Relationship between length variations in Ser/Asp-rich repeats in phosphophoryn and
in vitro precipitation of calcium phosphate

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Footnotes:

Abbreviations

PP: phosphophoryn, DSPP: dentin sialophosphoprotein, SDrr: serine/asparatic acid rich repeats, rPP: recombinant PP proteins, DGI: dentinogenesis imperfecta, DD: dentin dysplasia, DMP-1: dentin matrix protein-1, HA: hydroxyapatite, IDPs: intrinsically disordered proteins, DSP: dentin sialoprotein

Abstract

Objective

Phosphophoryn (PP) is generated from the proteolytic cleavage of dentin sialophosphoprotein (DSPP). PP which contains tandem serine/asparatic acid rich repeats (SDrr) is known to enhance dentin mineralization. The nucleotide sequences coding SDrr are identified in the DSPP genes of toothed animals and the length variations of SDrr between intra- and inter-species have

been reported. However, it remains unknown about the relationship between the length variations in SDrr and the functions of PP in matrix mineralization.

Design

By utilizing a mammalian expression system, we generated several recombinant PP proteins (rPP) containing SDrr of different lengths and analyzed their effects on the precipitation of calcium phosphate with an *in vitro* gel diffusion system.

Results

rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr, which possessed shortened SDrr that accounted for 62.4 and 36.5 % the length of SDrr in full-length rPP (rPP-full), respectively, induced the precipitation of calcium phosphate similar to that of rPP-full at the same molar concentration, whereas rPP- Δ SDrr, in which SDrr were flipped, did not. Furthermore, rPP- Δ 63.5 SDrr significantly increased the accumulation of calcium compared with rPP-full at adjusted concentrations so that the same amounts of SDrr were embedded. The results of an ELISA analysis indicated that the amounts of rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr secreted from transfected cells were 5.2- and 7.1-fold greater than that of rPP-full, respectively.

Conclusions

The generated rPP- Δ 63.5 SDrr which can be substituted for rPP-full may be a candidate for a therapeutic molecule to facilitate hard tissue generation such as reparative dentin formation.

Key words

phosphophoryn (PP), *in vitro* gel diffusion system, serine/aspartic acid-rich repeats (SDrr), recombinant protein preparation

Introduction

Phosphophoryn (PP) (alternatively referred to as dentin phosphoprotein or dentin phosphophoryn), the carboxyl-terminal cleaved product of dentin sialophosphoprotein (DSPP), contains repeat sequences of serine/aspartic acid (there are over 200 tandem copies in humans and approximately 100 copies in mice). Most of the serine residues in serine/aspartic acid-rich repeats (SDrr) are phosphorylated; therefore, PP may be one of the most acidic proteins in the human body [1, 2]. Functional analyses in genetically altered mouse models mainly elucidated the function of DSPP as an inducer of mineralization in the extracellular matrix [3-5]. Many

point mutations in the human *DSPP* gene have been linked to dentinogenesis imperfecta (DGI) and dentin dysplasia (DD), which are the two most common hereditary diseases affecting dentin [6-9]. *Dspp* null mice were previously shown to display severe tooth defects due to widened predentin and dentin hypomineralization [3], which are similar to those reported in patients with DGI and DD. The teeth of these patients have irregular mineralization fronts and widened predentin concomitant with a narrow dentin width. Their dentin also contains ectopic unmineralized spots. These findings suggest that the conversion of predentin to dentin is impaired by a lack of DSPP as well as dentin maturation.

A recent study demonstrated that the ancestor DSPP gene was duplicated from ancestor dentin matrix protein-1 (DMP-1) in toothed animals [10], and the nucleotide sequences coding SDrr assigned to the DSPP gene after duplication because DMP-1 lacks long repeats [11]. Thus SDrr are considered to be essential for tooth mineralization however, each toothed animal has its own SDrr length [12]. Length polymorphisms in SDrr in intra-species have been reported in humans and porcine [2, 13]. In humans, one DSPP haplotype, which has the longest SDrr, was found to be 144 amino acids longer than that with the shortest SDrr among the 100 chromosomes obtained from the donors without any dental abnormality [2]. It currently remains unknown whether length variations between the intra- and inter-species of SDrr influence matrix mineralization.

An *in vitro* gel diffusion system is a good tool for mimicking the *in vivo* extracellular matrix environment [14-19]. Several studies utilizing an *in vitro* gel diffusion system examined the effects of PP isolated from dentin extracts at several concentrations and showed diverse effects for hydroxyapatite (HA) formation and growth. PP was previously shown to induce the formation of HA at relatively low concentrations and inhibit it at non-physiologically high concentrations [16].

In the present study, we used several recombinant PP proteins with various SDrr lengths and an *in vitro* gel diffusion system to determine whether length variations in SDrr affected the functions of PP in the precipitation of calcium phosphate.

Materials and methods

Preparation and purification of recombinant proteins

Mouse PP nucleotide sequences were cloned into the pCEP4-Mul-PURD expression vector (a

kind gift from Dr. Yoshi Yamada, NIDCR/NIH) [20, 21] such that the amino-termini of recombinant proteins were 6xHis-tagged. As described previously [22], long-repeat nucleotide sequence-coding SDrr in PP were easily released from circular DNA during the transformation process. By utilizing repeat sequence exclusion during transformation into *E. coli*, we transformed the PP pBS-sk (+) vector into HST08 competent cells (Takara Bio Inc., Otsu, Japan) with a general protocol and obtained the PP- Δ 37.6 SDrr, PP- Δ 63.5 SDrr, and PP- Δ SDrr vector, in which SDrr were differentially flipped. These shortened nucleotide sequences were further cloned into the pCEP4-Mul-PURD expression vector. Recombinant full-length PP (rPP-full), PP- Δ 37.6 SDrr (rPP- Δ 37.6 SDrr), PP- Δ 63.5 SDrr (rPP- Δ 63.5 SDrr), and PP- Δ SDrr (rPP- Δ SDr) were purified as described previously with slight modifications [22]. The PP, PP- Δ 37.6 SDrr, PP- Δ 63.5 SDrr, and PP- Δ SDrr vector were transfected into 293EBNA cells utilizing X-tremeGENE (Roche, Indianapolis, IN) and incubated for 24 hr. The transfection medium was replaced with growth medium containing puromycin (5 μ g/ml), and transfected cells were then cultured for 3 days. Surviving cells were routinely cultured with puromycin (0.5 μ g/ml) and used in the expression of the His-tagged recombinant PP (rPP) protein. To collect supernatants containing recombinant proteins, these puromycin-resistant cells were cultured with growth medium containing 10% fetal bovine serum (FBS) until confluent. The medium was replaced with new growth medium containing 5% FBS and 1x Insulin-Transferrin-Selenium (ITS) (BD Biosciences) and then cultured for 3 days. The growth medium was replaced with serum-free collection medium containing 1x ITS and then cultured for 5 days. The collection medium was collected and replaced every 24 hr during a 5-day period. rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr were purified from the collection medium with same procedure as follows. All procedures were generally performed in a cold room (4°C). Non-serum medium containing recombinant proteins was added to anion-exchange columns (Q Sepharose Fast Flow, GE Healthcare Japan, Tokyo, Japan) equilibrated with 20 mM Tris-HCl (pH 7.0), and these columns were then washed completely with 0.3 M NaCl/20 mM Tris-HCl buffer. Recombinant proteins were eluted from these columns with 0.6 M NaCl/20 mM Tris-HCl buffer. The eluents were further mixed with an equal amount of 20 mM Tris-HCl to decrease the NaCl concentration to 0.3 M. Solutions containing recombinant proteins in 0.3 M NaCl/20 mM Tris-HCl buffer were then added to the next anion-exchange columns (Hi Trap Q column, GE Healthcare Japan), which had been washed completely with 0.3 M NaCl/20 mM Tris-HCl buffer. Recombinant proteins were eluted from the columns with 0.36 M NaCl/20 mM Tris-HCl buffer. Eluents were then dialyzed against His-binding buffer (50 mM NaH₂PO₄, 150 mM NaCl, 10 mM imidazole, pH 8.0), incubated with TALON Magnetic Beads (Clontech Inc., Mountain View, CA) to ultimately purify the proteins via the chelation of His-tags and cobalt ions, and finally eluted with 250 mM imidazole/DPBS. These eluents were dialyzed against

DPBS and lyophilized. As shown in supplemental Figure, the SDrr in rPP-full was composed of 356 amino acids (from Ser⁹⁶ to Asp⁴⁵¹). The shortened SDrr in rPP-Δ37.6, rPP-Δ63.5 SDrr, and rPP-ΔSDrr were composed of 222, 130, and 3 amino acids, respectively. Therefore, the lengths of SDrr in rPP-Δ37.6, rPP-Δ63.5 SDrr, and rPP-ΔSDrr accounted for 62.4, 36.5, and 0.8 % the length of SDrr in rPP-full, respectively. rPP-ΔSDrr was identical to the recombinant protein described as rPP-ΔSSD in a previous study [22].

Gelatin gel preparation

The *in vitro* gel diffusion system was performed following a previously described procedure [16, 19]. rPP-full, rPP-Δ37.6 SDrr, rPP-Δ63.5 SDrr, and rPP-ΔSDrr were mixed with HA powder (0.5 mg/ml) (diameter < 200 nm) (Sigma-Aldrich, St. Louis, MO) in preparation buffer (150mM Tris-HCl and 0.01% sodium azide, pH 7.4) overnight at 4 °C. The next day, 1600 μl of 10 % (w/v) gelation gel (Type A, bloom 225, MP Biomedicals, Santa Ana, CA, USA) in preparation buffer was pre-heated at 50 °C for 2 hrs and poured into a 6-cm height plastic tube, which was made by cutting a disposable pipette (10 ml, Becton, Dickinson and Company, Franklin Lakes, NJ), and then kept at 4 °C to solidify the gelatin gel inside. The recombinant protein-HA complex or recombinant protein alone was further mixed with an equal amount of pre-warmed 20 % (w/v) gelatin gel in preparation buffer to generate 10 % gelatin gel containing the recombinant protein-HA complex or recombinant protein alone. One hundred microliters of 10 % gelatin gel containing the recombinant protein-HA complex or recombinant protein alone was then added onto solid 10 % gelatin gel and the pipette was kept at 4 °C to solidify the layer containing the recombinant protein (unless otherwise noted, this layer was called the embedded-layer in the present study). A total of 1300 μl of liquid 10 % (w/v) gelation gel in preparation buffer was added onto the embedded-layer to completely fill the inner space of the pipette. In the resultant 6-cm height pipette, there was a 32-mm layer containing 10% gelatin gel, a 2-mm layer containing 10% gelatin gel with the recombinant protein-HA complex or recombinant protein alone (embedded-layer), and a 26-mm layer containing 10% gelatin gel.

Measurement of the amounts of calcium and phosphorus in gelatin gel slices

The 6-cm height pipette was placed on a flat desk sideways and both ends were connected to connection tubes (MasterFlex™ PharMed® BPT tube, Yamato Scientific Co., Ltd., Tokyo, Japan). Calcium ion solution (100 mM CaCl₂, 150 mM Tris-HCl, 0.01% sodium azide, pH 7.4) was supplied to the 6-cm height pipette from one end at a distance of 26 mm from the edge of the embedded-layer, and phosphorus solution (100 mM (NH₃)₂HPO₄, 150 mM Tris-HCl,

0.01% sodium azide, pH 7.4) was supplied from the other edge at a distance of 32 mm from the embedded-layer. Both calcium and phosphorus solutions were circulated from a 4-L reservoir through solution-sending tubes (MasterFlex 7554-80) by a perista pump. All procedures were generally performed at 24°C. After 24 and 120 hrs of circulation, the 6-cm height pipette was disconnected from the connection tubes, the gelatin gel was removed, and several 3-mm layers obtained at 0, 17, 25.5, 31.5, 40, and 57-mm distances from calcium boundary for Figure 1A and B and the embedded-layer for Figure 1D, 2B, 4A, and 4B were sliced and collected. The collected gel slices were incubated with 150 μ l of 2N HCl at 50°C for 120 min to extensively hydrolyze gelatin. The total amount of the hydrolyzed solution was then measured to quantify the amount of the collected gelatin gel by substituting 150 μ l from the total amount. The amount of precipitated calcium phosphate was then quantified by the QuantiChrom™ Calcium Assay kit (BioAssay Systems, Hayward, CA, USA) for calcium content and by the Malachite Green phosphate Assay kit (BioAssay Systems) for phosphorus content, respectively. To determine the amount of calcium in the hydrolyzed solution, we used serially-diluted (20 μ M to 5 mM) calcium solution, which was made by diluting the control solution provided in the kit with 5 mM NaOH solution in order to obtain the standard line. The absorbance of the hydrolyzed solution was applied to the standard line in order to determine the amount of calcium included in hydrolyzed samples. Since the QuantiChrom™ Calcium Assay kit was suitable for measuring calcium ion concentrations in the range of 20 μ M to 5 mM, the hydrolyzed solution was 10-fold diluted with 5 mM NaOH solution to fit this range before absorbance was measured. The amount of phosphorus in the hydrolyzed solution was measured by the Malachite Green phosphate Assay kit in a similar manner to that of calcium. Since the Malachite Green phosphate Assay kit was suitable for measurements in the range of 20 nM to 40 μ M phosphorus, the hydrolyzed solution was 600-fold diluted with 5 mM NaOH solution before absorbance was measured. The amounts of both calcium and phosphorus were obtained by multiplying by the dilution factors and dividing by the volume of gelatin gel collected, and were indicated as mM units.

SDS-PAGE, Stains-All staining, and Western Blotting

All protein samples were reduced with DTT and loaded onto NuPAGE Bis-Tris (Life Technologies) in MOPS buffer. To efficiently detect acidic proteins, the gel was washed and fixed with 25% isopropanol for 3 hrs. Fixative solution was replaced every 20 min. The gel was stained overnight with 0.025% Stains-All (Sigma) containing 30 mM Tris-HCl, 7.5% formamide, and 25% isopropanol at pH 8.8 and then washed with deionized water until the

protein bands were visible. Separated proteins were transferred onto a PVDF membrane for immunodetection by anti-PP as described previously [22].

Enzyme-linked immunosorbent assay (ELISA)

Four kinds of puromycin-resistant cells, which stably expressed rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr, were cultured with growth medium containing 10% fetal bovine serum (FBS) until confluent. The growth medium was replaced with serum-free collection medium and then cultured for 24 hrs. The collection medium was collected and the wells of an ImmobilizerTM Nickel-chelate clear plate (Thermo Scientific Nunc, Waltham, MA) were coated with 100 μ l of collection medium overnight at 4°C. An ELISA assay was performed according to the manufacturer's protocol. The amounts of captured 6xHis tagged recombinant proteins were detected with 3, 3', 5'-Tetramethyl Benzidine substrate solution (TMB, Pierce, Rockford, IL) for 30 min at room temperature. After the addition of 2 N HCl to stop the colorimetric reaction, optical density was measured at 450 nm using a microtiter plate reader (Multiskan, Thermo Scientific Japan, Tokyo, Japan). In order to convert absorbance to the amounts of 6xHis tagged recombinant proteins in the medium, serially-diluted purified rPP- Δ SDrr (0 to 800 ng/ml) was utilized to obtain a standard line.

Statistical analyses

Statistical analyses were performed by Dunnett's test for Figures 1 and 2 and by a one-way analysis of variance (ANOVA), followed by Tukey's test for Figure 3 and Dunnett's test for Figure 4. Standard samples have been described in the figure legends.

Results

Dose-dependent effects of rPP-full for the precipitation of calcium phosphate.

We evaluated the concentrations of calcium and phosphorus at several positions in the gelatin gel after 24 or 120 hrs of circulation in a diffusion system. As shown in Figure 1A, after 24 hrs of circulation, the concentration of calcium was nominal in the embedded-layer (mesh layer) in both the double diffusion system (black dotted line) and single diffusion system (silver dotted line). In contrast, after 120 hrs of circulation, the concentration of calcium in the

embedded-layer was approximately 20 mM and the polygonal line showed a gradient in the double diffusion system (black solid line) and single diffusion system (silver solid line). The similar concentrations of calcium in the double and single diffusion systems at 24 and 120 hrs indicated that phosphorus did not interrupt the infiltration of calcium into the gelatin gel. A similar infiltration pattern was obtained for phosphorus, as shown in Figure 1B. The concentration of phosphorus in the embedded-layer was approximately 20 mM and the polygonal line showed a gradient after 120 hrs of circulation in the double diffusion system (black solid line) and single diffusion system (silver solid line). The similar concentrations of phosphorus between the double and single diffusion systems indicated that calcium did not interrupt the infiltration of phosphorus into the gelatin gel. Thus, this system was useful for examining whether rPP was able to induce the precipitation of calcium phosphate when low amounts of calcium and phosphorus were continuously supplied to the mineralization front, similar to *in vivo* extracellular matrix mineralization. Therefore, we collected gelatin gel samples after 120 hrs of circulation in all experiments in the present study.

To establish whether rPP-full was able to facilitate the precipitation of calcium phosphate in the *in vitro* gel diffusion system, we embedded serially-diluted rPP-full ($0 \sim 1.6 \times 10^2 \mu\text{M}$) into the embedded-layer with HA. After 120 hrs of circulation, the embedded-layer became blurred in the control gelatin gel ($0 \mu\text{M}$), in which only HA was embedded (Fig. 1C column 1). On the other hand, we found that the embedded-layer became opaque and was distinguishable from the surrounding area when rPP-full was embedded into the gelatin gel at 1.6 , 1.6×10 , and 1.6×10^2 μM concentrations (columns 3, 4, and 5), but the embedded-layer was not clearly distinguishable when rPP-full was embedded at $0.16 \mu\text{M}$ concentration (columns 2). As shown in Figure 1D, the precipitation of calcium phosphate was significantly greater at the $1.6 \mu\text{M}$ concentration of rPP-full than in the control gel. Therefore, we embedded rPP-full with HA in gelatin gels at a concentration of $1.6 \mu\text{M}$ in subsequent experiments.

rPP-full facilitated the precipitation of calcium phosphate with the co-embedding of HA in the double diffusion system.

In order to determine whether HA was essential for the rPP-full-dependent precipitation of calcium phosphate, we embedded rPP-full in the embedded-layer with or without HA. As shown in Figure 2A, we observed a distinguishable opaque layer when rPP-full was co-embedded with HA (column 2), as expected, whereas the embedded-layer remained blurred when rPP-full was embedded alone (column 4) similar to the control gelatin gel either with or without HA (column 1 and 3). Statistical analyses indicated that the precipitation of calcium phosphate was greater

with rPP-full with HA than with HA alone, rPP-full without HA, and without rPP-full nor HA. There was no apparent difference between rPP-full without HA and the group without rPP-full nor HA (Fig. 2B).

The generation of rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SD proteins had 62.4%, 36.5%, and nominal lengths of SDrr in rPP-full, respectively.

Difficulties have been associated with the generation and purification of rPP-full by a mammalian expression system due to its highly acidic nature, which is mainly attributed to SDrr [23]. Therefore, if mutated rPP, which possess shortened SDrr, exhibits similar inductive effects on the precipitation of calcium phosphate to those of rPP-full, it will be useful for future clinical applications. Thus, by utilizing this repeat sequence exclusion during transformation into *E. coli*, we generated rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr proteins having different lengths of SDrr, to examine the relationship between SDrr length and the precipitation of calcium phosphate. As shown in Figure 3A, each protein could be visualized by Stains-all staining. The concomitant bands identified by the anti-PP antibody were shown in Figure 3B. The SDrr lengths of rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr were 62.4 and 36.5 % that of rPP-full, respectively, and rPP- Δ SDrr had nominal SDrr repeats. The deduced amino acid sequences of rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr were shown in supplemental Figure. By utilizing the ELISA plate to quantify 6xHis-tagged recombinant proteins, we examined the amounts of these recombinant proteins in the supernatants of cells stably expressing recombinant proteins and found that rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr secreted 5.2-, 7.1-, and 11.1-fold more recombinant proteins than rPP-full, respectively. The amounts of recombinant proteins appeared to have been decreased when longer SDrr were contained in rPP (Fig. 3C).

rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr markedly affect the precipitation of calcium phosphate, similar to rPP-full.

Using rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr, we investigated whether length variations of SDrr affected the influence of rPP on the precipitation of calcium phosphate. As shown in Figure 4A, when rPP were embedded with HA at the same molar concentration (1.6 μ M), both rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr induced the precipitation of calcium phosphate, similar to rPP-full; however, the induction ability of rPP- Δ SDrr was significantly lower than that of rPP. We then embedded adjusted amounts of rPP-full (1.6 μ M), rPP- Δ 37.6 SDrr (2.67 μ M), and rPP- Δ 63.5 SDrr (4.85 μ M) and analyzed their impact on the precipitation

of calcium phosphate. These adjusted concentrations were selected based on the length of SDrr. For example, rPP-Δ37.6 SDrr possessed 62.4 % SDrr in rPP-full; therefore, it was embedded at a concentration of 2.6 (1.6/0.624) μM. Under this condition, as shown in Figure 4B, rPP-Δ63.5 SDrr had a significant inductive effect on the accumulation of calcium and induced the accumulation of phosphorus slightly more than rPP-full, whereas rPP-Δ37.6 SDrr tended to induce calcium and phosphorus accumulation compared with rPP-full; however, the inductive effects of rPP-Δ37.6 SDrr for both calcium and phosphorus were not significant.

Discussion

In the present study, we generated several recombinant mouse PP proteins that possessed different lengths of SDrr and found that rPP-Δ37.6 SDrr and rPP-Δ63.5 SDrr affected the precipitation of calcium phosphate similar to rPP-full, whereas rPP-ΔSDrr, which lacked SDrr repeats, did not. Moreover, we found that rPP-Δ63.5 SDrr significantly increased the most accumulation of calcium when the same amounts of SDrr were embedded. In addition, since the shortening of SDrr repeats increased the amounts of proteins produced by mammalian cells, our results suggest that rPP-Δ63.5 SDrr is a useful therapeutic molecule for facilitating mineralization *in vivo*.

An *in vitro* gel diffusion system is a good model for modifying *in vivo* extracellular matrix mineralization [14-19]. Based on previous findings [16, 19], we constructed an *in vitro* gel diffusion system and analyzed the effects of various PP-related recombinant proteins with or without HA on the precipitation of calcium phosphate. When the recombinant proteins were co-embedded with HA, the concentration of HA was 0.2488 mM in the gelatin gels and therefore the concentrations of calcium and phosphorus from embedded HA were estimated 2.488 mM and 1.492 mM, respectively. After 120 hrs of circulation, the amounts of precipitated calcium and phosphorus were more than 50 and 30 mM, respectively, when rPP were embedded at a concentration of 1.6 μM as shown in Fig. 1, 2, and 4. Therefore the amounts of calcium and phosphorus from embedded HA were less than 5 % of total amounts, respectively. Since PP isolated from a dentin extract was previously shown to have positive and negative effects on *in vitro* HA nucleation and growth by promoting at lower concentrations (0.01~1 μg/ml) and inhibiting at higher non-physiological concentrations (100 μg/ml) [16], we first examined the dose-dependent effects of rPP-full in a gel diffusion system (Fig. 1). rPP-full at a concentration of 1.6 μM (100 ng/ml) co-embedded with HA significantly induced the precipitation of calcium phosphate more than HA alone, whereas there was no significant difference in the precipitation between HA alone and rPP-full at the amounts of 0.16 or 1.6 x 10² μM with HA. Thus, the

active range of rPP-full for the precipitation of calcium phosphate was close to that of PP isolated from a dentin extract [16].

We quantified the amounts of calcium and phosphorus separately throughout this study. After 120 hrs of circulation, we found that the calcium/phosphorus ratio of all samples examined converged within a 15 % of the ideal molar ratio of 1.67, when rPP were embedded at a concentration of 1.6 μ M with HA (data not shown). This result implied that certain amounts of amorphous calcium phosphate may have been converted to HA in the gelatin gel. Moreover, unlike rPP-full, which required HA co-embedding (Fig. 2), PP isolated from a dentin extract has been shown to initiate the formation of HA in an *in vitro* gel diffusion system without HA [18]. The difference between rPP-full and PP from a dentin extract may be explained by the phosphorylation degree. Since PP in dentin is known to be highly phosphorylated [13], rPP-full used in this study may have been phosphorylated to a lesser extent and, thus, was unable to initiate the formation of HA alone.

Yamakoshi *et al.* recently proposed that PP should be classified into intrinsically disordered proteins (IDPs) due to its high net charge and low hydrophobicity [13, 24]. IDPs generally do not adopt a defined three-dimensional structure [25]. Previous studies showed that PP was able to bind to HA *in vitro* [26-28]. Furthermore, the conformation of PP was proposed to be influenced by its concentration and also the existence of the substrate [18, 27]. Therefore rPP appears to form an adequate structure that facilitates the precipitation of calcium phosphate by binding to HA. rPP was only able to induce the precipitation of calcium phosphate when HA was co-embedded, which was consistent with the *in vivo* finding that the re-expression of dentin sialoprotein (DSP), which is the other proteolytic cleavage product of DSPP, in DSPP null mice, which lacked PP only, restored the initiation, but not the maturation of impaired dentin mineralization observed in DSPP null mice [5].

Marschall *et al.* demonstrated that only small amounts of PP-related proteins were secreted from transfected mammalian cells [23]; therefore, the purification of a recombinant PP protein by a mammalian expression system was considered to be difficult. We found that the amounts of recombinant proteins secreted from recombinant protein-expressing cells were inversely proportional to the length of SDrr (Fig. 3). The amount of rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr were 5.2- and 7.1-fold greater than that of rPP-full at the molar ratio, respectively. However, the mRNA expression levels of transfected genes in the cells expressing rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr were approximately 1.4-fold higher or lower than those of cells expressing rPP-full (data not shown). These results suggested that the lower amounts of rPP-full secreted

were attributed to the synthesis, folding, or secretion of recombinant proteins rather than mRNA stability or transcriptional difficulty. rPP-full, rPP- Δ 37.6 SDrr, and rPP- Δ 63.5 SDrr had similar effects on the precipitation of calcium phosphate (Fig. 4A). This result was further validated by the finding that if the same amounts of SDrr were embedded with HA, rPP- Δ 63.5 SDrr significantly increased the accumulation of calcium (Fig. 4B). This result indicates that SDrr may be more effective if SDrr repeats are degraded into small fragments *in vivo*. Meprin 1 β was recently identified as an enzyme that degraded porcine PP *in vitro* [29]. Meprin 1 β -dependent PP degradation appeared to be more potent when free calcium concentrations were low. Therefore, a certain amount of PP may be degraded in the *in vivo* local extracellular microenvironment in which the concentration of calcium is low, with PP becoming a more effective molecule for mineral accumulation to compensate for the low amount of calcium and maintain the calcium/phosphorus molar ratio.

Each toothed animal has different SDrr lengths, which are apparently longer than that of rPP- Δ 63.5 SDrr [12]. In the present study utilizing an *in vitro* gel diffusion system, we found that rPP- Δ 63.5 SDrr was able to recapitulate the functions of rPP-full. Therefore, length variations in SDrr in inter-species may not result in different abilities of individual PP for tooth mineralization. This may explain why teeth in one species with longer SDrr are not always as hard as that of another species with shorter SDrr. Future *in vivo* studies examining the role of rPP- Δ 63.5 SDrr in hard tissue regeneration such as pulp capping are warranted to determine the usefulness of rPP- Δ 63.5 SDrr as a therapeutic molecule.

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Figure legends

Figure 1. Dose-dependent effects of rPP-full on the precipitation of calcium phosphate.

(A and B) The amounts of calcium and phosphorus in each position of a gelatin gel. Both calcium and phosphorus or either one circulated in gelatin gels for 24 and 120 hrs. The amounts of calcium and phosphorus were evaluated at each position of the gelatin gel. The area at which rPP-full were embedded (the embedded-layer) is shown as a mesh layer. (C) Representative images of the embedded-layer with hydroxyapatite after 120 hrs of circulation. After 120 hrs of circulation, the embedded-layer became blurred in the control gelatin gel (0 μ M), in which only HA was embedded (column 1). The embedded-layer is shown as a mesh layer. (D) The different concentrations ($0 \sim 1.6 \times 10^2 \mu$ M) of rPP-full are embedded into gelatin gel with hydroxyapatite and analyzed the amounts of calcium and phosphorus in embedded-layer after 120 hrs of circulation. Each value represents the mean of triplicate determinations; bars mean $\pm$ SD. Statistical analyses were performed by Dunnett's test. * $p < 0.05$ indicates significantly higher than control gels (0 μ M).

Figure 2. rPP-full facilitated the precipitation of calcium phosphate into gelatin gels when hydroxyapatite was co-embedded.

(A) Representative images of the embedded-layer after 120 hrs of circulation. The embedded-layer is shown as a mesh layer. (B) The effects of rPP-full on the accumulation of calcium and phosphorus with or without hydroxyapatite (HA). Each value represents the mean of triplicate determinations; bars mean $\pm$ SD. Statistical analyses were performed by Dunnett's test. * $p < 0.05$ indicates significantly higher than control gels (protein free with hydroxyapatite).

Figure 3. The generation of rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr proteins with medium, short, and nominal lengths of SDrr, respectively.

(A and B) The purified rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr proteins were loaded onto SDS-PAGE gels and visualized with Stains-all staining (A) or identified by anti-PP (B). The molecular sizes of these bands (A) were concomitant with the protein bands identified by anti-PP (B). Lane M, molecular weight marker. (C) The amounts of rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr proteins in the supernatants of transfected cells were quantified by ELISA. Each value represents the mean of triplicate determinations; bars mean $\pm$ SD. Statistical analyses were performed a one-way analysis of variance (ANOVA), followed by Tukey's test. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ indicate significantly different between the indicated samples. The asterisks above the SD bars indicate significantly higher than rPP-full expressing cells.

Figure 4. rPP- Δ 37.6 SDrr and rPP- Δ 63.5 SDrr affected the precipitation of calcium phosphate, similar to rPP-full.

(A) A comparative analysis of the effects of rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and

rPP- Δ SDrr on the precipitation of calcium phosphate. rPP-full, rPP- Δ 37.6 SDrr, rPP- Δ 63.5 SDrr, and rPP- Δ SDrr (1.6 μ M) were embedded into gelatin gels with hydroxyapatite and the amounts of calcium and phosphorus in the embedded-layer were evaluated. (B) The adjusted amounts of rPP-full (1.6 μ M), rPP- Δ 37.6 SDrr (2.6 μ M), and rPP- Δ 63.5 SDrr (4.4 μ M) were embedded into gelatin gels and the amounts of calcium and phosphorus in the embedded-layer were analyzed. These adjusted concentrations were selected based on the length of SDrr in order to embed equal amounts of SDrr. The embedded-layer is shown as a mesh layer. Each value represents the mean of triplicate determinations; bars mean $\pm$ SD. Statistical analyses were performed by a one-way analysis of variance (ANOVA), followed by Dunnett's test. * $p < 0.05$ indicates significantly higher than rPP-full-embedded gels.

Figure 1

Figure 2

Figure 3

Figure 4