogenetic protein-1 (BMP-1)\(^1\) is the prototype of a family of metalloproteinases involved in morphogenesis in a broad range of species (1). These proteinases mediate morphogenetic effects in part by biosynthetic processing of precursors into the mature functional forms of proteins necessary to formation of the extracellular matrix. For example, they provide the procollagen C-proteinase activity that excises the C-propeptides of procollagens I-III, to yield the major fibril-forming components of the extracellular matrix (ECM) (2-6). They also participate in the biosynthetic processing of the minor fibrillar collagens V and XI (6-8), which in turn further regulate the physical properties of types I and II collagen fibers (9,10). The BMP-1/Tolloid-like proteinases have also been shown to process a precursor to produce the small leucine-rich proteoglycan biglycan (11), a molecule which positively regulates bone growth, influences type I collagen fibril morphology, and which also may influence dentin mineralization (12-14). The BMP-1/Tolloid-like proteinases have also been implicated in the biosynthetic processing of laminin 5 (15,16), type VII collagen (17) and shown to proteolytically activate lysyl oxidase (18), an enzyme required for covalent crosslinking of collagen and elastin fibers. These metalloproteinases may thus be central regulators in the formation of ECM.

Type I collagen is the major organic component of mineralized ECM, and serves as the template upon which mineral is deposited in tissues such as bone and dentin. Noncollagenous proteins of the small integrin binding ligand N-linked glycoprotein (SIBLING) family, which includes dentin sialophosphoprotein (DSPP) and dentin matrix protein-1 (DMP1), are secreted into the ECM during assembly and mineralization of these tissues, and are thought to initiate mineralization through their acidic calcium...
binding domains (19,20). In fact, genetic studies of human defects in biomineralization and analysis of transgenic mice have identified DMP1 and DSPP as important mediators of mineralization in bone and dentin (21-23), although the mechanisms of action of these proteins in vivo remain to be clearly defined. The carboxy-terminal domain of DSPP, designated the dentin phosphoprotein (DPP), and the carboxy-terminal domain of and DMP1, have both been shown to stimulate hydroxyapatite crystal formation in vitro (24,25). Thus, it has been proposed that in vivo proteolytic processing of full-length DSPP and DMP1 occurs, to generate functional carboxy-terminal cleavage fragments that can be found in the extracts of mineralized tissue (26,27). While the proteinase(s) responsible for the specific cleavage of these proteins have not been identified, several predicted proteolytic sites have been mapped based on sequencing of DSPP and DMP1 fragments extracted from dentin and bone, respectively. The amino acid sequence at the amino terminus of DPP from rat dentin was identified as Asp-Asp-Pro, suggesting that the major site of DSPP processing occurs between Gly^{447} and Asp^{448} (26). Based on analysis of DMP1-derived cleavage products extracted from rat bone, several processing sites were predicted. However, only one of these sites, between Ser^{196} and Asp^{197}, occurs within sequences strictly conserved in DMP1 across species (27). Although sequence identity between DSPP and DMP1 is low, the conservation of certain residues surrounding the two cleavage sites described above, within these two proteins, suggests the same protease(s) may process DSPP and DMP1. Moreover, these sequences exhibit similarities to cleavage sites within a number of the physiological substrates of the BMP-1/Tolloid-like proteinases (11), suggesting DSPP and DMP1 as candidate substrates for BMP-1/Tolloid-like proteinases.
In this study, we have generated recombinant DMP1 and tested it as a possible substrate for BMP-1/Tolloid-like proteinases. We demonstrate that BMP-1 cleaves mouse DMP1 to generate fragments similar to those previously isolated from mature rat bone. Among the BMP-1/Tolloid-like proteinases, BMP-1, mammalian Tolloid (mTLD), and mammalian Tolloid-like 1 (mTLL-1) all have similar activities in generating DMP1 fragments of the expected sizes, while mammalian Tolloid-like 2 (mTLL-2) has lesser activity in generating these fragments. Consistent with possible roles for the BMP-1/Tolloid-like proteinases in the physiological processing of DMP1, the NH\textsubscript{2}-terminal sequences of the cleavage products generated by BMP-1 matched those predicted from processing at the most well conserved site within DMP1. Moreover, using fibroblasts derived from Bmp1/Tll1 double null mouse embryos, we demonstrate that these proteinases are likely to contribute to DMP1 proteolysis \textit{in vivo}. We provide evolutionary evidence that sequences adjacent to this particular processing site may be critical for maintenance of a functional DMP1 gene, and discuss implications of the BMP1/Tolloid-like proteinases as functional mediators of biomineralization of bone and dentin ECM \textit{in vivo}.
EXPERIMENTAL PROCEDURES

Construction of DMP1 Expression Constructs - Mouse DMP1 coding sequences were amplified from mouse E15 whole embryo Marathon Ready cDNA (Clontech) by PCR using forward primer 5'-AGTCACTAGTC

TACCCATACGATGTTCCAGATTACGCTCTCCCAGTTGCCAGATACCACAAT-3’ and reverse primer 5’-

GCATGCGGCCGCTACTTGTCGTCATCGTCCTTGTAGTCGTAGCCGTCCTGACA

GTCATTGTC-3’, containing sequences 172-195 and 1609-1632, respectively, of the reported sequence (GenBank accession number U65020). The forward and reverse primers also contained SpeI and NotI restriction sites, respectively, to facilitate cloning, and sequences encoding NH₂-terminal hemaglutinin (HA) and COOH-terminal Flag epitopes, to facilitate detection of DMP1-derived proteolytic fragments. The PCR employed Advantage cDNA polymerase (Clontech) and denaturation at 94°C for 30 s, followed by 40 cycles of 94°C for 10 s, 55°C for 30 s, and 72°C for 3 min 30 s, and final extension at 72°C for 10 min. After digestion with SpeI and NotI, the 1.6 kb PCR product, which contained sequences encoding full-length DMP1 minus the signal peptide, was inserted, together with an AflII-NheI fragment encoding the BM40 signal peptide, between the the AflII and NotI sites of the expression vector pcDNA4TO (Invitrogen). In the resulting expression vector, DMP1 sequences are downstream of, and in frame with, the BM40 sequences, which were included to optimize secretion. Fidelity of the DMP1 expression vector was verified by DNA sequencing of the PCR insert and cloning junctions.
Expression of Recombinant DMP1-293T-Rex cells (Invitrogen) were maintained in growth medium consisting of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 1 mM L-glutamine, 10 IU/ml penicillin/streptomycin, 5 µg/ml blasticidin, and 10% fetal bovine serum (FBS) (HyClone). Cells at 80% confluence were transfected with 10 µg of the DMP1 expression vector per 100-mm tissue culture dish, using LipofectAMINE according to the manufacturer’s instructions (Invitrogen). For transient expression studies, cells were processed for protein production 24 h post-transfection as described below. For establishment of stable lines, cells were subcultured 1:4 at 48 h after transfection and selected in growth medium containing 200 µg/ml zeocin (Invitrogen). Cells were maintained under selection until colonies were visible (approximately 10 days), individual clones were isolated with cloning cylinders and amplified under selection in complete growth medium containing 200 µg/ml zeocin, and individual clones were processed for protein production as described below.

Protein Production - Growth medium was removed from cell cultures, cell layers were washed twice with phosphate buffered saline (PBS), and cells were incubated 15 min in serum-free DMEM at 37°C. Cell layers were washed with serum-free DMEM, and replaced with serum-free DMEM containing 1 µg/ml tetracycline and 40 µg/ml soybean trypsin inhibitor (SBTI) (Sigma). After 24 h, conditioned medium was harvested, cell debris was removed by centrifugation, and protease inhibitors were added to final concentrations of 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM N-ethylmaleimide (NEM), 1 mM p-aminobenzoic acid (PABA), 10 µg/ml leupeptin, and 5 mM EDTA (Sigma). Samples were stored at –80°C until further use.
**Immunoprecipitation** - To determine if the expressed DMP1 was intact, 1 ml conditioned medium from the DMP1 expression vector-transfected 293 T-Rex cells was incubated with 20 µl of FLAG BioM2 matrix (Sigma) for 2 h at 4°C. The matrix was pelleted by centrifugation, washed 3 times with buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl), and proteins were eluted with 50 µl buffer A containing 1 mg/ml Flag peptide (Sigma). SDS-PAGE 10x sample buffer containing 2-mercaptoethanol was added to eluted proteins, and the samples were boiled prior to SDS-PAGE.

**Western Blot Analysis** - Samples were subjected to reducing SDS-PAGE on 4-15% acrylamide gradient resolving gels, and electrotransferred to either Immobilon-P PVDF (Millipore) (for anti-Flag, anti-HA, or anti-PCPE1 immunoblots) or Trans-Blot nitrocellulose (Bio-Rad) (for anti-DMP1 immunoblot) membranes. Immunoblots were performed as previously described using antibodies specific for PCPE1 (28), HA or Flag epitopes (4), or rabbit polyclonal antiserum specific for DMP1 (29). For detection of DMP1, membranes were blocked in phosphate buffered saline containing 0.1% Tween-20 (TPBS) and 5% nonfat dry milk for 4 h., followed by incubation with a 1:1000 dilution of primary antibody in TPBS containing 5% milk for 12 h. Blots were washed six times for 10 min in TPBS, and probed with a 1:30000 dilution of goat anti rabbit IgG/horseradish peroxidase conjugate (Bio-Rad) for 1 h in TPBS/5% milk. The blots were washed six times for 10 min each in TPBS, and immunoreactive proteins were revealed using Supersignal peroxidase substrate (Pierce).

**Purification of Recombinant DMP1** - Conditioned media from transfected cells were applied to a column of 2 ml of settled DEAE-cellulose (DE52, Whatman), washed with 50 mM Tris-HCl pH 7.5, and eluted with a gradient of 0-500 mM NaCl in 50 mM
Tris-HCl containing protease inhibitors (1 mM PMSF, 1 mM NEM, 1 mM PABA, 40 
µg/ml SBTI). Fractions were prepared for reducing SDS-PAGE, eluted samples were 
resolved by SDS-PAGE and proteins were visualized with Stains-All (Sigma). Fractions 
with the greatest enrichment of DMP1 protein were used both for in vitro cleavage assays 
and amino acid sequence analysis as described below.

In Vitro Enzyme Assays - Approximately 750 ng of recombinant DMP1 was 
incubated alone or in combination with 1 pmol recombinant BMP-1, mTLD, mTLL-1, or 
mTLL-2 containing C-terminal Flag epitopes (4) in a 50 µl total volume of 50 mM Tris-
HCl pH 7.5, 150 mM NaCl, 5 mM CaCl₂ containing protease inhibitors (0.5 mM PMSF, 
0.5 mM NEM, 0.5 mM PABA, 20 µg/mL SBTI) and incubated 20 h at 37°C. Reactions 
were stopped by the addition of 10x SDS-PAGE sample buffer containing 2-
mercaptoethanol, and boiling samples for 5 min. Samples were subjected to SDS-PAGE 
and stained with Stains-All as described above.

Amino Acid Sequence Analysis - Approximately 3 µg purified recombinant DMP1 
was incubated with 150 ng of BMP-1 at 37°C for 20 h, and the reaction was quenched 
and prepared for SDS-PAGE as above. Products were resolved by SDS-PAGE on a 10% 
resolving gel and electrotransferred to Sequi-Blot PVDF membrane (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 using a Perkin Elmer/ Applied Biosystems Division 
Procise494 HT Protein Sequencing System.

Mouse Embryo Fibroblasts (MEF’s) – MEF’s were isolated from 13.5-days 
postconception embryos as described (6). Cells were maintained in growth medium
consisting of DMEM, 1 mM L-glutamine, 10 IU/ml penicillin/streptomycin, and 10% FBS, and were immortalized by routine serial passage. To detect endogenous DMP1 protein in MEF’s, wild-type or Bmp1/Tll1 doubly null cells at 80% confluence were treated with 10 nM dexamethasone, 10 mM \( \beta \)-glycerophosphate, and 100 \( \mu \)g/ml ascorbic acid in growth medium containing DMEM, 1 mM L-glutamine, 10 IU/mL penicillin/streptomycin, and 10% FBS for a period of 12 days. Cell layers were washed twice with PBS, and incubated in serum-free DMEM for 15 min at 37°C. Media were replaced with serum-free DMEM containing 40 \( \mu \)g/ml SBTI, 10 nM dexamethasone, 10 mM \( \beta \)-glycerophosphate, and 100 \( \mu \)g/ml ascorbic acid. Media were harvested at 48 h into protease inhibitors, cell debris was removed by centrifugation, and media were concentrated by ethanol precipitation as previously described (6).
RESULTS

BMP-1/Tolloid-like Metalloproteinases as Candidate Enzymes for the Cleavage of Dentin Sialophosphoprotein and Dentin Matrix Protein-1 - Proteolytic processing of DSPP and DMP1 has been suggested to occur in bone and dentin, based on the isolation of fragments of these proteins from demineralized extracts of these tissues. However, alignment of DMP1 amino acid sequences from a number of mammalian species demonstrates strict cross-species conservation of sequences (Fig. 1A, also see Fig. 5) around only one of the four predicted (27) cleavage sites. In addition, although pairwise sequence comparison of mouse DMP1 and DSPP sequences yielded an overall identity of only ~20% (data not shown), the highest level of conservation between the two proteins (42%) is found between 38 amino acid residues surrounding the conserved DMP1 cleavage site and a stretch of 38 residues surrounding the DSPP cleavage site which has the highest cross-species conservation of sequences when DSPP molecules are compared (Fig. 1A). This conservation of sequences suggested that these sites in particular might be of functional importance, and thus represent true sites of in vivo proteolytic processing, rather than artifactual cleavage which might occur during extraction from tissues.

The amino acid sequences surrounding the conserved predicted cleavage sites of DMP1 and DSPP exhibit similarities to the amino acid sequences surrounding cleavage sites used by the BMP-1/Tolloid-like proteinases in their known substrates, including procollagens I-III, V and VII; the proteoglycan probiglycan, prolysyl oxidase, laminin 5, and Chordin (Fig 1B). In particular, the presence of aspartate residues at P1’ positions,
and of residues with aromatic side chains or methionines at P2-P4 positions, are the most common features of cleavage sites in substrates of the BMP-1/Tolloid-like proteinases. These similarities suggested that BMP-1 or related proteinases might have the requisite specificity to process DSPP and DMP1 at the conserved predicted sites.

**Expression and Characterization of Recombinant Dentin Matrix Protein-1-**

Previous studies which have reported the isolation of DSPP and DMP1 from tissues have yielded only fragments of these proteins, complicating the study of their proteolytic processing and identification of the responsible proteinase(s). In order to examine the ability of BMP-1/Tolloid-like proteinases to generate physiological forms of DMP1 from the full-length protein, an expression plasmid was generated for the production of full-length DMP1, differing from native murine DMP1 only by replacement of the native signal peptide with the BM40 signal peptide (for optimal secretion), and by addition of NH\textsubscript{2}- and COOH-terminal HA and Flag epitopes, respectively, to facilitate detection of the protein and proteolytic fragments. Transient transfection of 293 T-rex cells with the expression construct resulted in secretion of a ~85-90 kDa protein species that reacted with both Flag and HA-specific antibodies (Fig. 2A), whereas immunoblots failed to detect any reactive protein species in conditioned medium from cells transfected with the empty pcDNA4TO vector. The molecular weight predicted by the murine DMP1 amino acid sequence, minus signal peptide sequences, is 52,225. However, although recombinant DMP1 produced in *E. coli* was reported to be ~90 kDa (28), DMP1 from mammalian tissues is both glycosylated and heavily phosphorylated, and biochemical estimates of the apparent molecular mass of post-translationally modified DMP1 from tissues have ranged from 150 - 200 kDa (29,30). To further characterize the recombinant
DMP1 in the current study, DMP1 was immunoprecipitated from the conditioned media of transfected 293 T-rex cells using Flag-specific antibody, and then probed on immunoblots with HA-specific antibody. As can be seen (Fig. 2B), anti-Flag antibody precipitated ~85-90 kDa DMP1 that was recognized on immunoblots by HA-specific antibody. Thus, at least some of the ~85-90 kDa DMP1 protein is full-length, retaining both NH$_2$- and COOH-termini, and it is highly unlikely that any of the ~85-90 kDa forms represent a mix of DMP1 fragments of similar size, some of which retain NH$_2$-termini and some of which retain COOH-termini. As can be observed in Fig. 2B, the recombinant DMP1 sometimes migrated as a doublet, the nature of which is further characterized below.

Despite the persistence of the Flag epitope, recombinant DMP1 bound poorly to anti-Flag affinity matrix (data not shown). However, as in previous studies which have employed ion exchange chromatography for the enrichment of dentin phosphoproteins (26,27), DEAE-cellulose allowed for the enrichment and concentration of recombinant DMP1, detectable by staining with Stains-All (Fig. 2C). As the major product of this enrichment corresponded to intact DMP1, this material was employed for subsequent enzyme cleavage assays.

**BMP-1/Tolloid-like Proteinases Process DMP1 at the Conserved Predicted Physiological Site** - To determine whether the mammalian BMP-1/Tolloid-like proteinases are capable of processing DMP1, purified recombinant DMP1 was incubated alone or with purified recombinant BMP-1. While DMP1 was stable and, in the absence of added proteinase, remained intact after prolonged incubation at 37°C, BMP-1 efficiently cleaved recombinant DMP1 to generate two species of approximately 53 kDa
and 30 kDa, as determined by 4-15% acrylamide gel SDS-PAGE and Stains-All staining (Fig. 3A). These sizes are similar to the previously observed 57 and 37 kDa forms, isolated from rat dentin, which correspond to the COOH- and NH2-terminal fragments of DMP1, respectively (27).

To determine which of the mammalian BMP-1/Tolloid-like proteinases might be capable of cleaving DMP1, cleavage assays were performed by separately incubating recombinant DMP1 with equimolar amounts of each of the four mammalian members of this family of metalloproteinases: BMP-1, mTLD, mTLL-1, and mTLL-2. Under the assay conditions used, all four enzymes were capable of generating DMP1 cleavage fragments of identical size, with no evidence of additional cleavages or non-specific degradation by any of the proteinases (Fig. 3B). The 10% acrylamide SDS-PAGE gel of Fig. 3B, shows a ~85-90 kDa doublet for full-length DMP1, cleavage of which produces a doublet of 53 and 51 kDa forms and a single species of 33 kDa. The four mammalian BMP-1/Tolloid-like proteinases have been previously observed to have differing efficiencies in cleaving various substrates (4,11,16-18). In the present study, mTLL-2 is seen to have lesser activity against DMP1 than do BMP-1, mTLD, or mTLL-1 (Fig. 3B).

To identify the site(s) at which BMP-1 cleaves the DMP1 precursor protein, a scaled up cleavage reaction was performed in order to generate sufficient DMP1-derived fragments to allow for NH2-terminal sequencing of the 53 and 51 kDa forms. Edman degradation of the 53 kDa and 51 kDa products yielded the amino acid sequences DDPESTRSDR and DDPESTRSD, respectively (Fig. 3C), both corresponding to the same NH2-terminus, resulting from cleavage between Ser212 and Asp213 of DMP1. As noted above, the latter is one of four possible in vivo DMP1 processing sites, predicted by
analysis of DMP1 cleavage products extracted from bone, and is the only one of the four sites located within DMP1 sequences strictly conserved across species (27).

Comparison of DMP1 Processing in Wild Type and Bmp1/Tll1 null MEF’s-

Comparison of cleavage patterns of proteins in wild type MEF cultures and in MEF’s derived from mouse embryos lacking both the Bmp1 gene, which produces alternatively spliced mRNAs that encode BMP-1 and mTLD, and the Tll1 gene, which encodes mTLL-1, has led to identification and verification of several in vivo substrates of the BMP-1/Tolloid-like proteinases (6,8,11,18). We were unable to detect endogenous full-length DMP1 or DMP1 fragments by immunoblot analysis of conditioned media from wild type or Bmp1/Tll1 doubly null MEF’s cultured under normal growth conditions (data not shown). However, as previous studies have demonstrated that expression of DMP1 is associated with mineralizing ECM in cell cultures (31), we employed conditions favoring ECM mineralization and osteoblastic differentiation of fibroblasts, and were able to detect a doublet centered at ~150 kDa in conditioned medium from Bmp1/Tll1 doubly null MEF’s that reacted strongly with DMP1-specific antibody (Fig 4). In contrast, conditioned media from wild type cells showed dramatically reduced or absent levels of DMP1 protein, although immunoblots of control proteins showed similar protein loading in the two lanes (Fig. 4), as did staining for total proteins (not shown). These observations suggest that one or more of the BMP-/Tolloid-like proteinases contribute to proteolytic processing of DMP1 in vivo, or at least in cultured MEF’s producing mineralized ECM.
DISCUSSION

Previous studies of the BMP-1/Tolloid-like proteinases have shown them to play integral roles in formation of the vertebrate ECM via the biosynthetic processing necessary to produce the mature functional forms of collagens I-III, V, VII, and XI; lysyl oxidase; biglycan; and laminin 5. Additionally, proteinases of the BMP-1/Tolloid family have been shown to cleave Chordin, an antagonist of the TGF-β-like BMP’s. Here, we expand the known range of activities of these enzymes by demonstrating that BMP-1-like proteinases cleave DMP1 at a single site that appears to correspond to a site employed in the physiological cleavage of DMP1 in vivo.

DMP1 sequences across species are quite divergent, and comparisons among a variety of species suggest that the DMP1 gene is one of the most rapidly evolving genes examined to date (32-34). However, one of the most conserved regions in the DMP1 protein is localized at the cleavage site demonstrated in the current study to be utilized by the BMP-1/Tolloid-like proteinases (Fig. 5, asterisk). In fact, of the four sites in the DMP1 amino acid sequence predicted to be in vivo cleavage sites, based on the NH$_2$-terminal sequences of DMP1 fragments extracted from bone (27), the site cleaved by BMP-1-like proteinases is the only one that occurs within sequences strictly conserved across species (Fig. 5). Thus, based on the above observations, it is tempting to speculate that necessary cleavage of the DMP1 protein by the BMP-1/Tolloid-like proteinases has exerted selective pressure on the evolution of DMP1. Closer inspection of residues adjacent to the scissile bond supports this idea, as residues at P3, P2, P1’, and P2’ positions are invariant in all species examined (Fig. 5, amino acids 210, 211, 213, and
214, respectively), while at the P1 position, eutherian mammals have an invariant serine residue (Fig. 1A). Even in evolutionarily more distant animals, which lack a P1 serine residue, such as birds, reptiles and marsupials (33) this residue is replaced by glycine, an amino acid found at the P1 site of scissile bonds in known BMP-1/Tolloid-like substrates such as prolyl oxidase, the pro-α1(III) procollagen chain, and the laminin 5 γ2 chain (Fig. 1A). Taken together, these data suggest that while much of the DMP1 sequence is highly mutable, selective pressure has maintained a high level of sequence conservation around the BMP-1 cleavage site within DMP1, underscoring the potential importance of DMP1 cleavage by the BMP-1/Tolloid-like proteinases.

In addition to the above observations, DMP1 processing is shown to be compromised in MEF cultures derived from Bmp1/Tll1 null embryos, offering further evidence that products of these two genes are likely to contribute to proteolytic processing of DMP1 in vivo. The BMP-1/Tolloid-like proteinases appear to play central roles in the formation of mineralized tissues, via biosynthetic processing of type I collagen, biglycan, and lysyl oxidase; and enhancement of BMP signaling, all of which are involved in bone growth and dentin formation. Perhaps these proteinases also affect mineralized tissues via the additional, novel role as mediators of processing of DMP1.

Although the functional significance of proteolytic processing of DMP1 and DSPP remains largely unknown, it has been speculated that the processing of these proteins to produce specific proteolytic products represents biosynthetic events (26,27). In vitro, we have shown that the BMP-1/Tolloid-like proteinases are capable of processing DMP1 into discrete cleavage fragments analogous to those extracted from demineralized bone. In contrast, although processing of DMP1 appeared to differ in
cultures of wild type and Bmp1/Tll1 doubly null MEF’s, with detection of high molecular weight DMP1 in Bmp1/Tll1 doubly null MEF media that was essentially absent in wild type MEF media, we were unable to detect discrete cleavage fragments in either wild type or doubly null media samples. Perhaps DMP1 cleavage products are stabilized in vivo by incorporation into insoluble matrix, while in MEF cultures, where neither high molecular DMP1 nor DMP1 cleavage products were detectable in cell layer ECM (data not shown), such cleavage products are susceptible to further, nonspecific proteolysis. It is also possible that such fragments are stabilized in vivo by association with other molecules not available in MEF cultures.

This study describes for the first time the expression of recombinant DMP1 via eukaryotic cells. Previous studies examining DMP1 have reported variable molecular weights for the DMP1 protein depending on the source, ranging from 90 kDa for E. coli-expressed recombinant rat DMP1 (29) to >150 kDa for endogenous DMP1 isolated from rat dentin (30). We suggest that the 293 T-rex cells used in this study do not have the appropriate cellular machinery to synthesize DMP1 with the extensive posttranslational modifications predicted for DMP1 (27,35), since the migration of the ~85-90 kDa recombinant protein used here is similar to that seen for E.coli expressed DMP1 (25). In contrast, the doublet of DMP1 centered at ~150 kDa found in the media of Bmp1/Tll1 doubly null MEF’s suggests that these cells are capable of extensive posttranslational modification of DMP1, similar to that which occurs in vivo. It should be noted that although the molecular weight predicted by the murine DMP1 amino acid sequence is 52,225, it is not surprising that a mobility suggestive of a ~90 kDa protein was observed by SDS-PAGE for versions of DMP1 with minimal post-translation modification, since
the intrinsic high charge density of similar proteins have previously been shown to interfere with assumption of true random-chain conformation (36).

Bone and dentin contain mineralized matrices whose main organic components are type I collagen and non-collagenous proteins of the SIBLING family such as DMP1 and DSPP. This study demonstrates proteolytic processing of DMP1 by the BMP-1/Tolloid-like proteinases \textit{in vitro} and provides evidence for this activity \textit{in vivo}. Previous biochemical studies have shown that the COOH-terminal domains of both DMP1 and DSPP can nucleate hydroxyapatite crystals \textit{in vitro} and initiate calcium mineralization (24,25), and it is possible that excision of such COOH-domains may contribute to the regulation of mineralization \textit{in vivo}. DSPP is cleaved at a physiological site similar to that observed for DMP1 (Fig. 1B), thus it may also be processed by BMP-1/Tolloid-like proteinases. Consistent with the possibility that BMP-1/Tolloid-like proteinases process DMP1 and DSPP \textit{in vivo} is evidence that these three types of protein are similarly localized within tissues. BMP-1 sequences were originally isolated from demineralized bone extracts (37), while, although originally characterized in dentin, proteolytic fragments of DMP1 and DSPP have been recovered from demineralized bone extracts as well (27,38). Additionally, BMP-1/Tolloid-like proteinases (28) and the DSPP fragment DPP (39,40) have both been found to associate with collagen fibrils, while \textit{in situ} hybridization analyses have shown relatively high levels of expression of both BMP-1/Tolloid-like proteinases and DMP1 in ossification centers during the development of the mineralized skeleton (4,11,31,41). These observations suggest that the BMP-1/Tolloid-like proteinases are correctly situated to mediate DMP1 and DSPP processing \textit{in vivo}. 

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It has been suggested (27) that the cell membrane protease PHEX might contribute to processing of DMP1, as PHEX is expressed at high levels in bones and teeth and has a demonstrated preference for cleavage sites with aspartates in the P1’ position (42,43). Although we do not exclude the possibility that PHEX is involved in DMP1 processing, it should be noted that PHEX is thought likely to cleave oligopeptide, rather than protein substrates (43,44), and that PHEX does not cleave the matrix extracellular phosphoglycoprotein MEPE (44), which has been noted to have homology to DMP1 (43).

Induction of bone and dentin formation are mediated in part by the actions of the TGF-β-like BMP’s (45,46), which are themselves activated by BMP-1/Tolloid-like proteinases through inactivation of at least one known BMP antagonist, Chordin (4,47,48). Additionally, it is generally accepted that a functional collagenous matrix is important for normal mineralization of bone and dentin, as is suggested by analysis of bone and dentin formation in oim mutant mice (49,50) and in the clinical presentation of bone and dentin defects in patients with osteogenesis imperfecta (51), a genetic disorder associated with type I collagen gene mutations. Although further study is required, it is tempting to speculate that the BMP-1/Tolloid-like proteinases, as physiological processing enzymes for both structural and regulatory components of ECM, may coordinate BMP-mediated growth factor signaling with ECM biosynthesis and with mineralization through its diverse activities against a variety of biological substrates. In this capacity, the BMP-1/Tolloid-like proteinases could be of critical importance in the correct assembly of specialized hard tissues such as bone and dentin.
REFERENCES


The abbreviations used are: BMP, bone morphogenetic protein; C-propeptide, carboxy-terminal propeptide; ECM, extracellular matrix; SIBLING, small integrin-binding ligand N-linked glycoprotein; DSPP, dentin sialophosphoprotein; DMP1, dentin matrix protein-1; DPP, dentin phosphoprotein; mTLD, mammalian Tolloid; mTLL, mammalian Tolloid-like; E15, embryonic day 15; PCR, polymerase chain reaction; HA, hemaglutinin; BM40, osteonectin/SPARC/BM40; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PBS, phosphate buffered saline; SBTI, soybean trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride, NEM, N-ethylmaleimide, PABA, p-aminobenzoic acid, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel
electrophoresis; PVDF, polyvinylidene difluoride; PCPE, procollagen C-proteinase enhancer protein; TPBS, PBS containing 0.1% Tween-20; DEAE, diethylaminoethyl; MEF, mouse embryo fibroblast; P1’, P2, P4 cleavage site residues amino-terminal to (nonprimed) and carboxy-terminal to (primed) the cleaved bond; oim, osteogenesis imperfecta murine.
Fig. 1. **BMP-1 is a candidate enzyme for proteolytic cleavage of DMP1 and DSPP.**

A, alignment DMP1 and DSPP sequences from several mammalian species showing primary sequence identity around the putative BMP-1 cleavage site. A dash (-) indicates the predicted sites of proteolytic processing. Amino acids identical to those in the mouse proteins are indicated by asterisks (*), and residues conserved between mouse DSPP and DMP1 are indicated with vertical lines. B, alignment of mouse DMP1 and DSPP cleavage sites with proteolytic processing sites identified in known substrates of the BMP-1/Tolloid-like proteinases. Sites of proteolysis are indicated with a dash (-), and conserved aspartate, methionine, and residues with aromatic side chains are shown in boldface.

Fig. 2. **Characterization and purification of recombinant DMP1 produced by transfected 293 T-rex cells.** A, western blot analysis using antibodies specific to Flag or HA epitopes to analyze the proteins secreted into the medium of 293 T-rex cells transiently transfected with DMP1 expression construct (+) or empty pcDNA4TO vector (-). B, western blot analysis using HA-specific antibodies on conditioned medium samples from empty vector (-) or DMP1-transfected (+) 293 T-Rex cells subjected to immunoprecipitation using Flag-specific antibodies. Positions of molecular weight markers are shown at right. C, Stains-All staining of fractions of recombinant DMP1 purified and concentrated by DEAE-cellulose chromatography. The NaCl gradient increases from left to right as indicated by the black triangle, and a vertical arrow indicates the fraction used for subsequent enzyme assays. The major band in this fraction was confirmed as DMP1 by Western blotting (data not shown) and by Edman
degradation/NH$_2$-terminal sequencing of cleavage products (see Fig. 3). Positions of molecular weight markers are shown at left, in kilodaltons. Start, starting material; IB, immunoblot; IP, immunoprecipitation.

Fig. 3. **Cleavage of recombinant DMP1 by BMP-1/Tolloid-like proteinases.** A, Recombinant DMP1 is shown at zero time (0 h) and after 20 h incubation at 37°C either alone (DMP1) or in the presence of recombinant human BMP-1 (DMP1 + BMP-1). Processing was monitored by the disappearance of the 85-90 kDa intact DMP1 and the appearance of intermediates at 53 kDa and 30 kDa, using Stains-All staining. B, processing of DMP1 incubated separately (DMP1) or together with (+) BMP-1, mTLD, mTLL-1, or mTLL-2, using Stains-All to resolve protein bands. Molecular weights of size markers are indicated in kilodaltons at left. C, schematic representation of recombinant DMP1 and processing intermediates generated by incubation with BMP-1 is shown as are relative positions of the intermediates on 10% SDS-PAGE and the designations given to them are indicated at right. An alignment of NH$_2$-terminal sequences of intermediates resulting from BMP-1 cleavage, as determined by Edman sequencing, with sequences surrounding the cleavage site predicted from the mouse DMP1 cDNA is also shown (27). The predicted physiological cleavage site in this region of DMP1 is indicated with a vertical arrow.

Fig. 4. **Processing of DMP1 in mineralizing cultures of MEF’s from wild type and Bmp1/Tll1 doubly null embryos.** Western blot analyses using antiserum specific for DMP1 were employed to detect DMP1 in conditioned medium from Bmp1/Tll1 doubly
null (-/-) or wild type (+/+ ) MEF’s (upper panel). To show similar levels of loaded proteins in each lane, identical samples were subjected to Western blot analysis using an antiserum specific for the unrelated PCPE1 protein (lower panel).

Fig. 5. Divergence of DMP1 amino acid sequences. From a previously reported alignment of DMP1 amino acid sequences from ten species (33), with additional alignment of the amino acid sequence of DMP1 from *N. leporinus* (34), a consensus DMP1 sequence was derived. The fraction of sequences differing from the derived consensus was plotted as a function of amino acid position within the mouse DMP1 protein, and is presented graphically. Vertical arrows indicate the DMP1 processing sites predicted from Ref. 27, and an asterisk (*) indicates the position of the BMP-1/Tolloid-like proteinase cleavage site identified in the current study.
A

Bov DMP1- HW********D************************--***GAY**E**N
Hum DMP1- HW******D*E********L*********---*****I**E**N
Rat DMP1- Y********E******************----***G********
Mus DMP1- QRVGGGSEGQSSHGDBGSEFDDEGMQS-DDPESTRSDRGH
        ||   ||   ||   ||||   ||   ||   ||   |
Mus DSPP- GHSRIGSSSSNSDGHSYEFDDDESMQG-DDPSSSDESNGS
Rat DSPP- ******************D********---***N********
Hum DSPP- ***NT**D*******Y***D***K******---***N********SN

B

Small integrin binding ligand N–linked glycoposphophoproteins
Mouse DMP1
Mouse DSPP
Known BMP-1/Tolloid–like substrates
pro α1(I)                  RYYRA–DDA
pro α2(I)                  DFYRA–DQP
pro α1(II)                 QYMRA–DQA
pro α1(III)                APYYG–DEP
pro α2(V)                  PEFTE–DQA
pro α1(VII)                PSYAA–DTA
Mouse laminin 5 γ2          DCYSG–DEN
Human prolysyl oxidase     DRMVG–DDP
Human probiglycan          DFMLE–DEE
Mouse chordin (N–terminal site) HRSYS–DRG
Mouse chordin (C–terminal site) DPMQA–DGP
Figure 2

A

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<th>mDMP1</th>
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B

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<tr>
<td>IB: α HA</td>
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C

Start → NaCl
Figure 4

IB: α DMP1

IB: α PCPE1

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Figure 5

Divergence of DMP1 sequences

Amino acid position (mDMP1)

Divergence from consensus sequence
Bone morphogenetic protein-1/tolloid-like proteinases process dentin matrix protein-1
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