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Research Article

DSP-PP C-Terminal Conservation Is Crucial for Accurate DSP-PP Precursor Cleavage

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Abstract

Dentin Sialoprotein (DSP) and Phosphophoryn (PP), acidic proteins critical to dentin mineralization, are translated from a single transcript as a DSP-PP precursor that undergoes proteolytic processing to generate DSP and PP. Because of the difficulty in obtaining large amounts of DSP-PP, we used a Sf9-baculovirus expression system to yield large amounts of DSP-PP_{240} recombinant protein, a variant form of rat DSP-PP. Previous evidence stated that DSP-PP_{240} produced by baculovirus-infected Sf9 cells can be cleaved accurately into DSP and PP by the endogenous processing enzyme Sf9 Tolloid-Related 1 (TLR1), a homolog for human Bone Morphogenic Protein 1 (BMP1) and the proposed protease to cleave DSP-PP in human. It was also discovered via mass spectrometric analysis that the specific cleavage occurred at the site: $SMQG^{447}|D^{448}DPN$. In addition, we reported that any mutations within the DSP-PP P4 to P4'cleavage site can block, impair or accelerate DSP-PP cleavage, which suggest that its BMP1 cleavage site is highly conserved to regulate its cleavage efficiency. Furthermore, mutations outside of the DSP-PP P4 to P4' cleavage site can impair or accelerate DSP-PP cleavage. Here, we investigate the role of the highly conserved DSPP C-terminal region in DSP-PP cleavage. We generated a DSP-PP C-terminal mutation by substituting the terminal two aspartate residues for two histamine residues (DD/HH-DSP-PP). To test the impact of the DD/HH mutant on DSP-PP cleavage, we used the Sf9 expression system's endogenous TLR1 and exogenous recombinant BMP1. The DD/HH mutation was shown to block DD/HH-DSP-PP cleavage into DSP and PP by both TLR1 and BMP1 *in vitro*. Taken together, these evidence supports our hypothesis that the C-terminal peptides $D^{686}D^{687}$ actively participates in controlling DSP-PP cleavage and that C-terminal conservation is critical for proper DSP-PP precursor cleavage by TLR1 and BMP1.

Keywords: Conservations of DSP-PP C-terminal; DSP-PP Precursor Protein Cleavage; TLR1; BMP1

Introduction

The major component of mineralized dentin tissue was found to be collagen type I. Besides the collagenous proteins, acidic non collagenous proteins were identified and Dentin Sialoprotein (DSP) and phosphophoryn (PP) were found to be the two most abundant acidic non collagenous proteins in dentin [1,2], which were postulated to play significant roles during tissue mineralization. PP was identified in 1967 by Veirs and Perry [3]. PP is an extremely acidic protein and well established as a mineral nucleator for dentin mineralization [4-6]. DSP was identified in 1981 [7]. Since the reports of DSP-PP cDNAs from rat, mouse, human and porcine, and that DSP and PP are derived from a DSP-PP gene [8-14], numerous efforts have focused on the DSP-PP cleavage site and on the protease responsible for DSP-PP cleavage. Despite intensive investigations since 2001, there was no direct evidence proving that $G^{447}|D^{448}$ is the DSP-PP cleavage site and only Western blot analysis to substantiate the claim that BMP1 can correctly cleave DSP-PP. The understanding of this crucial process was rather limited due to a lack of large quantities of DSP-PP precursor protein readily available from tissues or cultured cells [15]. To overcome this limitation, we recently described a method using the Sf9 baculovirus expression system, in which we infected Sf9 cells with baculovirus, a family of viruses known to infect insects and represents a robust method for producing recombinant proteins, containing recombinant DSP-PP_{240} to yield large amounts of DSP-PP_{240} precursor protein.

We reported that DSP-PP_{240} produced by baculovirus-infected Sf9 cells can be cleaved accurately into DSP and PP by the endogenous processing enzyme Sf9 Tolloid-Related 1 (TLR1), a

homolog for human Bone Morphogenic Protein 1 (BMP1), the proposed protease to cleave DSP-PP in human. It was also discovered via mass spectrometric analysis that the specific cleavage occurred at the site: SMQG^{447}|D^{448}DPN [15,16]. In addition, we reported that any mutations within the DSP-PP P4 to P4'cleavage site can block, impair or accelerate DSP-PP cleavage [17,18], which suggest that its BMP1 cleavage site is highly conserved to regulate its cleavage efficiency. Furthermore, mutations outside of the DSP-PP P4 to P4' cleavage site can impair or accelerate DSP-PP cleavage [17], which suggest that these distal conserved residues may participate in exo-site interactions or affect conformation at the cleavage site that is important for DSP-PP catalysis.

The C-terminal 18 residues of DSP-PP$_{240}$ are most highly conserved among the sequences of all six-mammalian species (Figure 1). Strong conservation of this region suggests the possible presence of a functional domain. We hypothesized that the C-terminal peptide D^{686}D^{687} in DSP-PP actively participates in controlling DSP-PP cleavage. Here, we investigate the role of the highly conserved DSPP C-terminal region in DSP-PP cleavage. We generated a DSP-PP C-terminal mutation by substituting the terminal's two aspartate residues for two histamine residues (DD/HH-DSP-PP). To test the impact of the DD/HH mutant on DSP-PP cleavage, we used the Sf9 expression system's endogenous TLR1 and recombinant BMP1.

Figure 1: Alignment of DSP-PP$_{240}$ C-terminal sequences from marsupial and placental mammals. The heavy bar indicates the residues flanking the cleavage site between DSP and PP from P$_4$ to P$_4$'. Mutations (i.e., D^{686}D^{687}/H^{686}H^{687}) were generated in the C-terminal (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.

Materials and Methods

Site-Directed Mutagenesis

Mutations were created in the DSP-PP$_{240}$ cDNA in the vector pGEM7Z (+) using 35-nucleotide sense and antisense mutagenic primers following the Strata gene quick change site-directed mutagenesis protocol (Stratagene, La Jolla, CA). The 35-nucleotide sense (5'AACCACTCAACCAGTCATCATTAGAGCAGAGAA CC3') and antisense (5'GGTTCTCTGCTCTAATGATGACTGG TTGAGTGGTT3') mutagenic primers were used to generate the D^{686}D^{687}/H^{686}H^{687} mutant from DSP-PP$_{240}$ cDNA (Figure 1). These two mutant sites are located at the C-terminal conserved region of DSP-PP precursor protein (Figure 1). Mutations were verified by DNA sequence analysis, and mutated cDNAs were sub cloned into the baculovirus recombination vector pVL1392 to generate infectious baculovirus containing the mutated cDNA.

Baculovirus Expression System

The mutant cDNA from site-directed mutagenesis was verified by DNA sequence analysis and sub cloned into the baculovirus recombination vector pVL1392 to generate infectious baculovirus containing the mutated cDNA. The baculovirus was then used to infect Sf9 cells to allow the expression of the mutated recombinant protein.

Partial Purification of Mutated Recombinant Proteins using Polyanion Extraction

Recombinant acidic proteins DSP-PP precursor, DSP, and PP are soluble in 5% TCA, which was used to isolate the recombinant proteins. DSP-PP related a recombinant protein in 5% TCA was then neutralized with 3M Tris. HCl pH 8.8 and precipitated with 1M $CaCl_2$. The pellet was re-suspended in 0.1 M EDTA.

Recombinant DSP-PP$_{240}$ Protein Preparation for Human BMP1 Cleavage Studies

Secretion of TLR1 protease from Sf9 cells diminished 3 days after infection. After 3 days, fresh Grace Medium was added to the culture of baculovirus-infected Sf9 cells and incubated at 28°C for an additional 4 days. This new 4-day Condition Medium (CM), called CM$_{3\text{-}7d}$, was mostly unprocessed due to the lack of TLR1 and was used as a substrate for cleavage reactions by BMP1.

Results

Wild-Type (wt) Recombinant DSP-PP$_{240}$ Precursor Protein was Cleaved into DSP$_{430}$ and PP$_{240}$ in 4 day Sf9 Culture Medium.

wt DSP-PP$_{240}$ cDNA in baculovirus vector expresses a recombinant DSP-PP$_{240}$ precursor protein, which was cleaved into DSP$_{430}$ and PP$_{240}$ by TLR1 in 4 day sf9 culture medium (Figure 2).

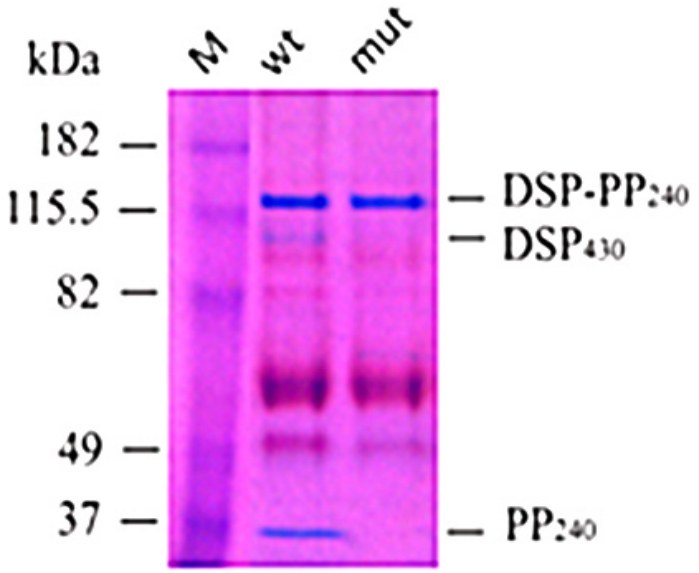

Figure 2: Recombinant wild type (wt) DSP-PP$_{240}$ and D^{686}D^{687}/H^{686}H^{687}mutant DSP-PP$_{240}$ protein expression. M: size marker. Wild type (wt) DSP-PP$_{240}$ cDNA in baculovirus expression vector: DSP-PP$_{240}$; precursor band, cleaved products DSP$_{430}$ and PP$_{240}$ bands were detected in 4d sf9 culture medium. D^{686}D^{687}/H^{686}H^{687}mutant DSP-PP$_{240}$ cDNA: Only DSP-PP$_{240}$ precursor band was detected in mutant lane. Neither cleaved product DSP$_{430}$ nor PP$_{240}$was found in the mutant lane.

Mutant DSP-PP$_{240}$ cDNA (see Figure 1) in baculovirus vector expresses only a recombinant DSP-PP$_{240}$ precursor protein. No DSP$_{430}$ and PP$_{240}$ cleavage products were detected. Thus mutant DSP-PP$_{240}$ was not cleaved at all (Figure 2) by TLR1 in the culture medium.

Can longer incubation of mutant DSP-PP$_{240}$ from 4d Sf9 culture medium enhance cleavage by TLR1 in the medium?

Mutant DSP-PP$_{240}$ in 4d Sf9 culture medium showed only DSP-PP$_{240}$ precursor protein. No DSP$_{430}$ or PP$_{240}$ cleaved products were detected (Figure 3, Lane 2). Previously we reported that TLR1 is the protease responsible for wt DSP-PP$_{240}$ cleavage and the longer incubation time with conditional medium enhanced wt DSP-PP$_{240}$ precursor protein cleavage into DSP$_{430}$ and PP$_{240}$. Here we tested whether longer incubation of mutant DSP-PP$_{240}$ with TLR1 in the Sf9 culture medium would affect the cleavage. As shown in (Figure 3 Lane 3), only mutant DSP-PP$_{240}$ precursor protein was present. Thus longer incubation did not enhance mutant DSP-PP$_{240}$ cleavage. Thus TLR1 in the culture medium could not cleave mutant DSP-PP$_{240}$ precursor protein.

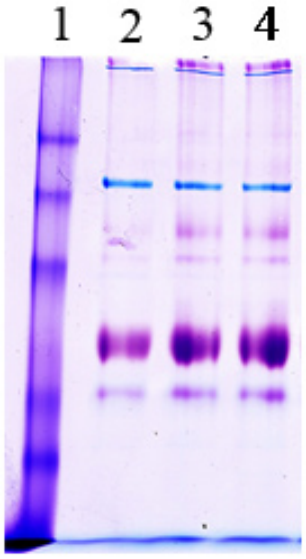

Figure 3: Effect of endogenous TLR-1 and-exogenous BMP1 on recombinant D^{486}D^{487}/H^{486}H^{487} mutant DSP-PP$_{240}$ protein expression. Lane 1: size marker. Lane 2: Recombinant DSP-PP$_{240}$ Mutant protein from 4d Sf9 Culture Medium (CM). Lane 3: Mutant DSP-PP$_{240}$ in baculovirus expression vector from 4d Sf9 culture medium CM was incubated at 28^{o}C for an additional 24 hours. Lane 4**:** Mutant DSP-PP$_{240}$ in baculovirus expression vector from 4d Sf9 culture medium was incubated with BMP1 (170ng/ml) for 24 hours at 28^{o}C.

Can BMP1 Cleave Mutant DSP-PP$_{240}$ from 4d Sf9 Culture Medium?

The TRL1 in the culture medium did not enhance mutant DSP-PP$_{240}$ cleavage. 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 [16]. We further tested whether the addition of human recombiant BMP1 protein could affect mutant DSP-PP$_{240}$ precursor protein cleavage. As shown in (Figure 3), Lane 4, the addition of BMP1 into culture medium did not cleave the mutant DSP-PP$_{240}$ precursor protein. In other words, the presence of both BMP1 and TLR1 did not lead to DSP-PP$_{240}$ cleavage.

Can human recombinant BMP1 cleave wild type and mutant DSP-PP$_{240}$ in CM$_{3\text{-}7d}$ which contains no or low amount of TLR1 protease?

Recombinant wt and mutant DSP-PP$_{240}$ proteins (see Figure 1) were from 3-7d condition medium (CM$_{3\text{-}7d}$). contains no or low endogenous Sf9 TLR1[16]. From Figure 4 wt lane, we see no

cleavage of recombinant wt DSP-PP_{240} derived from $CM_{3\text{-}7d}$ due to this lack of protease. When the wt $CM_{3\text{-}7d}$ substrate was incubated with BMP1 (170 g/ml) at 37°C for 24 hours, DSP-PP_{240} precursor protein was greatly reduced and the cleaved product PP_{240} appeared (Figure 4, wt+BMP1 lane). Recombinant mutant DSP-PP_{240} from $CM_{3\text{-}7d}$ culture medium only showed uncleaved DSP-PP_{240} precursor protein (Figure 4 Lane mutant). Recombinant mutant DSP-PP_{240} $CM_{3\text{-}7d}$ was incubated with BMP1 (170 g/ml), no DSP-PP_{240} cleavage occurred and no cleaved product PP_{240} appeared (Figure 4, Lane mutant+BMP1). Thus BMP1 could not cleave recombinant mutant protein in $CM_{3\text{-}7d}$ medium.

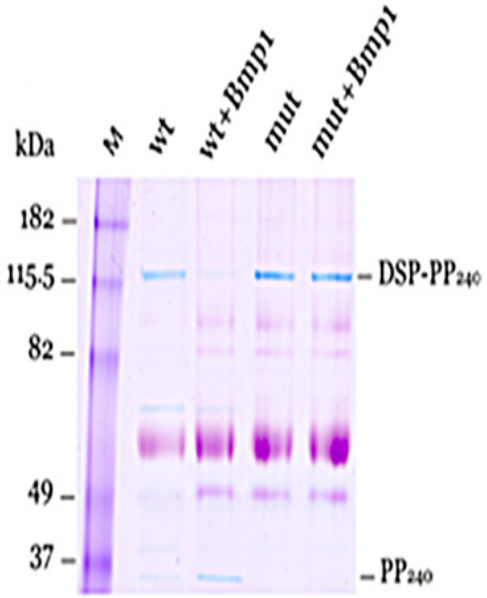

Figure 4: Effect of BMP1 on the cleavage of recombinant $CM_{3\text{-}7d}$ substrates (containing no or few TLR-1) from wt and mutant. M: size marker. wt: wild type DSP-PP_{240} precursor substrate(control) derived from $CM_{3\text{-}7d}$ Sf9 condition medium. wt+Bmp1: wild type recombinant DSP-PP_{240} proteins from $CM_{3\text{-}7d}$ was incubated with BMP1 (170 ng/ml at 28°C) at 37°C for 24 hours. mut: recombinant mutant $D^{686}D^{687}/H^{686}H^{687}$ DSP-PP_{240} from $CM_{3\text{-}7d}$ medium. mut+Bmp1**:** mutant DSP-PP_{240} from $CM_{3\text{-}7d}$ medium was incubated with BMP1 (170 ng/ml at 28°C) at 28°C for 24 hours.

Discussion

Previously we reported that wt DSP-PP_{240} cDNA in baculovirus expression system expresses DSP-PP_{240} precursor protein which undergoes cleavage to generate cleavage products DSP_{430} and PP_{240} via the TLR1 protease present in Sf9 culture medium. Incubation of the recombinant DSP-PP_{240} precursor protein in the presence of TLR1 results in further DSP-PP_{240} cleavage. The exact cleavage site was established as SMQG|DDPN [16].

The C-terminal 18 residues of DSP-PP_{240} are highly conserved among the sequences of all seven mammalian species (Figure 1). Strong conservation of this region suggests the possible presence of a functional domain. Indeed, C-terminal mutation (i.e., D^{687} to M) generated a new, more slowly migrating minor band just above PP_{240}, suggesting the possibility that a novel upstream cleavage site was activated [17]. Since single C-terminus mutation $D^{687}M$ affected DSP-PP_{240} precursor protein cleavage, we further examined the conservation of $D^{686}D^{687}$ on DSP-PP_{240} precursor protein cleavage. We mutated $D^{686}D^{687}$ to $H^{686}H^{687}$ and found these double mutations totally blocked DSP-PP_{240} cleavage in Sf9 baculovirus expression system. This double mutant DSP-PP_{240} precursor protein was not able to cleave by endogenous TRL1 secreted by Sf9 cells into the culture medium. Mutant DSP-PP_{240} recombinant protein substrate from $CM_{3\text{-}7d}$ contains low level of TRL1 or no TRL1 was added with human recombinant BMP1 to test whether BMP1 is able to cleave the mutant DSP-PP_{240} substrate. We found that wt DSP-PP_{240} can be cleaved by BMP1, in contrast mutant DSP-PP_{240} was not. Both single C-terminal $D^{687}M$ and double C-terminal $D^{686}D^{687}/H^{686}H^{687}$ mutants affect DSP-PP_{240} precursor protein cleavage. We speculate that these C-terminal D^{686} and D^{687} residues may participate in exosite interactions or affect conformation at the cleavage site that is important for DSP-PP catalysis. The conservation of C-terminal region likely play a crucial role in controlling proper DSP-PP precursor protein cleavage. Future work needs to further dissect the mechanism how C-terminal conservation in substrate conformation and possible excite interactions with BMP1.

In summary, wt DSP-PP_{240} can be cleaved by both TLR1 and BMP1 to generate DSP_{430} and PP_{240}. The C-terminal double mutation of $D^{686}D^{687}/H^{686}H^{687}$ completely shut down the cleavage process. Thereby this finding affirms our hypothesis that the C-terminal peptides $D^{686}D^{687}$ actively participates with BMP1 in controlling DSP-PP cleavage and that they must be conserved for the cleavage process to take place normally.

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