Porcine Dentin Sialoprotein Is a Proteoglycan with Glycosaminoglycan Chains Containing Chondroitin 6-Sulfate*

Yasuo Yamakoshi‡, Jan C-C. Hu‡, Makoto Fukae§, Takanori Iwata‡, Jung-Wook Kim‡, Hengmin Zhang‡, and James P. Simmer¶

From the ‡University of Michigan Dental Research Laboratory, Ann Arbor, Michigan 48108, the §Department of Biochemistry, School of Dental Medicine, Tsurumi University, 2-1-3 Tsurumi, Tsurumi-ku, Yokohama 230, Japan, and the ¶Seoul National University, College of Dentistry, Department of Pediatric Dentistry & Dental Research Institute, 28-2 Yongon-Dong, Chongno-Gu, Seoul, Korea 110-768

Dentin sialoprotein (DSP) is a glycoprotein that is critical for proper tooth dentin formation, but little is known about the nature of its carbohydrate attachments and other post-translational modifications. We have isolated DSP from pig dentin and demonstrate that it is a proteoglycan. Polyclonal antibodies were raised in chicken against recombinant pig DSP, and used to identify native DSP in fractions of tooth dentin proteins extracted from developing pig molars. Amino acid analyses and characterization of lysylendopeptidase cleavage products confirmed that the purified protein was DSP, and that Arg391 is at the DSP C terminus. On SDS-PAGE and on urea gels, DSP appeared as a smear extending from 280 to 100 kDa, but in the presence of β-mercaptoethanol the top of the DSP smear disappeared. The high molecular weight material was likely comprised of covalent DSP dimers connected by a disulfide bridge at Cys205. Oligosaccharides were released from DSP following N- and O-linked glycosidase digestions, but these digestions had little effect on the apparent molecular weight of DSP on SDS-PAGE, when compared with the significant reduction following chondroitinase ABC digestion. Glycosaminoglycans with assorted glycosaminoglycan (GAG) cleavage specificities coupled with Western analyses of the cleaved GAG “stubs” demonstrated that the DSP GAG attachments contain chondroitin 6-sulfate, but not keratan sulfate, heparan sulfate, chondroitin, or chondroitin 4-sulfate. DSP binds biotin-labeled hyaluronic acid, and such binding is inhibited by the addition of unlabeled hyaluronic acid. We conclude that DSP is a proteoglycan and that GAG attachments are the predominant structural feature of porcine DSP.

The dentin sialophosphoprotein gene (DSPP)1 on human chromosome 4q21.3 encodes the two major noncollagenous proteins in tooth dentin: dentin sialoprotein (DSP) and dentin phosphoprotein (DPP) (1, 2), and these proteins are critical for proper dentin formation. As of this writing, eight mutations have been identified in the human DSPP gene that cause hereditary dentin defects (3–7). In each case, the phenotype is inherited in an autosomal dominant pattern and is usually limited to the teeth. Occasionally the condition is associated with progressive neurosensory high frequency hearing loss (DFN3A/DGII1, MIM 605594). In addition, DSPP-null mice developed dentin defects in the absence of other symptoms (8). Whereas it is known that DSPP gene products are essential for normal dentin biomineralization, DSP structural features are only partially characterized, and its functions are unknown.

Much of our current understanding of the structure and function of dentin sialoprotein comes from studies of the rat protein (9–11). Rat DSP is the N-terminal portion of DSPP and is generated by proteolytic cleavages, primarily after Tyr421 but also following His406 (12). The molecular mass of the major rat DSP component, which contains 29.6% carbohydrate, was determined by sedimentation equilibrium analysis to be 52.57 kDa (13), although the glycoprotein has an apparent molecular mass of 95 kDa on SDS-PAGE (14). Most recently, higher molecular mass forms of rat DSP (designated HMW-DSP) have been detected. Rat HMW-DSP has 10.3 phosphates per molecule whereas DSP has 6.2. The majority of the carbohydrates attached to the protein backbone are by N-linked glycosylations (15). It has been proposed that the rat HMW-DSP may contain GAG chains and/or considerable amounts of sulfate, but these ideas have not been tested experimentally (16).

DSPP is expressed primarily during dentin formation (17, 18), and in bone (19–21). The extracellular matrices of dentin and bone share a similar inventory of noncollagenous proteins, but show differences in the relative quantities of specific protein components and in the way they are processed by proteases. DSP and DPP are the only extracellular constituents that appear to be more abundant in dentin than in bone (22). DSPP mRNA has also been detected in the inner ear. The finding of progressive neurosensory hearing loss in some DGI patients suggests that DSPP gene products may play an important role in processes not yet identified (5), and these processes may or may not involve biomineralization. Rat DSP is not an effective nucleator of hydroxyapatite (HA) formation in vitro, and has low affinity for seed crystals (23), suggesting that

* This work was supported by United States Public Health Service Research Grants DE12769, DE15846, and DE11301 from the NIDCR, National Institutes of Health, Bethesda, MD 29892. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

† To whom correspondence should be addressed: University of Michigan Dental Research Laboratory, 1210 Eisenhower Place, Ann Arbor, MI 48108. Tel.: 1-734-975-9318; Fax: 1-734-975-9329; E-mail: jjsimmer@umich.edu.

1 The abbreviations used are: DSPP, dentin sialophosphoprotein; GST, glutathione S-transferase; Ab, antibody; biHyA, biotin-labeled hyaluronic acid; CBP, Coomassie Brilliant Blue; Ch, chondroitin; Chstubs; ACh-4S, chondroitin 4-sulfate stubs; CS, chondroitin sulfate; ACh-6S, chondroitin 6-sulfate stubs; HPr, heparan sulfate; DPP, dentin phosphoprotein; DSP, dentin sialoprotein; DS, dermatan sulfate; HA, hydroxyapatite; HPLC, high performance liquid chromatography; biHyA, hyaluronic acid; KS, keratan sulfate; rDSP, recombinant pig DSP; rP172, recombinant pig amelogenin; RP-HPLC, reverse phase high performance liquid chromatography; GAG, glycosaminoglycan.
DSP is unlikely to regulate mineralization directly. Given the fact that the function of dentin sialophosphoprotein is currently unknown, isolating DSP from other organisms besides rat and characterizing its structural properties might highlight conserved structural features and provide insights into its potential functions.

Previously we cloned and characterized a cDNA encoding porcine DSP (24). This provided us with the entire porcine DSP-derived amino acid sequence, and the mRNA code needed for the expression of recombinant protein. Here we report the expression of recombinant porcine DSP in bacteria and the generation of a polyclonal antibody (Ab) able to specifically detect DSP in dentin extracts. We have used this Ab to devise methods to isolate native DSP from developing pig teeth. Finally, we have performed a number of procedures to characterize the post-translational modifications of porcine DSP, and prove that porcine DSP is proteoglycan, with chondroitin 6-sulfate being the predominant glycan attachment. We provide evidence that DSP forms covalent dimers, connected by a disulfide bridge. We have identified the proteolytic cleavage site uniquely SalI and NotI restriction sites at the 5' and 3'-ends of the DSP coding region. The amplification product was purified from an agarose gel, ligated into the TA cloning vector (pCR2.1-TOPO, Invitrogen, Carlsbad, CA), and transfected into bacteria. The recombinant plasmid was isolated and confirmed by DNA sequencing. The pig DSP coding sequence was excised with SalI and NotI, and subcloned into the pGEX4T-1 vector (Amersham Biosciences) restricted with the same sequence was excised with SalI and NotI, and subcloned into the Carlsbad, CA), and transfected into bacteria. The recombinant plasmid was isolated and confirmed by DNA sequencing. The pig DSP coding sequence was excised with SalI and NotI, and subcloned into the pGEX4T-1 vector (Amersham Biosciences) restricted with the same enzymes. The expression construct was transformed into E. coli strain BL21 (DE3) (Stratagene, La Jolla, CA). The bacteria were cultured in N2YPY medium, and DSP expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. The bacteria were cultured for 5 additional hours, and the cell pellet was collected by centrifugation. The cell pellet was resuspended in BugBuster reagent (Novagen, Madison, WI) and Protease Inhibitor Mixture II (Calbiochem). The cell free extract was centrifuged at 10,000 g at 4 °C and 3000 g. This supernatant fraction was loaded onto a Superdex 200 column (1.6 cm × 60 cm, Amersham Biosciences), and DSP eluted in the second peak eluted at 0.2 ml of fraction at 4 °C. Protein was detected by monitoring the absorbance at 280 nm. DSP eluted in the third peak, which was concentrated by a YM-3 membrane, and further fractionated by hydroxyapatite affinity chromatography using a Beckman System Gold HPLC system equipped with a Bio-Scale CHT5 Ceramic Hydroxyapatite, Type I (Beckman, Fullerton, CA) with a YM-3 membrane (Amicon, Beverly, MA), and was lyophilized and stored at −80 °C.

Expression of DSP in Bacteria Using the pGEX4T-1 Vector—The pig DSP cDNA (24) was amplified by PCR using the primers 5′-AGTCGACTCTAGGATCCCGTCGAC-3′ (forward) and 5′-AGCCGGCCGCTTCTGAAATACCTAAGTCTT (reverse), which introduced unique SalI and NotI restriction sites at the 5′- and 3′-ends of the DSP coding region. The amplification product was purified from an agarose gel, ligated into the TA cloning vector (pCR2.1-TOPO, Invitrogen, Carlsbad, CA), and transfected into bacteria. The recombinant plasmid was isolated and confirmed by DNA sequencing. The pig DSP coding sequence was excised with SalI and NotI, and subcloned into the pGEX4T-1 vector (Amersham Biosciences) restricted with the same enzymes. The expression construct was transformed into E. coli strain BL21 (DE3) (Stratagene, La Jolla, CA). The bacteria were cultured in N2YPY medium, and DSP expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a final concentration of 1.0 mM. The bacteria were cultured for 5 additional hours, and the cell pellet was collected by centrifugation. The cell pellet was resuspended in BugBuster reagent (Novagen, Madison, WI) and Protease Inhibitor Mixture II (Calbiochem). The cell free extract was centrifuged at 10,000 g at 4 °C and 3000 g. This supernatant fraction was loaded onto a Superdex 200 column (1.6 cm × 60 cm, Amersham Biosciences), and DSP eluted in the second peak eluted at 0.2 ml of fraction at 4 °C. Protein was detected by monitoring the absorbance at 280 nm. Both DSP and DPP eluted in the third peak, which was concentrated by a YM-3 membrane, and further fractionated by hydroxyapatite affinity chromatography using a Beckman System Gold HPLC system equipped with a Bio-Scale CHT5 Ceramic Hydroxyapatite, Type I (Beckman, Fullerton, CA) with a YM-3 membrane (Amicon, Beverly, MA), and was lyophilized and stored at −80 °C.

Purification of Recombinant DSP—The lyophilized E extract (100 mg) was dissolved in 10 ml of 50 mM Tris-HCl/6 M urea buffer (pH 7.4), and fractionated by an anion exchange chromatography with a Q-Sepharose Fast Flow column (1.6 cm × 20 cm, Amersham Biosciences). The column was equilibrated with 50 mM Tris-HCl, 6 M urea buffer (pH 7.4) (buffer A) and 50 mM Tris-HCl, 6 M urea buffer containing 50 mM Tris-HCl (buffer B) at a flow rate of 0.2 ml/min at 4 °C. Three fractions were collected and specific IgY antibodies were purified by the affinity chromatography. The titer of this purified Ab, as estimated by enzyme-linked immunosorbent assay (ELISA), was 1:17,000. This polyclonal antibody was used in subsequent Western blot analyses to identify DSP-containing fractions from developing porcine molars.

Preparation—Tooth germs of permanent molars were surgically extracted with a hammer and chisel from the maxillae and mandibles of 16 six-month-old pigs, within minutes of each animal's termination at the Michigan State University Meat Laboratory (East Lansing, MI). The soft tissue was removed, and the hard tissue was quickly frozen in dry ice. All subsequent preparation steps were carried out at 4 °C or on ice or on dry ice. Dental enamel was removed by scraping 55 molars with a curette. Each molar crown was separately embedded in paraffin with the dentin side facing up.

Extraction of Porcine DSP from Developing Pig Teeth—All extraction steps were carried out at 4 °C on ice and Protease Inhibitor Mixture Set III (1 mM AEBSF, 0.8 μM aprotinin, 50 μM bestatin, 15 μM E-64, 20 μM leupeptin, and 10 μM pepstatin) (Calbiochem) and 1 mM 1,10-phenanthroline (Sigma) were added into the buffer during the extraction. Dentin was first washed with Milli-Q water for 1 day. Dentin buffer (G extract) was obtained by extraction with 50 mM Tris-HCl/4 M guanidine buffer (pH 7.4) for each of 5 days and then combined. Then “E” extract was obtained by sequential extraction with 50 mM Tris-HCl/4 M guanidine/0.5 M EDTA buffer (pH 7.4) for 7 days, changing the buffer everyday, and then combined. The G and E extracts were centrifuged at 7,000 × g, and then desalted and concentrated using the combination of a Spectra/Por 3 membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA) with a YM-3 membrane (Amicon, Beverly, MA), and were lyophilized and stored at −80 °C.

Isolation and Purification of Porcine DSP—The lyophilized E extract (100 mg) was dissolved in 10 ml of 50 mM Tris-HCl/6 M urea buffer (pH 7.4), and fractionated by an anion exchange chromatography with a Q-Sepharose Fast Flow column (1.6 cm × 20 cm, Amersham Biosciences). The column was equilibrated with 50 mM Tris-HCl, 6 M urea buffer (pH 7.4) (buffer A) and 50 mM Tris-HCl, 6 M urea buffer containing 50 mM Tris-HCl, 6 M urea buffer (pH 7.4) at a flow rate of 0.2 ml/min at 4 °C. Protein was detected by monitoring the absorbance at 280 nm. Both DSP and DPP eluted in the third peak, which was concentrated by a YM-3 membrane, and further fractionated by hydroxyapatite affinity chromatography using a Beckman System Gold HPLC system equipped with a Bio-Scale CHT5 Ceramic Hydroxyapatite, Type I (Beckman, Fullerton, CA) with a YM-3 membrane (Amicon, Beverly, MA), and was lyophilized and stored at −80 °C.

SDS-PAGE—SDS-PAGE was performed using PAGE® Gold Prestained (4–20% gradient, 10% or 7.5% Tris-glycine gels) (Cambrex Bio Science Rockland Inc., Rockland, ME). SDS-PAGE-urea gels contained 10% polyacrylamide and 8 M urea. Samples were dissolved in laemmli sample buffer (Bio-Rad) with or without 2% β-mercaptoethanol and electrophoresis was carried out using a current of 20 mA for 1.5 h. The gels were stained with Bio-Safe Coomassie (Bio-Rad), Silver Stain Plus (Bio-Rad), or Stains-all (Sigma). The apparent molecular masses of protein bands were estimated by comparison with SeeBlue® Plus2 Pre-Stained standards (Invitrogen).

Western Immunoblots—Western immunoblots of proteins on electrophoresed gels were electrotransferred onto Hybond-P membrane (Amersham Biosciences). Polyclonal for porcine DSP and monoclonal for each of glycosaminoglycan antibodies were used at dilution of 1:500. In the Far-Western analysis of hyaluronic acid binding, biotin-labeled hyaluronic acid (1:1000) was detected using a horseradish peroxidase-streptavidin conjugate (1:5000, Sigma). The membranes were immunostained by the chemiluminescence detection method with RCL Advance®. Western blotting detection kit (Amersham Biosciences).
incubation period, three volumes of ice-cold ethanol were added to the reaction mixture, which was then centrifuged for 10 min at 10,000 g. Both supernatant and pellet were evaporated to dryness, and the dried samples were stored at −80 °C. An aliquot (20 μg) of the pellet was electrophoresed on 4–20% Tris-glycine gradient gel, and the degradation pattern was confirmed by Stains-all staining and Western immunoblots using the polyclonal anti-DSP antibody.

Release of O-linked Oligosaccharide Chains by O-Glycosidase Digestion—The pellet (~0.2 mg) after the glycopeptidase A digestion in 250 mM sodium phosphate buffer (pH 5.0) was incubated with 5 million units of sialidase Au, 0.5 milliunits of O-α-(1,3,4)-nucleotidase, and keratanase II in 10 mM sodium acetate buffer (pH 6.0) at 37 °C for 24 h. An aliquot (20 μg) of the pellet was electrophoresed on 4–20% Tris-glycine gradient gel, and the degradation pattern was treated as described for the glycopeptidase A digestion.

The fluorescence labeling for both N- and O-linked oligosaccharides after glycopeptidase A and O-glycanase digestions was previously described (25). The sample was applied to a column of Sephadex G-15 (1.5 × 35 cm, Amersham Biosciences) equilibrated with 10 mM ammonium acetate buffer (pH 6.0) and was eluted with the same buffer. The effluent was continuously monitored by a fluorescence monitor (FP-2020, JASCO, Tokyo, Japan) using an excitation wavelength of 315 nm and an emission wavelength of 400 nm.

Glycosaminoglycanase Digestion of DSP—Four glycosaminoglycanase-free chondroitinase ABC, heparitinase I, keratanase I, and keratanase II (Seikagaku America) were purchased from Seikagaku America. Each glycosaminoglycanase digestion of glycopeptidase A and O-glycanase-treated DSP was separately carried out with 0.2 unit of protease-free chondroitinase ABC in 10 ml of 40 mM Tris-Cl/40 mM sodium acetate buffer (pH 8.0), 0.005 units of the heparitinase I in 50 mM sodium acetate buffer (pH 7.0) containing 5 mM CaCl2, 0.05 units of the keratanase I in 10 mM sodium acetate buffer (pH 6.0) containing 5 mM NaCl, 0.05% (v/v) Tween 20 (pH 5.8) at room temperature unless otherwise stated. The sample was applied to a 100% on the precycled cartridge filter. Ultrapure water was used as a blank and the column was equilibrated with 10 mM ammonium acetate buffer (pH 6.0) and was eluted with the same buffer. The effluent was continuously monitored by a fluorescence monitor (FP-2020, JASCO, Tokyo, Japan) using an excitation wavelength of 315 nm and an emission wavelength of 400 nm.

Panel A

S 1 2 3 4 5 6 7 8

A B

C

1 2 3 4 5 6 7 8

D

1 2 3 4 5 6 7 8

E

1 2 3 4 5 6 7 8

F

1 2 3 4 5 6 7 8

G

1 2 3 4 5 6 7 8

H

1 2 3 4 5 6 7 8

I

1 2 3 4 5 6 7 8

J

1 2 3 4 5 6 7 8

K

1 2 3 4 5 6 7 8

L

1 2 3 4 5 6 7 8

M

1 2 3 4 5 6 7 8

N

1 2 3 4 5 6 7 8

O

1 2 3 4 5 6 7 8

P

1 2 3 4 5 6 7 8

Q

1 2 3 4 5 6 7 8

R

1 2 3 4 5 6 7 8

S

1 2 3 4 5 6 7 8

T

1 2 3 4 5 6 7 8

U

1 2 3 4 5 6 7 8

V

1 2 3 4 5 6 7 8

W

1 2 3 4 5 6 7 8

X

1 2 3 4 5 6 7 8

Y

1 2 3 4 5 6 7 8

Z

FIG. 1. Isolation of recombinant pig DSP. Panel A, SDS-PAGE (4–20% gradient gel) comparing expression and affinity purification fractions. Lanes: S, molecular mass standard; 1, uninduced crude extract; 2, induced crude extract; 3, insoluble cell lysate; 4, flow-through of affinity column; 5, wash; 6, elute from reduced glutathione; 7, beads after elution. Panel B, SDS-PAGE (4–20% gradient gel) showing the isolation of recombinant porcine DSP after cleavage by thrombin. Lanes: I, intact fusion protein (GST-DSP) prior to cleavage by thrombin (apparent molecular mass = 97 kDa); 2, phenyl column peak 2 following thrombin cleavage; phenyl column peak 3 following thrombin cleavage. 2 mg of the material shown in lane 2 were used to generate polyclonal antibodies in chicken. Panel C, chromatogram of the thrombin digest eluting from the phenyl column. Left scale is absorbance at 230 nm; right scale is percent buffer B (80% acetonitrile).

Optimum amounts of RP2, rpDSP or recombinant pig amelogenin (rP172) used in this assay were determined by coating with a range of protein concentrations (5–800 pmol per well) and binding bHyA in the absence of competitor. On this basis, 600 pmol per well of RP2, 250 pmol per well of rpDSP or 800 pmol per well of rP172 were used in the competition assay.

Automated Edman Degradation—Automated Edman degradation was performed with an Applied Biosystems Procise 494 cLC protein sequencer. The purified peptide samples were suspended in 20 μl of 100 mM trifluoroacetic acid and loaded 100% on the precycled cartridge filter.

RESULTS

Expression of Recombinant Pig DSP—The first goal in the characterization of porcine dentin sialoprotein was to isolate the protein from developing teeth. Polyclonal antibodies were raised against recombinant pig DSP and used to identify DSP in the various dentin fractions. Recombinant porcine DSP was expressed as a GST-fusion protein in bacteria (Fig. 1). The intact GST-DSP fusion protein was purified by HPLC and affinity chromatography (Fig. 1A). Recombinant DSP was excised from the GST domain by thrombin and purified (Fig. 1, B and C). The final recombinant porcine DSP product had an apparent molecular mass of 65 kDa (Fig. 1B, lane 2). The identity of the purified protein was confirmed by N-terminal sequencing, which gave the sequence GSPFPGRLIPVPQI (the DSP N-terminal sequence is underlined), as expected. 2 mg of recombinant pig DSP were used to generate specific DSP polyclonal antibodies in chicken.

Isolation of Porcine DSP from Developing Molars—Dentin proteins were extracted from developing porcine molars (Fig. 2A) using sequential guanidine (G extract) and guanidine/EDTA (E extract) extractions (26). DSP was identified as a wide band above 250 kDa by Western blot analyses in the E extract (Fig. 2B, lane E). The E extract was first fractionated by anion exchange chromatography using a Q-Sepharose column, which sorted the E extract into 3 well-defined chromatographic peaks (Fig. 2C). DSP was identified in the third peak (Fig. 2D, lane Q3), the contents of which were further fractionated over an HA column. The HA column divided the Q3 component into

DSP Is a Proteoglycan

Hyaluronic Acid (HyA) Binding Assay—HyA binding assay determined the amount of biotin-labeled hyaluronic acid (bHyA) binding to DSP protein immobilized on a microtiter plate in the presence of competing unlabelled HyA. All dilutions, incubations, and washes for the HyA binding assay were performed using a Beckman Model 7300 automatic amino acid analyzer.

Hyaluronic Acid (HyA) Binding Assay—HyA binding assay determined the amount of biotin-labeled hyaluronic acid (bHyA) binding to DSP protein immobilized on a microtiter plate in the presence of competing unlabeled HyA. All dilutions, incubations, and washes for the HyA binding assay were performed using a Beckman Model 7300 automatic amino acid analyzer.
3 parts (Fig. 3B, left). After passing the HA2 fraction over with a protein G column to remove immunoglobulins, the three HA fractions were analyzed by SDS-PAGE and immunostained with the DSP Ab (Fig. 3A). Two major DSP components were detected on the Western blot at ~280 and 140 kDa. We associated the appearance of the lower DSP band at 140 kDa with the removal of immunoglobulins, which may have blocked immunodetection of this band in previous fractions. This band was not prominent in the previous fractions (compare lanes E and Q3 in Fig. 2, B and C with lane HA2 in Fig. 3A). The smaller immunostained bands, having apparent molecular masses in the 25–30 kDa range (Fig. 3A, HA2), also immunostained in negative controls in which only the secondary antibody was included (Fig. 3D, S2–S3). Fraction HA2 was further separated by size exclusion chromatography (Fig. 3, C and D) into four parts. The DSP components were in the first fraction (S1). Several Stains-all-positive proteins, which were not detected by the DSP Ab, were now sufficiently concentrated by the fractionation procedures to be detected in each of the four size exclusion fractions. Stains-all is a cationic carbocyanine dye that stains most proteins red or pink, while Ca^{2+}-binding proteins stain dark blue or purple (27).

Porcine DSP Is a Covalent Dimer—The two large DSP components in S1 were partially separated using a TSK-gel phenyl
5PW RP-HPLC column (Fig. 4A). As DSP was concentrated in the final fractionation, it became detectable by Stains-all. The first peak to elute (RP1, 2.8 mg), when analyzed under nonreducing conditions (minus β-mercaptoethanol); contained predominantly a smear between 100 and 150 kDa and a band at 280 kDa (Fig. 4B, lanes RP1), while RP2 (1.6 mg) contained predominantly a smear between 130 and 180 kDa, and a band at 280 kDa (Fig. 4B, lanes RP2). These two fractions (RP1 and RP2) were reduced with β-mercaptoethanol and analyzed by SDS-PAGE and by Western blotting (Fig. 4B, plus lanes). The 280 kDa DSP could not be detected in either of the reduced fractions. The amino acid compositions of RP1 and RP2 were similar to each other and to the predicted composition of DSP from pig and other mammals, suggesting that the fractions were comprised mostly of DSP protein (Table I). When DSP was denatured with urea, DSP appeared as a smear extending from 280 to 100 kDa. When both β-mercaptoethanol and urea were added, and the samples were run on a urea gel, the top of the smear disappeared (Fig. 4B, right). We interpret these findings to indicate that the higher molecular mass material contains DSP dimers covalently linked by a disulfide bridge. As porcine DSP has only one cysteine residue per molecule, the two DSP monomers are necessarily joined at Cys205.

Porcine DSP Is a Proteoglycan—The nature of DSP carbohydrate attachments was investigated. The RP1 and RP2 fractions were separately digested with N-glycosidase. This digestion did not produce a noticeable difference in the DSP profile on SDS-PAGE and on Western blots (Fig. 5A, lanes 1 versus 2); however, carbohydrate chains were released from both DSP fractions and could be detected following fluorescent labeling (Fig. 5B, left). The N-linked glycosylations released from RP1 differed from those released by RP2 (compare Fig. 5B, left top to left bottom). We interpret these results to indicate that porcine DSP has N-linked glycosylations, but that these glycosylations do not explain the substantial discrepancy between the DSP apparent molecular mass (100–280 kDa), and the apparent molecular mass of the recombinant protein (65 kDa), or the predicted molecular mass of the unmodified protein (39 kDa).

Following N-linked glycosidase digestion, the RP1 and RP2 DSP fractions were separately digested with enzymes to remove fucose and sialic acid (which can interfere with cleavage by O-glycosidase) and with O-glycosidase, to remove O-linked oligosaccharides. As in the case of the N-linked glycosylations, no differences were observed in the apparent mobility of DSP on SDS-PAGE and on Western blots (Fig. 5A, lanes 2 versus 3). Carbohydrate chains released by O-glycosidase digestion from both DSP fractions could be detected following fluorescent labeling, but the amount was less than the amount released by N-glycosidase digestion (Fig. 5B, right). We interpret these results to indicate that porcine DSP contains O-linked carbo-
hydrate, but less than the amount of N-linked carbohydrate.

Following the N- and O-linked glycosidase digestions of the RP1 and RP2 DSP fractions were separately digested with chondroitinase ABC. This enzyme partially removes glycosaminoglycan chains and had a profound effect on the apparent mobility of DSP on SDS-PAGE and Western blots (Fig. 5A, lanes 3 versus 4). When visualized by immunostaining with an antibody specific for the cut ends (or “stubs”) left behind by cleavage of chondroitin 6-sulfate, the apparent molecular masses of the two DSP smears from the RP1 fraction centered around 30 and 50 kDa, which was smaller than the corresponding smears, centered around 40 and 65 kDa, from the RP2 fraction digest (compare Fig. 5A, lanes 4 for RP1 and RP2).

**Table 1**

| Human | Mouse | Rat | Pig | RP1 | RP2 |
|-------|-------|-----|-----|-----|-----|
| Aax   | 196*  | 150 | 151 | 186 | 188 | 179 |
| Thr   | 54    | 63  | 65  | 54  | 53  | 51  |
| Ser   | 99    | 127 | 123 | 100 | 93  | 90  |
| Glx   | 160   | 169 | 170 | 151 | 199 | 176 |
| Pro   | 29    | 44  | 42  | 51  | 35  | 44  |
| Gly   | 146   | 148 | 158 | 137 | 167 | 166 |
| Ala   | 38    | 53  | 63  | 67  | 65  | 74  |
| Cys   | 2     | 2   | 2   | 3   | 3   | —   |
| Val   | 49    | 46  | 37  | 43  | 36  | 31  |
| Met   | 7     | 2   | 7   | 5   | —   | —   |
| Ile   | 47    | 42  | 35  | 38  | 35  | 32  |
| Leu   | 22    | 32  | 30  | 35  | 26  | 39  |
| Tyr   | 13    | 7   | 7   | 8   | —   | —   |
| Phe   | 4     | 7   | 5   | 5   | 7   | —   |
| His   | 31    | 39  | 37  | 30  | 30  | 32  |
| Lys   | 74    | 44  | 40  | 49  | 38  | 52  |
| Arg   | 27    | 23  | 28  | 35  | 28  | 34  |

*The numbers refer to residues per thousand.

Antibodies specific for dermatan sulfate and keratan sulfate, and for the stubs left behind by cleavage of chondroitin, chondroitin 4-sulfate, and heparan sulfate were negative, but gave positive results for controls (Fig. 5C). We interpret these results to mean that DSP is a proteoglycan with a large and variable amount of chondroitin 6-sulfate attachments, and that such attachments constitute a major structural feature of porcine DSP.

**Hyaluronic Acid Binding**—The finding that porcine DSP is a proteoglycan suggested that it might participate in the formation of very high molecular mass proteoglycan assemblies built upon a backbone of hyaluronic acid. The binding of proteoglycans to hyaluronic acid is often stabilized by a link protein that binds to both hyaluronic acid and the proteoglycan, so that in the absence of link protein aggregates are smaller and less stable (28). Increasing amounts of rpDSP, rP172, and the RP2 fraction were coated onto microtiter plates and then incubated with biotin-labeled hyaluronic acid (bHyA). Unbound bHyA...
was removed and the amount of bound bHyA determined by measuring the absorbance at 405 nm and plotted (Fig. 6A). DSP and rpDSP bound to the bHyA, but recombinant amelogenin (rP172) did not. The binding of DSP and rpDSP to bHyA was specific and could be prevented by the addition of unlabeled hyaluronic acid. Panel B, RP2 (600 pmol per well), rpDSP (250 pmol per well), or rP172 (800 pmol per well) were coated on separate plates, and bHyA binding at pH 5.8 was measured in the presence of competing unlabeled HyA. Values are plotted as mean absorbance (n = 3 ± S.E. This shows that the biotin-labeled hyaluronic acid bound specifically to DSP, as the binding could be reversed by the addition of unlabeled hyaluronic acid. Panel C, Far-Western analysis showing DSP fraction RP2, recombinant DSP, recombinant amelogenin (negative control) and hyaluronic acid binding protein (positive control) transblotted from SDS-PAGE to nitrocellulose and affinity stained using a biotin-labeled hyaluronic acid probe. DSP, rpDSP (arrow), and HABP were all positively stained. Amelogenin was negative.

Identification of the DSP C Terminus—Endoproteinase Lys-C digestion of pig RP2 generated DSP cleavage products that could be resolved into 10 chromatographic peaks by C-18 RP-HPLC (Fig. 7A). These cleavage products were characterized by N-terminal sequencing (Fig. 7B). The N terminus of seven different digestion products were characterized. Only DSP sequences were observed. The locations of the digestion products in the porcine DSP primary sequence are underlined in Fig. 7C. The protein sequence obtained from peak 10 (GIEIAAPR) did not end in lysine and the sequence chromatograms showed an abrupt loss of signal, indicating that the end of the peptide was reached. This suggests that Arg391 is at the DSP C terminus. This conclusion was further supported by the isolation and characterization from pig dentin of a 19 kDa DSPP cleavage product that has Ser392 at its N terminus (data not shown). After eliminating the 15 amino acid signal peptide, the porcine DSP protein, in the absence of any post-translational modifications, has 376 amino acids, a predicted molecular mass of 39 kDa, and an isoelectric point of 4.4. We were not able to obtain protein sequence for the DSP N terminus or for any DSP peptide containing the predicted N terminus. The DSP sequence for the peptide in peak 9 (NEDVGXAXQ) gave blank cycles for Asn170 and Ser172, suggesting that these residues are likely to be modified, by glycosylation and phosphorylation, respectively.

**DISCUSSION**

Proteoglycans have been proposed to play important roles in the mineralization of tooth dentin. It is believed that the predominant proteoglycans in predentin and dentin are chondroitin sulfate-rich decorin and biglycan and keratan sulfate-rich lumican and fibromodulin, but other proteoglycans, such as versican are also present (29). In a situation analogous to the relationship between osteoblasts, osteoid and bone, the surface of the dentin forming cell (odontoblast) is separated from the mineralized extracellular matrix by an unmineralized layer of predentin. The profile of proteoglycans changes during the transition of predentin to dentin, supporting the idea that some proteoglycans inhibit, whereas others promote and guide the mineralization process (30). Based upon the calcium- and hydroxyapatite-binding properties of glucuronic acid-rich and iduronic acid-rich glycosaminoglycans and proteoglycans (31), it has been proposed that proteoglycans containing dermatan sulfate within predentin act as inhibitors of mineralization and that highly sulfated ChS proteoglycans function in the control of mineralization (30).

Among the noncollagenous proteins, DPP is considered to be the most abundant, and DSP the second most abundant macromolecules in the dentin matrix (16). DSP and DPP, however, are
Cys205 is conserved in human DSP, but not in rodents. Mouse bond did not contribute perceptibly to the intensity of monomer. Comprised of DSP covalent dimers connected at the lone cysteine, 280 kDa to disappear, suggesting that the 280-kDa band was Arg391.

Western analyses migrating at roughly 280 and 140 kDa, re-arrange into two fractions, that appeared as wide bands on DSP protein expressed from the DSP-only transcript. C-terminal cleavage product (VSL) that would have been present dopeptidase cleavage products, we did not detect the specific CBB or Stains-all. In the partial characterization of DSP lysylen-ination, although we also did not confirm it. DPP is easier to isolate and read. No evidence opposing this expectation, although we also did not confirm it. DPP is easier to isolate and concentrates into discrete bands on SDS-PAGE that are readily detected by Stains-all. DSP is difficult to isolate and forms a smear on SDS-PAGE that does not readily stain with CBB or Stains-all. The two final DSP fractions (RP1 and RP2) show porcine DSP to be a concentrated smear proteoglycan on SDS-PAGE that stains weakly with Stains-all. Both fractions have amino acid compositions that match what is predicted for DSP (Table 1). The distribution of the DSP smears on SDS-PAGE is not altered perceptibly following successive cleavages by N- and O-glycosidases, although DSP has eight potential N-linked glycosylation sites (Table 1). The DSP chondroitin 6-sulfate chains might function to bind the single cysteine residue (Cys205) that may form a disulfide bond with DPP. The finding that porcine DSP has major chondroitin 6-sulfate attachments supports a re-evaluation of its potential roles in dentin biomineralization. The functions of proteoglycans are often closely coupled to the roles of their glycosaminoglycan chains. Glycosaminoglycans (GAGs) are highly extended, un-branched, negatively charged repeating disaccharide units that increase the viscosity and structural integrity of extracellular matrices. Some proteoglycans, such as aggrecan, interact with hyaluronic acid and a link protein to form large aggregates. The glycosaminoglycan chains of such aggregates generate a large osmotic swelling pressure and increase resistance to compressive forces.

The DSP chondroitin 6-sulfate chains might function to bind and organize water molecules and contribute to the hydration of dentin, as aggrecan does for cartilage (34). The hydration of dentin is important clinically. Devital or endodontically treated teeth dry out over time and become brittle, requiring the placement of crown-type restorations to prevent subsequent tooth fractures. Another potential function of the DSP chondroitin 6-sulfate chains could be to mediate specific binding to other macromolecules. The negative charges of the DSP chondroitin...
sulfate chains would be expected to repel negatively charged proteins, such as DPP. Interactions, both positive and negative, with other proteins could play a role in matrix assembly. In addition, the binding of signaling molecules such as growth factors to DSP GAG chains might give it a role in tooth development. The binding of heparan sulfate chains to growth factors to DSP GAG chains might give it a role in tooth development. The binding of heparan sulfate chains to growth factors to DSP GAG chains might give it a role in tooth development.

We have found that DSP isolated from the forming dentin of developing pig teeth binds hyaluronic acid in vitro, even in the absence of a link protein. This finding suggests that DSP might contribute to large molecular assemblies built upon a backbone of hyaluronic acid. The molecular basis for the in vitro binding of DSP to hyaluronic acid cannot be predicted from the DSP primary sequence. Some extracellular hyaluronan-binding proteins contain a link module (~100 amino acids), which is involved in hyaluronic acid binding (38). Others contain a BXX7 cluster of amino acids (two basic residues B separated by 7 non-acidic residues X) (39). Porcine DSP contains neither a link module nor a BXX7 domain. A protein-protein BLAST search of the nonredundant protein databases hits DSP sequences from other organisms without matching any known proteoglycans, let alone proteoglycans known to bind hyaluronic acid. Despite this, there can be no doubt that porcine DSP is a proteoglycan, although its binding to hyaluronic acid in vivo remains to be demonstrated. We suspect that the lack of amino acid sequence homology between DSP and other proteoglycans discouraged the pursuit of biochemical analyses that would have previously demonstrated the presence of glycosaminoglycan chains in DSP isolated from other organisms.

The isolation of porcine DSP and the finding of major structural features not previously characterized will facilitate future studies to more fully characterize the DSP structure, determine its relative abundance to DPP in dentin, and to explore the function of its proteoglycan attachments.

Acknowledgments—We thank Tom Forton, manager of the Michigan State University Meat Laboratory and members of the Michigan State University Department of Animal Science for their kind assistance in obtaining fresh developing molars from pigs slaughtered at their facility. We also thank Dr. Myron Crawford, director of W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University, and Nancy Williams for the amino acid analysis and protein sequencing. We thank Dr. John D. Bartlett for his critical review of the manuscript.

REFERENCES
1. Ritchie, H. H., and Wang, L. H. (1996) J. Biol. Chem. 271, 21685–21698
2. MacDougall, M., Simmons, D., Lu, X., Nydegger, J., Feng, J., and Gu, T. T. (1997) J. Biol. Chem. 272, 835–842
3. Malmgren, B., Lindskog, S., Elgadi, A., and Norgren, S. (2004) Hum. Genet. 114, 491–498
4. Kim, J.-W., Nam, S.-H., Jang, K.-T., Lee, S.-H., Kim, C.-C., Hahn, S.-H., Hu, J.-C., and Simmer, J. P. (2004) Hum. Genet. 115, 248–254
5. Xiao, S., Yu, C., Chu, X., Yuan, W., Wang, Y., Bu, L., Fu, G., Qian, M., Yang, J., Shi, Y., Hu, L., Han, B., Wang, Z., Huang, W., Liu, J., Chen, Z., Zhao, G., and Kong, X. (2001) Nat. Genet. 27, 201–204
6. Zhang, X., Zhao, J., Li, C., Gao, S., Qiu, C., Liu, P., Wu, G., Qian, B., Lo, W. H., and Shen, Y. (2001) Nat. Genet. 27, 151–152
7. Rajpar, M. H., Koch, M. J., Davies, R. M., Melody, K. T., Kielty, C. M., and Dixon, M. J. (2002) Hum. Mol. Genet. 11, 2559–2565
8. Sreenath, T., Thayagarajan, T., Hall, B., Longenecker, G., D’Souza, R., Hong, S., Wright, J. T., MacDougall, M., Sacz, J., and Kulikarni, A. B. (2003) J. Biol. Chem. 278, 24874–24880
9. Ritchie, H. H., Hou, H., Veis, A., and Butler, W. T. (1994) J. Biol. Chem. 269, 3698–3702
10. Butler, W. T., Brunn, J. C., Qin, C., and McKee, M. D. (2002) Connect. Tissue Res. 43, 301–307
11. Butler, W. T., Ritchie, H. H., and Bronckers, A. L. (1997) Ciba Found. Symp. 205, 107–115
12. Qin, C., Cook, R. G., Orkiszewski, R. S., and Butler, W. T. (2001) J. Biol. Chem. 276, 904–909
13. Butler, W. T., Brunn, J. C., D’Souza, R. N., Farach-Carson, M. C., Habponen, R.-P., Schroenhofer, R. E., Seyer, J. M., Somerman, M. J., Foster, R. A., Tomana, M., and van Dijk, S. (1992) Matrix 12, 343–351
14. Butler, W. T., Brinn, J. C., Dimuzio, M. T., and Linde, A. (1981) Coll. Relat. Res. 1, 187–189
15. Qin, C., Brunn, J. C., Baba, O., Wygant, J. N., McIntyre, B. W., and Butler, W. T. (2003) Eur. J. Oral Sci. 111, 235–242
16. Qin, C., Baba, O., and Butler, W. T. (2004) Crit. Rev. Oral Biol. Med. 15, 126–136
17. Butler, W. T. (1987) Methods Enzymol. 145, 290–303
18. Butler, W. T. (1992) Oper. Dent. 5, (suppl.) 18–23
19. Qin, C., Brunn, J. C., Cadena, E., Ridall, A., Tsuji-mura, H., Nagatuku, H., Nagai, N., and Butler, W. T. (2002) J. Dent. Res. 81, 392–394
20. Butler, W. T., Brunn, J. C., and Qin, C. (2003) Connect. Tissue Res. 44, 171–178
21. Qin, C., Brunn, J. C., Cadena, E., Ridall, A., and Butler, W. T. (2003) Connect. Tissue Res. 44, 179–183
22. Qin, C., Brunn, J. C., Jones, J., George, A., Ramachandran, A., Gorski, J. P., and Butler, W. T. (2001) Eur. J. Oral Sci. 109, 133–141
23. Boskey, A., Speakman, L., Tan, M., Doty, S. B., and Butler, W. T. (2000) Calcif. Tissue Int. 67, 472–478
24. Yamakoshi, Y., Hu, J. C., Liu, S., Zhang, C., Oida, S., Fukue, H., and Simmer, J. P. (2003) Eur. J. Oral Sci. 111, 60–67
25. Yamakoshi, Y. (1995) Calcif. Tissue Int. 56, 323–330
26. Termine, J. D., Belcourt, A. B., Miyamoto, M. S., and Conn, K. M. (1980) J. Biol. Chem. 255, 9769–9772
27. Campbell, R. P., MacLennan, D. H., and Jorgensen, A. O. (1983) J. Biol. Chem. 258, 11267–11273
28. Neame, P. J., and Barry, F. P. (1994) Experientia Suppl. 70, 53–72
29. Embrey, G., Hall, R., Waddington, R., Septier, D., and Goldberg, M. (2001) Crit. Rev. Oral Biol. Med. 12, 331–349
30. Waddington, R. J., Hall, R. C., Embrey, G., and Lloyd, D. M. (2003) Matrix Biol. 22, 153–161
31. Embrey, G., Rees, S., Hall, R., Rose, K., Waddington, R., and Shells, P. (1998) Eur. J. Oral Sci. 106, 267–273
32. Ritchie, H. H., and Li, X. (2001) Eur. J. Oral Sci. 109, 342–347
33. Winzen, U., Cole, G. J., and Halper, W. (2003) J. Biol. Chem. 278, 30106–30114
34. Kiani, C., Chen, L., Wu, Y. J., Yee, A. J., and Yang, B. B. (2002) Cell Res. 12, 19–32
35. Kramer, K. L., and Yost, H. J. (2003) Annu. Rev. Genet. 37, 461–484
36. Young, M. F., Bi, Y., Ameye, L., and Chen, X. D. (2002) Glycoconjug. J. 19, 257–292
37. Takano, Y., Sakai, H., Watanabe, E., Ida, Oh, N., Jyayawenna, C. K., Arai, K., Kasa, Y., Nakano, Y., Shuda, Y., Sakamoto, Y., and Terasima, T. (2003) J. Electron Microsc. 52, 573–580
38. Kohda, D., Morton, C. J., Parker, A. A., Hatanaka, H., Inagaki, F. M., Campbell, I. D., and J. A. (1996) Cell 86, 767–775
39. Yang, B. B., Yang, B. L., Savani, R. C., and Turley, E. A. (1994) EMBO J. 13, 286–296
40. Gu, K., Chang, S., Ritchie, H. H., Clarkson, B. H., and Rutherford, R. B. (2000) Eur. J. Oral Sci. 108, 35–42