



Published in final edited form as:

Connect Tissue Res. 2012 ; 53(4): 307–312. doi:10.3109/03008207.2011.650301.

Partial Blocking of Mouse DSPP Processing by Substitution of Gly⁴⁵¹-Asp⁴⁵² Bond Suggests the Presence of Secondary Cleavage Site(s)

Qinglin Zhu^{1,2,*}, Monica Prasad^{1,*}, Hui Kong¹, Yongbo Lu¹, Yao Sun¹, Xiaofang Wang¹, Albert Yamoah¹, Jian Q. Feng¹, and Chunlin Qin¹

¹Department of Biomedical Sciences, Baylor College of Dentistry, Texas A&M Health Science Center, Dallas, Texas 75246

²Department of Operative Dentistry and Endodontics, The Fourth Military Medical University, School of Stomatology, Xi'an, Shaanxi 710032, China

Abstract

Dentin sialophosphoprotein (DSPP) in the extracellular matrix of dentin is cleaved into dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), which originate from the NH₂-terminal and COOH-terminal regions of DSPP, respectively. In the proteolytic processing of mouse DSPP, the peptide bond at Gly⁴⁵¹-Asp⁴⁵² has been shown to be cleaved by bone morphogenetic protein 1 (BMP1)/Tolloid-like metalloproteinases. In the present study, we generated transgenic mice expressing a mutant DSPP in which Asp⁴⁵² was substituted by Ala⁴⁵². Protein chemistry analyses of extracts from the long bone of these transgenic mice showed that the D452A substitution partially blocked DSPP processing *in vivo*. When the full-length form of mutant DSPP (designated "D452A-DSPP") isolated from the transgenic mice was treated with BMP1 *in vitro*, a portion of the D452A-DSPP was cleaved, suggesting the presence of secondary peptide bond(s) that can be broken by BMP1. To identify the potential secondary DSPP cleavage site(s), site-directed mutagenesis was performed to generate nine DNA constructs expressing DSPP bearing substitutions at potential scission sites. These different types of mutant DSPP made in eukaryotic cell lines were treated with BMP1 and the digestion products were assessed by Western immunoblotting. All of the mutant DSPP molecular species were partially cleaved by BMP1, giving rise to a protein band on SDS-PAGE similar to that of normal DSP. Taken together, we concluded that in addition to the peptide bond Gly⁴⁵¹-Asp⁴⁵², there must be a cryptic cleavage site or sites close to Asp⁴⁵² in the mouse DSPP that can be cleaved by BMP1.

Keywords

DSPP; BMP1; Proteolytic Processing; Posttranslational Modification; Biomineralization

Introduction

In addition to type I collagen, dentin contains a set of non-collagenous proteins (NCPs), which include osteonectin, osteocalcin, osteopontin (OPN), bone sialoprotein (BSP), dentin

Correspondence to: Chunlin Qin, DDS, PhD, Department of Biomedical Sciences, Baylor College of Dentistry, 3302 Gaston Avenue, Room 400, Dallas, TX 75246, cqin@bcd.tamhsc.edu Phone: 214-828-8292; Fax: 214-874-4538.

*These two authors contributed equally to this work.

DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

matrix protein 1 (DMP1), dentin sialophosphoprotein (DSPP) and matrix extracellular phosphoglycoprotein (MEPE). OPN, BSP, DMP1, DSPP, MEPE belong to the SIBLING (Small Integrin-Binding Ligand, N-linked Glycoprotein) family [1], with similar properties in their genomic organization, posttranslational modification and tissue localization. These SIBLING proteins secreted into the extracellular matrix (ECM) during the formation and mineralization of dentin and bone are believed to play key biological roles in the formation of these tissues[2].

MacDougall et al. [3] and Feng et al. [4] discovered that the nucleotide sequences for mouse dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) reside on the same gene coding for a single mRNA named DSPP. The full-length cDNA of mouse DSPP revealed a 934-amino acid open reading frame, including a 17-amino acid signal peptide. This transcript would result in a translational protein product termed dentin sialophosphoprotein (DSPP) that would be specifically cleaved into two proteins, DSP and DPP. Human genetic studies have shown the association of mutations in the *DSPP* gene with dentinogenesis imperfecta (DGI), an autosomal dominant disorder causing dentin hypomineralization and significant tooth decay [5], [6]. *Dspp* ablation studies showed that *Dspp*-deficient mice have defects in the mineralization of dentin [7] and bone [8]. The predentin in the *Dspp* null mice is hypomineralized (widening of predentin), giving rise to phenotypic changes similar to the manifestations of DGI in humans. This information indicates that DSPP is critical for dentin mineralization.

Although DSP and DPP are derived from the same protein precursor, their biochemical properties are very different. DSP, a sialic acid-rich glycoprotein with few or no phosphate groups [9], [10], accounts for 5–8% of the dentin non-collagenous proteins. This protein shares overall characteristics with other sialoproteins (OPN and BSP) present in dentin and bone. Recently we discovered that the majority of the NH₂-terminal fragment of rat or mouse DSPP is present as the proteoglycan (PG) form containing two glycosaminoglycan (GAG) chains [11], [12]. DPP is the most abundant non-collagenous protein in the ECM of dentin, accounting for as much as 50% of the NCPs [13]. DPP contains a large number of aspartic acid (Asp) and serine (Ser) residues, with the majority of Ser being phosphorylated [14], [15]. The Asp and phosphoserine (Pse) residues are mostly present in the repeating sequences of DSS and DS. The rat DPP occurs in three forms with different levels of phosphorylation; the highly phosphorylated DPP has approximately 200 phosphates attached to the molecule [14]. The repeating sequences of (Asp-Pse-Pse)_n and (Asp-Pse)_n fit well with the purported function of DPP in the nucleation and modulation of hydroxyapatite crystal formation [2].

The NH₂-terminal sequence of rat DPP determined by Edman degradation was Asp-Asp-Pro-Asn for the highly phosphorylated form of rat DPP [14], and to be Asp-Asp-Pro in human [16]. The beginning of the DPP portion of mouse DSPP was Asp-Asp-Pro [3]. The NH₂-terminal sequences for rat [14], [17], [18] and mouse DPP [3], [4] establish that the cleavage sites of DSPP are NH₂-terminal to residue 448 in rat or residue 452 in mouse DSPP. Earlier protein chemistry work in our laboratory indicated that proteolytic processing of rat DSPP to DSP and DPP involves the cleavage of the peptide bonds Gly⁴⁴⁷-Asp⁴⁴⁸, Tyr⁴³⁸-Asp⁴³⁹ and His⁴²³-Ser⁴²⁴[14], [19]. Recent studies indicated that the proteolytic processing of DSPP was catalyzed by bone morphogenetic protein 1 (BMP1)/Tolloid-like metalloproteinases [20], [21] and the astacin proteases [22]. However, the cleavage sites involved in the proteolytic processing of DSPP by BMP1 are not totally clear and the cleavage of DSPP by BMP1 has not been verified by *in vivo* studies.

The aims of this study were: 1) to examine if substitution of residue Asp⁴⁵² by Ala⁴⁵² blocks the *in vivo* processing of mouse DSPP, 2) to test if the D452A substitution blocks the

cleavage of DSPP by BMP1, and 3) to determine if DSPP has peptide bonds, other than the one at the NH₂-terminus of Asp⁴⁵² in the mouse DSPP, which can be cleaved by BMP1.

Materials and Methods

Generation of Transgenic Mice Expressing Normal DSPP or D452A-DSPP

Site-directed mutagenesis was performed on pcDNA3-DSPP construct to generate the cDNA encoding a mutant DSPP in which Asp⁴⁵² was substituted by Ala⁴⁵² (designated “D452A-DSPP”) [20]. A pBCKS construct containing a 3.6-kb rat *Col 1a1* promoter upstream of the mouse DMP1 cDNA [23] was used to generate the targeting transgene. The normal DSPP cDNA and D452A-DSPP cDNA were inserted downstream of the 3.6-kb rat *Col 1a1* promoter in pBCKS construct, respectively. The restriction enzymes SacII and Sall (New England Biolabs) were used to release an 8.6-kb fragment containing the 3.6-kb rat *Col 1a1* promoter, 1.6-kb intron 1 of the *Col 1a1* gene, 3-kb mouse DSPP cDNA and a SV40 later poly A tail from the construct. This 8.6-kb fragment, referred to as the “normal DSPP transgene” or “D452A-DSPP transgene”, was used for pronuclear injection at the University of Texas Southwestern Medical Center at Dallas to generate transgenic founders in the C57BL/6J background. We obtained four independent founders expressing the normal DSPP transgene (Tg) and three founders expressing DSPP-D452A Tg. DSPP mRNA in the long bone of the transgenic mice was detected using reverse transcription polymerase chain reaction (RT-PCR) analyses. The protocol of animal work was approved by the Animal Welfare Committee of Baylor College of Dentistry of the Texas A&M University Health Science Center.

Quantitative Real-time Reverse Transcription PCR (qRT-PCR) and Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The qRT-PCR analyses were performed to measure the expression levels of DSPP in the long bone of the transgenic mice. The primers used for qRT-PCR were: forward, 5′AACTCTGTGGCTGTGCCTCT-3′ (in exon 3) and reverse, 5′TATTGACTCGGAGCCATTCC-3′ (in exon 4). The qRT-PCR reactions were performed using Brilliant SYBR Green QPCR Master Mix (Applied Biosystems; Foster City, California) and the Stratagene MX4000 Real-Time PCR Detection System. GAPDH was used as the control. The qRT-PCR conditions were: initial denaturation at 95°C for 10 min, followed by 45 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 30 s. The mean values from triplicate analyses were then calculated and compared. RT-PCR was performed to examine the expression of the exogenous DSPP transgene in bone and tooth. The primers used for RT-PCR were: forward, 5′-AGCTTGGTACCGAGCTCGGATCCCC (in the linker region between rat *Col 1a1* promoter and DSPP cDNA) and reverse, 3′-TCATTGTGTTCTTCAGCAGTGTTC (in exon 4). The reaction conditions were: 95°C for 30 s, 56 °C for 30 s and 72 °C for 30 s, 30 cycles.

Extraction of NCPs and Detection of DSPP-Related Proteins

NCPs were extracted from the long bones of 3-week-old normal DSPP Tg mice or D452A-DSPP Tg mice, as previously described [24]. The NCPs in the extracts were separated by a 10 ml Q-Sepharose (Amersham Biosciences) column connected to a fast protein liquid chromatography (FPLC) system (GE Healthcare). The gradient employed for the Q-Sepharose anion-exchange chromatography was 0.1–0.8 M NaCl in 6 M urea solution at pH 7.2. NCPs including DSPP-related proteins eluted into 120 0.5 ml fractions. The full-length form of DSPP and its processed fragments were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting. For Western immunoblotting, we used the polyclonal anti-DSP antibody. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) was employed as the secondary antibody for the

Western immunoblotting analysis. The blots were incubated in the chemiluminescent substrate CDP-star (Ambion) for 5 min and exposed to X-ray films (Kodak).

Substitutions of Amino Acid Residues at Potential DSPP Cleavage Sites

To identify the peptide bonds that were potential DSPP cleavage sites, we performed site-directed mutagenesis to substitute candidate residues in the mouse DSPP cDNA [20]. Totally, we generated nine mutant-DSPP constructs in which Asp residues at candidate sites were substituted with Ala. These substitution constructs (each representing one mutant DSPP) include: 1) D452A-DSPP [20], 2) H227A-D452A-DSPP, 3) E443A-D452A-DSPP, 4) D445A-D452A-DSPP, 5) D446A-D452A-DSPP, 6) D445A-D446A-D452A-DSPP, 7) D452A-D453A-DSPP, 8) D452A-D487A-DSPP, and 9) D452A-D491A-DSPP. The constructions of D452A-DSPP, H227A-D452A-DSPP and E443A-D452A-DSPP were based on our previous protein chemistry work [19], and the designs for the other six mutant constructs were based on the understanding of the sequences known to be cleaved by BMP1. These nine pcDNA-DSPP constructs were used to transfect human embryonic kidney 293-EBNA (HEK293-EBNA) cells using lipofectamine 2000 (Invitrogen). The cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (Invitrogen). The medium was replaced with serum-free medium 16–18 hrs after transfection, and then the transfected cells were cultured for an additional 72 hrs. The medium was collected and analyzed for DSPP and/or its processed fragments.

Digestion of DSPP by BMP1

The cell culture medium or the protein sample from the chromatographic fractions containing the full-length form of DSPP was dialyzed for 72 hours at 4°C and then lyophilized. The lyophilized powder was reconstituted in BMP1 digestion buffer [25]. The prepared protein samples were incubated with recombinant human BMP1 (R&D Systems) for 21 hrs at 37°C. The digestion products were analyzed by Western immunoblotting using the polyclonal anti-DSP antibody [20].

Results

Expression of Normal DSPP and D452A-DSPP Transgene in the Transgenic Mice The expression of the normal DSPP and D452A-DSPP transgene in the transgenic mice was confirmed by qRT-PCR and RT-PCR, using RNAs extracted from the long bone of three lines of normal DSPP transgenic mice and four lines of the D452A-DSPP transgenic mice. Although different expression levels for the transgene were observed among the transgenic mice, they all contained higher levels of DSPP mRNA compared to the wild type mice (Fig. 1a), indicating that the transgene was active. It should be noted that the expression of endogenous DSPP in the long bone of wild type animals is very low [26]. The primers for RT-PCR were designed specifically to detect exogenous transgene as shown in Figure 1b, whereas the endogenous DSPP transcripts could not be detected using these primers (Fig. 1b).

Full-length DSPP and its Fragments in the ECM Extracts of Mouse Long Bone

We extracted NCPs from the long bone of line 4 (Fig. 1a) for D452A-DSPP analysis and that of line 7 (Fig. 1a) for analyzing the normal DSPP. Q-Sepharose chromatography separated the long bone extracts into 120 fractions (Fig. 2). Stains-All staining and Western immunoblotting were used to detect normal DSPP as well as D452A-DSPP and to determine if the mutant D452A-DSPP was processed into DSP and DPP fragments. All of the chromatographic fractions from the bone extracts that might potentially contain DSPP-related products were analyzed; to illustrate the results, fraction 34 was shown in this report. Both the fragment (DSP) and the full-length form of DSPP were observed in the long bone

extracts of D452A-DSPP Tg as well as in the normal DSPP Tg mice. The ratio of full-length form of DSPP to DSP in the D452A-DSPP Tg mice was much greater than that in the normal DSPP-Tg mice. Significant amounts of the full-length DSPP were detected in the D452A-DSPP Tg mice, while very weak protein bands matching the NH₂-terminal fragment of DSPP were observed, indicating that D452A-DSPP was present mainly as the full-length form. Only a small portion of the mutant D452A-DSPP was cleaved *in vivo* (Fig. 3).

BMP1 Completely Cleaves Normal DSPP, and Partially Cleaves D452A-DSPP

The *in vitro* enzyme assay experiments showed that the full-length form of DSPP extracted from the wild type C57BL/6 mice was completely processed by BMP1, whereas the full-length form of DSPP extracted from the long bone of D452A-DSPP Tg mice was partially cleaved (Fig. 4).

Substitutions of Amino Acid Residues at Potential DSPP cleavage Sites Were Unable to Completely Block DSPP Processing

Western immunoblotting analyses showed that the culture medium of the HEK-293 EBNA cells transfected with the normal pcDNA3-DSPP or the mutant constructs contained significant amounts of full-length DSPP and/or its fragments. When treated with BMP1, the full-length form of normal DSPP disappeared, whereas the majority of the full length-form of mutant D452A-DSPP was still present, indicating that this substitution partially blocked the proteolytic processing of DSPP. Nevertheless, a portion of mutant DSPP were cleaved suggesting that there might be unidentified “secondary” or “cryptic” cleavage site(s), in addition to the peptide bond at the NH₂-terminus of Asp⁴⁵². To uncover the secondary cleavage sites, we generated and analyzed eight more DNA constructs expressing DSPP with substitutions at the potential secondary cleavage sites. None of the substitutions could completely block DSPP processing (Fig. 5).

Discussion

DSPP belongs to the SIBLING family. Although details concerning their precise functions are unknown, the common features of SIBLING proteins indicated that they may share some common mechanisms in the biomineralization of dentin and bone. DMP1, DSPP, MEPE are all cleaved into NH₂-terminal and COOH-terminal fragments, indicating that the proteolytic processing of these proteins may play important roles in the formation of dentin and bone. We have hypothesized that the proteolytic processing of DSPP and DMP1 is an activation step converting the protein precursors to functional fragments. This hypothesis has been proven in our previous study showing that failure to process DMP1 into fragments leads to its loss of function in osteogenesis [27] and dentinogenesis [28].

Several research groups have performed studies to analyze the cleavage sites during DSPP processing. Earlier work in our group revealed that three peptide bonds were cleaved during the processing of rat DSPP [14], [19]. An *in vitro* study showed that matrix metalloproteinases (MMP-2 and MMP-20) cleaved the NH₂-terminal portion of porcine DSPP (namely, DSP and dentin glycoprotein) at multiple sites, yielding several low molecular-weight fragments [29]. Another *in vitro* study [30] showed that a recombinant rat DSP-PP (a shorter form of the rat DSPP variants) underwent self-processing; one of the self-cleavages appeared to be at Gly⁴⁴⁷-Asp⁴⁴⁸, which corresponds to Gly⁴⁵¹-Asp⁴⁵² in the mouse DSPP. In a previous investigation, we and another group have found that BMP1 is responsible for the proteolytic processing of DSPP into DSP and DPP [20], [21]. The astacin proteases were also reported to cleave DSPP to generate DPP [22]. We have found that the substitution of Asp⁴⁵² to Ala⁴⁵² could block the proteolytic processing of DSPP in HEK293-EBNA cells [20]. In this study, we observed that the D452A-DSPP from transfected

HEK293-EBNA cells was partially processed when treated with recombinant human BMP1 *in vitro*, while normal DSPP was completely cleaved by BMP1 [20].

In this study, transgenic mice were generated to express normal DSPP or D452A-DSPP (mutant) driven by the type I collagen promoter. A great level of variation in the transgene expression was observed among different transgenic mouse lines (particularly with regard to the expression of the normal DSPP transgene). In the generation of these transgenic mice, the normal DSPP or D452A-DSPP transgene (cDNA) was randomly inserted into the mouse genome. The variation of in the expression level of the transgene was likely caused by the difference in the localization of the cDNA inserted in the mouse genome. Different insertion sites might have either enhancing or inhibiting effects on the transcription of the exogenous genes due to the local genomic environment. In the D452A-DSPP transgenic mice, the D452A substitution could not completely block the proteolytic processing of mouse DSPP and the full-length D452A-DSPP isolated from the mutant mice were also partially cleaved when treated with BMP1 *in vitro*. As we were performing the mutation analyses, another group reported that DSPP was processed into DSP and DPP by BMP1, and a combined substitution of M449I and Q450E could completely block DSPP processing *in vitro* [21]. In the present study, we also included M449I and Q450E substitutions to test if BMP1 could digest DSPP with the double mutations; we found that this combination of mutations (M449I+Q450E) was unable to completely block DSPP processing. These results showed strong evidence that there must be secondary, cryptic cleavage site(s), in addition to Gly⁴⁵¹-Asp⁴⁵².

According to the sequence characteristics of the known substrates of BMP1, the presence of aspartate residues at P1 positions and of residues with aromatic side chains or methionines at P2–P4 positions are the most common features of the cleavage sites in the substrates of the BMP1/Tolloid-like proteinases [25]. Based on these rules, we searched the sequence surrounding the Asp⁴⁵² and found several amino acid sequence clusters showing some degree of similarity to those known cleavage-sequences of BMP1. We did site-directed mutagenesis to substitute these potential secondary cleavage sites. The different types of mutant DSPP molecular species made by HEK293-EBNA cells were treated with BMP1 *in vitro* to see if the cleavage of these mutant DSPP molecules was totally blocked. However, we found that none of the combined mutations, containing potential secondary cleavage site mutations and the D452A substitution could completely block DSPP processing. Interestingly, in the Western immunoblotting analyses, after the dialysis and freeze-drying of the mutant DSPP samples, we detected a certain portion of cleaved fragment (DSP) even though these samples were not treated with BMP1 (Fig. 5), whereas no DSP fragments could be detected before dialysis and freeze-drying (data not shown). One interpretation is that before the dialysis and freeze-drying, the concentration of the endogenous BMP1 in the medium was too low to initiate the cleavage reaction. After the dialysis, freeze-drying and reconstitution, the concentration of the endogenous BMP1 from the culture medium was increased and the salt concentration was lowered, which helped to increase the BMP1 activity [21]. Another possibility is that besides BMP1, other enzymes in the astacin family may also cleave DSPP [22], or perhaps DSPP might have undergone self-processing [30] at sites other than the NH₂-terminus of Asp⁴⁵².

It should be noted that the molecular weight of the newly formed DSP fragments by BMP1 digestion was consistent with those of the endogenous DSP generated from the HEK-293-EBNA cells transfected with normal DSPP construct, indicating that the unidentified secondary cleavage site(s) must be very close to Asp⁴⁵² in mouse DSPP.

Acknowledgments

This work was supported by Grant DE005092 (to CQ) from the National Institutes of Health and Grant 30700943 from National Natural Science Foundation of China.

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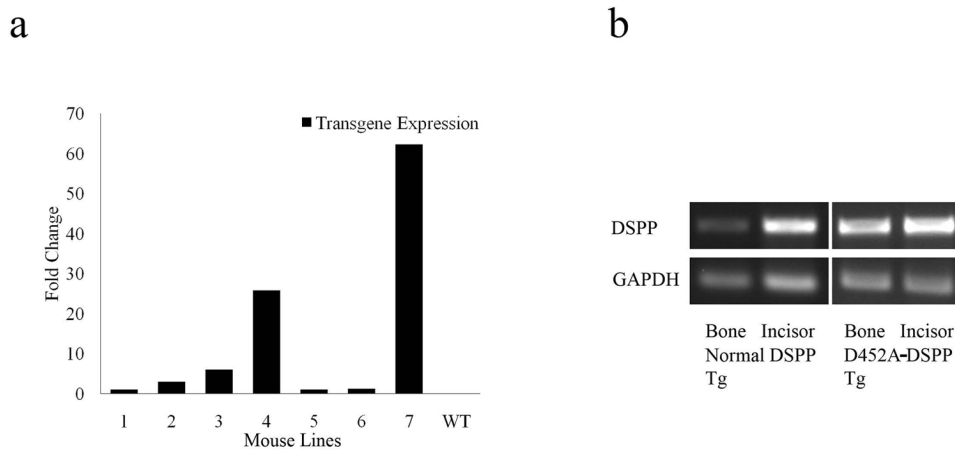


Fig. 1. Expression of normal-DSPP and D452A-DSPP in the bone of transgenic mice. a: Real time PCR showing the different expression levels in the long bone from four lines of D452A-DSPP transgenic mice (1–4) and three lines of normal DSPP transgenic mice (5–7). The expression level of line 5 was set as 1. b: RT-PCR demonstrating the expression of normal DSPP transgene as well as D452A-DSPP transgene in the long bone and incisor of the transgenic mice. Note that only the exogenous DSPP mRNAs could be detected using the primers specifically designed for the transgenic transcripts

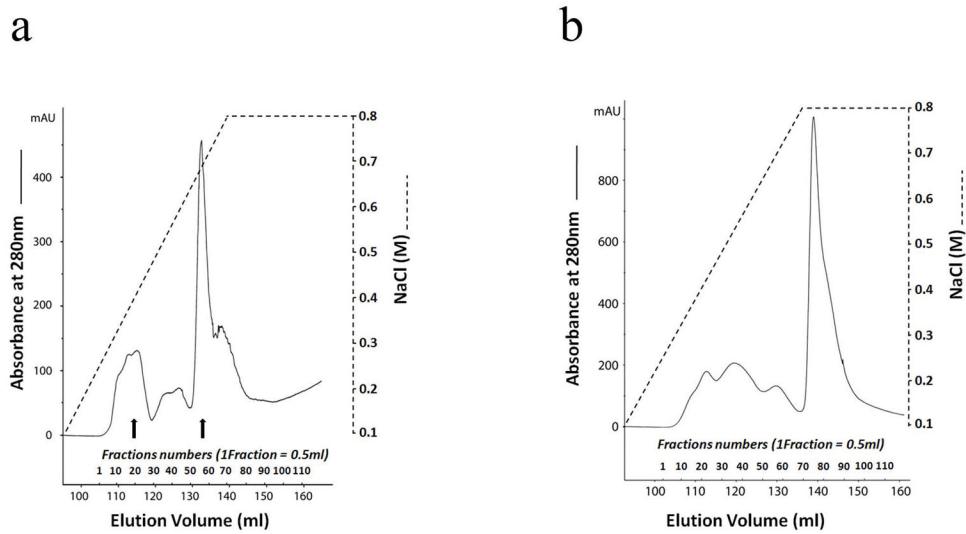


Fig. 2. FPLC profile of Q-Sepharose chromatography for acidic proteins extracted from the mouse long bone. Protein extracts were separated and collected into continuous 120 fractions for biochemistry analyses. a: FPLC chromatogram for bone extracts from D452A-DSPP transgenic mice. b: FPLC chromatogram for bone extracts from wild type mice. Western immunoblotting and Stains-All staining illustrated that the fractions between the two arrows (fractions 20 to 58) in (a) contained DSPP and its fragments, DSP and DPP.

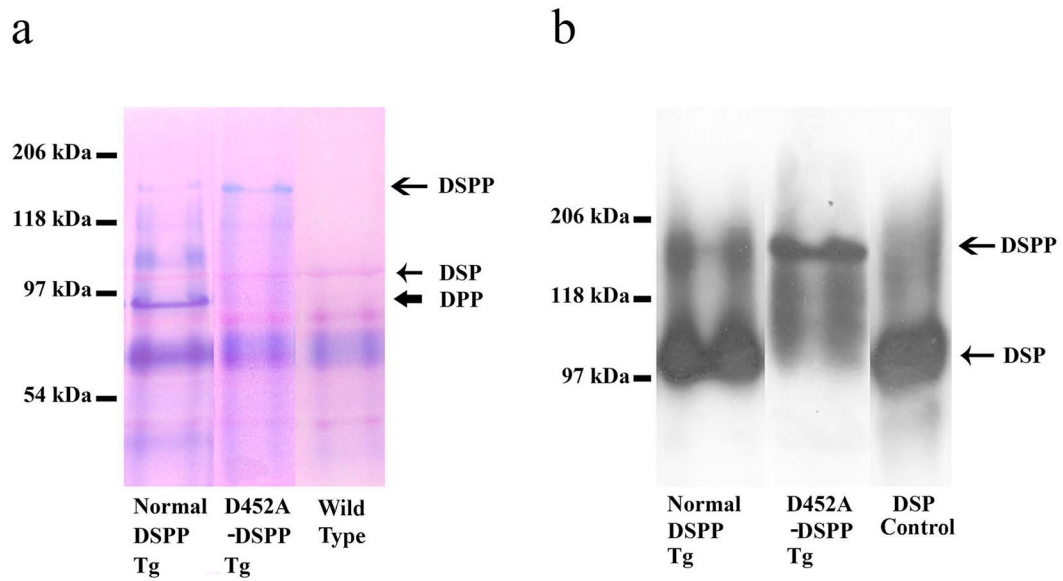


Fig. 3.

Fraction 34 from the Q-Sepharose chromatography separations was used to demonstrate the acidic proteins between the three types of mice. We loaded the normal DSPP lane and D452A DSPP lane with equal volume (60 μ l) of the sample from the fraction 34 of the long bone extracts of normal DSPP or D452A-DSPP transgenic mice and for each sample, the non-collagenous proteins were extracted from the long bone of six mice. a: acidic proteins (including the SIBLING members) were visualized by Stains-All staining (blue and purple bands). An extra blue band (DPP) and weak staining of full-length form of DSPP were detected in the long bone extracts of normal-DSPP transgenic mice, whereas a weak staining of DPP and a stronger full-length form of DSPP were detected in the D452A-DSPP transgenic mice. The normal DSPP sample was from transgenic mice expressing the normal DSPP. The D452A-DSPP was from the transgenic mice expressing D452A-DSPP. The wild type sample was from the C57BL/6J mice. DSP (less acidic than DPP) is much less sensitive to Stains-All staining. The purple protein band indicated by the thin short arrow was likely to be DSP. In each lane, 60 μ l of sample was loaded. b: DSPP-related proteins in the long bone of the transgenic mice (Western blotting). The ratio of DSP to DSPP in the normal-DSPP transgenic mice is much greater than that in the D452A-DSPP transgenic mice, indicating that the D452A substitution effectively blocked DSPP processing *in vivo*. Normal DSPP or D452A-DSPP lane: 60 μ l of sample. DSP lane: 0.1 μ g of rat DSP

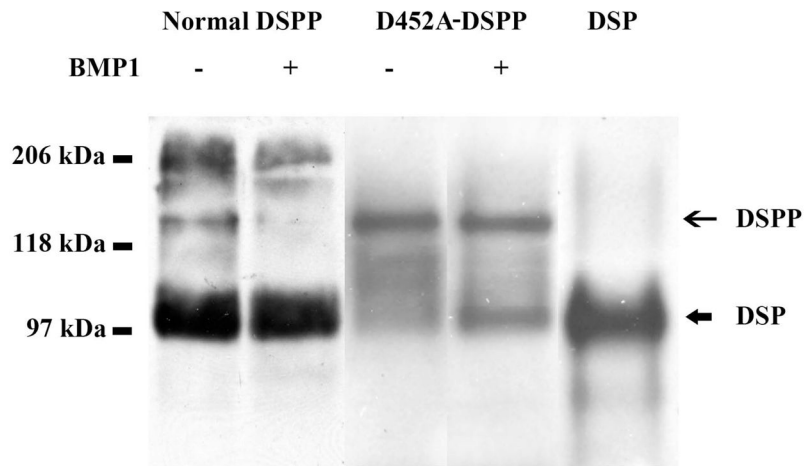
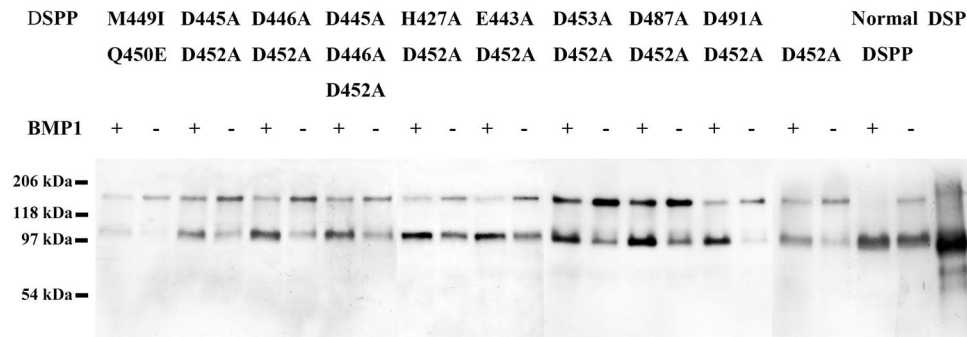


Fig. 4. Full-length form of DSPP from the long bone of the D452A-DSPP transgenic mice as well as from wild type mouse dentin was treated with BMP1. DSPP-related proteins were detected by Western immunoblotting using anti-DSP polyclonal antibody. The full-length form of normal DSPP was totally processed, whereas the full-length form of D452A-DSPP were partially cleaved, indicating that D452A substitution could partially block DSPP processing by BMP1. The differences in the ratio of full-length DSPP to DSP indicated that more DSPP molecules were cleaved into DSP in the wild type mice than in the transgenic mice expressing the mutant D452A-DSPP. Normal DSPP or D452A-DSPP lane: 60 μ l of sample. DSP lane: 0.1 μ g of rat DSP.



NH₂-AHSNLG**H**SRIGSSNSDGHDSY**E****F****D****D**ESMQG**D****D**PKSSDESNGSDESDTNSESANES
 GSRGDASYTS**D**ESS **D****D****D**NDS**D**SHA-COOH

Fig. 5. Mutant mouse DSPP from HEK-293 EBNA cells transfected with pcDNA3-mutant DSPP constructs was treated with BMP1. DSPP-related proteins were identified using the anti-DSP polyclonal antibody in Western immunoblotting analyses. All of these different types of DSPP proteins were partially cleaved by BMP1 digestion, implying that BMP1 cleaves DSPP at secondary, unidentified peptide bond(s), in addition to that at the NH₂-terminus of Asp⁴⁵². The sample for each lane was equal to 1 ml of culture medium (concentrated by dialysis and lyophilization). 0.1 μg of rat DSP was loaded in DSP lane. The substitution sites we designed in the mouse DSPP linker region were shown in bold and enlarged font