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

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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 probably play a further role in formation of bone through activation of transforming growth factor β-like BMPs. 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) 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 carboxy-terminal 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 type 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 that positively regulates bone growth, influences type I collagen fibril morphology, and 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) and type VII collagen (17) and shown to proteolytically activate lysyl oxidase (18), an enzyme required for covalent cross-linking 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 family, which includes dentin sialophosphoprotein (DSP) 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 bimineralization and analysis of transgenic mice have identified DMP1 and DSP 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 carboxyl-terminal domain of DSP, designated the dentin phosphoprotein, and the carboxyl-terminal domain of 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 DSP and DMP1 occurs, to generate functional carboxyl-terminal cleavage fragments that can be found in the extracts of mineralized tissue (26, 27). Whereas the protease(s) responsible for the specific cleavage of these proteins has not been identified, several predicted proteolytic sites have been mapped based on sequencing of DSP and DMP1 fragments extracted from dentin and bone, respectively. The amino acid sequence at the amino terminus of dentin phosphoprotein from rat dentin was identified as Asp-Asp-Pro, suggesting that the major site of DSP processing occurs between Gly⁴⁴⁷ and Asp⁴⁴⁸ (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 Ser¹⁹⁶ and Asp¹⁹⁷, occurs within sequences strictly conserved in DMP1 across species.
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(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 that 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 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 NH2-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 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/Tll1 proteinases as functional mediators of biomineralization of bone and dentin ECM in vivo.

EXPERIMENTAL PROCEDURES

Construction of DMP1 Expression Constructs—Mouse DMP1 coding sequences were amplified from mouse embryonic day 15 whole embryo Marathon Ready cDNA (Clontech) by PCR using forward primer 5’-AGTCTCATGACTACCATACCATATATGTGATTTGTCTCCTGGCCAGATTACAACAT-3’ and reverse primer 5’-GCATGCGGCCGCTCACTTGTGCATGCTGTATGTGGTTAAGGCATATGGC-3’, containing sequences 172–195 and 1609–1632, respectively, of the reported sequence (GenBank™ accession number U650290). The forward and reverse primers included SpeI and NotI restriction sites, respectively, to facilitate cloning and sequences encoding NH2-terminal hemagglutinin (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 AhfNheI fragment encoding the osteonectin/SPARC/BM40 signal peptide, between the Ahf and NotI sites of the expression vector pcDNA4TO (Invitrogen). In the resulting expression vector, DMP1 sequences are downstream of and in frame with the osteonectin/SPARC/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.

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 AhfNheI fragment encoding the osteonectin/SPARC/BM40 signal peptide, between the Ahf and NotI sites of the expression vector pcDNA4TO (Invitrogen). In the resulting expression vector, DMP1 sequences are downstream of and in frame with the osteonectin/SPARC/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.

Purification of Recombinant DMP1—Conditioned medium from transiently transfected 293 T-REx cells (Invitrogen) containing sequences 172–195 of DMP1 cloned into an expression vector (pcDNA4TO) was harvested, cell debris was removed by centrifugation, and the samples were boiled prior to SDS-PAGE. 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 of recombinant BMP-1, mTLD, mTLL-2, or mTLD-like 1 (mTLD-L1) (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 PerkinElmer Life Sciences/Applied Biosystems Division Procise494 H-IT Protein Sequencing System.

Western Blot Analysis—Samples were subjected to reducing SDS-PAGE on 4–15% acrylamide gradient resolving gels and electrotransferred to either Immobilon-P polyvinylidene difluoride (Millipore Corp.) (for anti-FLAG, anti-HA, or anti-procollagen C-proteinase enhancer protein 1 immunoblot) or Trans-Blot nitrocellulose (Bio-Rad) (for anti-DMP1 immunoblot) membranes. Immunoblots were performed as previously described using antibodies specific for proteinase C-proteinase enhancer protein 1 (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 (TBPS) and 5% nonfat dry milk for 4 h, followed by incubation with a 1:1000 dilution of primary antibody in TBPS containing 5% milk for 12 h. Blots were washed six times for 10 min in TBPS and probed with a 1:30,000 dilution of goat-anti-rabbit IgGhorseradish peroxidase conjugate (Bio-Rad) for 1 h in TBPS plus 5% milk. The blots were washed six times for 10 min each in TBPS, and immunoreactive proteins were revealed using SuperSignal peroxidase substrate (Pierce).

Amino Acid Sequence Analysis—Approximately 3 μg of 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 a nitrocellulose 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 PerkinElmer Life Sciences/Applied Biosystems Division Procise494 H-IT Protein Sequencing System.

Embryo Fibroblasts (MEFs)—MEFs were harvested from 13.5-day postconception embryos as described (6). Cells were maintained in growth medium consisting of DMEM, 1 mM t-glutamine, 10 IU/ml penicillin/streptomycin, and 10% fetal bovine serum and were immortalized by routine serial passage. To detect endogenous DMP1 protein in MEFs, wild type or Bmp1/Tll1 double null cells at 80% confluence were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10 mM N-ethylmaleimide, 1 mM p-amino benzoic acid, 10 μg/ml leupeptin, and 5 μM EDTA (Sigma). Samples were stored at −80 °C until further use.

Immunoprecipitation—To determine whether the expressed DMP1 was intact, 1 μl of conditioned medium from the DMP1 expression vector-transfected 293 T-REx cells was incubated with 20 μl of FLAG antibody (Sigma) for 2 h at 4 °C. The samples were boiled prior to SDS-PAGE and washed three times with buffer A (50 mM Tris-HCl, pH 7.5, 150 mM NaCl), and proteins were eluted with 50 μl of buffer A containing 1 mg/ml FLAG peptide (Sigma). SDS-PAGE 10% sample buffer containing 2-mercaptoethanol was added to eluted proteins, and the samples were boiled prior to SDS-PAGE.
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 type.

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 that 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 profibiglycan; prolyl 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 methionine 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 that 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 osteonectin/SPARC/BM40 signal peptide (for optimal secretion), and by the addition of NH2- and COOH-terminal HA (for optimal secretion), and by the addition of NH2- and COOH-terminal HA (for optimal secretion), and by the addition of NH2- and COOH-terminal HA (for optimal secretion), and by the addition of NH2- and COOH-terminal HA (for optimal secretion).

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Fig. 2. Expression and Characterization of Recombinant Dentin Matrix Protein-1. A, molecular weight of recombinant DMP1 produced in *Escherichia 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 to 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,
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Fig. 2. Characterization and purification of recombinant DMP1 produced by transfected 293 T-REx cells. A, Western blot (IB) 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 (IP) using FLAG-specific antibodies. Positions of molecular mass markers are shown at the 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/NH2-terminal sequencing of cleavage products (see Fig. 3). Positions of molecular mass markers are shown on the left, in kilodaltons. Start, starting material; IB, immunoblot; IP, immunoprecipitation.

at least some of the ~85–90-kDa DMP1 protein is full-length, retaining both NH2 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 NH2 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 that 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 stain (Fig. 2C). Since 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. Whereas 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 ~53 and 30 kDa, as determined by 4–15% acrylamide gels 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 nonspecific degradation by any of the proteinases (Fig. 3B). The 10% acrylamide SDS-PAGE gel of Fig. 3B shows an ~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 does 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- 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 MEFS—Comparison of cleavage patterns of proteins in wild type MEF cultures and in MEFS 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 MEFS cultured under normal growth conditions (data not shown). However, since 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 MEFS 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-1/Tolloid-like proteinases contribute to proteolytic processing of DMP1 in vivo or at least in cultured MEFS 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 transforming growth factor-β-like BMPs. 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 NH2-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, since residues at P3, P2, P1′, and P2′ positions are invariant in all species examined (Fig. 5, amino acids 210, 211, 213, and 214, respectively), whereas 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 scissors 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 whereas 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 MEFs, 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, whereas in MEF cultures, where neither high molecular weight DMP1 nor DMP1 cleavage products were detectable in cell layer ECM (data not shown), such cleavage products are susceptible to further, non-specific 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 MEFs suggests that these cells are capable of extensive posttranslational modification of DMP1, similar to that which occurs \textit{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 an \(90\)-kDa protein was observed by SDS-PAGE for versions of DMP1 with minimal post-translation modification, since the intrinsic high charge densities of similar proteins have previously been shown to interfere with the assumption of true random chain conformation (36).

Bone and dentin contain mineralized matrices whose main organic components are type I collagen and noncollagenous proteins of the small integrin-binding ligand \(N\)-linked glycoprotein 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), whereas, 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 (26) and the DSPP fragment dentin phosphoprotein (39, 40) have both been found to associate with collagen fibrils, whereas \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}.

It has been suggested (27) that the cell membrane protease PHEx might contribute to processing of DMP1, since 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 transforming growth factor-\(\beta\)-like BMPs (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 \textit{oim} (osteogenesis imperfecta murine) 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

**Fig. 5. Divergence of DMP1 amino acid sequences.** From a previously reported alignment of DMP1 amino acid sequences from 10 species (33), with additional alignment of the amino acid sequence of DMP1 from \textit{Noctilio 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 murine DMP1 protein and is presented graphically. The \textit{vertical arrows} indicate the DMP1 processing sites predicted from Ref. 27, and an \textit{asterisk} indicates the position of the BMP-1/Tolloid-like protease cleavage site identified in the current study.
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 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.