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The LPV Motif is Essential for the Efficient Export of Secretory DMP1 from the Endoplasmic Reticulum

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Abstract

Dentin matrix protein 1 (DMP1) is found abundantly in the extracellular matrices of bone and dentin. Secretory DMP1 begins with a tripeptide of leucine-proline-valine (LPV) after the endoplasmic reticulum (ER)-entry signal peptide is cleaved. The goal of this study was to determine the role of the LPV motif in the secretion of DMP1. A series of DNA constructs was generated to express various forms of DMP1 with or without the LPV motif. These constructs were transfected into a preosteoblast cell line, the MC3T3-E1 cells, and the subcellular localization and secretion of various forms of DMP1 were examined by immunofluorescent staining and Western-blotting analyses. Immunofluorescent staining showed that the LPV-containing DMP1 variants were primarily localized in the Golgi complex, whereas the LPV-lacking DMP1 variants were found abundantly within the ER. Western-blotting analyses demonstrated that the LPV-containing DMP1 variants were rapidly secreted from the transfected cells, as they did not accumulate within the cells, and the amounts increased in the conditioned media over time. In contrast, the LPV-lacking DMP1 variants were predominantly retained within the cells, and only small amounts were secreted out of the cells over time. These results suggest that the LPV motif is essential for the efficient export of secretory DMP1 from the ER to the Golgi complex.

Keywords

DMP1; Endoplasmic reticulum; Golgi complex; Protein secretion

Introduction

Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) are two members of the SIBLING (Small Integrin-Binding LIgand N-linked Glycoprotein) family. In addition to the common features of the SIBLING family (Fisher et al., 2001), DMP1 and DSPP share a similar posttranslational modification and processing mechanism. Both are cleaved at the X-Asp bonds by bone morphogenetic protein 1/tolloid-like metalloproteinases, which gives rise to a glycosylated amino (N)-terminal fragment and a phosphorylated and acidic

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carboxyl (C)-terminal fragment (Qin et al., 2003; Steiglitz et al., 2004; Qin, 2005; Qin et al., 2006; Sun et al., 2010; von Marschall and Fisher, 2010; Sun et al., 2011; Zhu et al., 2012). DSPP is cleaved into an N-terminal fragment called dentin sialoprotein (DSP) and a C-terminal fragment known as dentin phosphoprotein (DPP) (Zhu et al., 2010; Qin et al., 2004; Sun et al., 2010). DSP is a proteoglycan containing two glycosaminoglycan chains (Zhu et al., 2010), whereas DPP is a highly phosphorylated and acidic protein (Butler et al., 1983; Qin et al., 2004). DMP1 is processed into a 37 kDa N-terminal fragment and a 57 kDa C-terminal fragment (Qin et al., 2003). The 37 kDa fragment is a proteoglycan containing a single chondroitin sulfate chain (Qin et al., 2006), while the 57 kDa fragment is a phosphorylated and acidic protein (Qin et al., 2003).

Human genetic studies have demonstrated that mutations in one allele of the *DSPP* gene cause dentinogenesis imperfecta (DGI) type I (formerly termed “type II”) (OMIM 125490), type III (OMIM 125500), or mild dentin dysplasia (DD) type II (OMIM 125420). To date, more than 40 *DSPP* mutations have been identified in patients suffering from DGI/DD. These mutations have been classified into three types: 1) mutations in the endoplasmic reticulum (ER)-entry signal peptide coding region; 2) mutations in the DSP coding region; and 3) mutations in the DPP coding region (McKnight et al., 2008; Maciejewska and Chomik, 2012). It is of particular interest that most of the disease-causing mutations identified in the DSP coding regions result in changes in the first three amino acids (isoleucine-proline-valine or “IPV”) of the mature DSPP (Von Marschall et al., 2012).

DSPP begins with a highly conserved “IPV” tripeptide (or motif) after the ER-entry signal peptide cleavage site; this IPV motif is essential to the transportation of DSPP from the ER to the Golgi complex with assistance from a hypothetical IPV receptor (von Marschall et al., 2012). Most disease-causing mutations in the DSP coding region result in a change within the “IPV” motif and are referred to as “IPV mutations”, such as the substitution of the proline (P) residue with leucine (L) (Li et al., 2012). In addition, skipping exon 3 due to a splice site mutation may also be classified as an IPV mutation (von Marschall et al., 2012). The IPV mutations cause an accumulation of mutant DSPP protein in the ER, which may eventually form cation (Ca^{2+})-dependent aggregates in the ER, thereby interfering with ER homeostasis (von Marschall et al., 2012).

DMP1 has a tripeptide of leucine-proline-valine (LPV) similar to that of DSPP after the ER-entry signal peptide cleavage site. Although several mutations in DMP1 causing Autosomal Recessive Hypophosphatemic Rickets/osteomalacia (ARHR) have been identified in humans (Feng et al., 2006; Farrow et al., 2009; Koshida et al., 2010; Gannage-Yared et al., 2014), none of these mutations affects the LPV motif of DMP1. In addition, we previously showed that the phosphorylated acidic 57 kDa C-terminal fragment lacking the intact LPV motif was secreted *in vitro* and *in vivo* and rescued the skeletal and serum biochemical abnormalities of *Dmp1*-null mice (Lu et al., 2009; Lu et al., 2011). Therefore, it remains to be determined whether the LPV motif of DMP1 is essential for its secretion.

In this study, we generated various DNA constructs expressing different forms of DMP1 with or without the intact LPV motif and examined the subcellular localization and secretion

of these DMP1 variants in the MC3T3-E1 cells. We found that the LPV motif is required for the efficient export of secretory DMP1 from the ER to the Golgi complex.

Materials and methods

DNA Constructs

Six DNA constructs were generated to express DMP1 variants in order to determine the role of the “LPV” motif in the secretion of DMP1: 1) a construct expressing the full-length DMP1 with a hemagglutinin (HA) tag inserted after the proteolytic cleavage site (referred to as “LPV-DMP1”) (Siyam et al., 2012); 2) a construct expressing the 37 kDa N-terminal fragment of DMP1 with an HA tag attached to the C-terminal end (LPV-37K); 3) a construct expressing the 57 kDa C-terminal fragment of DMP1 fused to the first amino acid residue, leucine (L), of the mature full-length DMP1 (LDD-57K); 4) a construct expressing the 57 kDa C-terminal fragment fused to the first 40 amino acid residues of the mature full-length DMP1 (LPV-N40-57K); 5) a construct expressing the 57 kDa C-terminal fragment fused to the first three amino acid residues (LPV) of the mature full-length DMP1 (LPV-57K); 6) a construct expressing the full-length DMP1 with the proline (P) residue (the second amino acid residue of the mature full-length DMP1) substituted by a leucine (L) residue (LLV-DMP1). Except for the LPV-37K construct, all the DNA constructs expressed DMP1 or DMP1-related protein with an HA tag inserted at the same location as the LPV-DMP1. All the constructs were generated by polymerase chain reaction (PCR) or by site-directed mutagenesis using the QuickChange mutagenesis kit (Agilent Technologies, Inc., Santa Clara, CA) and were confirmed by DNA sequencing.

Cell culture

MC3T3-E1 preosteoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were grown in α -minimum essential media (MEM) supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% CO₂ at a temperature of 37 °C.

DNA transfection

DNA was transfected into cells using X-tremeGENE 9 reagent (Roche, Indianapolis, IN) according to the manufacturer’s instructions. For immunofluorescent staining, MC3T3-E1 cells were transiently transfected with 0.6 μ g of various DMP1-expressing constructs in a 24-well plate; on the next day, the transfected cells were trypsinized and replated into 8-well chamber slides. Twenty-four hours after replating, the transfected cells were processed for immunofluorescent staining. For Western-blotting analyses, the MC3T3-E1 cells in a 6-well plate were transiently transfected with a total of 2 μ g of a construct expressing LPV-DMP1, LLV-DMP1, LPV-57K or LDD-57K. Total cell lysates and conditioned media were harvested at 6, 12 and 24 hours (hrs) after transfection and analyzed by Western-blotting analysis. In addition, MC3T3-E1 cells transfected with an empty expression vector (pCDNA3) were used as a control for Western-blotting analysis.

Immunofluorescent staining

Immunofluorescent staining was performed as described previously (Siyam et al., 2012). Briefly, the transfected cells were incubated with mouse anti-HA monoclonal antibody (Covance Inc., Dallas, TX; 1:2000) together with rabbit anti-GM130 polyclonal antibody (Abcam, Cambridge, MA; 1:1000) for Golgi complex or rabbit anti-calreticulin polyclonal antibody (Santa Cruz Biotechnology, Inc., Dallas, TX; 1:1000) for endoplasmic reticulum (ER), followed by incubation with Alexa Fluor 555 conjugated goat anti-mouse (Invitrogen, Grand Island, NY; 1:1000) and Alexa Fluor 488 conjugated goat anti-rabbit IgG(H+L) (Invitrogen, Grand Island, NY; 1:1000). The nuclei were counterstained with DAPI. The fluorescent-stained cells were imaged under a Nikon Eclipse TE2000-U fluorescence microscope (Nikon Instruments Inc., Melville, NY).

Western-blotting analysis

Western-blotting analysis was carried out as described previously (Lu et al., 2009). Briefly, 5 µg of the total cell lysates and the total proteins extracted by strataclean resin (Agilent Technologies, Inc., Santa Clara, CA) from 500 µl of conditioned medium were electrophoresed on an 8% SDS-PAGE gel and transferred onto PVDF membrane (EMD Millipore). The membranes were blocked in 5% milk (LabScientific, Highlands, NJ) for 1 hour at room temperature and then immunoblotted with mouse anti-HA monoclonal antibody (Covance Inc., Dallas, TX; 1:1000) overnight at 4°C, followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology, Inc., Dallas, TX; 1:1000) for 2 hrs at room temperature. The immunostained protein bands were detected with ECLTM Chemiluminescent detection reagents (Pierce Biotechnology, Inc., Rockford, IL) and imaged using a CL-XPosure film (Pierce Biotechnology, Inc., Rockford, IL).

Results

Localization of LPV-DMP1 and LPV-37K in the Golgi complex

Secretory proteins first enter the ER, and they are then exported from the ER to the Golgi complex, where they undergo post-translational modifications, processing and packaging into secretory vesicles to be secreted out of the cells (Harter and Wieland, 1996; Hong, 1998). The ER consists of a network of flattened membrane tubules that extend throughout the cytoplasm, whereas the Golgi complex is a very confined intracellular organelle located near the nucleus.

We previously generated a construct, named as “LPV-DMP1”, expressing the full-length DMP1 with an HA tag inserted after the proteolytic cleavage sites, so that the HA-tag would label either the full-length DMP1 (before cleavage) or the 57 kDa C-terminal fragment (after cleavage) (Siyam et al., 2012) (Fig. 1). To characterize the secretion of DMP1 along the secretory pathway, we transfected the LPV-DMP1-expressing construct into the MC3T3-E1 cells. Co-immunofluorescent staining showed that the LPV-DMP1 protein was primarily co-localized with GM130 protein, a resident protein in the Golgi complex, suggesting that the LPV-DMP1 protein was mainly localized in the Golgi complex (Fig. 2). We then generated a construct expressing a 37 kDa N-terminal fragment in which an HA tag was attached to

the C-terminal end of the 37 kDa fragment (referred to as “LPV-37K”). We transfected the LPV-37K-expressing construct into MC3T3-E1 cells; co-immunofluorescent staining showed that the LPV-37K protein was also co-localized with the GM130 protein, a distribution pattern similar to LPV-DMP1 protein (Fig. 2). These observations suggested that the 37 kDa N-terminal fragment contains all the elements that are necessary for directing the normal secretion of DMP1.

Localization of LDD-57K in the ER

Next, we examined the subcellular localization of the 57 kDa C-terminal fragment. We previously generated a construct expressing the 57 kDa C-terminal fragment of DMP1 in which the 57 kDa C-terminal fragment was fused to the first amino acid residue (leucine) of the mature DMP1 and showed that this 57 kDa C-terminal fragment had been detected in the conditioned medium when the construct was transfected into HEK293 EBNA cells (Lu et al., 2009). To determine whether the 57 kDa C-terminal fragment had a subcellular localization similar to the full-length LPV-DMP1, we generated a construct expressing the 57 kDa C-terminal fragment with an HA tag inserted at the same location as the LPV-DMP1 (designated as LDD-57K) (Fig. 1). We then transfected this LDD-57K-expressing construct into the MC3T3-E1 cells; co-immunofluorescent staining showed that the LDD-57K protein was primarily co-localized with calreticulin, a resident protein in the ER, but was barely co-localized with GM130 (Fig. 3). These results demonstrated that the LDD-57K protein was mainly retained within ER, suggesting that LDD-57K lacked the elements required for its efficient ER export.

Localization of LPV-N40-57K and LPV-57K in the Golgi complex

To further define the amino acid sequence of DMP1 that assures the efficient ER export of DMP1, we generated a construct expressing the 57 kDa C-terminal fragment fused to the first 40 amino acid residues of mature full-length DMP1 (referred to as “LPV-N40-57K”) (Fig. 1). In addition, we generated a construct expressing the 57 kDa C-terminal fragment fused to the first three amino acid residues (LPV) of the mature full-length DMP1 (called “LPV-57K”) (Fig. 1). We transfected these two constructs into the MC3T3-E1 cells; co-immunofluorescent staining showed that both LPV-N40-57K and LPV-57K were predominantly co-localized with GM130 (Fig. 4). These data suggested that the first tripeptide, LPV, of the mature full-length DMP1 was essential and sufficient for the efficient export of DMP1 from the ER to the Golgi complex.

Localization of LLV-DMP1 in the ER

Previous human genetic studies showed that mutations resulting in the substitution of the proline (P) residue with a leucine (L) residue in the first tripeptide, IPV, of the mature human DSPP caused retention of DSPP in the ER and led to DGI (Li et al., 2012; von Marschall et al., 2012). To determine whether a similar mutation would affect the secretion of DMP1, we generated a construct expressing the full-length DMP1 with the proline residue (the second amino acid residue of the LPV motif) substituted by a leucine residue (designated as “LLV-DMP1”) (Fig. 1). When we transfected this construct into the MC3T3-E1 cells, co-immunofluorescent staining showed that the LLV-DMP1 protein was primarily co-localized with calreticulin, but was barely co-localized with GM130 (Fig. 5). These

findings demonstrated that the LLV-DMP1 protein was mainly retained within the ER, suggesting that a single amino acid substitution in the LPV motif interfered with the ER exit of DMP1.

The LPV motif was required for the efficient secretion of DMP1

To further determine whether the LLV-DMP1 was able to secret from the cells, we transfected the constructs expressing LPV-DMP1 or LLV-DMP1 into the MC3T3-E1 cells, and then analyzed the total cell lysates and conditioned media harvested at 6 hrs, 12 hrs and 24 hrs after transfection (Fig. 6). Western-blotting analysis showed that three clusters of protein bands of ~130 kDa, ~100 kDa and ~57 kDa were detected at 12 hrs and 24 hrs in the cell lysates and in the conditioned media from the cells transfected with the LPV-DMP1-expressing construct. The protein band of 100 kDa corresponded to the full-length DMP1, whereas the 130 kDa band represented a glycosylated form of DMP1 and the 57 kDa band was the processed C-terminal fragment. Notably, the intensities of these protein bands were similar in the cell lysates harvested at 12 hrs and 24 hrs after transfection, but they were much stronger in the conditioned media collected at 24 hrs than at 12 hrs. In contrast, while three similar clusters of protein bands were observed at both 12 hrs and 24 hrs in the cell lysates from the cells transfected with the LLV-DMP1-expressing construct, they were detectable only at 24 hrs in the conditioned medium. Furthermore, the intensities of these protein bands were much stronger in the cell lysates harvested at 24 hrs than at 12 hrs. These data suggested that the LPV motif was required for the efficient secretion of DMP1, and that the substitution of the proline (P) residue by a leucine (L) residue caused an accumulation of the mutant DMP1 within the cells over time.

Similarly, we determined the secretion of LPV-57K and LDD-57K in the MC3T3-E1 cells (Fig. 7). Western-blotting analysis showed that a protein band of ~57 kDa was detected at both 12 hrs and 24 hrs in the cell lysates and in the conditioned media from the cells transfected with the LPV-57K-expressing construct. The intensity of the LPV-57K protein band appeared to be similar in the cell lysates at 12 hrs and 24 hrs, but it was much stronger in the conditioned media harvested at 24 hrs than at 12 hrs. Similar protein band was observed at both 12 hrs and 24 hrs in the cell lysates from cells transfected with the LDD-57K-expressing construct, but it was only weakly detected at 24 hrs in the conditioned media. In addition, the intensity of the LDD-57K protein band was much stronger in the cell lysates harvested at 24 hrs than at 12 hrs, and it was also much stronger than the LPV-57K protein in the cell lysates at the same time point. These findings further supported the belief that the LPV motif was required for the efficient secretion of DMP1.

Discussion

In this study, we determined the role of the LPV motif (or the first three amino acid residues of the mature full-length DMP1) in the export of DMP1 variants from the ER to the Golgi complex. We showed that the LPV-containing DMP1 variants were barely detectable in the ER and that the loss of the LPV motif interfered with the export of DMP1 variants from the ER to the Golgi complex. Nevertheless, we noticed that a small amount of the LPV-lacking DMP1 variants, such as LDD-57K and LLV-DMP1, was secreted out of the cells over time.

In addition, the secreted LLV-DMP1 and LDD-57K showed a pattern similar to their LPV-containing counterparts, LPV-DMP1 and LPV-57K, respectively. These findings suggest that the LPV motif is necessary and sufficient to assist the efficient ER export of DMP1.

Larry Fisher hypothesized that there might be an “IPV receptor” that binds to the “IPV-like” motif found in DSPP protein and other secreted proteins including DMP1 (von Marschall et al., 2012). The IPV receptor may assist the movement and/or packaging of the proteins into transport vesicles that transport the ER-derived proteins to the Golgi complex (von Marschall et al., 2012). Based on this hypothesis, LPV-DMP1 may exit the ER at a very rapid rate with the assistance of the hypothetical “IPV receptor”. Both LDD-57K and LLV-DMP1, however, can still exit the ER by non-selective bulk flow or other unknown mechanisms (Pelham, 1989; Klumperman, 2000), but at a much slower rate. The slow secretion of LDD-57K may explain why it lacked the LPV motif but still rescued the serum biochemical and skeletal defects of *Dmp1*-null mice (Lu et al., 2011).

The effects of the ER-retained mutant proteins may depend on the chemical properties of the retained molecule. We found that the transgenic expression of the LDD-57K protein did not have any apparent skeletal defects in mice (Lu et al., 2009). However, human DSPP mutations that change the IPV motif of mature DSPP result in DGI [reviewed in (Maciejewska and Chomik, 2012)]. The difference in the pathogenesis of LDD-57K and IPV mutant DSPP may be attributed to the difference in their chemical properties. The 57 kDa C-terminal fragment of DMP1 contains 41 phosphates, whereas DPP contains a large number of repeating sequences of aspartate (Asp)-phosphoserine (Pse) and Asp-Pse-Pse; it can have as many as 209 phosphoserines (Butler et al., 1983; Qin et al., 2003; Qin et al., 2004). The large number of Asp and Pse residues makes the DPP extremely acidic, with an isoelectric point estimated to be 1.1 for rat DPP (Jonsson et al., 1978). It has been hypothesized that the mutant DSPP protein may form cation (Ca^{2+})-dependent aggregates in the ER, thereby interfering with ER homeostasis (von Marschall et al., 2012). In addition, the function of secreted mutant proteins may also contribute to the final phenotype. We demonstrated that the secreted 57 kDa C-terminal fragment was functional, as it was able to rescue the serum biochemical and skeletal abnormalities of *Dmp1*-null mice (Lu et al., 2011). It has been shown that the secreted IPV-lacking DSPP protein is glycosylated and proteolytically processed in a way that is similar to the normal DSPP protein (von Marschall et al., 2012); however, whether such secreted mutant DSPP protein has normal function remains to be determined.

The proline residue at the +2-position (P^{+2}) from the signal peptide cleavage site is found in many secretory proteins, soluble Golgi proteins and membrane proteins (Supplemental Table 1). However, the amino acid residues at the +1 and +3 positions show great variations; they can be a hydrophobic amino acid or a positively or negatively charged amino acid. Tsukumo et al. found that the P^{+2} residue was essential for the ER export of Golgi-localized soluble proteins (Tsukumo et al., 2009). Therefore, they hypothesized that the P^{+2} residue may function as an ER export signal for soluble Golgi proteins (Tsukumo et al., 2009). However, Larry Fisher’s group demonstrated that the “IPV” motif was required for the secretion of matrix proteins such as DSPP and OPN, and that when the valine (V) residue at the +3 position was replaced with a charged aspartic acid residue (D), the mutant protein

was largely retained within the cells (von Marschall et al., 2012). Therefore, the amino acids at both the +1 and +3 positions can also influence the binding of the +P⁺² to a hypothetical ER exit receptor. Nevertheless, we cannot exclude the possibility that different receptors may exist to assist the ER export of different proteins.

In summary, we have shown that the first tripeptide (LPV) of the mature DMP1 is required for the efficient export of DMP1 from the ER. It remains to be determined whether there is a receptor that binds to the LPV motif to facilitate the ER exit of DMP1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

- Butler WT, Bhowm M, DiMuzio MT, Cothran WC, Linde A. Multiple forms of rat dentin phosphoproteins. *Archives of biochemistry and biophysics*. 1983; 225:178–186. [PubMed: 6614917]
- Farrow EG, Davis SI, Ward LM, Summers LJ, Bubbear JS, Keen R, Stamp TC, Baker LR, Bonewald LF, White KE. Molecular analysis of DMP1 mutants causing autosomal recessive hypophosphatemic rickets. *Bone*. 2009; 44:287–294. [PubMed: 19007919]
- Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, Yu X, Rauch F, Davis SI, Zhang S, Rios H, Drezner MK, Quarles LD, Bonewald LF, White KE. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet*. 2006; 38:1310–1315. [PubMed: 17033621]
- Fisher LW, Torchia DA, Fohr B, Young MF, Fedarko NS. Flexible structures of SIBLING proteins, bone sialoprotein, and osteopontin. *Biochemical and biophysical research communications*. 2001; 280:460–465. [PubMed: 11162539]
- Gannage-Yared MH, Makrythanasis P, Chouery E, Sobacchi C, Mehawej C, Santoni FA, Guipponi M, Antonarakis SE, Hamamy H, Megarbane A. Exome sequencing reveals a mutation in DMP1 in a family with familial sclerosing bone dysplasia. *Bone*. 2014; 68:142–145. [PubMed: 25180662]
- Harter C, Wieland F. The secretory pathway: mechanisms of protein sorting and transport. *Biochimica et biophysica acta*. 1996; 1286:75–93. [PubMed: 8652612]
- Hong W. Protein transport from the endoplasmic reticulum to the Golgi apparatus. *Journal of cell science*. 1998; 111(Pt 19):2831–2839. [PubMed: 9730976]
- Jonsson M, Fredriksson S, Jontell M, Linde A. Isoelectric focusing of the phosphoprotein of rat-incisor dentin in ampholine and acid pH gradients. Evidence for carrier ampholyte-protein complexes. *Journal of chromatography*. 1978; 157:235–242. [PubMed: 29908]
- Klumperman J. Transport between ER and Golgi. *Current opinion in cell biology*. 2000; 12:445–449. [PubMed: 10873822]
- Koshida R, Yamaguchi H, Yamasaki K, Tsuchimochi W, Yonekawa T, Nakazato M. A novel nonsense mutation in the DMP1 gene in a Japanese family with autosomal recessive hypophosphatemic rickets. *J Bone Miner Metab*. 2010; 28:585–590. [PubMed: 20213538]
- Li D, Du X, Zhang R, Shen B, Huang Y, Valenzuela RK, Wang B, Zhao H, Liu Z, Li J, Xu Z, Gao L, Ma J. Mutation identification of the DSPP in a Chinese family with DGI-II and an up-to-date bioinformatic analysis. *Genomics*. 2012; 99:220–226. [PubMed: 22310900]

- Lin SX, Zhang Q, Zhang H, Yan K, Ward L, Lu YB, Feng JQ. Nucleus-targeted Dmp1 transgene fails to rescue dental defects in Dmp1 null mice. *International journal of oral science*. 2014; 6:133–141. [PubMed: 25105818]
- Lu Y, Qin C, Xie Y, Bonewald LF, Feng JQ. Studies of the DMP1 57-kDa functional domain both in vivo and in vitro. *Cells Tissues Organs*. 2009; 189:175–185. [PubMed: 18728349]
- Lu Y, Yuan B, Qin C, Cao Z, Xie Y, Dallas SL, McKee MD, Drezner MK, Bonewald LF, Feng JQ. The biological function of DMP-1 in osteocyte maturation is mediated by its 57-kDa C-terminal fragment. *J Bone Miner Res*. 2011; 26:331–340. [PubMed: 20734454]
- Maciejewska I, Chomik E. Hereditary dentine diseases resulting from mutations in DSPP gene. *Journal of dentistry*. 2012; 40:542–548. [PubMed: 22521702]
- McKnight DA, Suzanne Hart P, Hart TC, Hartsfield JK, Wilson A, Wright JT, Fisher LW. A comprehensive analysis of normal variation and disease-causing mutations in the human DSPP gene. *Human mutation*. 2008; 29:1392–1404. [PubMed: 18521831]
- Pelham HR. Control of protein exit from the endoplasmic reticulum. *Annual review of cell biology*. 1989; 5:1–23.
- Qin C, Baba O, Butler WT. Post-translational modifications of sibling proteins and their roles in osteogenesis and dentinogenesis. *Crit Rev Oral Biol Med*. 2004; 15:126–136. [PubMed: 15187031]
- Qin, C.; Baba, O.; Brunn, JC.; McKee, MD.; Bonewald, L.; Butler, WT. Dentin matrix protein 1 (DMP1) and dentin sialophosphoprotein (DSPP) share unique properties including tissue localization, proteolytic processing, and high molecular weight forms. In: SODEK, J.; LANDIS, W., editors. *Proceedings of the 8th ICCBMT*. Banff, Alberta, Canada: University of Toronto Press; 2005. p. 4
- Qin C, Brunn JC, Cook RG, Orkiszewski RS, Malone JP, Veis A, Butler WT. Evidence for the proteolytic processing of dentin matrix protein 1. Identification and characterization of processed fragments and cleavage sites. *J Biol Chem*. 2003; 278:34700–34708. [PubMed: 12813042]
- Qin C, Huang B, Wygant JN, McIntyre BW, McDonald CH, Cook RG, Butler WT. A chondroitin sulfate chain attached to the bone dentin matrix protein 1 NH2-terminal fragment. *J Biol Chem*. 2006; 281:8034–8040. [PubMed: 16421105]
- Siyam A, Wang S, Qin C, Mues G, Stevens R, D'Souza RN, Lu Y. Nuclear localization of DMP1 proteins suggests a role in intracellular signaling. *Biochemical and biophysical research communications*. 2012; 424:641–646. [PubMed: 22813642]
- Steiglitz BM, Ayala M, Narayanan K, George A, Greenspan DS. Bone morphogenetic protein-1/Tolloid-like proteinases process dentin matrix protein-1. *J Biol Chem*. 2004; 279:980–986. [PubMed: 14578349]
- Tsukumo Y, Tsukahara S, Saito S, Tsuruo T, Tomida A. A novel endoplasmic reticulum export signal: proline at the +2-position from the signal peptide cleavage site. *The Journal of biological chemistry*. 2009; 284:27500–27510. [PubMed: 19656946]
- Sun Y, Chen L, Ma S, Zhou J, Zhang H, Feng JQ, Qin C. Roles of DMP1 processing in osteogenesis, dentinogenesis and chondrogenesis. *Cells Tissues Organs*. 2011; 194:199–204. [PubMed: 21555863]
- Sun Y, Lu Y, Chen S, Prasad M, Wang X, Zhu Q, Zhang J, Ball H, Feng J, Butler WT, Qin C. Key proteolytic cleavage site and full-length form of DSPP. *Journal of dental research*. 2010; 89:498–503. [PubMed: 20332332]
- von Marschall Z, Fisher LW. Dentin sialophosphoprotein (DSPP) is cleaved into its two natural dentin matrix products by three isoforms of bone morphogenetic protein-1 (BMP1). *Matrix Biol*. 2010; 29:295–303. [PubMed: 20079836]
- von Marschall Z, Mok S, Phillips MD, McKnight DA, Fisher LW. Rough endoplasmic reticulum trafficking errors by different classes of mutant dentin sialophosphoprotein (DSPP) cause dominant negative effects in both dentinogenesis imperfecta and dentin dysplasia by entrapping normal DSPP. *Journal of bone and mineral research: the official journal of the American Society for Bone and Mineral Research*. 2012; 27:1309–1321.

- Zhu Q, Prasad M, Kong H, Lu Y, Sun Y, Wang X, Yamoah A, Feng JQ, Qin C. Partial Blocking of Mouse DSPP Processing by Substitution of Gly(451)-Asp(452) Bond Suggests the Presence of Secondary Cleavage Site(s). *Connect Tissue Res.* 2012
- Zhu Q, Sun Y, Prasad M, Wang X, Yamoah AK, Li Y, Feng J, Qin C. Glycosaminoglycan chain of dentin sialoprotein proteoglycan. *Journal of dental research.* 2010; 89:808–812. [PubMed: 20400719]

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Fig. 1. Schematic representations of constructs expressing DMP1 variants

The construct LPV-DMP1 expressed a full-length DMP1 including the 37 kDa amino-terminal fragment (37K) and the 57 kDa carboxyl-terminal fragment (57K). The construct LPV-37K expressed the 37 kDa amino-terminal fragment of DMP1. The construct LDD-57K expressed the 57 kDa carboxyl-terminal fragment of DMP1 fused to the leucine (L) residue – the first amino acid residue immediately after the signal peptide cleavage site. The construct LPV-N40-57K expressed the 57 kDa carboxyl-terminal fragment fused to the first 40 amino acid residues (N40) after the signal peptide cleavage site. The construct LPV-57K expressed the 57 kDa carboxyl-terminal fragment fused to the first three amino acid residues (LPV) after the signal peptide cleavage site; and the construct LLV-DMP1 expressed a full-length DMP1 with the proline (P) residue (the second amino acid residue after the signal peptide cleavage site) substituted by a leucine (L) residue. SP, ER entry signal peptide; CL, the key cleavage site; LPV, the first three amino acid residues (leucine-proline-valine) after the signal peptide cleavage site; DD, the first two amino acid residues (aspartate-aspartate) of the 57 kDa carboxyl-terminal fragment; LE, leucine-glutamate residues introduced by linker DNA. The black box “**I**” marks the position of hemagglutinin (HA) tag.

Fig. 2. Subcellular localization of LPV-DMP1 and LPV-37K

Constructs expressing LPV-DMP1 (A) or LPV-37K (B) were transiently transfected into MC3T3-E1 cells. The transfected cells were then incubated with a mouse anti-HA monoclonal antibody for LPV-DMP1 and LPV-37K (red) and a rabbit anti-GM130 polyclonal antibody for Golgi complex (green). The nuclei were counterstained with DAPI (blue). The merged images showed that the LPV-DMP1 (A) and LPV-37K (B) proteins were primarily localized in the Golgi complex in the transfected cells. The arrows indicate the transfected cells; the asterisks mark the non-transfected cells. Scale bars = 20 μm.

Fig. 3. Subcellular localization of LDD-57K

Constructs expressing LDD-57K were transiently transfected into MC3T3-E1 cells. The transfected cells were then incubated with a mouse anti-HA monoclonal antibody for LDD-57K (red) and a rabbit anti-GM130 polyclonal antibody (A) for Golgi complex (green) or a rabbit anti-calreticulin polyclonal antibody (B) for endoplasmic reticulum (green). The nuclei were counterstained with DAPI (blue). The merged images showed that LDD-57K protein was barely detected in the Golgi complex (A), but had predominantly accumulated in the endoplasmic reticulum (B). The arrows indicate the transfected cells; the asterisks mark the non-transfected cells. Scale bars = 20 μm.

Fig. 4. Subcellular localization of LPV-N40-57K and LPV-57K

Constructs expressing LPV-N40-57K (A) or LPV-57K (B) were transiently transfected into MC3T3-E1 cells. The transfected cells were then incubated with a mouse anti-HA monoclonal antibody for LPV-N40-57K and LPV-57K (red) and a rabbit anti-GM130 polyclonal antibody for Golgi complex (green). The nuclei were counterstained with DAPI (blue). The merged images showed that both LPV-N40-57K (A) and LPV-57K (B) proteins were primarily found in the Golgi complex. The arrows indicate the transfected cells; the asterisks mark the non-transfected cells. Scale bars = 20 μm .

Fig. 5. Subcellular localization of LLV-DMP1

Constructs expressing LLV-DMP1 were transiently transfected into MC3T3-E1 cells. The transfected cells were then incubated with a mouse anti-HA monoclonal antibody for LLV-DMP1 (red) and a rabbit anti-GM130 polyclonal antibody (A) for Golgi complex (green) or a rabbit anti-calreticulin polyclonal antibody (B) for endoplasmic reticulum (green). The nuclei were counterstained with DAPI (blue). The merged images showed that the LLV-DMP1 protein was barely found in the Golgi complex (A), but had mainly accumulated in the endoplasmic reticulum (B). The arrows indicate the transfected cells; the asterisks mark the non-transfected cells. Scale bars = 20 μm.

Fig. 6. Secretion of full-length DMP1 in MC3T3-E1 cells

MC3T3-E1 cells were transiently transfected with pCDNA3 empty vector (Ctrl) or constructs expressing LPV-DMP1 or LLV-DMP1. The total cell lysates and conditioned media were harvested at 6 hrs, 12 hrs and 24 hrs after transfection, and analyzed by Western-blotting with mouse anti-HA monoclonal antibody, and the blot for the cell lysates was then stripped and probed with mouse monoclonal β -actin antibody. The positions of DMP1 proteoglycan (PG) form (~130 kDa), full-length DMP1 (~100 kDa) and C-terminal 57 kDa fragment (~57 kDa) are indicated. The arrow indicates a non-specific protein found in all conditioned medium samples. Note that the DMP1 PG form overlaps the non-specific protein, particularly in the conditioned medium of 24 hrs.

Fig. 7. Secretion of 57 kDa C-terminal fragment in MC3T3-E1 cells

MC3T3-E1 cells were transiently transfected with pCDNA3 empty vector (Ctrl) or constructs expressing LPV-57K or LDD-57K. The total cell lysates and conditioned media were harvested at 6 hrs, 12 hrs and 24 hrs after transfection, and analyzed by Western-blotting with mouse anti-HA monoclonal antibody, and the blot for the cell lysates was then stripped and probed with mouse monoclonal β -actin antibody. The position of the 57 kDa C-terminal fragment is shown. The arrow points to a non-specific protein present in all conditioned medium samples.