Proteolytic Processing of Dentin Sialophosphoprotein (DSPP) Is Essential to Dentinogenesis*

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Background: In dentin and bone, dentin sialophosphoprotein (DSPP) is processed into the NH2-terminal and COOH-terminal fragments.

Results: The blocking of DSPP processing leads to hypomineralization defects in dentin, similar to those of Dspp-deficient mice.

Conclusion: The proteolytic processing of DSPP is an activation step essential to dentinogenesis.

Significance: This study represents major progress in understanding how DSPP functions in dentinogenesis.

Dentin sialophosphoprotein (DSPP) mRNA was first identified by cDNA cloning using a mouse odontoblast cDNA library in 1997 (1). However, dentin sialoprotein (DSP) and dentin phosphophosphoprotein (DPP), the cleaved products of the DSPP protein, were discovered much earlier and were believed to be separate entities until the single DSPP transcript was discovered (1, 2, 3). Human genetic studies have shown that DSPP mutations are associated with dentinogenesis imperfecta (DGI), an autosomal dominant inherited disease characterized by dentin hypomineralization and significant tooth decay (4–11). Animal studies revealed that Dspp knock-out (Dspp-KO) mice manifest hypomineralization defects in dentin. The widened predentin with irregular dentin mineralization in the Dspp-KO mice resembles the dentin defects of human DGI (12). These findings from human subjects and mouse models indicate that DSPP is critical for the formation and mineralization of dentin. However, the exact mechanistic steps by which DSPP functions in dentinogenesis remain largely unknown.

In dentin and bone, DSP is proteolytically processed into the NH2-terminal and the COOH-terminal fragments (1, 13, 14). The NH2-terminal fragment of DSP in vivo mineralization analyses (22). However, informa-

dentin phosphophosphoprotein (DPP), the cleaved products of the DSP protein, were discovered much earlier and were believed to be separate entities until the single DSPP transcript was discovered (1, 2, 3). Human genetic studies have shown that DSPP mutations are associated with dentinogenesis imperfecta (DGI), an autosomal dominant inherited disease characterized by dentin hypomineralization and significant tooth decay (4–11). Animal studies revealed that Dspp knock-out (Dspp-KO) mice manifest hypomineralization defects in dentin. The widened predentin with irregular dentin mineralization in the Dspp-KO mice resembles the dentin defects of human DGI (12). These findings from human subjects and mouse models indicate that DSPP is critical for the formation and mineralization of dentin. However, the exact mechanistic steps by which DSPP functions in dentinogenesis remain largely unknown.

In dentin and bone, DSP is proteolytically processed into the NH2-terminal and the COOH-terminal fragments (1, 13, 14). The NH2-terminal fragment of DSP encoded by the 5′ portion of the DSPP transcript exists in two forms: the core protein form known as “dentin sialoprotein” (DSP) and the proteoglycan form referred to as “DSP-PG” (15–19). The COOH-terminal fragment of DSP encoded by the 3′ region of the DSPP transcript is found in only one form, referred to as “dentin phosphophosphoprotein” (DPP).

DSP isolated from the extracellular matrix (ECM) of rat den-
tin migrates at ~95 kDa on 5–15% SDS-PAGE (20). DSP accounts for 5–8% of the non-collagenous proteins (NCPS) in the ECM of rat dentin (21), while DSP-PG appears to be more abundant than DSP (15, 19). The two glycosaminoglycan (GAG) chains of DSP-PG isolated from rat dentin are made of chondroitin-4-sulfate, and the two GAG chains of mouse DSP-PG are attached to S242 and S254 in the mouse DSP sequence (17). In porcine dentin, the DSP-PG GAG chains appear to be made of chondroitin-6-sulfate (19). DSP, which contains few or no phosphates, has no significant effect on the formation and growth of hydroxyapatite (HA) crystals according to in vitro mineralization analyses (22). However, information regarding the effects of DSP-PG on the formation and

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3 The abbreviations used are: DSPP, dentin sialophosphoprotein; DSP, dentin phosphoprotein; DGI, dentinogenesis imperfecta; DSP-PG, proteoglycan form of DSPP; ECM, extracellular matrix; NCPS, non-collagenous proteins; GAG, glycosaminoglycan; BMP1, bone morphogenetic protein 1; β-ME, β-mercaptoethanol; H&E, hematoxylin & eosin; IHC, immunohistochemistry; SEM, scanning electron microscopy; MMA, methyl-methacrylate.
growth of HA crystals is lacking. In vivo studies involving the transfer of a transgene encoding the NH$_2$-terminal fragment of DSPP into the Dspp-KO background indicate that this fragment might regulate the initiation of dentin mineralization but not the maturation of mineralized dentin (23).

DSPP, which accounts for as much as 50% of the NCPs in the ECM of rat dentin (24), contains large amounts of aspartic acid (Asp) and serine (Ser) residues, with the majority of Ser being phosphorylated (25, 26). The Asp and phosphorylated Ser (Pse) residues are mostly present in the repeating sequences of (Asp-Pse-Pse)n and (Asp-Pse)n (1, 13, 14, 27–29). The high levels of Asp and Pse give rise to a highly phosphorylated (25) and very acidic protein with the isoelectric point estimated to be 1.1 for rat DPP (30). DPP has a relatively high affinity to calcium (31, 32) and is believed to have a direct role in controlling the rate and/or site of dentin mineralization (3, 33, 34). Several in vitro mineralization studies have indicated that DPP is an important initiator and modulator in the formation and growth of HA crystals (35–37).

The remarkable chemical differences between the NH$_2$-terminal fragment (including DSP and DSP-PG) and the COOH-terminal fragment (DPP) of DSPP suggest that these molecular variants may perform different functions in biomineralization although they are derived from the same mRNA. Studies have shown that significant amounts of DSP, DSP-PG, and DPP are present in the ECM of dentin, whereas a very minor quantity of the full-length form of DSPP is found in the dentin (16, 38). The abundance of DSPP fragments, along with the scarcity of full-length DSPP in the dentin, suggests that the processed fragments of DSPP may be the functional forms directly involved in biomineralization.

Previous in vitro studies by our group and others have shown that bone morphogenetic protein 1 (BMP1)/Tolloid-like metalloproteinases cleave mouse DSPP at the NH$_2$ terminus of Asp$^{452}$, while substitutions of Asp$^{452}$ or two residues that are immediately NH$_2$-terminal to Asp$^{452}$, block the processing of this protein partially or completely (38, 39, 40). More recently, we generated transgenic mice expressing a mutant DSPP in which Asp$^{452}$ was replaced by Ala$^{452}$; the transgene expressing we generated transgenic mice expressing a mutant DSPP in this protein partially or completely (38, 39, 40). More recently, transfer of a transgene encoding the NH$_2$-terminal fragment of DSPP into the 3.6-kb rat Col 1a1 promoter, which allows the expression of DSPP in the mouse bone (40). In the present study, we systematically characterized the dentin of mice expressing D452A-DSPP in the wild type background, indicating that the D452A substitution effectively blocked the proteolytic process-

EXPERIMENTAL PROCEDURES

Generation of Dspp-KO/D452A-Tg and Dspp-KO/normal-Tg Mice—The generation of transgenic mice expressing the transgene encoding D452A-DSPP or the transgene encoding normal DSPP in the wild type (WT) background has been described in our previous report (40). In these transgenic mice, the D452A-DSPP or normal DSPP transgene is downstream to the 3.6-kb rat Col 1a1 promoter, which drives the expression of the transgenes in type I collagen-expressing tissues, including bone and dentin. The mouse lines showing the highest expression level of D452A-DSPP (i.e. line 4 in Zhu et al., Ref. 40) or of normal DSPP (line 7 in Zhu et al., Ref. 40) in the long bone were crossbred with Dspp knock-out (Dspp-KO) mice (strain name: B6; 129-Dspptm1Kul/Mmnc; MMRRC, UNC, Chapel Hill, NC). The first crossbreeding generated Dspp-Tg:Dspp$^{+/−}$ mice. Then, the Dspp-Tg:Dspp$^{+/−}$ mice were mated with Dspp$^{−/−}$ mice to generate mice expressing the D452A-DSPP or normal DSPP transgene in the Dspp-KO background (i.e. without the endogenous Dspp gene). The mice expressing the D452A-DSPP transgene in the Dspp-KO background are referred to as Dspp-KO/D452A-Tg mice while those expressing the normal DSPP transgene in the Dspp-KO background are called Dspp-KO/normal-Tg mice. The polymerase chain reaction (PCR) primers for detecting the Dspp transgene were: forward, 5′-CCAGTTAGTACCACTGGAAAGAGAC-3′; reverse, 5′-TCATGGTTGGTGTACTTTGCATGC-3′ (the expected PCR products when using mouse genomic DNA as the template were 521 bp for the transgene and 676 bp for the endogenous Dspp gene). The primers used to identify the endogenous Dspp alleles were: forward, 5′-GTATCTTCTAC-
GCTGTTGCTTC-3’; reverse, 5’-TGTGTTTGCTTCATCGAGA-3’ (expected PCR product from the endogenous Dspp, 489 bp). The primers specific to the Dspp null allele (containing LacZ gene) in the Dspp-KO mice were: forward, 5’-GTATCTTCATGGCTGTTGCTTC-3’ from the Dspp sequence; reverse, 5’-CCTCTTCGCTATTACGCCAG-3’ from the LacZ sequence (expected size of PCR product, 389 bp). The animal protocols used in this study were approved by the Animal Welfare Committee of Texas A&M Health Science Center Baylor College of Dentistry (Dallas, TX). Multiple approaches were used to characterize the mandibles of the following four types of mice: 1) Dspp-KO/D452A-Tg mice, 2) Dspp-KO/normal-Tg mice, 3) Dspp-KO mice, and 4) WT mice (C57/BL6J mice).

Expression Levels of the DSPP Transgenes in Teeth—Quantitative real-time PCR was performed to evaluate the relative levels of DSPP mRNA in the incisors of the 1-month-old Dspp-KO/D452A-Tg, Dspp-KO/normal-Tg and WT mice. For real-time PCR analyses, total RNA was extracted from the mouse incisors with an RNeasy mini kit (Qiagen, Germantown, MD). The RNA (1 μg/per sample) was reverse-transcribed into cDNA using the QuantiTect Rev Transcription Kit (Qiagen). The DSPP primers used for real-time PCR were: forward, 5’-AACTCTGTGGCTGTGCCTCT-3’ (in exon 3) and reverse, 5’-TATTGACTCGGAGCCATTCC-3’ (in exon 4). The real-time PCR reactions were performed as we previously reported (41).

Extraction and Isolation of Noncollagenous Proteins (NCPs) from Mouse Dentin and Detection of DSPP-related Proteins—The NCPs, including DSPP-related proteins in the dentin, were extracted from the incisors of the 3-month-old mice. Detailed protocols for the extraction of NCPs from mouse incisors have been described in our previous publications (38). The incisor extracts were separated into 118 fractions (0.5 ml/fraction) by Q-Sepharose ion-exchange chromatography (Amersham Biosciences; Uppsala, Sweden) with a gradient ranging from 0.1 to 0.8 M NaCl in 6 M urea solution (pH 7.2). Equal amount of sample (60 ul) from each of the fractions that potentially contained DSPP-derived products was loaded onto 5–15% SDS-PAGE. The dotted arrows denote DSPP, while the hollow arrows indicate DSP; their identities as DSPP and DSP were confirmed by Western immunoblotting (see Fig. 3). The major blue bands in fractions 47–65 in the WT and Dspp-KO/normal-Tg mice (solid arrows) was primarily made of DPP, although these bands also contained a small amount of bone sialoprotein (BSP), which was confirmed by anti-BSP Western immunoblotting (data not shown). It should be noted that no anti-DPP antibodies are available to detect DPP in Western immunoblotting analyses. Note the abundance of DSP and DPP in the samples from the WT and Dspp-KO/normal-Tg mouse incisors as well as the large amounts of full-length DSPP in the samples from the Dspp-KO/D452A-Tg mice. M, molecular weight standard; DSP, pure DSP isolated from rat dentin; DPP, pure DPP isolated from rat dentin.
3% β-mercaptoethanol (β-ME) and then loaded onto 5–15% SDS-PAGE. Stains-all staining was performed to visualize all the acidic NCPs in each fraction, and Western immunoblotting was used to detect the DSPP-related proteins in the fractions containing these molecules. For Western immunoblotting, we used the polyclonal anti-DSP antibody [20] at a dilution of 1:2000. Alkaline phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich) was employed as the secondary antibody for the Western immunoblotting analyses. The blots were incubated in a chemiluminescent substrate CDP-star (Ambion, Austin, TX) for 5 min and exposed to x-ray films. The image J software program was employed to scan the positive bands and measure their densities and areas for calculating the ratio of DSP to DSPP in each analysis.

Plain X-ray Radiography and Micro-computed Tomography (μ-CT)—The mandibles from the 3-month-old and 6-month-old mice were dissected from the four groups of mice and analyzed with the Faxitron MX-20 Specimen Radiography System (Faxitron x-ray Corp., Buffalo Grove, IL). For the μ-CT analyses, the mandibles were scanned using μ-CT35 imaging system (Scanco Medical, Basserdorf, Switzerland), as we previously described [42]. In the μ-CT program, a scan of the whole mandible in 7.0-μm slice increments was selected for three-dimensional reconstructions to assess the shape and structure of the mouse mandibles.

Histology and Immunohistochemistry—Under anesthesia, the Dspp-KO/D452A-Tg, Dspp-KO/normal-Tg, Dspp-KO and WT mice at the ages of postnatal 3 and 6 months were perfused from the ascending aorta with 4% paraformaldehyde in 0.1M phosphate buffer. The mandibles were dissected and further fixed in the same fixative for 24 h, and then decalcified in 8% EDTA containing 0.18 M sucrose (pH 7.4) at 4 °C for approximately 2 weeks. The tissues were subsequently processed for paraffin embedding, and serial sections of 5 μm were prepared.

FIGURE 3. Western immunoblotting to detect DSPP and DSP in the NCP extracts from mouse dentin. Western immunoblotting with polyclonal anti-DSP antibodies was used to detect DSPP and DSP in the dentin extracts from the four types of mice. The partially purified rat dentin extract (0.1 μg) containing rat DSPP and DSP was used as a positive control (Ctrl). The Western immunoblotting results from a representative fraction (fraction 29, 60 μl) are shown here. While DSP (hollow arrow) and DSPP (dotted arrow) were detected in the dentin extracts from the Dspp-KO/D452A-Tg, Dspp-KO/normal-Tg, and WT mice, the ratios of DSP to DSPP among these three types of samples were remarkably different. Based on our integrated calculation from triplicate analyses (n = 3) using the image J program, we estimated that the ratio of DSP to DSPP in the Dspp-KO/D452A-Tg mice was 1:10, while that in the Dspp-KO/normal-Tg mice was 15:1. In other words, if the quantity of DSP was used for normalization, the amount of DSPP in the Dspp-KO/D452A-Tg mice would be ~150 times that of the full-length protein in the Dspp-KO/normal-Tg mice. The findings from both Stains-All and Western immunoblotting analyses showed that the D452A substitution effectively blocked the proteolytic processing of DSP in the mouse teeth.

FIGURE 4. Anti-DSP immunohistochemistry. The specimens were from the first mandibular molars of four types of mice at postnatal 3 months. The samples from the Dspp-KO mice (B) were used as negative controls. Anti-DSP activity was observed in the dentin matrix of the WT (A), Dspp-KO/normal-Tg (C), and Dspp-KO/D452A-Tg (D) mice. The signal for the anti-DSP antibody in the dentin of the Dspp-KO/D452A-Tg mice was weaker than in the WT or Dspp-KO/normal-Tg mice. Bar: 50 μm.
The sections were either stained with hematoxylin & eosin (H&E) or used for immunohistochemistry (IHC) analyses. For the IHC analyses, the anti-DSP-2C12.3 monoclonal antibody (43) was used at a dilution of 1:800 to detect DSPP and DSP. The anti-biglycan antibody (a gift from Dr. Larry Fisher of the Craniofacial and Skeletal Diseases Branch, National Institutes of Health, Bethesda, MD) was used at a 1:1000 dilution to detect biglycan. Mouse IgG of the same concentration as that of the primary antibody was the negative control. All the IHC experiments were carried out using the M.O.M. kit and DAB kit (Vector Laboratories; Burlingame, CA) according to the manufacturer’s instructions.

Backscattered and Resin-casted Scanning Electron Microscopy (SEM)—The dissected mandibles in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer solution (pH 7.4) at room temperature. Four hours later, the fixation buffer was replaced with 0.1 M cacodylate solution. The specimens were then dehydrated in ascending concentrations of ethanol and embedded in methyl-methacrylate (MMA) resin (Buehler, Lake Bluff, IL). The surfaces of the dentin tissues of interest were polished using a micro cloth with Metadi Supreme Polycrystalline diamond suspensions of decreasing sizes (0.1 μm, 0.25 μm, and 0.05 μm; Buehler, Lake Bluff, IL). The samples were then washed in the ultrasonic wash and placed in the vacuum system overnight. For backscattered SEM, the surfaces of the teeth embedded in MMA were polished and coated with carbon. For the resin-casted SEM, the dentin surface was acid etched with 37% phosphoric acid for 2–10 s and washed with 5.25% sodium hypochlorite for 5 min. The samples were then coated with gold and palladium as described previously (44). A FEI/Philips XL30 Field emission environmental SEM (Philips, Hillsboro, OR) was used to perform the SE analyses.

Double Fluorochrome Labeling of the Dentin—Double fluorescence labeling was performed, as we described previously, to analyze the mineral deposition rate of the dentin in the mouse incisors (42, 45). Briefly, calcein (green) label (Sigma Aldrich) was injected into the abdominal cavities of the 5-week-old mice.

### FIGURE 5. Plain x-ray analyses (A–H) and the µ-CT analyses (I–P) of mandibles from 3- and 6-month-old mice.

At postnatal 3 months, the WT mice had evenly distributed and well mineralized dentin (A). The mandibular molars in the Dspp-KO mice (B) had an enlarged pulp chamber and thinner dentin compared with the WT mice. The tooth defects in the Dspp-KO/D452A-Tg mice (D) were similar to those of the Dspp-KO mice, whereas the teeth of the Dspp-KO/normal-Tg mice (C) resembled those of the WT mice. At postnatal 6 months, the teeth in the Dspp-KO/normal-Tg mice (G) also appeared the same as those in the WT mice (E), while the teeth of the Dspp-KO/D452A-Tg mice (H) resembled the Dspp-KO mouse teeth (F). In the µ-CT analyses of mandibles, the teeth of the Dspp-KO (I, N) and Dspp-KO/D452A-Tg (L, P) mice had similar dental defects, which included enlarged pulp chambers and thinner dentin. The WT (I, M) and Dspp-KO/normal-Tg (K, O) mice had normal dental structures. Bar: 200 μm.
at 5 mg/kg. One week later, 20 mg/kg of Alizarin Red label (Sigma Aldrich) was administered intraperitoneally. The mice were sacrificed 48 h after the injection of Alizarin Red label. The mandibles were dissected and fixed in 70% ethanol for 48 h and dehydrated through ascending concentrations of ethanol (70–100%) and embedded in MMA. Sections (10 μm thick) were cut and viewed under epifluorescent illumination using a Nikon E800 microscope interfaced with Osteomeasure histomorphometry software (version 4.1, Atlanta, GA). The distance between the two fluorescence labels of the incisor dentin (cross section of incisors under the mesial root of the first molar) was determined, averaged and divided by seven to calculate the mineral deposition rate, expressed as μm/day.

RESULTS

The dentin and bone of the transgenic mice expressing the normal DSPP transgene or the D452A-DSPP transgene in the wild type background were the same as those of normal mice. In the following section, we describe in detail the dentin phenotypes of the Dspp-KO/D452A-Tg mice and Dspp-KO/normal-Tg mice in comparison with the Dspp-KO mice and WT mice. We noticed that the Dspp-KO and Dspp-KO/D452A-Tg mice also had alveolar bone defects. In this report, we did not include a description of the non-dentin defects in these Dspp-mutant mice.

Expression of the DSPP Transgenes in the Teeth—Real-time PCR analyses using the mouse incisor RNA as the template revealed that the expression level of the normal DSPP transgene in the Dspp-KO background was ~16-fold of the transcription level of the endogenous Dspp in the WT mice, while the expression level of D452A-DSPP transgene was about 13-fold of the endogenous Dspp in the WT mice (Fig. 1).

Extraction and Separation of NCPs and Detection of DSPP-related Proteins—Stains-All staining and Western immunoblotting were used to visualize the DSPP-derived proteins in the dentin of the Dspp-KO/normal-Tg, Dspp-KO/null-Tg and WT mice. All the chromatographic fractions from the dentin extracts that might contain DSPP-related products were analyzed by SDS-PAGE with Stains-All staining (version 4.1, Atlanta, GA). The distance between the two fluorescence labels of the incisor dentin (cross section of incisors under the mesial root of the first molar) was determined, averaged and divided by seven to calculate the mineral deposition rate, expressed as μm/day.
KO/D452A-Tg and Dspp-KO/normal-Tg mice (Fig. 3). The ratios of DSP (hollow arrow) to DSPP (dotted arrow) varied dramatically between the samples from the Dspp-KO/D452A-Tg mice and the Dspp-KO/normal-Tg mice. The ratio of DSP to DSPP in the Dspp-KO/D452A-Tg mice was 1:10, while that in the Dspp-KO/normal-Tg mice was 15:1, indicating that the full-length form of DSPP in the former mice was ~150-fold greater than in the latter. The findings from both the Stains-All and Western immunoblotting analyses indicate that the Dspp-KO substitution effectively blocked the proteolytic processing of DSPP in the mouse teeth.

Anti-DSP Immunostaining—Anti-DSP reactivity was observed in the odontoblasts and the dentin matrix of the WT, Dspp-KO/D452A-Tg, and Dspp-KO/normal-Tg mice (Fig. 4). In the matrix, the anti-DSP signals were primarily detected around the dentinal tubules in both the Dspp-KO/normal-Tg mice (Fig. 4C) and Dspp-KO/D452A-Tg mice (Fig. 4D). The presence of anti-DSP activity in the dentin matrix of the Dspp-KO/D452A-Tg mice indicated that the uncleaved DSPP, like its processed fragments, was also secreted into the ECM (Fig. 4D).

Plain X-ray and μ-CT Analyses—Plain x-ray radiography (Fig. 5, A–H) and μ-CT (Fig. 5, I–P) analyses were performed to reveal the dentin structure in the 3- and 6-month-old mice. These analyses showed enlarged pulp chambers with very thin dentin in the Dspp-KO mice (Fig. 5, B, F, J, and N). The expression of normal DSPP transgene fully rescued the defects of enlarged pulp and thin dentin in the Dspp KO mice (Fig. 5, C, G, K, and O), whereas the D452A-DSPP transgene failed to reverse the dentin defects of the Dspp-KO mice (Fig. 5, D, H, L, and P).

Histological Analysis—At postnatal 3 and 6 months, the pulp chamber in the mandibular molars of the Dspp-KO mice was remarkably larger and the predentin zone was much wider than in the WT mice (Fig. 6, A, B, E, and F). While the structure of the dentin–pulp complex in the Dspp-KO/normal-Tg mice (Fig. 6, C and G) was similar to that of the WT mice, the structure of the Dspp-KO/D452A-Tg mice (Fig. 6, D and H) resembled the one in the Dspp-KO mice. The histology findings confirmed x-ray data showing that the normal DSPP transgene rescued the Dspp-KO dentin defects while the mutant transgene did not.

Anti-biglycan Immunostaining—Biglycan immunostaining (Fig. 6, I–L) was performed to show the predentin zone since in the teeth, this proteoglycan is primarily localized in the predentin. The biglycan immunostaining analyses clearly showed that the predentin zone in the Dspp-KO (Fig. 6F) and Dspp-KO/D452A-Tg mice (Fig. 6L) was much wider and more irregular.
than that in the WT (Fig. 6J) or Dspp-KO/normal-Tg mice (Fig. 6K).

**Backscattered SEM**—Backscattered SEM analyses (Fig. 7, A–H) indicated that the dentin in the Dspp-KO mice (Fig. 7, B and F) and Dspp-KO/D452A-Tg mice (Fig. 7, D and H) contained more areas that were unmineralized or hypomineralized and resembled interglobular dentin. In the backscattered SEM images, the white areas represent the regions with greater amounts of mineral (higher level of mineralization), while the black areas indicate less mineralization (i.e. unmineralized or hypomineralized). The dentin in the Dspp-KO (Fig. 7, B and F) and Dspp-KO/D452A-Tg mice (Fig. 7, D and H) contained more hypomineralized areas compared with the WT (Fig. 7, A and E) and Dspp-KO/normal-Tg (Fig. 7, C and G) mice.

**Resin-casted SE**—The resin-casted SE analyses (Fig. 7, I–L) revealed that the dentin in the WT (Fig. 7I) and Dspp-KO/normal-Tg mice (Fig. 7K) had well organized and evenly distributed dentinal tubules of similar thickness, whereas in the Dspp-KO (Fig. 7J) and Dspp-KO/D452A-Tg (Fig. 7L) mice, the dentinal tubules were disorganized and collapsed in some areas.

**Double Fluorochrome Labeling**—In the WT (Fig. 8A) and Dspp-KO/normal-Tg mice (Fig. 8C), the two labeled zones were regular and evenly distributed. In the Dspp-KO (Fig. 8B) and Dspp-KO/D452A-Tg mice (Fig. 8D), the zones of fluorochrome labeling appeared irregular and diffused; in certain areas, the boundary between the two labels appeared blurry. In the double fluorochrome labeling analyses, the distance between the green (first) labeling and red (second) labeling represented the mineral deposition of the dentin matrix during the period between the two injections (7 days). The quantitative analyses of the distance between the two labels (Fig. 8E) indicated that the mineral deposition rates in the dentin of the Dspp-KO mice and Dspp-KO/D452A-Tg mice had a remarkably lower mineral deposition rate compared with the WT and Dspp-KO/normal-Tg mice.

**DISCUSSION**

In the ECM of dentin and bone, DSPP is mainly present as the processed NH₂-terminal and COOH-terminal fragments (including DSP, DSP-PG, and DPP); only a minor amount of full-length DSPP could be detected in the dentin of wild type rat
or mouse (38). Based on the abundance of DSPP fragments and scarcity of its full-length form in the dentin, along with the observed roles of DPP in the nucleation and modulation of apatite crystal formation, we hypothesized that the conversion of DSPP to its fragments by proteolytic processing may be an activation event, converting an inactive precursor to active forms, and this activation step may represent one of the controlling mechanisms in dentin formation (16, 40, 46). In this study, we generated Dspp-KO/D452A-Tg mice lacking the endogenous Dspp gene but expressing the transgenic D452A-DSPP protein, in which Asp\(^{52}\) was replaced by Ala\(^{452}\). The dentin of the Dspp-KO/D452A-Tg mice was compared with that of the Dspp-KO mice, WT mice and Dspp-KO/normal-Tg mice that lacked the endogenous Dspp gene but expressed the transgenic expression of normal DSPP protein. These analyses showed that the D452A substitution effectively blocked the proteolytic processing of this protein in dentin and led to the inactivation of this molecule in dentinogenesis. The findings in the present investigation lend strong support to our hypothesis that the proteolytic processing of DSPP is an activation event, essential to its biological function in biomineralization.

A small portion (10%) of D452A-DSPP was cleaved in the Dspp-KO/D452A-Tg mice. Previously, we showed that substitutions of Asp\(^{52}\) and other residues close to this residue could not totally block the cleavage of DSPP by BMP1 in vitro, suggesting the presence of a secondary (cryptic) cleavage site that is currently unidentified (40). Nevertheless, the dentin defects in the Dspp-KO/D452A-Tg mice were very similar to those in the Dspp-KO mice; this observation indicates that the cleavage of DSPP at this cryptic cleavage site has a very limited effect on DSPP activation.

Immunohistochemistry with the monoclonal anti-DSP antibody revealed positive signals for DSP in the dentin matrix of the Dspp-KO/D452A-Tg mice, indicating that the uncleaved full-length DSPP was also secreted into the ECM of dentin. The anti-DSP activity in the dentin of the Dspp-KO/D452A-Tg mice was weaker than in the Dspp-KO/normal-Tg mice or WT mice. The relatively weaker signal for the anti-DSP antibody in the dentin of the Dspp-KO/D452A-Tg mice may be attributed to the difference in the degree of exposure of the epitopes (antigenic determinants); i.e. the epitopes of the processed fragment (DSP) may be more easily exposed and readily recognized by the anti-DSP antibody than the same antigenic determinants wrapped up in the full-length form of DSPP.

In addition to dentin and bone, DSPP has also been found in certain soft tissues such as the salivary glands, cartilage, liver, kidney, and brain (41, 47). It appears that the DSPP-derived products in the non-mineralized tissues may have posttranslational modifications different from those in the dentin. For example, the majority of DSPP in the condylar cartilage was not cleaved (47), and DSP in the non-mineralized tissues may be devoid of any carbohydrate moieties (41). These variations in the posttranslational modifications of DSPP suggest that the biological role of DSPP in the non-mineralized tissue might differ from that in dentin and bone, in which the cleavage of the full-length protein into its fragment forms is essential to its biological function in the mineralization of these two tissues.

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