The Efficiency of Dentin Sialoprotein-Phosphophoryn Processing Is Affected by Mutations Both Flanking and Distant from the Cleavage Site

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Background: Proper processing of DSP-PP precursor protein is required for normal dentin mineralization.

Results: Mutation of conserved residues near and distant from the cleavage site blocks, impairs, or accelerates cleavage.

Conclusion: Residues near and distant from the cleavage site have evolved to regulate processing.

Significance: These data will be useful in understanding tissue development in tooth and other tissues expressing DSP-PP.

Normal dentin mineralization requires two highly acidic proteins, dentin sialoprotein (DSP) and phosphophoryn (PP). DSP and PP are synthesized as part of a single secreted precursor, DSP-PP, which is conserved in marsupial and placental mammals. Using a baculovirus expression system, we previously found that DSP-PP is accurately cleaved into DSP and PP after secretion into medium by an endogenous, secreted, zinc-dependent Sf9 cell activity. Here we report that mutation of conserved residues near and distant from the active site cleavage in DSP-PP, had dramatic effects on cleavage efficiency by the endogenous Sf9 cell processing enzyme. We found that: 1) mutation of residues flanking the cleavage site from P_4 to P_4' blocked, impaired, or enhanced DSP-PP cleavage; 2) certain conserved amino acids distant from the cleavage site were important for precursor cleavage; 3) modification of the C terminus by appending a C-terminal tag altered the pattern of processing; and 4) mutations in DSP-PP had similar effects on cleavage by recombinant human BMP1, a candidate physiological processing enzyme, as was seen with the endogenous Sf9 cell activity. An analysis of a partial TLR1 cDNA from Sf9 cells indicates that residues that line the substrate-binding cleft of Sf9 TLR1 and human BMP1 are nearly perfectly conserved, offering an explanation of why Sf9 cells so accurately process mammalian DSP-PP. The fact that several mutations in DSP-PP significantly modified the amount of PP_240 product generated from DSP-PP_240 precursor protein cleavage suggests that such mutations may affect the mineralization process.

In the developing tooth germ, polarized odontoblasts create an optimal environment for dentin mineralization (i.e. the conversion of predentin to dentin). Type I collagen is actively secreted at the odontoblast cell border into predentin (1). Mineralization is then initiated by the secretion of noncollagenous proteins, exemplified by dentin sialoprotein (DSP) and phosphophoryn (PP), the latter a highly negatively charged phosphoprotein capable of binding Ca^{2+} and enabling the formation of hydroxyapatite (2, 3). The ultimate result of these biochemical events is the formation of dentin, the major mineralized tissue of teeth.

DSP and PP are highly conserved in vertebrates and are synthesized as part of a single secretory precursor protein, DSP-PP (also referred to as DSPPP) (4–6), in mammals from opossum (Ensembl Transcript ID: ENSMODT00000026398) to humans (7, 8). The importance of the DSP-PP precursor protein for tooth development is evident from the linkage of mutations in the DSP-PP gene to dentinogenesis imperfecta II, affecting 1 in 7,000 newborns resulting in teeth having amber, gray, or purple discoloration (9, 10). Within 2–4 years, the enamel is chipped away, leaving poorly formed dentin that is more susceptible to decay than enamel.

Recently, a Tyr-to-Asp mutation in the presumptive signal peptide hydrophobic core of the DSP-PP precursor protein has been linked to patients with dentinogenesis dysplasia (11). Likely, the mutated signal peptide is not able to direct translocation of the DSP-PP precursor protein into the lumen of the endoplasmic reticulum. Thus, modifications in protein processing and secretion can also lead to clinical abnormalities. Moreover, proper DSP-PP precursor protein processing may also be critical for normal tissue development during early tooth development as well as other yet to be determined developmental programs in bone, salivary gland, and kidney (4, 12).

Previous work by our group demonstrated that the DSP-PP precursor protein could, for the first time, be produced in quantities necessary for chemical analysis by using a baculovirus

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expression system (4). The DSP-PP precursor protein is secreted into the conditioned medium of infected Sf9 (Spodoptera frugiperda) insect cells. It then undergoes processing at the physiological cleavage site by a Zn$^{2+}$ metalloproteinase (most likely Tolloid-related-1 protein, TLR1), which is also secreted by Sf9 cells. The resulting stable peptides correspond to the physiological cleavage products DSP and PP. Mass spectrometric (MS and MS/MS) analysis identified the cleavage site for generating mature DSP and PP proteins, **G**$^{447}$→**D**$^{448}$, which is highly conserved in mammals (13). We hypothesize that this conserved DSP-PP cleavage domain (P$_4$P$_3$P$_2$P$_1$ → P$_1$P$_2$P$_3$P$_4$) (14) is necessary for proper cleavage. Mutations in this domain are likely to affect proper DSP-PP240 cleavage. Substitutions outside this cleavage domain that affect DSP-PP precursor cleavage are expected to identify residues important for cleavage, either because they are important for conformation and exosite interactions or because they participate in peptide catalysis.

Here we have created mutations in conserved residues within and outside of the immediate vicinity of the cleavage site in DSP-PP$_{240}$ and assessed the processing of these mutant precursors by the endogenous Sf9 cell processing enzyme. We found that nearly all mutations of conserved residues from P$_3$ to P$_1$ affected cleavage. However, although some substitutions impaired or blocked cleavage, several others actually increased the cleavage efficiency. Mutation of certain conserved residues outside of the P$_4$P$_3$P$_2$P$_1$ region also had significant effects on processing. We also found that the effects of mutations of DSP-PP$_{240}$ on cleavage by purified recombinant human BMP1, a candidate physiological processing enzyme (5, 6, 15), paralleled the effects on cleavage by the Sf9 cell processing activity (13) explained by the high degree of conservation between the substrate-binding clefts of the candidate Sf9 cell and human processing enzymes.

**EXPERIMENTAL PROCEDURES**

**Baculovirus Expression System**—Recombinant rat DSP-PP$_{240}$ precursor protein was produced by a baculovirus expression system containing DSP-PP$_{240}$ cDNA, which represents one of three endogenous DSP-PP transcripts (16, 17). The DSP-PP$_{240}$ cDNA contains a 17-amino acid leader sequence, the DSP-PP$_{240}$ coding sequence, a stop codon, and a 200-bp non-coding sequence. Mutants of DSP-PP$_{240}$ were generated using site-directed mutagenesis (see below under "Site-directed Mutagenesis"). Construction and expression of DSP-PP$_{240}$ cDNAs and the mutated cDNAs using the pVL1392 baculovirus expression system were as described (4).

The pVL1392-DSP-PP$_{240}$ cDNA and mutated pVL1392-DSP-PP$_{240}$ cDNA constructs were individually co-transfected with a linearized BaculoGold baculovirus DNA (BaculoGold transfection kit, Pharmingen) (18) into insect Sf9 cells to obtain virus stocks. Stocks were passaged at least three times to obtain sufficient virus titers for experiments. To produce recombinant proteins, insect Sf9 cells were infected with recombinant virus stock at a multiplicity of 10 and grown in Grace’s insect cell medium (Invitrogen) supplemented with 10% FBS to a density of 2×10$^6$ cells in T-25 flask. For studies of cleavage by the endogenous Sf9 cell activity, supernatants were harvested on day 4 after infection and partially purified using a polymeran extraction protocol (see below).

**Site-directed Mutagenesis**—Mutations were created in the DSP-PP$_{240}$ cDNA in the vector pGEM7Z(+) using 32-nucleotide sense and antisense mutagenic primers (supplemental Table 1) following the Stratagene QuickChange site-directed mutagenesis protocol (Stratagene, La Jolla, CA). Mutations were verified by DNA sequence analysis, and mutated cDNAs were subcloned into the baculovirus recombination vector pVL1392 to generate infectious baculovirus containing the mutated cDNA.

**Partial Purification of Recombinant DSP-PP$_{240}$ Proteins and the Mutated Recombinant Proteins Using Polyacrylamide Gel Electrophoresis**—This protocol takes advantage of the finding that acidic proteins such as the DSP-PP$_{240}$ precursor proteins, DSP and PP, are soluble in 5% trichloroacetic acid (TCA) (4). The 4-day supernatant from Sf9 cells infected with baculovirus encoding wild type (WT) or mutant forms of DSP-PP$_{240}$ was treated with 5% TCA to precipitate the majority of culture medium proteins. The TCA-soluble portion was neutralized and precipitated with 1.0 M CaCl$_2$ as described previously (4). This precipitate was dissolved in one tenth volume of 0.1 M EDTA and stored at −20°C.

**Recombinant DSP-PP$_{240}$ Protein Preparation for Human BMP1 Cleavage Studies**—WT DSP-PP$_{240}$ secreted by Sf9 cells is processed by a zinc-dependent enzyme secreted by Sf9 cells (13). However, secretion of this activity diminished 3 days after infection. To assess cleavage of DSP-PP$_{240}$ by exogenously added protease, medium from baculovirus-infected Sf9 cells was removed 3 days after infection and replaced with fresh Grace’s medium. The cells were then incubated for an additional 4 days. The conditioned medium collected at the end of this period (CM4–7d) contained DSP-PP$_{240}$ that was mostly unprocessed (13) and that could therefore be used as a substrate for cleavage reactions by exogenously added protease. In cleavage experiments, CM4–7d from cells expressing WT or mutant DSP-PP$_{240}$ was incubated with an equal volume of Grace’s medium (no BMP1 control) or noninfected Sf9 3-day CM containing endogenous proteolytic activity (13) or with an equal volume of Grace’s medium containing BMP1 (33 and 170 ng/ml final concentration respectively). After a 24-h incubation, samples were prepared as described above. Human recombinant BMP1 was from R&D Systems (Minneapolis, MN).

**Polyacrylamide Gel Electrophoresis**—Native PAGE was performed using 7.5% polyacrylamide gels. Samples were dissolved in sample buffer lacking SDS. Electrophoresis was carried out at 60 mA for 45 min, with the BenchMark$^{	ext{T}}$ prestained protein ladder as a marker (Invitrogen). Gels were stained with Stains-All (Sigma) and were air-dried in a cellophane membrane overnight. Stains-All is characteristic in staining acidic proteins (i.e., neutral proteins (such as BSA) appear orange-red.

**Quantification of DSP-PP$_{240}$ and PP$_{240}$ Proteins**—Dried gels were scanned to TIFF files, and ImageJ (National Institutes of Health) was used to quantify the intensity of Stains-All-stained bands. The stained protein band images were converted to grayscale and inverted, and rectangular areas were used to integrate the intensity of the DSP-PP$_{240}$ and PP$_{240}$ bands. These
**DSP-PP Cleavage Site**

![Diagram of DSP-PP cleavage site](image)

**FIGURE 1. Alignment of DSP-PP240 junction peptide and C-terminal sequences from marsupial and placental mammals.** The heavy bar indicates the residues flanking the cleavage site between DSP and PP (G447 ↓ D448) as defined for DSP-PP240 expressed in SF9 cells (13) from P2 to P3. Mutations were generated both within P1-P2 and outside of P1-P4 (see arrows). Purple highlights amino acid residues perfectly conserved in all seven species. Yellow highlights residues identical in six or seven species. Blue highlights residues identical in four species. The alignment for the C-terminal 18 amino acids is shown for the six placental mammalian species.

Intensity values were then used to calculate the ratio of PP240:D240 (PP240+DSP-PP240). Each experiment was conducted at least three times with comparable results. Plotted PP240:D240 (PP240+DSP-PP240) ratios represent the mean of at least three experiments, and error bars represent S.E. (n = 3). Data were analyzed and plotted using KaleidaGraph (Synergy Software, Reading, PA).

Preparation of Fast Protein Liquid Chromatography (FPLC)-purified Recombinant PP240 Mutants—20 ml of condition medium containing recombinant DSP-PP240 mutants was collected and partially purified as described previously. The precipitate was dissolved in one tenth volume (2 ml) of 0.1M EDTA followed by dialysis in 1 liter of 25 mM Tris-HCl, pH 8.1, with three buffer changes. The dialyzed sample was resuspended in 200 μl of autoclaved water. 50 μl of the resuspended sample was loaded onto a UnoQ1 column, which contains quaternary ammonium functional groups. The sample was eluted with a gradient of buffer A (25 mM Tris-HCl, pH 8.1) and buffer B (25 mM Tris-HCl, pH 8.1, and 2 mM NaCl). PP240 mutants were eluted at fractions 21–22 (1 ml/fraction) at 0.3M NaCl and were detected at a wavelength of 214 nm. Fractions 21–22 were combined and dialyzed in 1 liter of 25 mM Tris-HCl, pH 8.1, and then followed with three buffer changes. The dialyzed samples were lyophilized for Edman degradation analyses for N-terminal amino acid sequence analyses via Edman degradation—The N-terminal amino acid sequences of the mutated proteins were determined by Procise eLC Model 492 (Applied Biosystems, Foster City, CA) at the Beckman Institute, California Institute of Technology, Pasadena, CA using reagents and methods recommended by the manufacturer.

**RESULTS**

Alignment of DSP-PP Amino Acid Sequences from Seven Mammalian Species Reveals Conservation of the Processing Site and Other Regions—Alignment of DSP-PP peptide sequences of seven mammalian species from marsupials (opossum) to humans (Fig. 1), representing 180 million years of evolutionary divergence, illustrates that amino acid sequences surrounding the purported cleavage site Gly447 ↓ Asp448 ASPROAPN (G447 ↓ D448 DPN) between DSP and PP are highly conserved. The alignment also shows that the C-terminal region of DSP (from Ser430 to Gly447) and the C-terminal 18 residues of PP are highly conserved. Mutations were generated within the cleavage domain (P1 to P4) to test the importance of the conservation in the cleavage site. Mutations at conserved sites in DSP (Try438 and PP (Try481, Asp484, and Asp687), which are positioned outside of the DSP-PP cleavage domain, were also generated to test their effects on DSP-PP240 precursor protein cleavage. Furthermore, the C-terminal addition of a 6-amino acid sequence (MS and MS/MS) was also examined to test the effect of a C-terminal tag on cleavage. The effects of the mutations on processing by the endogenous SF9 cell activity are shown in representative Stains-All-stained native polyacrylamide gels in Figs. 2, 4, A-C, and 5A and summarized in Figs. 3, 4D, and 5B.

G447 ↓ D448 Is the First Cleavage Site—Our previous data on cleavage of rat DSP-PP240 in SF9 cell medium, MS and MS/MS data on the N terminus of rat PP240 produced in SF9 cells, and alignments of DSP-PP junctions (13) suggest that G447 ↓ D448 is the initial cleavage site cleaved by a BMP1-like processing protease. Gly is found at P1 in all cases except in opossum, where there is a nonconservative substitution of Arg. To determine whether the conservation of the putative P1 position is critical for proper DSP-PP240 cleavage, we generated mutations in the rat DSP-PP240 cDNA sequence that changed Gly447 to Val, Ala, and Arg, the last of these mimicking the marsupial sequence. Baculovirus encoding WT DSP-PP240 and the three P1 substitutions was used to infect SF9 cells, and medium collected 4 days after infection was analyzed. The G447V and G447A mutations completely blocked processing (Fig. 2A, lanes 2 and 3). Even at 11 days after infection, these two mutant precursor proteins remained uncleaved (data not shown). Remarkably, at 4 days after infection, DSP-PP240 precursor protein with the G447R substitution (Fig. 2A, lane 4) exhibited nearly as extensive processing as WT DSP-PP240 precursor protein (Fig. 2A, lane 8). Taken together, these results strongly support the conclusion that G447 ↓ D448 is the initial cleavage site, that conservation of P1 position is critical for proper DSP-PP240 cleavage, and that...
either of the two residues, Gly or Arg, found at the putative P1 position in mammalian DSP-PP sequences will support processing.

P2 and P3 Are Critical for DSP-PP240 Precursor Protein Cleavage—Both P2 and P3 residues in DSP-PP precursor proteins are highly conserved from opossum to human (Fig. 1). The change from the hydrophilic P2 residue Gln446 to the basic residue Lys (Fig. 2A, lane 5) and from the hydrophobic P3 residue Met445 to the basic residue Lys (Fig. 2A, lane 6) completely blocked DSP-PP240 precursor protein cleavage and resulted in production of stable, unprocessed DSP-PP240 precursor protein in the medium. These data indicate that Gln446 at P2 and Met445 at P3 are both critical for DSP-PP240 precursor protein cleavage.

P4 Mutation Ser444 to Ala (S444A) Enhanced DSP-PP240 Cleavage—We expected that mutation of Ser444 to Ala at the putative P4 site would not significantly affect DSP-PP240 cleavage because this replacement neither changes the main chain conformation nor imposes strong electrostatic or steric effects. However, we found that not only did this mutation not block DSP-PP240 cleavage; it actually accelerated DSP-PP240 cleavage (Fig. 2A, lane 7).

Mutations in the P1’ Position Block DSP-PP240 Cleavage—Asp at P1’ is conserved in all known BMP1 substrate cleavage sites. Several substitutions (Asp448 to Ala, Glu, and Asn) were made at the putative P1’ site in DSP-PP240. Each of these substitutions completely blocked cleavage of DSP-PP240 when assessed 4 days after infection. The fact that even the conservative substitution of Glu for Asp448 abrogated cleavage is consistent with recognition of Asp at P1’ being a primary determinant of processing specificity (Fig. 2B, lane 2). Similarly, changing Asp448 to a hydrophobic residue (Ala) (Fig. 2B, lane 4) or to a hydrophilic residue (Asn) (Fig. 2B, lane 3) also blocked DSP-PP240 cleavage. However, after 7 days, the Ala448 mutant precursor protein exhibited a small amount of PP240 cleavage product (data not shown). This may result from the fact that although the Ala side chain lacks the β-carboxylate, which might be required for optimal interaction with the S1 subsite of the processing enzyme, the Ala methyl side chain cannot be excluded from the site, unlike the larger Glu side chain, and lacks potential unfavorable interactions of the neutral Asn side chain. Nevertheless, the effects of these substitutions are consistent with the conclusion that Asp at P1’ is a crucial determinant in protein processing.

Mutations at the Putative P3’ Position Modify the DSP-PP240 Cleavage—Asp449, at the putative P3’ site, is highly conserved in DSP-PP from all seven mammalian species (Fig. 1). However, P2’ exhibits considerable variability in other BMP1 substrates, with Asp, Glu, and the small residues Ala and Ser predominating (see table of BMP1/Tolloid cleavage sites in the MEROPS database (peptidase M12.005)). If the presence of an acidic residue at P2’ were important for cleavage, we would expect that substitution of Glu at 449 would have little effect on the DSP-PP cleavage; it actually accelerated DSP-PP240 cleavage. However, after 7 days, the Ala448 mutant did not block DSP-PP240 cleavage. Thus, mutations at the putative P3’ site in DSP-PP240 precursor protein cleavage (Fig. 2B, lane 7). We also expected that mutating the Asp449 to Ala might inhibit DSP-PP cleavage. On the contrary, the D449A mutant also showed accelerated DSP-PP240 cleavage (Fig. 2B, lane 8). In contrast, the Asp449 to Asn mutation strongly reduced DSP-PP240 cleavage such that faint PP240 was observed at 4 days after viral infection (Fig. 2B, lane 6). Thus, mutations at the putative P3’ residue, Asp449, had dramatic and distinct effects on generating large amounts of PP240 product from D449E DSP-PP240 precursor protein cleavage.

Mutations at the Putative P1’ Position Accelerate DSP-PP240 Cleavage—Pro (Pro450 in rat DSP-PP240) is highly conserved at the putative P1’ site among the seven mammalian species (Fig. 1). Because of conformational constraints imposed by the Pro
reported to be the major C-terminal residue of a mature form of the DSP protein (19) and a candidate P1 residue for cleavage. The Y438A mutant expressed in SF9 cells exhibited a processing efficiency (Fig. 4C) similar to that of WT DSP-PP240 (Fig. 4C), suggesting that Tyr438 is not a crucial residue for DSP-PP240 processing and therefore not a real candidate for the P1 residue. Mutation of another conserved site, D484A, also resulted in a processing efficiency (Fig. 4C) similar to that of WT DSP-PP240 (Fig. 4C).

**Mutations of the Distal Residue Tyr481 to Ser and Ala Also Generated More PP240 Products from the Mutated DSP-PP240 Precursor Proteins**—Tyr481 is located 33 amino acids distal to the putative P1′ and is conserved among all mammalian species except opossum (Fig. 1). When Tyr481 was changed to Ser (Fig. 4A) or Ala (Fig. 4B), more PP240 was generated from the mutated precursors. Thus, mutations in conserved residues outside of the proposed P1-P1′ cleavage site can also affect DSP-PP precursor cleavage.

**Effects of Mutation of the Conserved C Terminus of DSP-PP240 and of Adding a C-terminal MSMSMS Tag on DSP-PP240 Precursor Cleavage**—The C-terminal 18 residues of DSP-PP240 are highly conserved among the sequences of all seven mammalian species (Fig. 1). Strong conservation of this region suggests the possible presence of a functional domain. To assess the possible influence of these conserved sequences on processing, we determined the effect of substituting Met for the conserved C-terminal Asp residue (Asp687 in rat DSP-PP240). This substitution did not block production of PP240 but did result in the appearance of a new, more slowly migrating minor band just above PP240 (Fig. 5A, D687A lane), suggesting the possibility that a novel upstream cleavage site was activated.
DSP-PP Cleavage Site

FIGURE 5. Effect of a C-terminal mutation and a C-tag modification on DSP-PP240 processing. Medium from infected Sf9 cells was harvested 4 days after virus infection. A, mutation of Asp687 to Met (D687M) and addition of C-terminal MSMSMS tag (C-tag). M, molecular mass marker. WT: wild type DSP-PP240. B, quantification of effects on cleavage of the D687M mutation and the C-terminal MSMSMS tag. Error bars represent S.E. (n = 3).

FIGURE 6. Cleavage of WT and mutant forms of DSP-PP240 by recombinant hBMP1. In each panel, M indicates molecular mass marker. Grace: WT DSP-PP240 substrate sample (CM4–7d) or mutant DSP-PP240 substrate incubated 24 h with Grace medium. Sf9 CM: WT or mutant substrate (CM4–7d) incubated 24 h with Sf9 3-day CM (containing endogenous proteolytic activity). BMP1(33 ng): WT or mutant substrate (CM4–7d) incubated 24 h with hBMP1 (33 ng/ml); BMP1(170 ng/ml): WT or mutant substrate (CM4–7d) incubated 24 h with hBMP1 (170 ng/ml). For details, see “Experimental Procedures.” A, cleavage of WT DSP-PP240. B, no cleavage of G447A mutant. C, cleavage of D449E mutant. D, cleavage of DSP-PP240 C-terminally tagged with MSMSMS.

The addition of MSMSMS after the C-terminal Asp687 of DSP-PP240 resulted in a dramatic increase in the efficiency of processing and also in a large change (decrease) in the mobility of the PP-related product (Fig. 5A, C-tag lane). Because the shift in mobility is larger than might be expected from the mass of the added residues (655 Da), it is possible that addition of the tag has altered the cleavage site or resulted in post-translational modifications.

**Human BMP1 Exhibits Similar Specificity to the Endogenous Sf9 Cell Activity in Cleaving DSP-PP240 Precursor Protein Mutants**—Previously, we showed that the hBMP1, a candidate physiological processing enzyme, could cleave rat DSP-PP240 secreted by Sf9 cells yielding DSP350 and PP240 products that co-migrated with the products of cleavage by the endogenous Sf9 cell activity (13). This assay relied on the fact that the processing activity secreted by Sf9 cells decreased after 3 days of infection with DSP-PP240-expressing baculovirus. When medium of infected cells was replaced after 3 days of infection, and infected cells were incubated for an additional 4 days, the DSP-PP240 secreted during that period remained largely uncleaved and could be used as a substrate (termed CM4–7d) for cleavage by exogenously added enzyme (13). As found previously (13), when recombinant hBMP1 was added to CM4–7d from cells expressing WT DSP-PP240 precursor protein, all of the precursor was cleaved in a 24-h incubation, yielding an expected amount of the PP240 cleavage product (Fig. 6A). In a control incubation lacking hBMP1, the DSP-PP240 precursor protein remained largely uncleaved (Fig. 6A, Grace medium control). WT CM4–7d incubated with Sf9 CM showed weak cleavage due to the endogenous proteolytic activity in insect cells. At lower BMP1 concentration (33 ng/ml), protein showed a smaller amount of PP240 product. At higher BMP1 concentration (170 ng/ml), protein showed a larger amount of PP240 product. G447A DSP-PP240 precursor protein was not cleaved in all incubation conditions such as Grace medium, Sf9 CM, and 33 and 170 ng/ml BMP1 (Fig. 6B). The D449E mutant was cleaved completely by 170 ng/ml hBMP1 after a 24-h incubation (Fig. 6C).

Finally, DSP-PP240 C-terminally tagged with MSMSMS was also cleaved to completion by incubation with hBMP1 (170 ng/ml) for 24 h (Fig. 6D). The product again exhibited slower mobility than WT PP240. These results provide evidence that hBMP1 responds to mutations in DSP-PP240 in the same way as the endogenous Sf9 cell activity and that hBMP1 and the endogenous activity cleave DSP-PP240 at the same site.

**N-terminal Peptide Sequence of Recombinant Mutant PP Proteins Generated from S444A, D449E, P450A, and C-tag PreProteins**—We have used FPLC to purify the PP240 from these mutants for Edman degradation analyses. The cleavage PP240 products from S444A, D449E, and P450A mutants showed the same mobility as that from the wild type. Edman degradation revealed the N-terminal sequence of the S444A mutant as DDPNSS, the sequence of the D449E mutant as DEPNS, and the sequence of the P450A mutant as DDANS (Table 1). The C-tag (a 6-residue tag, Met-Ser-Met-Ser-Met-Ser) showed a slower mobility than WT PP240. However, the Edman degradation showed that the C-tag PP cleaved product also has DDPNS as the N terminus. Taken together, these four mutants are all cleaved at the G\(^{447}\)\(\text{D}^{448}\) site.

**DISCUSSION**

DSP-PP precursor protein must undergo multiple post-translational modifications, including signal peptide cleavage, Asn-linked glycosylation, phosphorylation, and proteolytic processing between the DSP and PP domains, to produce mature DSP and PP proteins required for dentin mineralization. Recent research on DSP-PP has focused on the identification of its initial cleavage site, as well as on the identification of the protease responsible for cleavage, to better understand DSP-PP precursor protein processing during normal as well as pathological dentin mineralization.

Previously, we used an insect cell Sf9 expression system to produce substantial amounts of secreted rat DSP-PP240 and...
showed that the precursor was processed to stable products, DSP430 and PP240 (4). From the spectrum obtained by MS and MS/MS analysis of isolated tryptic fragments of the PP240 band obtained by gel electrophoresis, we determined a molecular mass of 7,700 Da, which agreed well with the peptide 448–523 and was consistent with DDPSN as the N terminus of PP240. From this result, we proposed that the DSP-PP cleavage site was G447-D448 (4).

Others have based their identification of cleavage products on gel mobility of immuno-stained or Stains-All-stained bands separated by SDS-PAGE (5, 6, 15, 19–21). However, the exact cleavage site of the WT mouse DSP-PP precursor protein in cultured cells has not been determined by direct sequencing or mass spectrometry. For example, Qin et al. (19), using purified rat DSP protein and trypsin digestion, identified a peptide product ending with Tyr438 (numbered Tyr421 in their work) and concluded that Y438-D439 was the major DSP-PP cleavage site. However, here we find that introduction of a Y438A mutation in DSP-PP240 precursor protein had no effect on cleavage, providing evidence that Y438-D439 is not a cleavage site. Our studies demonstrate conclusively that rat DSP-PP240 is cleaved exclusively at G447-D448DPN by an endogenous Sf9 cell processing enzyme (13). More recently, Sun et al. (5) reported that a Y448A mutation blocked cleavage of recombinant mouse DSP-PP in cultured human cells system and concluded that the key cleavage site is G447-D448. Additional studies devoted to identifying the exact DSP-PP cleavage site by Steiglitz et al. (20), von Marschall and Fisher (6), and Yamakoshi and co-workers (15, 21) have been summarized by Ritchie et al. (13).

More recently, we demonstrated that cleavage depended on a secreted zinc-dependent activity produced by Sf9 cells. Furthermore, we used MS and MS/MS to analyze a smaller, chymotryptic fragment of the PP240 band, which permitted direct determination of the N-terminal amino acid sequence by ion trap/fragmentation MS/MSMS as D448DPNSSDE SNGSDGSDDANSEAIENGHGDASY. No other peptide sequence was obtained by gel electrophoresis, we determined a molecular mass of 7,700 Da, which agreed well with the peptide 448–523 and was consistent with DDPSN as the N terminus of PP240.

Here we created mutations of conserved residues flanking and outside of the DSP-PP cleavage site (P4 to P4') and determined their effects on cleavage by the endogenous Sf9 cell processing enzyme. We found that most of the selective mutations that we generated within the P4 to P4' cleavage domain altered DSP-PP cleavage. A striking finding is that although mutation of certain conserved residues (P6, P2', P1', P1'') blocked cleavage by the Sf9 processing enzyme, mutation of others (P4 and P5') accelerated cleavage. Remarkably, mutation of one site (P2') accelerated or blocked cleavage depending on the nature of the substitution. One interpretation of these results is the effect of evolution of the processing site in DSP-PP is a consequence of the fact that the physiological processing enzyme, if it is BMP1, is an enzyme with many different substrates. Although comparison of the cleavage sites in different types of BMP1 substrates yields a very limited consensus sequence (22), with only the P1', Asp being highly conserved, cleavage sites (P4-P4') in a particular type of substrate, such as DSP-PP, are nevertheless very highly conserved, possibly because the enzyme cannot evolve to satisfy the distinct requirements of each substrate. An analogous situation is seen in the case of plasminogen activation where the activation site that is cleaved alternatively by tissue plasminogen activator or urokinase does not represent a “perfect” cleavage site for either enzyme (23, 24).

Mutation of the P1 Gly447 to Ala or Val completely blocked cleavage, supporting the conclusion that G447-D448 is the initial cleavage site and that conservation of the P1, position is critical for proper DSP-PP cleavage. However, cleavage was unaffected in the G447R mutant. This is striking in that Arg at P1 is a naturally occurring variation in mammalian DSP-PP sequences, found in the marsupial opossum. Therefore, it appears that either Gly or Arg at P1 will support processing, obviously necessitating an alternative binding mode for the propyl guanidinium side chain of Arg. An unexpected finding was that several substitutions in conserved residues within the P4-P4' cleavage domain actually accelerated cleavage by the Sf9 cell processing enzyme. Mutation of the P2 Ser444 to Ala substantially increased the cleavage product PP240 (Fig. 2A, lane 7). This is somewhat surprising in that both Ser and Ala have small side chains. It is possible that the large increase in cleavage product PP240 observed is due to the hydroxyl alone, but it should be noted that Ser444 is a potential casein kinase phosphorylation site. Different substitutions at P2' had dramatically different effects on DSP-PP cleavage; cleavage of D449A and D449E mutants was accelerated, whereas cleavage of D449N was strongly reduced. These effects are difficult to explain without structural information. For example, the effect of the Ala substitution is consistent with loss of unfavorable steric interactions, and indeed, the isosteric substitution Asn is inhibitory, perhaps consistent with the loss of a compensatory favorable interaction with the β-carboxylate. The Glu substitution at P2', which yields a large increase in cleavage product PP240 of any of the mutant forms of DSP-PP240 tested, provides a favorable carboxylate moiety and is bulkier and might be expected to compound steric clash. Alternatively, the additional β-methylene group may permit conformational flexibility that allows the favorable interaction and avoids the steric clash. Indeed, among well characterized substrates of BMP1 (see table of BMP1/Tolloid cleavage sites in the MEROPS database (peptidase M12.005)), several have Glu at P2' (human proglycin and prodecorin and mouse laminin5γ2) and others have Gln at P2' (Pro-α1 collagen I and Pro-α2 collagen I and V). As mentioned under “Results,” the change from hydrophilic residue Gln446 to basic residue Lys446 at P2' (Fig. 2A, lane 5) and hydrophobic residue Met445 to basic residue Lys445 at P3' (Fig. 2A, lane 6) completely blocked the DSP-PP240 precursor protein cleavage. In concert with this, von Marschall and Fisher (6) reported that a M445Q446 to E445F mutation blocked DSP-PP

### TABLE 1

<table>
<thead>
<tr>
<th>Mutants</th>
<th>N-terminal sequence</th>
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<tbody>
<tr>
<td>S444A</td>
<td>DDPNS</td>
</tr>
<tr>
<td>D449E</td>
<td>DEPNSS</td>
</tr>
<tr>
<td>P450A</td>
<td>DDDANS</td>
</tr>
<tr>
<td>C-tag</td>
<td>DDPSN</td>
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precursor cleavage as demonstrated by Western blot analyses. Therefore, P_2 Gln^{446} and P_3 Met^{445} are critical for DSP-PP_{240} precursor protein cleavage.

We also produced a set of mutations in conserved regions lying outside of the P_4-P_4' cleavage domain. One (i.e. Y438A) did not affect DSP-PP_{240} cleavage (see discussion above). Mutation of the conserved amino acid Asp^{484} also did not affect DSP-PP cleavage (Fig. 4C). However, mutation of another conserved residue, Tyr^{483} (Y481S and Y481A), located 33 amino acids distal to the P_4' site, dramatically increased DSP-PP cleavage efficiency (Fig. 4, A and B), indicating, surprisingly, that residues quite distant from the cleavage site can affect recognition by the processing enzyme. Substitution of Met for the C-terminal residue, Asp^{487}, resulted in a slight enhancement of cleavage but also the appearance of a small amount of a slower migrating band (Fig. 5A). Thus, the conservation of these two sites (Tyr^{483} and Asp^{487}) is critical for proper DSP-PP cleavage and suggests that the C terminus may participate in exosite interactions or affect conformation at the cleavage site that is important for DSP-PP catalysis.Appending a 6-residue tag (the C-tag) generated a large amount of C-tag PP product (Fig. 5A). This fusion mutation also resulted in a band that migrated considerably more slowly than WT PP_{240}. This observation raises the question of whether this simply reflects the effect of the tag on the mobility of PP_{240} or an actual alteration in the cleavage site. From Edman degradation analysis, the N-terminal sequence of the major C-tag PP protein is DDPNS. Thus, the C-tag mutant is cleaved at the G^{447}↓D^{448} site. The slower mobility of C-tag PP product may be due to a post-translational modification.

Most of our mutants were generated from the DSP-PP_{240} transcript. Previously, we reported three rat DSP-PP transcripts (i.e. DSP-PP_{240}, DSP-PP_{252}, and DSP-PP_{271}), which generate different PP isoforms (16, 17). To test whether mutants generated from a longer isoform (i.e. DSP-PP_{252}) would be cleaved at the same DSP-PP_{240} cleavage site, we used a DSP-PP_{252} transcript, which generated recombinant DSP-PP_{523} precursor protein with an apparent molecular mass of 200 kDa. The apparent molecular mass for the cleavage product, PP_{523}, is 90 KDa (supplemental Fig. 1), which migrated at the same position as native highly phosphorylated protein (data not shown), which is equivalent to isoform PP_{523} (17). When we generated G447V (supplemental Fig. 1) and G447A (data not shown) mutants, we found that both of these DSP-PP_{523} mutants produced DSP-PP_{523} precursor proteins that were not able to undergo further cleavage. This observation is identical to what we observed in DSP-PP_{240} mutants G447V and G447A. The G447R mutant is cleavable in both DSP-PP_{523} and DSP-PP_{240} precursor proteins. Likely, other mutants in DSP-PP_{523} will show the same cleavage patterns as we have found with DSP-PP_{240}.

The most likely advantage of regulated DSP-PP proteolysis during dentinogenesis is that the timely release of mature DSP and mature PP is critical for proper dentinogenesis timing. It is well accepted that collagen expression occurs as a first step in dentin mineralization. Following collagen maturation, PP becomes available to initiate mineralization. Thus, accelerated or delayed cleavage of the DSP-PP precursor protein would certainly affect the temporal relationship between collagen and PP during the mineralization process, which could cause improper dentin formation.

Purified hBMP1 appears to cleave baculovirus-expressed WT rat DSP-PP_{240} at the same site as the endogenous Sf9 cell activity. This conclusion is further supported by the fact that the PAGE mobility of the primary, Stains-all-stained product of cleavage of MSMSMS-tagged DSP-PP_{240} is shifted identically to the product of cleavage by the endogenous Sf9 cell activity (Fig. 6D). Another example is found with the P_4 mutant G447A DSP-PP_{240} which was resistant to cleavage by the endogenous activity and was also completely resistant to cleavage by hBMP1 (Fig. 6B). Thus, at a qualitative level at least, mutations in DSP-PP_{240} had similar effects on cleavage by the endogenous Sf9 protease activity and by hBMP1.

The fact that the Sf9 cell activity cleaves DSP-PP_{240} with high selectivity at the physiological cleavage site (i.e. immediately upstream from the known N terminus of phosphophoryn) suggests that the Sf9 enzyme is homologous to the physiological processing enzyme. The fact that mutations of conserved residues in the immediate vicinity of the cleavage site (P_4-P_4') have dramatic effects on cleavage by the Sf9 enzyme (i.e. blocking or accelerating cleavage) suggests that the substrate-binding domain of the enzyme must be very highly conserved. From this work and other published work, the mammalian Tolloid homologs, BMP1/TLD, TLL1, and TLL2 (22), have emerged as strong candidates for the physiological DSP-PP processing enzyme. The BMP1/TLD family of processing proteases comprises a set of molecules with complex multidomain structures. C-terminal to the relatively small astacin-related catalytic domain (~200 residues) is found variable arrangements of complement C1r/C1s, Uegf, BMP1 (CUB) and EGF domains that may influence substrate recognition, although little is known of how this occurs (25).

We have shown that Sf9 cells express an RNA for a homolog of Drosophila Tolloid-related-1 protein, which we refer to as Sf9 TLR1. This RNA sequence corresponds to the most highly conserved 120 residues of the 202 residue TLR1 astacin catalytic domain (13). We propose that Sf9 TLR1 is the DSP-PP_{240} processing activity secreted by Sf9 cells and that it represents an extremely good model for the mammalian physiological processing enzyme. First, within that part of the astacin catalytic domain corresponding to our cloned Sf9 TLR1 cDNA, both Sf9 and Drosophila TLRI sequences are significantly more similar to the human Tolloid homologs (65–75% identity) than is Drosophila Tolloid (TLD) (51–54% identity) (supplemental Fig. 2A). This analysis holds up when comparing the full 202-residue dTLRI and dTLD catalytic domains with the human sequences as well (65–67% identity for dTLRI versus 52–54% identity for dTLD) (supplemental Fig. 2B). The Sf9 TLR1 sequence is also more similar to the mammalian BMP1/TLD, TLL1, and TLL2 sequences (71–75% identical) than is Drosophila TLRI (65–69% identity) (supplemental Fig. 2A). Even more strikingly, conservation between Sf9 TLR1 and the human Tolloid family is near perfect within the catalytic cleft. The high resolution (1.27Å) structure of the hBMP1 catalytic domain has a deep cleft for binding substrate peptides (supplemental Fig. 3). Nearly all of the residues that form the walls of this cleft are encoded within the cloned Sf9 TLR1 cDNA (supplemental Fig. 4).
Moreover, of 22 residues encoded by the Sf9 TLR1 cDNA that correspond to residues that line the hBMP1 substrate-binding cleft, 20 are identical between Sf9 TLR1 and hBMP1 and the other two represent conservative substitutions (Tyr188 to Phe and Met252 to Leu) (supplemental Figs. 3 and 4). Therefore, the substrate-binding clefts of Sf9 TLR1 and hBMP1 are predicted to be nearly identical. It is therefore not surprising that the two enzymes respond similarly to alterations in substrate sequence.

Why should the TLR1/BMP1 catalytic cleft be so highly conserved? One possible explanation is that the large number of different substrates that must be cleaved imposes a significant evolutionary constraint on a processing enzyme. Alterations in substrate-interacting residues demand compensatory alterations in the many genes encoding the many substrates. A similar degree of conservation within the substrate-binding cleft is seen in the kexin/furin family of proprotein processing pro tease (26), enzymes that also cleave a wide range of substrates (27, 28). Indeed, analysis of heterologous processing of mammalian substrates by insect processing enzymes has been a useful tool in understanding specificity in this family, for example, in the analysis of the substrate specificity of the Drosophila furin homologs, Dfurin1 and Dfurin2, using human von Willebrand factor precursor and other mammalian proproteins as substrates (29–31).

These results clearly demonstrate that the production of DSP-PP240 precursor proteins by infected Sf9 cells is a unique means of overcoming problems with low yield of DSP-PP expression found with mammalian cells, as well as a valid means of understanding the interactions between BMP1 and the DSP-PP precursor protein. The analysis of the mutations we have generated in rat DSP-PP240 provides strong evidence that the conservation of the cleavage domain SMQG ↓ DDP (N/K) is critical for the proper cleavage of DSP-PP precursor protein and that mutations outside of this cleavage domain can also lead to altered cleavage. These results may also help explain the ability of BMP1 and related enzyme proteins to process very specifically a wide variety of different extracellular proteins despite there being a limited consensus cleavage sequence (22). Certainly, these data will be useful in understanding tooth development and possibly the development of other tissues expressing DSP-PP (12).

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The Efficiency of Dentin Sialoprotein-Phosphophoryn Processing Is Affected by Mutations Both Flanking and Distant from the Cleavage Site

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