Dentin matrix protein-1 (DMP1) is a mineralized tissue matrix protein synthesized by osteoblasts, hypertrophic chondrocytes, and ameloblasts as well as odontoblasts. DMP1 is believed to have multiple in vivo functions, acting both as a signaling molecule and a regulator of mineralization. Using a cell-free system, in vitro, we evaluated the action of DMP1 in the regulation of hydroxylapatite (HA) formation and crystal growth. The non-phosphorylated recombinant protein acted as an HA nucleator, increasing the amount of mineral formed in a gelatin gel HA growth system relative to protein-free controls. The recombinant protein phosphorylated in vitro had no detectable effect on HA formation and growth. In contrast, phosphorylated bovine DMP1 expressed in marrow stromal cells with an adenovirus vector containing 29.7 phosphates/mol was an effective inhibitor of HA formation and growth. The native full-length protein appeared to be absent or present in only small amounts in the extracellular matrix of bones and teeth. However, two highly phosphorylated fragments representing the N- and C-terminal portions of DMP1 have been identified, apparently arising from proteolytic cleavage of four X–Asp bonds. The highly phosphorylated C-terminal 57-kDa fragment (containing 42 phosphates/mol), like the non-phosphorylated DMP1, was an HA nucleator. These data suggest that, in its native form, DMP1 inhibits mineralization, but when cleaved or dephosphorylated, it initiates mineralization. These in vitro data are consistent with the findings in the DMP1 knockout mouse.
protein might be a nucleator. In cultures, DMP1 acts as a signaling molecule enhancing osteoblast mineralization when overexpressed (13). However, the function of the phosphorylated form of the protein is not known. Furthermore, intact, highly phosphorylated DMP1 has yet to be isolated from bone or dentin. Fully phosphorylated peptides corresponding to the C and N termini of full-length DMP1 were recently isolated from rat bone (14). Although these peptides are heterogeneous in molecular size, they appear to be derived from cleavage at four X-Asp bonds located in close proximity.

In this study, using a dynamic gelatin gel system to monitor mineral formation and growth (15), we compare the effects on mineralization of recombinant DMP1 with and without post-translational modifications. Bovine DMP1 expressed using an adenovirus system in human marrow stromal cells, and the phosphorylated C-terminal fragment of DMP1 isolated from rat bone. We demonstrate that the concentration-dependent in vitro actions of these proteins are a function of size and phosphorylation.

**EXPERIMENTAL PROCEDURES**

All chemical reagents were acquired from Fisher unless otherwise indicated. HA seed crystals were prepared previously (16) by conversion of amorphous calcium carbonate at pH 8. Synthetic HA was characterized by x-ray diffraction as detailed below.

**Protein Preparations**—Four proteins were used in these studies: rat recombinant DMP1 (rDMP1) expressed in *Escherichia coli*, phosphorylated rDMP1, “native” bovine DMP1 (bDMP1) expressed in human marrow stromal cells, and a 57-kDa C-terminal fragment of DMP1 isolated from rat bone. Details of the preparation and purification of the recombinant protein prepared in *E. coli* appear elsewhere (17). Purified rDMP1 was phosphorylated in vitro. Batches of 500 μl (0.3–0.5 μg) were mixed with 100 ng of casein kinases I and II (Upstate Biotechnology, Inc., Lake Placid, NY). The reaction was initiated by the addition of 20 μl of 1× ATP, carried out overnight at 37 °C, and terminated by the addition of an inhibitor mixture (casein kinase I and II assay kit, Upstate Biotechnology, Inc.). Samples were then purified by 10% SDS-PAGE. Phosphorylated rDMP1 was stored in phosphate-buffered saline at −70 °C until used. Prior to use, the protein was dialyzed against 0.15 M Tris (pH 7.4) to remove the excess phosphate.

bDMP1 was made by infecting bone marrow stromal cells with full-length bDMP1 cDNA cloned into replication-deficient adenovirus (Ad5) constructs using the cytomegalovirus promoter as described (18). Briefly, adenovirus was plaque-selected and propagated on HEK293 cells (American Type Culture Collection CRL1573). Viral particles were purified by twice banding on CsCl, and viral titers were evaluated by plaque formation of virus dilutions on HEK293 cells. rDMP1 was generated by infecting subconfluent normal human marrow stromal fibroblasts with 10,000 plaque-forming units/cell. Harvested serum-free medium from the infected human marrow stromal cells was subjected to anion-exchange chromatography. Proteins were purified by diluting the medium from normal human marrow stromal fibroblasts 1:1 with 40 mM phosphate buffer (pH 7.4) and loading directly onto a 0.9 × 2.0-cm column packed with TocoyPearl TSK QAE resin. A linear salt gradient to 2.0 M NaCl was employed to separately purify the proteins (~95% purity as measured by SDS-PAGE). Solutions were desalted; a stock solution of 50 μg/ml was prepared in Tris buffer (pH 7.4), and solutions for analysis were prepared by serial dilution with Tris buffer (pH 7.4).

The 57-kDa peptide from the C-terminal end of DMP1 was isolated from rat long bones as described (14). In brief, after ion-exchange separation of the EDTA extract containing bone extracellular matrix proteins, subfractions rich in the 57-kDa fragment were first gel-filtered using a Bio-Gel A-15m column (Bio-Rad). Next, fractions enriched in the 57-kDa fragment were passed over a Bio-Gel A-50m column (Bio-Rad), providing a preparation of the 57-kDa fragment free of osteopontin, bone sialoprotein, the 37-kDa fragment, and other known bone proteins. SDS-PAGE with Stains-All staining showed that the 57-kDa protein migrated as a broad band with an average molecular mass of 57 kDa on 7.5% gels. Parallel phosphate and amino acid analyses were performed on phosphorylated peptides isolated from the 57-kDa fragment. For mineralization analyses, the lyophilized 57-kDa protein was resuspended in Tris buffer (pH 7.4).

**Qualitative Phosphoamino Acid Analyses of In Vitro Phosphorylated Bacterial rDMP1 and Native Human Marrow Stromal Cell-expressed rDMP1**—Ten μg of rDMP1 and 14 μg of bDMP1 were subjected to partial acid hydrolysis for 6 h at 110 °C in Pyrex-sealed tubes under vacuum in 4 × HCl to determine the phosphoamino acid content. One-third of each sample was further hydrolyzed in 6 × HCl for an additional 18 h at 110 °C for total amino acid analysis. The samples were dried in vacuo, suspended in borate buffer in the presence of AccQFluor reagent, and derivatized and analyzed following the AccQ-Tag method (Waters-Millipore Corp., Milford, MA) as recommended by the manufacturer. The AccQFluor-derivatized phosphoamino acids were identified by analyzing initially the standard mixture of AccQFluor derivatives of phosphoserine, phosphothreonine, and phosphotyrosine (100 pmol of each alone), followed by analysis of the AccQFluor derivatives of the rDMP1 and bDMP1 acid hydrolysates. Comparison of the elution times for these standard phosphoamino acids and the peaks for the unknown means to determine the absence or presence of particular phosphoamino acids and their quantitative levels. The total amino acid content was determined similarly using standard AccQFluor derivatives of naturally occurring amino acids. The data from phosphoamino acid analysis and the total amino acids were used to calculate quantitatively the phosphorylation states of rDMP1 and bDMP1.

**Mineralization Assays**—De novo HA formation and growth were monitored in the dynamic collagen gel HA growth system (15). A double diffusion system, Ca<sup>2+</sup> and HPO<sub>4</sub><sup>2-</sup> ions, was circulated at room temperature and diffused into opposite ends of a 6-cm-long 10% gelatin gel (bloom 275). The pH of the gelatin solution (prepared by stirring at 50 °C by mixing 1× NaOH) was adjusted in an appropriate manner to position the gel on a ruler. The reaction was initiated by the addition of 100 μl of 200-fold concentrated HA seed crystals preincubated with the protein for 18 h at 4 °C. HA seed crystals (0.5 mg/ml) were added to the gel site where apatite formation occurred in the absence of added protein. The proteins were delivered in 0.15 mM Tris buffer (pH 7.4) and mixed with an equal volume of 10% gelatin so that the final gelatin concentration in the band was 10%. The experimental gels contained 0.1–25 μg/ml protein. Control gels prepared in a similar fashion contained only buffer. Once the gel had hardened after cooling at 20 °C for 30 min, it was covered without any interface with the 10% gelatin.

Calcium and phosphate solutions (100 mM each) were circulated from 200-cml antibiotic-infinity reservoirs, providing over a 200-cml span the osmolar contents of the 18 gels used for each experiment. Solutions were circulated at a continuous rate through this device in separate loops at opposite ends of the gel; when mineralization began, the CaP concentration in their respective sides adjacent to the precipitant band was 5.5 mM (19). Solution flow was maintained using a nitrogen pump. The device was not thermostatted; but the ambient temperature around the device was monitored, and experiments were not done if the temperature was outside the range of 20–25 °C. Physiologic temperatures could not be used because the gelatin began to melt at 35 °C. After 3.5 days, a thin opaque band (precipitant band) formed perpendicular to the direction of the flow. Gels were sampled at 3.5 days (de novo formation) or at 5 days (formation and growth). A measurement of crystal growth, preformed HA seed crystals (0.5 mg/ml) were added to the 100-μl bands along with buffer and/or protein. For these cases, the seed crystals were preincubated with the protein for 18 h at 4 °C.

All experiments were repeated at least in triplicate. For the de novo formation and growth studies, Ca<sup>2+</sup> and HPO<sub>4</sub><sup>2-</sup> accumulation in the precipitant band containing Tris buffer (control) was compared with experimental gels. Identical “single diffusion” gels, into which either Ca<sup>2+</sup> or HPO<sub>4</sub><sup>2-</sup> diffused, served as additional controls to correct for ion accretion due only to diffusion or to bonding of the ion to the matrix protein. Controls and experimental tubes were run parallel to each other. Comparisons were made with the controls to avoid errors inherent in experiments that may have varied in temperature, time span, solution flow, or reservoir concentrations.

Gels were removed from the apparatus, and the position at which the mineral was deposited was noted. The precipitant was used as a reference to position the gel on a ruler. The gel was sliced into seven slices with a device consisting of equally spaced (0.5 cm apart) wires. Either the entire band or a fragment band was removed and ground in a dry-ice–nitrogen diffraction, or the entire gel was cut into slices (0.30 ml). The Ca<sup>2+</sup> (20) and HPO<sub>4</sub><sup>2-</sup> (21) contents of each slice were determined following hydrolysis of the gel in 2 N HCl for 18 h at 110 °C. Ca<sup>2+</sup> and HPO<sub>4</sub><sup>2-</sup> accumulation in the precipitant band was calculated by subtracting the content of the comparable slice in the single diffusion gels from that in the single diffusion sample in the double diffusion gel.

**X-ray Diffraction Analysis**—X-ray powder diffraction was used to identify phases present at 5 days and to determine HA crystal size and perfection. Once the gels were cut, the slice with the precipitant band was heated to 50 °C and microcentrifuged for 20 s; the liquefied gelatin

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**Significance**

- The study provides insights into the role of DMP1 in mineralization processes in bone.
- The use of recombinant and native protein forms allows for a comparison of their effects on mineralization.
- The experimental setup, including the use of dynamic gelatin gels, helps in understanding bone formation and growth dynamics.
- The methodological approach, including the use of specific reagents and techniques, demonstrates the importance of phosphorylation in protein function.

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**References**


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**Authors' Note**

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RESULTS

This study has shown that DMP1 has a variety of effects on in vitro mineral deposition that appear to be dependent on concentration, extent of phosphorylation, and size. The recombinant protein expressed in E. coli functioned as an HA nucleator, losing its nucleation activity once phosphorylated. The more phosphorylated protein expressed in narrow stromal cells was an inhibitor. In contrast, the highly phosphorylated 57-kDa peptide was capable of nucleation. rDMP1 expressed in E. coli promoted HA formation and growth at 3.5 days relative to protein-free controls (Fig. 1A). There was no statistically significant effect when de novo formation and growth were analyzed at 5 days (Fig. 1B). However, when HA crystals were precoated with DMP1, there was a dose-dependent inhibition of mineral accumulation, although this effect was only marginally significant (p = 0.06) (Fig. 1C). There was no effect of rDMP1 on HA crystal size at 5 days as measured by x-ray line broadening analysis (Table I).

When the recombinant protein was phosphorylated in vitro, there was no significant effect on HA proliferation and growth at either 3.5 (Fig. 2A) or 5 (Fig. 2B) days. The phosphorylated recombinant protein also did not alter the HA crystal size as determined by line broadening analyses (Table I). Analysis of the extent of phosphorylation showed that there were 91.8 nmol of Ser(P)/mg of protein (9.6 Ser(P) residues/1000 residues), translating to 5.6 mol of Ser(P)/mol of DMP1, corresponding to ~10% of the potential phosphorylation sites.

In contrast, bDMP1 expressed in narrow stromal cells was a very effective inhibitor of de novo HA formation at 3.5 days (Fig. 3A). In these studies, the apparent crystal yield decreased with increasing concentrations of bDMP1. Based on line broadening analysis, the c axis crystallite particle length increased with increasing bDMP1 concentrations from 0 to 10 μg/ml, but decreased to control values at 25 μg/ml (Table I). In seeded growth experiments, bDMP1 showed a significant dose-dependent linear trend (p < 0.05) when yields were compared, but the observed values measured at each concentration were not statistically different from the control values measured at the same time (Fig. 3B). Analysis of the phosphoamino acid content of this protein showed 330 nmol of Ser(P)/mg of protein (35.5 Ser(P) residues/1000 residues), translating to 29.7 mol of Ser(P)/mol of bDMP1. It is noteworthy that the only phosphoamino acids detectable in both rDMP1 and bDMP1 are seryl residues. The data were corrected for both rDMP1 and bDMP1 analysis for partial hydrolysis and some cleavage of phosphate groups from the serine residues during partial acid hydrolysis. Comparison of in vitro phosphorylated rDMP1 with native bDMP1 indicated a substantial difference in the extent of phosphorylation between these two DMP1 forms, which is reflected in the differences in the mineral assays (see "Discussion").

Like the non-phosphorylated recombinant protein, the highly phosphorylated 57-kDa protein was an HA nucleator (Fig. 4). The HA crystals formed in the presence of the 57-kDa protein decreased in size with small increases in the concentration of the 57-kDa protein, but no detectable effect was noticed at 100 μg/ml (Table I).

DISCUSSION

This study has demonstrated that the cell-free in vitro actions of DMP1 on apatite mineral formation and growth depend on concentration, extent of phosphorylation, and protein size. Hohling et al. (8) have suggested that, because the predicted secondary structures of several extracellular matrix proteins (including that of DMP1) match the HA crystal faces, these are the proteins that bind and stabilize HA critical nuclei and HA...
crystals, facilitating their growth and proliferation. This postulate is consistent with our observations that non-phosphorylated rDMP1 acted as a mineralization promoter.

The partially phosphorylated form of the recombinant protein expressed in *E. coli* had no detectable effect on apatite formation and growth, in contrast to the native bovine protein expressed in human bone marrow cells, which acted as an effective inhibitor. The absence of effects of the phosphorylated recombinant protein may be due to incomplete protein phosphorylation, as phosphorylated bDMP1 did have an effect. Although it might be argued that the cloned protein is smaller than the intact bone protein (14), comparing the recombinant protein and the similarly sized phosphorylated recombinant protein argues for the effect of phosphorylation on protein conformation. Other phosphoproteins such as dentin phosphophoryn, osteopontin, and bone sialoprotein have little effect on *in vitro* apatite formation and growth when dephosphorylated or partially dephosphorylated (23–26). As in other systems, phosphorylation may change the conformation of the protein (27, 28) and may also expose anionic domains that can interact with calcium on the apatite surface. Thus, although

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**FIG. 2.** Effect of phosphorylated rDMP1 on HA formation and growth in a gelatin gel system. A, 3.5 days; B, 5.0 days. Values represent means ± S.D. for five experiments with three replicate gels in each experiment. Values were normalized to controls run at the same time (E/C). PrDMP1, phosphorylated rDMP1.

**FIG. 3.** Effect of bDMP1 expressed in human bone marrow stromal cells on HA formation and growth in a gelatin gel system. A, at 3.5 days, there was a significant dose-dependent inhibition of HA formation. B, seeded growth at 5 days was also inhibited. All data were corrected for single diffusion and show means ± S.D. for five experiments with three replicate gels in each experiment. Values were normalized to controls run at the same time (E/C). ***, p < 0.01 relative to the control.

**FIG. 4.** Effect of the highly phosphorylated 57-kDa fragment on HA formation and growth in a gelatin gel system. At 3.5 days, there was a significant increase in the amount of mineral formed in the presence of increasing amounts of DMP1. Data were corrected for single diffusion and show means ± S.D. for five experiments with three replicate gels in each experiment. Values were normalized to controls run at the same time (E/C). *, p < 0.05 relative to the control. 57K Protein, 57-kDa protein.
apatite nuclei are stabilized in the presence of totally non-phosphorylated DMP1, these interactions may be blocked upon partial phosphorylation.

Using two different systems in which DMP1 was immobilized on different surfaces, He et al. (9, 29) showed recently that rDMP1 acts as a promoter of HA formation, binding large amounts of calcium. The HA used in those studies was formed by growth on an amorphous calcium phosphate surface. Even when not immobilized, as in the present study, rDMP1 promoted apatite formation.

In contrast to the actions of non-phosphorylated DMP1 and partially phosphorylated DMP1, bDMP1 expressed in human marrow stromal cells was a significant inhibitor of in vitro mineralization. In parallel 5-day studies, the crystal size increased at 0.1–10 μg/ml, but decreased at 25 μg/ml. At the lowest concentrations, DMP1 inhibited crystal formation; but most likely, coating of existing crystals was not complete, and the crystals that were presented continued to grow at the expense of the smaller crystals. At higher concentrations, DMP1 most likely coated all the crystals present, preventing crystal growth. This presumably highly phosphorylated full-length protein should represent the actual DMP1 made in bone cells prior to protein degradation (14).

Although DMP1 may have other in situ functions in cell signaling and gene regulation (13, 30), our data suggest that intact phosphorylated DMP1 acts as a mineralization inhibitor. This conclusion is consistent with immunohistochemical observations showing a cellular distribution of DMP1 predominantly in chicken and rat osteocytes, rather than in osteoblasts (4), and around cell processes in bone and dentin (12). These localization studies, along with results from DMP1 knockout experiments (31), suggest that phosphorylated full-length DMP1 may represent a protective form, preventing pathologic calcification of cells and their processes. Similar functions have been attributed to matrix Gla protein and osteopontin (32, 33).

In the mouse lacking DMP1 expression (31), although there is no visible skeletal phenotype in the newborns, with age, there is extensive cartilage calcification, a disorganized mineralization pattern and abnormal dentin development, and increased radiographic bone mineral density. There is, however, decreased mineral content of the matrix as determined by Fourier transform infrared imaging and microcomputed tomography.2 Our present studies, along with earlier data, indicate that the highly phosphorylated C-terminal 57-kDa fragment can act as a nucleator and that this activity is liberated from the highly phosphorylated C-terminal 57-kDa fragment can act as a nucleator and that this activity is liberated from the highly phosphorylated C-terminal 57-kDa fragment can act as a nucleator and that this activity is liberated from the highly phosphorylated C-terminal 57-kDa fragment can act as a nucleator and that this activity is liberated from the highly phosphorylated C-terminal 57-kDa fragment can act as a nucleator.

In X-linked hypophosphatemic rickets in man and in rodent models (38), failure to properly mineralize the epiphysis and bone is linked to a defect in an endopeptidase known as PHEX. The substrate for PHEX has not been established. The recent observations of Qin et al. (14) that DMP1 fragments arise by cleavage of X–Asp bonds strongly suggest that the endopeptidase that cleaves DMP1 is the PHEX protein because this enzyme has a high preference for cleavage of X–Asp bonds. It is therefore interesting to speculate that PHEX removes or modifies one or more inhibitors of mineralization (e.g. DMP1) and then releases the 57-kDa fragment or other fragments, which then promote mineralization. These fragments may include members of the related SIBLING phosphoprotein family and not only DMP1.

A recent study demonstrated that DMP1 is cleaved by bone morphogenetic protein-1, a metalloprotease important for extracellular matrix development (39). The knowledge that there is at least one and perhaps more than one enzyme linked to bone development important for DMP1 processing may explain the contradictory data relating to the cell culture effects of overexpression of DMP1, resulting in increased mineralization (13) and the time-dependent changes in the knockout in which, in most cases, excessive mineralization is seen, but in others (presumably those in which other PHEX and bone morphogenetic protein-1 substrates are in lower abundance), mineralization is deficient. It will be important to characterize the mineralization alterations in mice in which these proteins are absent or overexpressed.

In conclusion, it appears that DMP1 activity in mineralization is dependent on size, concentrations, and state of phosphorylation. Thus, DMP1 may act as a signaling molecule, an inhibitor of mineralization, and, perhaps, before it is phosphorylated or after it is cleaved, a nucleator. Thus, DMP1 plays a crucial role in mineralization. This conclusion will be confirmed by the detailed analysis of mineral properties in the knockout mouse.3

REFERENCES