Studies of the DMP1 57-kDa Functional Domain both in vivo and in vitro

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Key Words

DMP1 • PHEX • Furin-like proprotein convertase • Transgenic mice

Abstract

Dmp1-null mice and patients with mutations in dentin matrix protein 1 (DMP1) resulting in autosomal recessive hypophosphatemic rickets display similar skeletal defects. As mutations were observed in the last 18 amino acids of DMP1 in 1 subset of patients and as fragments of intact DMP1, a 37-kDa N-terminal and a 57-kDa C-terminal fragment, have been purified from bone and dentin, we hypothesized that the cleaved 57-kDa C-terminal fragment is the essential functional domain of DMP1. To test this hypothesis, different forms of recombinant DMP1 were expressed in 293EBNA, CHO and 2T3 cells. The results showed that DMP1 was processed into a 37-kDa N-terminal and a 57-kDa C-terminal fragment in vitro in all cell lines examined. DMP1 processing in CHO cells was blocked by a furin protease inhibitor, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone, in a dose-dependent manner. Coexpression of PHEX, a potential upstream protease, had no apparent effect on DMP1 cleavage in 293EBNA cells, suggesting that PHEX may not be required for DMP1 processing. To test the in vivo role of the C-terminal fragment, transgenic mice overexpressing full-length DMP1 or the 57-kDa fragment controlled by the 3.6-kb Col1 promoter were generated. Overexpression of these transgenes had no effect on the wild-type skeleton, but on the *Dmp1*-null background showed expression in the osteoblast layer

and throughout the bone matrix leading to the rescue of the null bone phenotype. This suggests that the 57-kDa C-terminal fragment may be able to recapitulate the function of intact DMP1 in vivo.

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Introduction

Dentin matrix protein 1 (DMP1), an acidic phosphorylated extracellular matrix protein, was mapped to mouse chromosome 5q21 [George et al., 1994] and human chromosome 4q21:22 [MacDougall et al., 1996]. It is a member of the small integrin-binding ligand, N-linked glycoprotein family that shares similar features in biochemical properties and genomic organization [Fisher et al., 2001]. The *Dmp1* gene is highly expressed in odontoblasts in tooth and osteocytes in bone, with lower expression in osteoblasts and cartilage [George et al., 1993, 1995; Hirst

Abbreviations used in this paper

BMP1	bone morphogenetic protein 1
CHO cells	Chinese hamster ovary cells
DEC	decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone
DMP1	dentin matrix protein 1
FGF23	fibroblast growth factor 23
PHEX	phosphate-regulating gene with homologies to endopeptidase on the X chromosome

et al., 1997; MacDougall et al., 1998; Toyosawa et al., 2001; Feng et al., 2002].

Although full-length DMP1 cDNA has been cloned and sequenced in several mammalian and nonmammalian species [George et al., 1993; Hirst et al., 1997; MacDougall et al., 1998], the corresponding intact naturally occurring protein has not been isolated from mineralized tissues. However, 2 proteolytic fragments, a 37-kDa N-terminal fragment and a 57-kDa C-terminal fragment, have been purified from rat long bone and dentin extracts, suggesting that these may be the bioactive forms in vivo [Qin et al., 2003]. Phosphate analysis has indicated that the 57-kDa fragment is more highly phosphorylated than the 37-kDa fragment [Qin et al., 2003]. Boskey and her colleagues, using an in vitro cell-free system, showed that the highly phosphorylated 57-kDa fragment purified from rat bone acts as a hydroxyapatite nucleator [Tartaix et al., 2004]. In addition, the 57-kDa fragment contains almost all functional sequences and domains identified to date, including the RGD motif believed to be responsible for mediating cell attachment [Kulkarni et al., 2000], the nuclear localization signal [Narayanan et al., 2003], the ASARM peptide [Rowe, 2004] and the peptide functioning as nucleator [Gajjeraman et al., 2007]. More importantly, it has been discovered that loss of the last 18 amino acid residues of DMP1 results in autosomal recessive hypophosphatemic rickets in humans [Feng et al., 2006]. These observations point to the 57-kDa fragment as the functional domain of DMP1, supporting the hypothesis that full-length DMP1 may be an inactive precursor and that proteolytic processing is an activation step, converting the precursor into functional fragments with distinct yet critical roles in the mineralization of bone and dentin [Qin et al., 2004].

In vivo studies performed in our laboratory suggested that DMP1 plays an essential role in maintaining phosphate homeostasis and in the biomineralization of both teeth and bone. We previously generated *Dmp1*-null mice, in which exon 6 was replaced by the β -galactosidase gene [Feng et al., 2003]. These mice postnatally develop characteristics similar to the Hyp mouse, a murine homologue of human X-linked hypophosphatemia, such as the skeletal abnormalities of rickets and osteomalacia as well as similar serum biochemical profiles of elevated circulating serum fibroblast growth factor 23 (FGF23) levels accompanied by hypophosphatemia [Ling et al., 2005; Ye et al., 2005; Feng et al., 2006]. The phenotype of the Hyp mice is caused by a spontaneous 3'-deletion of the phosphate-regulating gene with homologies to endopeptidase on the X chromosome (*PheX*) [Eicher et al., 1976; Beck et

al., 1997]. PHEX is a member of the M13 family of type II transmembrane zinc-containing metalloendopeptidases [Tenenhouse and Sabbagh, 2002; Quarles, 2003].

FGF23 is a potent phosphaturic hormone that is produced by bone and regulates phosphate homeostasis and vitamin D metabolism in the kidney [Liu et al., 2003; Bai et al., 2004; Larsson et al., 2004; Shimada et al., 2004a, b]. PHEX has long been thought to be the protease involved in the processing/degradation of FGF23. Evidence in support of the direct enzyme-substrate relationship between FGF23 and PHEX has been controversial [Bowe et al., 2001; Campos et al., 2003; Liu et al., 2003; Benet-Pages et al., 2004], yet accumulating evidence tends to support the hypothesis that PHEX most likely directly or indirectly regulates FGF23 expression in bone [Liu et al., 2003, 2006]. The inactivating mutation of *PheX* in Hyp mice could be responsible for increased FGF23 expression in bone responsible for elevated circulating FGF23 levels [Liu et al., 2003]. Both PHEX and DMP1 are mainly and highly expressed in osteocytes [Miao et al., 2001; Toyosawa et al., 2001; Feng et al., 2002, 2003]. This colocalization of DMP1 and PHEX in bone suggests that PHEX may be the proteinase involved in the processing of DMP1. Therefore, it is tempting to propose that PHEX mediates cleavage of DMP1 into its active form and that this active DMP1 fragment then controls phosphate metabolism by regulating FGF23 expression.

In this study, we show that DMP1 is processed into 37-kDa N-terminal and 57-kDa C-terminal fragments in vitro. Although PHEX has been thought to be the protease responsible for DMP1 cleavage, the coexpression of PHEX in 293EBNA cells had no apparent effect on DMP1 cleavage. Rather, it was found that the processing of DMP1 in Chinese hamster ovary (CHO) cells was blocked by the protease inhibitor decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (DEC) in a dose-dependent manner, suggesting that a furin-like proprotein convertase is involved in DMP1 cleavage to generate the active 57-kDa C-terminal fragment. These theories are also being tested in vivo by targeted expression of either full-length DMP1 or the 57-kDa C-terminal fragment in *Dmp1*-null mice. Preliminary observations suggest that the 57-kDa fragment is the primary functional domain of DMP1.

Materials and Methods

Plasmids

The coding region of the full-length DMP1 has been previously used to generate transgenic mice under control of a 3.6-kb type I collagen promoter [Lu et al., 2007], and was subcloned into

the *EcoRI* site of the pCDNA3 expression vector, giving rise to a pCDNA3-DMP1 expression construct. Based on the cleavage sites identified in rat DMP1, we designed 2 C-terminal 57-kDa fragments: the 2 fragments differ in length by 45 amino acid residues. For generation of the pCDNA3-57^L construct, expressing the long 57-kDa fragment, and the pCDNA3-57^S construct, expressing the short 57-kDa fragment, a DNA sequence, consisting of the gene segment encoding DMP1 signal peptide (16 amino acid residues) in frame with the gene segment encoding the N terminus of each fragment was cloned into the pCDNA3 expression vector, downstream of the CMV promoter. The long 57-kDa fragment was then subcloned into a mammalian expression vector [Lu et al., 2007] containing the 3.6-kb rat type I collagen promoter plus a 1.6-kb intron 1 at *EcoRV* and *SalI* sites, giving rise to the Col1a1-57K transgene. For generation of the pCDNA3-37K construct expressing the short 37-kDa N-terminal fragment, a DNA sequence encoding DMP1 signal peptide and the short 37-kDa fragment was cloned into the pCDNA3 expression vector, downstream of the CMV promoter. A DMP1 construct with the cleavage site mutated at D197A (numbered if the signal peptide was excluded) was subcloned into the *EcoRI* site of the pCDNA3 expression vector, giving rise to the pCDNA3-mDMP1 construct. The murine PHEX cDNA (kindly provided by Dr. Shiguang Liu at Kansas University Medical Center, Kansas City, Kans., USA) has been previously used to overexpress PHEX in osteoblasts in transgenic mice [Liu et al., 2002]. The PHEX cDNA was subcloned into the *EcoRV* and *XbaI* sites of pCDNA3, termed pCDNA3-PHEX. All DMP1 expression constructs were confirmed by a combination of restriction enzyme digestion and DNA sequencing (ABI model 377) at the biotechnology support facility at the Kansas University Medical Center, Kansas City, Kans., USA.

Cell Culture and Transfection

293EBNA cells, a human kidney cell line, and CHO cells, derived from Chinese hamster ovary, were obtained from the American Type Culture Collection. Both types of cells were cultured in Dulbecco's modified Eagles's medium (with 4.5 g/l D-glucose, L-glutamine and sodium pyruvate; Mediatech Inc., Herndon, Va., USA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, Calif., USA). The osteoblast cell line 2T3, derived from transgenic mice expressing SV40 large T antigen under the control of the osteocalcin promoter (a gift from Dr. Stephen E. Harris, University of Texas Health Science Center at San Antonio, San Antonio, Tex., USA), was maintained in minimal essential medium, α -modified Eagle's medium, supplemented with 10% fetal bovine serum, 100 IU/ml penicillin and 100 μ g/ml streptomycin. Transfection was done using Lipofectamine 2000 reagents (Invitrogen) according to the manufacturer's instructions. All cell cultures were incubated at 37°C in a humidified 5% CO₂ atmosphere.

Assessment of DMP1 Processing in Various Cell Lines in vitro

To determine the DMP1 processing in vitro in cell lines, various forms of recombinant DMP1, including the pCDNA3 expression constructs, encoding either full-length wild type, mutant DMP1, a 37-kDa N-terminal fragment, or a 57-kDa-long or -short form of the C-terminal fragment, were transiently transfected into 293EBNA, CHO or 2T3 cells. For assessment of DMP1 cleavage by PHEX, 293EBNA cells were transiently co-

transfected with 1.5 μ g of full-length DMP1 or 57-kDa C-terminal fragment expression constructs with either 1.5 μ g of pCDNA3 empty constructs or 1.5 μ g of native PHEX expression constructs. For DMP1 cleavage inhibition, CHO or 2T3 cells were transiently transfected with 3 μ g of pCDNA3 vector, pCDNA-mDMP1, pCDNA3-57K or pCDNA-DMP1 constructs. Twenty-four hours after transfection, the medium was replaced with serum-free medium with addition of 0, 5, 10, 20, 40 or 50 μ M of DEC (BIOMOL International, Plymouth Meeting, Pa., USA), a furin-like proprotein convertase inhibitor, for 48 h. The growth medium was replaced with serum-free medium 24 h after transfection and the transfected cells were further cultured for an additional 48 h. The conditioned medium was collected and centrifuged at 14,000 g for 15 min to remove cells and cellular debris. The cell layers were washed with ice-cold PBS, and lysed with radioimmunoprecipitation assay buffer, containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40 and 0.5% sodium deoxycholate, and 0.1% SDS, supplemented with protease inhibitors, phenylmethanesulfonyl fluoride 0.1 mM (Sigma-Aldrich, St. Louis, Mo., USA), leupeptin 10 μ M (Sigma-Aldrich) and pepstatin 1 μ M (Sigma-Aldrich). The cell lysates were centrifuged at 14,000 g for 15 min. Recombinant DMP1 proteins in conditioned media or cell lysates were analyzed with Stains-All staining or Western immunoblotting.

SDS-PAGE, Stains-All Staining and Western Immunoblotting

The conditioned media or cell lysates were electrophoresed using 4–20% gradient polyacrylamide gels (Life Gels, Frenchs Forest, NSW, Australia). Recombinant DMP1 proteins were visualized with Stains-All staining as described previously [Qin et al., 2003] or Western immunoblotting using affinity-purified rabbit anti-mouse DMP1 peptide polyclonal antibody 784 against the N-terminal peptide aa100–aa120 (GLGPEEGQWGGPSKLDSD-EDS) and antibody 785 against the C-terminal peptide aa469–aa483 (AYHNKPIGDQDDNDC), at a dilution of 1:4,000. Recombinant PHEX was immunoblotted with the 13B12 anti-PHEX monoclonal antibody (kindly provided by Enobia Pharm Inc., Montreal, QC, Canada), as described previously [Ruchon et al., 2000]. The blot was then incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Bio-Rad, Hercules, Calif., USA) diluted to 1:10,000 in 15 ml of 5% Bio-Rad milk in 1× TBST for a minimum of 1 h. Immunostained bands were visualized using ECLTM Chemiluminescent Western Blotting Detection Reagents (Amersham Biosciences, Pittsburgh, Pa., USA), according to the manufacturer's instructions. Chemiluminescent bands were imaged using a CL-XPosure film (Pierce Biotechnology Inc., Rockford, Ill., USA).

Generation of Col1a1-DMP1 and Col1a1-57K Transgenic Mice

To test the function of the 57-kDa C-terminal fragment in vivo, 2 different transgenic mice were generated to express either a full-length DMP1, Col1a1-DMP1 or the long form of the 57-kDa fragment, Col1a1-57K, using the same 3.6-kb Col1a1 promoter, a promoter highly active in the osteoblast lineage. Col1a1-DMP1 transgenic mice were generated on a C57B/L6 genetic background at the University of Texas, Houston, Tex., USA and have been described earlier [Lu et al., 2007]. Col1a1-57K transgenic mice were generated similarly on a C57B/L6 genetic background. The transgenic mice were screened by PCR analysis using DNA extracted from tail biopsy as previously described [Lu

Fig. 1. Schematic representation of DMP1 expression constructs as well as Stains-All staining and Western blot analysis of recombinant DMP1. Full-length DMP1, cleavage site mutated at D197A, 37-kDa N-terminal fragment (aa1-aa196) (37^N) as well as long (57^L) and short (57^S) forms of 57-kDa C-terminal fragment (aa190-aa487 and aa234-aa487, respectively) were cloned downstream of the CMV promoter in the pCDNA3 expression vector (a). The endogenous DMP1 signal peptide aa1-aa16 (MKTVILL-VFLWGLSCAL) was linked to both 57-kDa fragments. Various pCDNA3-DMP1 expression constructs, including pCDNA3 vector (E), full-length DMP1 (FL), mutant (M), 37-kDa N-terminal fragment (37^N) as well as long and short forms of 57-kDa C-terminal fragment (57^L and 57^S , respectively) were transiently transfected into expression cell line 293EBNA cells using Lipofectamine 2000 reagent (Invitrogen). 24 h after transfection, the medium was replaced by serum-free medium and cultured for an additional 48 h. 20 μ l of conditioned medium was loaded on a 4–20% gradient gel and the proteins were visualized with Stains-All staining (b) or detected by Western blot (c) using antibody 784, which recognizes both the full-length DMP1 as well as the 37-kDa N-terminal fragment, or antibody 785, which recognizes both the full-length DMP1 as well as the 57-kDa C-terminal fragment.

et al., 2007]. Two of four independent Col1a1-DMP1 transgenic mouse lines and 3 of 10 independent Col1a1-57K transgenic mouse lines were partially characterized and crossed to *Dmp1*-null mice for rescue studies (see below). The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee of the University of Missouri at Kansas City, Kansas City, Mo., USA.

Expression of the Col1a1-DMP1 or Col1a1-57K Transgene in *Dmp1*-null Mice

We have previously described the breeding strategy to introduce the Col1a1-DMP1 transgene into *Dmp1*-null mice for re-expression of full-length DMP1 in mice lacking DMP1 [Lu et al., 2007]. Briefly, female Col1a1-DMP1 transgenic mice were first crossed with homozygous male *Dmp1*-null mice (viable and fertile) to generate female mice heterozygous for both

Col1a1-DMP1 transgene and *Dmp1* gene, Col1a1-DMP1^{+/-}; *Dmp1*^{+/-}. The double heterozygous female mice were further bred with *Dmp1*-null males to produce *Dmp1*-null mutants carrying the transgene, *Dmp1*^{-/-}; Col1a1-DMP1^{+/-}. The same breeding strategy was used to introduce the Col1a1-57K transgene into *Dmp1*-null mice to generate *Dmp1*-null mutants carrying the transgene, *Dmp1*^{-/-}; Col1a1-57K^{+/-}. The *Dmp1*-null mice as well as *Dmp1*-heterozygous mice are being used as the controls.

High-Resolution Radiography

The bone samples from Col1a1-DMP1 or Col1a1-57K transgenic mice as well as control mice were dissected free of muscle. The dissected bones were X-rayed on a Faxitron model MX-20 Specimen Radiography System with a digital camera (Faxitron X-Ray Corp., Buffalo Grove, Ill., USA).

Fig. 2. Effects of DEC, a specific furin inhibitor, on DMP1 processing in CHO and 2T3 cells. CHO (**a**) or 2T3 cells (**b**) were transfected with pCDNA3 vector, pCDNA-mDMP1, pCDNA-DMP1 or pCDNA3-57K constructs. 24 h after transfection, the medium was replaced with serum-free media containing either 0, 5, 10, 20, 40 or 50 μ M of DEC. 48 h later, the conditioned medium was harvested and analyzed by Western blot analysis using the DMP1 C-terminal antibody (785). E = pCDNA3 empty vector; M = pCDNA3-mDMP1, the cleavage site mutated at D197A; FL-DMP1 = pCDNA3-DMP1, expressing full-length DMP1; 57^L = pCDNA3-57K, expressing the DMP1 57-kDa C-terminal fragment.

In situ Hybridization and Immunohistochemistry

The expression of either Colla1-DMP1 or Colla1-57K transgene was analyzed in *Dmp1*-null mouse tibiae from 3-week-old mice by *in situ* hybridization and immunohistochemistry. Thereby, any signal generated by either method should reflect the level of transgene expression in *Dmp1*-null background. *In situ* hybridization was performed using digoxigenin-labeled DMP1 cRNA probe prepared from a 1.1-kb murine DMP1 cDNA fragment with an RNA Labeling Kit (Roche, Indianapolis, Ind., USA). The 1.1-kb cDNA fragment was obtained by PCR using the full-length DMP1 cDNA as a template with the following primers: forward primer, 5'-CTCCGCAGACACCACACAGTCC-3'; reverse primer, 5'-TAGCCGTCCTGACAGTCATTGTC-3'. *In situ* hybridization on paraffin sections was carried out essentially as described previously [Feng et al., 2002]. Immunostaining of DMP1 protein was performed as described previously [Lu et al., 2007] using the same DMP1 antibodies, 784 and 785, described in Western blot analysis.

Results

In vitro Cleavage of Recombinant Full-Length DMP1 into Fragments of the Expected Size

Although the intact native full-length DMP1 has not been isolated from the mineralized tissues, 2 proteolytic fragments, a 37-kDa N-terminal fragment and a 57-kDa C-terminal fragment, have been isolated from rat long bone and dentin extracts, suggesting that DMP1 might be processed into 2 fragments *in vivo* [Qin et al., 2003]. To test the cleavage hypothesis, various forms of DMP1 expression constructs were made (fig. 1a), including a recombinant full-length DMP1, a full-length DMP1 with a cleavage site mutated at amino acid 197 (D to A), a 37-kDa N-terminal fragment (aa1–aa196) and 2 different forms of the 57-kDa C-terminal fragment (aa190–aa487 and aa234–aa487). Both 57-kDa fragments used the native DMP1 signal peptide, aa1–aa16 (MKTVILLVFLWGLS-CA).

Stains-All staining was used to visualize the various forms of recombinant DMP1 expressed in 293EBNA cells (fig. 1b), since DMP1 is a highly acidic phosphorylated extracellular matrix protein. Stains-All stains phosphorylated proteins blue, while the nonphosphorylated proteins and background are stained pink (see online version for color figure). It is of note that lane E of the conditioned medium from the 293EBNA cells expressing the pCDNA3 empty vector has no blue bands. The recombinant DMP1 containing the single cleavage site mutation was not cleaved as expected. The intact full-length recombinant DMP1 was processed into 37- and 57-kDa fragments similar in size to the recombinant 57-kDa C-terminal fragment (both 57^L and 57^S) and the 37-kDa N-terminal fragment (37^N). The size of the recombinant long form of the 57-kDa C-terminal fragment (57^L) was almost identical to the cleaved 57-kDa fragment from the recombinant full-length DMP1. These findings suggest that in 293EBNA cells the recombinant DMP1 was processed at or close to the conserved cleavage site.

The results from Stains-All staining were further confirmed by Western blot analysis using antibody 784, which recognizes the 37-kDa N-terminal fragment as well as full-length DMP1, and antibody 785, which recognizes the 57-kDa C-terminal fragment as well as full-length DMP1 (fig. 1c). Antibody 784 did not react with the cleaved 57-kDa fragment and antibody 785 did not react with the cleaved 37-kDa fragment, validating the cleaved fragments as being either the amino- or carboxy-terminal fragments and showing that there was no cross-

Fig. 3. Coexpression of PHEX and DMP1 in 293EBNA cells. **a** 293EBNA cells were transfected with either pCDNA empty vector or pCDNA3-PHEX and conditioned media applied to SDS-PAGE and the membrane was probed with the 13B12 anti-PHEX monoclonal antibody to detect recombinant PHEX. **b** Conditioned media from 293EBNA cells cotransfected with combinations of pCDNA3-DMP1 or pCDNA3-57K, or pCDNA3 vector with pCDNA3-PHEX was applied to SDS-PAGE for Western blotting. The blot was probed with DMP1 antibody 785 to detect both the full-length DMP1 as well as the 57-kDa C-terminal fragment. E = pCDNA3 empty vector; PHEX = pCDNA3-PHEX, expressing PHEX; DMP1 = pCDNA3-DMP1, expressing full-length DMP1; 57^L = pCDNA3-57K, expressing the DMP1 57-kDa C-terminal fragment.

reactivity between the 2 antibodies. Recombinant DMP1 was also processed similarly in 2T3 cells, an osteoblastic cell line, and completely cleaved in CHO cells, as shown by Western blot analysis (see below), suggesting that this cleavage may not be unique to cells that undergo mineralization.

Furin-Like Proprotein Convertase Activity Is Required for DMP1 Processing

Mammalian furin-like proprotein convertases are calcium-dependent serine proteases involved in the endoproteolytic cleavage of precursor proteins to yield biologically active polypeptides [Bergeron et al., 2000]. Furin, a member of this family, has a ubiquitous tissue distribution and exists in 2 forms, a membrane and a shed form [Denault et al., 2002]. It has been demonstrated that furin can process a large number of precursor proteins in the trans-Golgi network/biosynthetic pathway and/or extracellularly within the extracellular matrix [Denault et al., 2002]. DEC is a potent inhibitor of furin-like proprotein convertases, inhibiting the cleavage of proprotein convertase substrates [Denault et al., 1995].

As DMP1 cleavage is apparently not tissue specific, experiments were performed to determine whether a furin-

like proprotein convertase is involved in DMP1 processing. Figure 2a shows the processing of DMP1 in the presence/absence of DEC. First, the cleavage efficiency of full-length DMP1 was compared to that of mutant DMP1 with a cleavage site mutated at amino acid residue 197 (D to A) in the absence of DEC. Western blot analysis showed that more than 95% of full-length DMP1 is cleaved in CHO cells with transient transfection of the CMV-DMP1 construct. In contrast, more than 99% of the mutated DMP1 remains intact, suggesting that the aspartic residue at position 197 is critical for DMP1 processing. Subsequently, the same experiment was performed in the presence of DEC at different concentrations. DEC inhibited DMP1 processing in a dose-dependent manner. At 5 μ M, more than 40% of cleavage is inhibited; at 50 μ M, DMP1 cleavage is completely blocked. Similarly, DMP1 processing was blocked by DEC in 2T3 cells, an osteoblast cell line (fig. 2b). These observations suggest that a furin-like proprotein convertase directly or indirectly processes DMP1.

PHEX Does Not Cleave DMP1 in vitro

Dmp1-null mice and Hyp mice manifest similar hypophosphatemic rickets/osteomalacia-like phenotypes. As PHEX is a type II transmembrane zinc-containing endopeptidase [Tenenhouse and Sabbagh, 2002; Quarles, 2003] and DMP1 is processed into a 37-kDa N-terminal fragment as well as a 57-kDa C-terminal fragment both in vivo [Qin et al., 2003] and in vitro, experiments were performed to determine the possible role of PHEX in the cleavage of DMP1 into its active fragments. 293EBNA cells were cotransfected with either full-length DMP1 or 57-kDa C-terminal fragment expression constructs and a native PHEX expression construct (fig. 3). A proportion of the full-length DMP1 was processed into a 57-kDa C-terminal fragment in the absence of PHEX, and coexpression of native PHEX had no effect on the processing of DMP1. In addition, PHEX does not further process 57-kDa C-terminal fragment either. This result suggests that DMP1 cleavage is independent of PHEX.

Overexpression of a Full-Length DMP1 or a 57-kDa C-Terminal Fragment Using the 3.6-kb Col1a1-DMP1 Transgene Has No Effect on the Skeleton

Figure 4 shows the constructs used for the generation of mice expressing the full-length or the 57-kDa fragment, Col1a1-DMP1 or Col1a1-57K, respectively, under the control of the 3.6-kb type I collagen promoter. Although *Dmp1* ablation in mice results in profound skeletal abnormalities postnatally [Ling et al., 2005; Ye et al., 2005], transgenic mice overexpressing either transgene

Fig. 4. Constructs used for the generation of full-length and 57-kDa fragment overexpression and rescue transgenic mice. The rat *Col1a1* promoter was used to drive either the full-length DMP1 cDNA, *Col1a1*-DMP1 (**a**), or a cDNA fragment encoding the 57-kDa-long C-terminal fragment, *Col1a1*-57K (**b**). The rat *Col1a1* promoter includes 3.6-kb 5'-flanking region, first exon and first intron [Kalajzic et al., 2002]. The transcription start site is indicated by the left arrow. The *Col1a1*-DMP1 transgene has been described earlier [Lu et al., 2007], and the *Col1a1*-57K transgene was used for generation of transgenic mice in this study. The signal peptide (SP) was in frame with the 57-kDa fragment to ensure the secretion of the 57-kDa fragment. The downward arrows indicate the 2 potential cleavage sites.

Fig. 5. Effects of overexpression of either the full-length DMP1 or a 57-kDa fragment using the *Col1a1* promoter in wild-type mice. **a** Overexpression of full-length DMP1. Representative radiographs of tibiae show no apparent phenotype in *Dmp1*-heterozygous mice overexpressing *Col1a1*-DMP1 transgene (HET+DMP1), compared to the age-matched *Dmp1* heterozygous mice (HET) at ages of 1, 2 and 5 months. **b** Overexpression of a 57-kDa fragment. Radiographs show no apparent phenotype in the lower limbs from 3-month-old *Col1a1*-57K transgenic mice (WT+57K), compared to those from the wild-type mice (WT) in the same litter.

Col1a1-DMP1 transgene expression

Col1a1-57K transgene expression

on the wild-type background displayed no apparent phenotype, as determined by radiographic examination of long bone (fig. 5). One possible explanation for this lack of effect could be that although DMP1 is required for normal skeletogenesis, the actual amount of DMP1 is not the limiting factor, but the presence of receptors or expression of upstream regulatory or downstream target molecules may be the limiting factor.

Next, the expression of the *Col1a1*-DMP1 or the *Col1a1*-57K transgene at both the mRNA and protein level was determined (fig. 6). These data confirmed expression in the osteoblast lineage on the *Dmp1*-null background and showed that expression of the transgene was actually higher than expression of the endogenous *Dmp1* gene (fig. 6). Transgene expression was highly elevated in osteoblasts on the bone surface compared to endogenous expression mainly in osteocytes in the wild-type bone. However, protein expression was elevated on the bone surface and in a lamellar pattern in the bone matrix unlike low or no expression in the osteoblast layer with protein expression along osteocyte canaliculi in wild-type bone. Preliminary data suggest that the 57-kDa fragment may rescue the *Dmp1*-null phenotype (data not shown).

Discussion

In this study, we first demonstrated that the recombinant full-length DMP1 was partially processed into a 37-kDa N-terminal and a 57-kDa C-terminal fragment in

293EBNA cells, and completely cleaved into these 2 fragments in 2T3 osteoblast cells and CHO cells. This processing was completely blocked when the residue at a conserved cleavage site (aspartic acid residue at position 197) was mutated to alanine. These findings not only support the DMP1 cleavage hypothesis [Qin et al., 2003], but also support the hypothesis that the protease(s) responsible for DMP1 cleavage is not tissue specific, that is, the cleavage may occur in several nonbone cell types.

Recently, bone morphogenetic protein 1 (BMP1)/tolloid-like proteinases have been shown to cleave recombinant murine DMP1 to generate fragments similar to those previously isolated from rat long bone [Qin et al., 2003; Steiglit et al., 2004]. Interestingly, BMP1 is synthesized as an inactive proenzyme (pro-BMP1), which comprises a signal peptide, a prodomain, a catalytic domain and protein interaction domains [Bond and Beynon, 1995]. The removal of the prodomain is required for the activation of pro-BMP1 into active BMP1. The prodomain is removed by a furin-like proprotein convertase in the trans-Golgi network, which can be blocked by DEC [Leighton and Kadler, 2003]. In this study, it was confirmed that recombinant murine DMP1 was cleaved into fragments in osteoblastic 2T3 cells and nonosteoblastic CHO cells. This cleavage process was blocked by DEC in a dose-dependent manner. This work supports the hypothesis that a furin-like proprotein convertase activates pro-BMP1 into active BMP1, which subsequently cleaves DMP1.

Accumulating evidence supports the hypothesis that PHEX may be the protease responsible for DMP1 cleavage to generate the 57-kDa C-terminal fragment. First, both PHEX and DMP1 are expressed in osteoblasts and osteocytes in bone. Second, Hyp mice and *Dmp1*-null mice manifest similar hypophosphatemic rickets/osteomalacia-like phenotypes. However, coexpression of both proteins in 293EBNA cells had no effect on DMP1 processing. This finding suggests that protease(s) other than PHEX cleave DMP1 into 2 fragments, but it cannot be excluded that other factor(s) may be required for PHEX to process DMP1 under normal physiological conditions.

Although overexpression of either a full-length DMP1 or a 57-kDa C-terminal fragment had no apparent effect on the skeleton, our preliminary data showed that reexpression of either transgene in *Dmp1*-null mice appears to rescue the skeletal abnormalities as well as the abnormal serum biochemical profile (data not shown). Detailed characterization of these mice is underway.

Fig. 6. Reexpression patterns of either full-length DMP1 or a 57-kDa fragment in *Dmp1*-null mice using the *Col1a1* promoter. *Col1a1*-DMP1 (**a**) or *Col1a1*-57K (**b**) transgene expression was analyzed in tibiae from 3-week-old mice by in situ hybridization (upper panels in **a** and **b**) and immunohistochemistry (lower panels in **a** and **b**). Both in situ hybridization and immunohistochemistry showed that expression of endogenous DMP1 in *Dmp1* heterozygous mice (HET) was found predominantly in the osteocytes (OC, white arrowheads) embedded in the bone matrix. No expression of endogenous DMP1 was detected in *Dmp1*-null mice (KO). In situ hybridization showed that both transgenes were highly expressed in osteoblasts (OB, black arrowheads). Immunohistochemical staining showed DMP1 protein was expressed in the canaliculi in the bone matrix in *Dmp1*-heterozygous mice and, as expected, there was no expression in the *Dmp1*-null mouse bone. However, DMP1 protein was highly expressed in the osteoblast layer and in lamellae within the bone in the 2 DMP1 transgenic mice, full-length DMP1 (KO+DMP1) or a 57-kDa fragment (KO+57K). **a** Scale bars = 20 μ m. **b** Scale bars = 50 μ m.

In summary, the current findings suggest that DMP1 is a proprotein which is most likely cleaved in the cell. However, this cleavage process is not tissue specific and PHEX may not be required. Furthermore, we have generated and characterized transgenic mice overexpressing the full-length and the 57-kDa fragment of DMP1 under the control of the 3.6-kb Col1 promoter separately. These transgenes are highly active in osteoblasts/osteocytes but wild-type mice overexpressing these

transgenes display no apparent phenotype, suggesting that the cleavage of DMP1 may not be a self-regulating step.

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