Synthesis of a Potentially Bioactive, Hydroxyapatite-Nucleating Molecule

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Abstract. A human phosphophoryn (PP) cDNA was previously cloned from immature root apex total RNA in our laboratory. This cDNA comprises 2,364 bp, encoding 788 amino acids. More than 80% of the sequences are arranged as $(DSS)_n$ ($n = 1−16$), DS, and NSS motifs. We hypothesize that the capability of PP to bind Ca^{2+} and nucleate hydroxyapatite may depend on these repeated sequences. Two polypeptides were synthesized based on the human PP cDNA sequence to test the hypothesis. One polypeptide has the amino acid sequence DDPNSSDESNGNDD (synthetic polypeptide 1, SP1), which is from the N-terminal end of PP; the other polypeptide, DSKSDSSKSESDSS (synthetic polypeptide 2, SP2), is the PP repeated sequence motif. Phosphorylation of the polypeptides was accomplished by reacting them with adenosine triphosphate and casein kinases I and II. The ability of these molecules to cause mineralization was tested in a steady-state agarose gel system. The results show that phosphorylated SP2 (P-SP2) precipitated approximately 60% of the total Ca + PO₄ precipitated by PP. P-SP1 precipitated about 23% of that precipitated by PP and was similar to the amount precipitated in the control gel, that is, without added peptides. Transmission electron microscopy and X-ray diffraction analysis showed that the precipitate formed in the P-SP2-containing gel was hydroxyapatite. The capability of P-SP2 to nucleate Ca + PO₄ and precipitate hydroxyapatite is a result of the repeated sequence motif, which contains a high percentage of phosphorylated serine. This molecule could be used in the repair and regeneration of dental tissue.

Key words: Human — Phosphophoryn — Polypeptide — Hydroxyapatite — Nucleation

The dentin mineralization process involves the formation and growth of hydroxyapatite (HA) crystals in an extracellular matrix. Type I collagen accounts for 90% of the extracellular matrix of dentin. The collagen fibers provide the framework for HA deposition and the subsequent crystal growth to form a mineralized matrix. This biomineralization process is highly regulated by a set of matrix proteins which are secreted by odontoblasts. The function of these dentin matrix proteins in dentin mineralization has been widely discussed [1−3]. Of these dentin matrix proteins, phosphophoryn (PP) is the most abundant noncollagenous protein. More than 80% of this protein's amino acid composition is aspartic acid and serine, and 85−90% of the serine residues are phosphorylated [4−6]. These unique characteristics make PP a very acidic protein. Early studies have suggested that PP is a mineral nucleator [7, 8] and may have the ability to regulate HA formation [9, 10]. Later studies have shown that PP affects *in vitro* mineralization in various ways. Linde and Lussi [11] reported that mineral formation could be induced at low supersaturation by covalently binding rat PP to agarose beads and, conversely, that mineral formation could be inhibited when PP was free in the solution. The concentration of PP has also been shown to affect HA formation and growth in a gelatin gel system: low concentrations (0.01−1 µg/mL) promoted HA formation and higher concentrations (100 µg/mL) inhibited HA growth [12, 13]. Another important acidic extracellular matrix protein, dentin matrix protein 1 (DMP-1), was shown to be expressed in the early stages of dentin mineralization, and the expression decreased after the appearance of mineral formation [14]. Thus, it is postulated that DMP-1 has a regulatory role in dentin mineralization. The amino acid composition of DMP-1 contains 28% glutamic acid and aspartic acid, which suggests that DMP-1 should have a great capacity to bind divalent cations such as calcium [15]. In an *in vitro* Ca binding assay and nucleating test, recombinant DMP-1 showed not only strong Ca binding ability but also the capability of assembling Ca and phosphate ions to facilitate HA crystal formation [16, 17]. A recent *in vitro* study of apatite growth in a gelatin gel system showed that the length and degree of phosphorylation of DMP-1 affected the initiation and regulation of mineral formation. DMP-1 initiates mineralization when it is dephosphorylated or cleaved but inhibits mineralization when it is in its native form [18].

Phosphorylation of acidic dentin matrix proteins such as DMP-1 and PP has been reported to affect crystal nucleation and subsequent mineral formation [8,
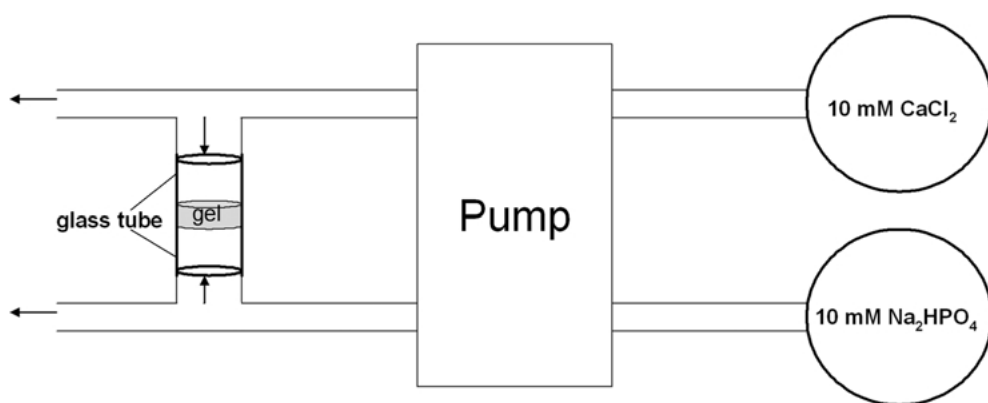
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Fig. 1. Schematic illustration of the steady-state agarose gel system. From two separate reservoirs containing 10 mM $CaCl_2$ and Na_2HPO_4, the solutions were pumped independently and flowed continuously at approximately 1 mL/hr through the apparatus. The system is not drawn to scale.

18−20]. More direct evidence using atomic force microscopy revealed that PP had a stronger binding capacity to enamel crystals than dephosphorylated PP [21]. Casein kinase I (CK1) and CK2 have been reported to be involved in the phosphorylation of PP [22−24]. The unique characteristic property of the $(DSS)_n$, $(DS)_n$, and $(NSS)_n$ (n ≫ 10) repeated motifs of PP have made it an ideal substrate for CK1 and CK2 kinase activity. These repeated motifs constitute > 80% of the PP amino acid sequence and are essential for its phosphorylation, Ca interaction, and subsequent effects on dentin mineralization [25, 26]. Two acidic phosphorylated, serine-rich proteins, DMP-2 and DMP-3, also have the repeated triplet DSS motif, with DMP-2 having many more repeated DSS than DMP-3 [25, 27]. DMP-2 and DMP-3 have been reported to be related to dentino-genesis imperfecta II [28].

DSP is another extracellular matrix protein that has been widely investigated. DSP and PP are the cleavage products from a large transcript encoded by the gene dentin sialophosphoprotein (*DSPP*) [29−31], but the amount of DSP found in dentin is much less than that in DPP. The DSP transcript is expressed in odontoblasts and transiently expressed in preameloblasts [32]. Immunohistochemical studies of DSP have shown that it is present in dentin and predentin [33−35]. These results indicate a role for DSP in dentin mineralization, but as yet this role has not been defined. A gelatin gel diffusion study has shown that DSP had only a limited effect on HA formation and growth [36], which may be because it has a low affinity for HA crystals [21].

The goal of this study was to explore whether the phosphorylated repeated motifs of PP, which consist of DSS, DS, and NSS, determine the ability of PP to nucleate HA and whether this ability could be replicated by a synthesized molecule. Different molecules were used in this experiment, including synthetic polypeptide 1 (SP1) from the N-terminal end of PP, SP2 from the repeated sequence of PP, human PP, and human DSP. SP1 and SP2 were phosphorylated by reacting them with adenosine triphosphate (ATP) and CK1/CK2 or used as unphosphorylated molecules. The ability of all these molecules to nucleate mineral was tested in a Ca and phosphate buffer steady-state gel system. The apatitic nature of the precipitate was determined by X-diffraction and energy-dispersive X-ray analysis (EDAX).

Materials and Methods

The steady-state agarose gel system consisted of two reservoirs, one filled with 10 mM $CaCl_2$ and the other with 10 mM Na_2HPO_4. Both solutions contained 150 mM NaCl, 0.01% NaN_3, and 10 mM Tris-HCl (pH 7.4) and were connected through glass tubes in which 10 μg of the test molecules were mixed with agarose gel at a final concentration of 2% (Fig. 1). The test molecules were prepared as follows.

PP. Open root apices from freshly extracted human immature third molar teeth were obtained and demineralized in 10% ethylenediaminetetraacetic acid (EDTA) plus protease inhibitors. After 1 week of extraction, the EDTA soluble fraction was collected and dialyzed against distilled water. Calcium chloride (1 M) was added to precipitate PP, the precipitation was re-dissolved in EDTA and run on a 7.5% polyacrylamide gel, and the PP band (140 kDa) was dissected from the gel. The band was eluted using an electroeluter (Bio-Rad, Richmond, CA) and desalted in a Microcon column (Millipore, Bedford, MA).

DSP. The protein was purified from human immature tooth dentin using Butler's three-step method [37]. The dentin was demineralized in 0.25 M EDTA plus 4 M guanidum-HCl (GdmCl) and protease inhibitors for 2 weeks. The EDTA/GdmCl extract was desalted and precipitated with 1 M $CaCl_2$ to remove DPP. The supernatant was dialyzed against 0.1 M formic acid to recover the acid soluble fraction. The acid soluble fraction was separated using a Sephadex G-50 (Sigma, St. Louis, MO, USA) column. This DSP-containing fraction was further purified in a Bio-Gel P-2 column (Bio-Rad, Richmond, CA, USA).

Both PP and DSP prepared by the methods described above resolved as a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels.

SP1: DDPNSSDESNGNDD. SP1 was synthesized based on the human PP N-terminal sequence (Invitrogen, Carlsbad, CA).

SP2: DSKSDSSKSESDSS. SP2 was synthesized based on the human PP repeated sequence motifs (Invitrogen).

P-SP1 and P-SP2. The SP1 and SP2 sequences were phosphorylated using a CK1 and CK2 phosphorylation kit (Upstate Biotechnology, Lake Placid, NY). The final concentration of 1 μg/μL was obtained by dissolving the polypeptide in assay dilution buffer. Ten microliters of the polypeptide solution were added to the microcentrifuge tube and mixed with 10 μL of CK1 (100 ng) plus 10 μL of 0.5 mM ATP. The mixture was incubated in a shaking water bath at 30°C for 10 min and boiled to arrest CK1 activity. Ten microliters of CK2 (100 ng) were then added to the mixture, which was incubated under the same conditions for 10 min and again boiled to stop the enzyme activity.

The control group in this experiment had the same concentration of agarose gel but no added peptide.

Experimental Procedure

The Ca and PO_4 solutions (pH 7.4) were pumped at a flow rate of 1 mL/hour through an apparatus which allowed Ca and phosphate to diffuse separately into the agarose gel from opposite sides. After 6 days at 37°C, gels were removed from the tubes and rinsed with distilled water before being analyzed for Ca and PO_4. The apatitic nature of the precipitate was also determined.

Ca and PO_4 Analysis

The precipitate formed a band in the gel, which was dissected from the gel and heated in a microwave. Ten microliters of the liquid gel were immediately added to 1 mL of reaction solution from a quantitative, colorimetric Ca determination kit (Sigma, St. Louis, MO). The reaction mixture was incubated at 37°C until the purple color developed. The intensity of the color was measured spectrophotometerically at 575 nm, and the amount of Ca was determined by comparing the absorbance to a Ca standard curve.

For PO_4 analysis, the precipitating bands in the gels were dissected, heated to dryness at 200°C, and dissolved in 1.5 mL of nitric acid. Samples were heated at 225°C until the volume was reduced to 0.1 mL before a molybdate-ascorbic acid solution was added. Phosphorus was determined by measuring the intensity of the color reaction using the method described by Chen et al. [38].

A center section, at the approximate interface of the diffusing Ca and PO_4 ions, was dissected from the gels that did not contain the precipitate and subjected to analysis as described above.

Crystal Analysis

The precipitate was dissected from the gel and heated by microwave as previously described. After the precipitate was suspended in methanol, 2 μL of it was pipetted onto transmission electron microscope (TEM) copper grids. TEM images were obtained using a JEOL 2010F analytical electron microscope (JEOL, Peabody, MA). The accelerating voltage was 200 kV and the vacuum of the system was 10^{-7} torr. The Ca/P ratio was based on electron dispersive spectroscopy (EDS) measurements using an EDAX r-TEM detector with EDAX acquisition software (EDAX, Mahwah, NJ, USA). Commercially available HA (Sigma) was used as a standard for the EDAX determination.

The apatitic nature of the precipitate was determined using an X-1 powder diffractometer (Scintag, Cupertono, CA). The diffraction patterns were compared to the instrument's standard HA pattern. To obtain accurate lattice parameters, a Rietveld refinement of the pattern produced by the P-SP2 precipitate was carried out based on high-resolution powder diffraction data [39] for pure HA.
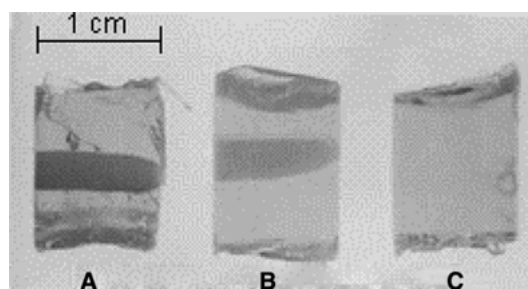

Fig. 2. Cross section of the gels which were removed from the glass tubes after 6 days of incubation. Liesgang rings were observed when PP (**A**) or P-SP2 (**B**) was added to the gel. The control gel with no peptides added showed no precipitation (**C**).

Results

In Figure 2, after 6 days of incubation in the steady-state gel system, the agarose gel containing PP (A) and P-SP2 (B) showed distinct precipitating bands: Liesgang rings. The Liesgang ring formed presumably at the interface of the Ca and PO_4 ions as they diffused through the gel. No such Liesgang ring was seen in the control group (C), which indicated that spontaneous precipitation did not occur in our steady-state gel system. The bands in the PP gel were more distinct than the one that contained P-SP2. Neither Liesgang ring formation nor obvious precipitation was seen in the DSP-, SP1-, SP2-, and P- SP1-containing gels.

Figure 3 shows the total amount of Ca + PO_4 in the gels with and without added peptides. The gel containing PP had the greatest amount of Ca + PO_4 at 55 μg, and the control gel had approximately 13 μg. The DSP-containing gel had 22 μg, which is 40% of that seen for the PP-containing gel and 169% of that for the control gel. The SP1-containing gel had approximately 18 μg of total Ca + PO_4, which is 32% and 138% compared to the PP-containing gel and control gel, respectively. The gels containing SP2 and P-SP1 had similar amounts of total Ca + PO_4 to that of the control gel and are approximately 23% of that seen in the PP-containing gel. The P-SP2-containing gel had approximately 33 μg, or 60% of the total Ca + PO_4 precipitated in the PP-containing gel and 253% of that in the control gel.

The TEM image of the precipitate from the P-SP2-containing gel is shown in Figure 4. The image shows small hexagonal crystals (see arrows), which resemble those of enamel HA. The Ca/PO_4 ratio was 1.58:1.62 when analyzed by EDS.

Figure 5 shows the X-ray diffraction result of the precipitates formed by PP (A) and P-SP2 (B) in the agarose gels. These X-ray diffraction patterns (A and B) were the same as that for the standard HA pattern (C). All had a major peak at 2.8 d Å and a minor one at 3.4 d Å (interplanar space). We concluded that the precipitates formed by P-SP2 and PP were HA. The Rietveld refinement showed that the lattice parameters of the HA
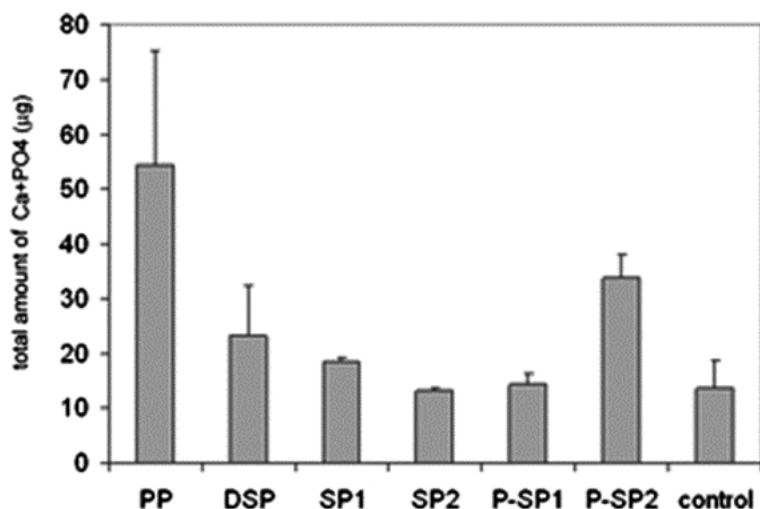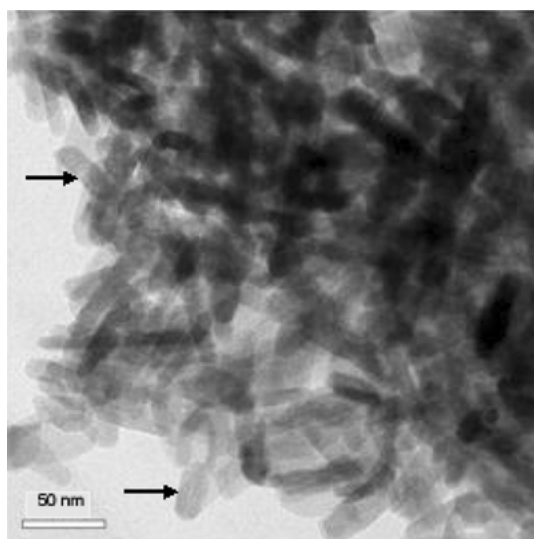
Fig. 3. Total precipitated amount of Ca + PO$_4$ in the gels containing different molecules. The control gel contained no peptides. The standard deviations shown are based on $n = 5$.

Fig. 4. TEM image of the precipitate in the P-SP2-containing gel, with *arrows* showing the individual HA crystals.

precipitate formed by P-SP2 were a = 9.392 Å, b = 9.392 Å, c = 6.851 Å.

Discussion

The technique of using part of the sequences from a whole protein to investigate the function of a polypeptide has been reported by several laboratories. Fujisawa et al. [40] synthesized a polyglutamic acid sequence which could be bound to HA, proving that the binding of osteonectin to HA was due to a region which contained glutamic acid-rich sequences. Tye et al. [41] used recombinant rat bone sialoprotein (rBSP) to investigate the role of two polyglutamic acid (poly [E]) regions involved in HA nucleation. Site-directed mutagenesis of the poly [E] domains was performed, and the HA-

nucleating ability was evaluated and compared to unmodified rBSP in a steady-state agarose gel system similar to ours. They successfully demonstrated that the second poly [E] domain of BSP was the one responsible for HA nucleation. To investigate PP's HA-nucleating ability, we synthesized two polypeptides based on the human PP sequence: one from the N terminus of PP which did not contain serine-rich sequences (SP1) and the other from the repeated motifs of PP which did contain the serine-rich sequences (SP2). The SP1 and SP2 effect on HA nucleation was similar to that of the control group even though SP2 had about 60% of its amino acids as serine residues. P-SP1, like SP1, had little or no effect on HA nucleation. However, P-SP2 promoted HA nucleation and formation. It was deduced that the percentage of serine residues in the molecule was not the factor affecting HA formation but rather their phosphorylation. Our results show that PP is very capable of HA nucleation in this system. P-SP2 also had this capability but not to the same extent as PP. The difference in their nucleating capabilities is probably due to the degree of phosphorylation, which is affected by the length of repeated motifs and their conformation arrangement. The phosphorylation of PP by CK1/2 is hierarchical. The initial phosphorylation of several of the serine residues quickly results in the remaining serines within the triple sets becoming substrate for the CK reaction [24]. The serines which were phosphorylated in SP2 based on the possible CK1 and CK2 phosphorylation sites are as follow: D-S^2-K-S^1-D-S^{2*}-S^{1*,2}-K-S^2-E-S-D-S^1-S (1 denotes the CK1 primary phosphorylation site and 1*, the secondary phosphorylation site; 2 denotes the CK2 primary phosphorylation site and 2*, the secondary phosphorylation site). P-SP2 was analyzed using mass spectrometry to determine how many serines were actually phosphorylated. Our result showed that there were five serines phosphorylated in the P-SP2 molecule, which was less than expected. The
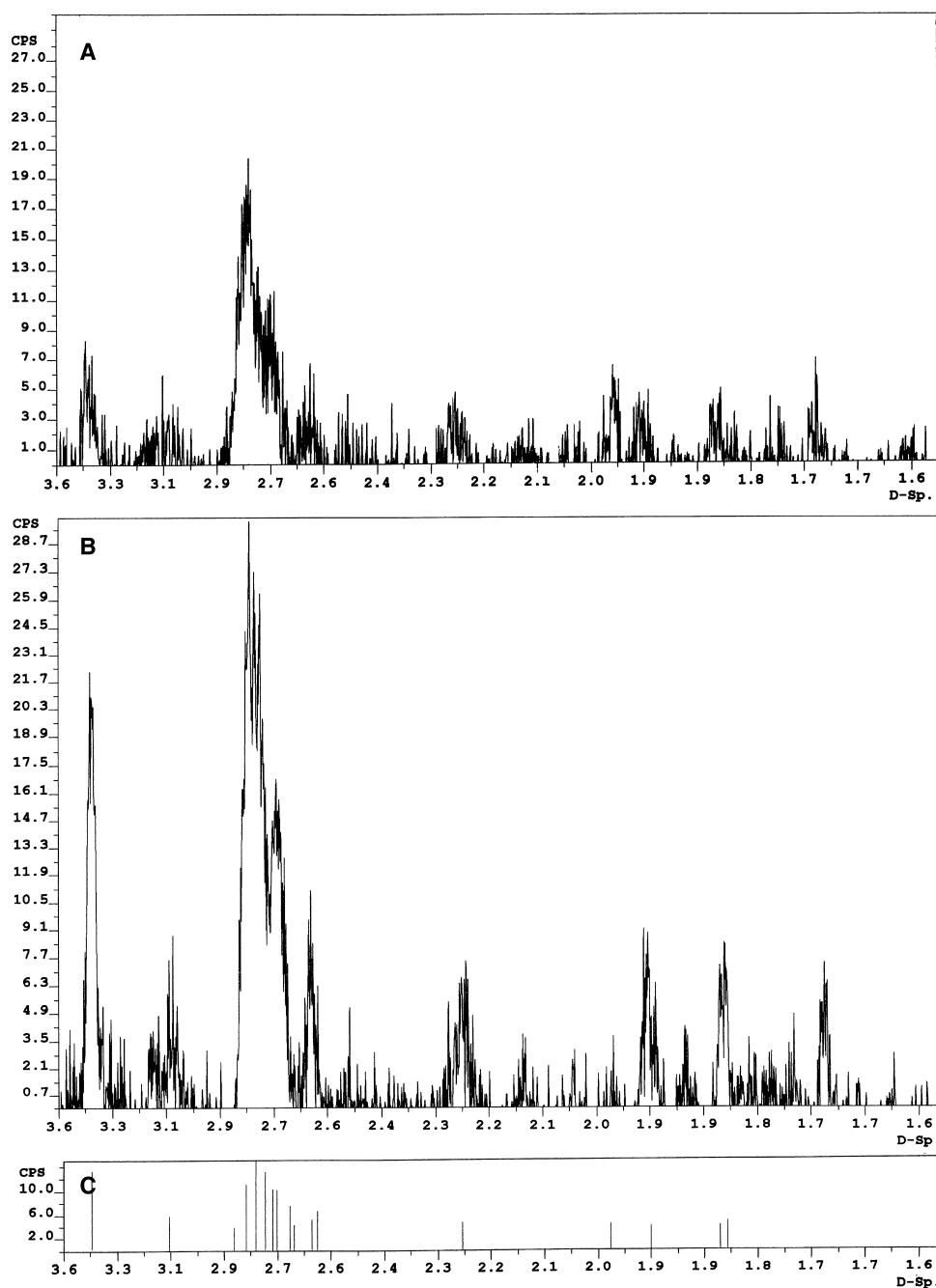
Fig. 5. XRD analysis of the precipitate formed in PP-containing gel (**A**) and in P-SP2-containing gel (**B**). *Vertical lines* show the diffraction profile of the HA standard used in the Scintag diffractometer (**C**).

reason for this is unclear. The short SP2 sequence may affect the phosphorylation of the CK1 and CK2 phosphorylation sites. Extending SP-2 would obviously increase the number of serine phosphorylation sites, thus resulting in a greater degree of nucleation.

Another possible factor that would affect biomineralization is conformational arrangement of the repeated motif. George et al. [26] used energy-minimized computation to analyze the DSS and DS repeated sequences' structure from the C terminus of DMP-2. The analysis showed that fully phosphorylated $(DS*S*)_{n>3}$ has two adjacent S′ phosphates in each triplet on opposite sides of the peptide chain and the D carboxyl groups are ar-

ranged opposite the adjacent S′ phosphate. Within the extended DSS arrangement, highly negatively charged SDS ridges are created. The DS arrangement is different from that of DSS because all the phosphates are on one side of the peptide chain and carboxyl on the other. Such SDS and DS arrangements will provide for Ca binding and mineral orientation. DMP-3 has a similar structure but shorter DSS repeated sequences [25]. It has been shown that DMP-2 and DMP-3 play an important role in dentinogensis [28]. Our SP2 has SDS and DS/KS/ES structures, but there is no continuous carboxyl-phosphate ridge, which may affect the efficiency of HA nucleation.

We have modeled the serine-rich motif of PP, SP2, as in β-sheet secondary structure using DeepView/ Swiss-Pdb Viewer v3.7 software (ExPASy, Basel, Switzerland). This model is very similar to previous models created by other authors but using different software [25, 26]. The distance between the hydroxyl groups of every two adjacent serines is 6.6 Å. When the serine of SP2 is phosphorylated, phosphate replaces the hydrogen of the hydroxyl group but leaves oxygen unchanged. The distance between two phosphate groups of adjacent serines should not deviate from 6.6 Å unless the repulsive force between the charged groups on the same side causes the trans extended chain to twist. This space is close to the HA crystalline unit cell dimensions (c = 6.85 Å). As the hydroxyl groups of the serine are phosphorylated in PP, it is possible that this motif presented in the β-sheet secondary structure may attract Ca ions, acting as the template for HA crystal formation.

Analysis of the precipitate in our experiment by X-ray diffraction (XRD) showed that HA was present but the Ca/phosphate ratio was between 1.58–1.62. There could be several reasons why the "ideal" Ca/phosphate ratio of 1.67 was not obtained in the XRD analysis. First, the standard HA purchased from Sigma may have a lower Ca/phosphate ratio due to the acid phosphate which remained in the product during the manufacturing process. Second, amorphous phases may have been present in the precipitate, but none was categorically identified by XRD analysis. Third, surface inclusions and impurities would also affect the stoichiometric HA ratio of the precipitate. Finally, inclusion of Ca carbonate in the precipitate may be another reason for the lower Ca/phosphate ratio. There also appears to be Liesgang ring or band formation at the interface where Ca-phosphorus ions meet in the gel. These bands are due to the reaction kinetics and the diffusion of the chemical species in the gel. They are created by depletion of the nucleating ions, which causes the local supersaturation to drop in the neighborhood of the precipitation site and the ring or band to form. The amount and type of precipitation are obviously dependent on the molecules involved in nucleation, as discussed previously, as well as the incubation time, pH, and ionic concentration at the precipitating interface. More work is needed to investigate these variables.

The mineral-nucleating system used in this experiment is similar to that designed by Hunter and Goldberg [42]. The Ca x PO_4 product in our system was above the physiological range and Hunter and Goldberg's spontaneous precipitation threshold. The ions diffusing into the gel followed Fick's law, and the rate of diffusion was dependent on the cross section of the glass tube, the distance the ions had to diffuse, and the nature of the gels. These factors may not have been exactly the same in our system as the one used by Hunter and Goldberg;

therefore, the efficiency of the ions diffusing into the gel may not be the same.

This study suggests that synthesized molecules, modeled on bioactive proteins, may be suitable for use in tissue regeneration and repair. Healy et al. [43–45] studied the effect of bone-derived cells growing on surfaces grafted with RGD-containing sequences of bone sialoprotein. Their work and ours demonstrate a great potential application for synthetic molecules in tissue engineering. Such molecules can be easily synthesized and do not have the immunological problems associated with nascent proteins and/or the quality/purification issues of recombinant molecules.

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