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Possible role of DMP1 in dentin mineralization

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ABSTRACT

Dentin Matrix Protein 1 (DMP1), the essential noncollagenous proteins in dentin and bone, is believed to play an important role in the mineralization of these tissues, although the mechanisms of its action are not fully understood. To gain insight into DMP1 functions in dentin mineralization we have performed immunomapping of DMP1 in fully mineralized rat incisors and *in vitro* calcium phosphate mineralization experiments in the presence of DMP1. DMP1 immunofluorescene was localized in peritubular dentin (PTD) and along the dentin-enamel boundary. *In vitro* phosphorylated DMP1 induced the formation of parallel arrays of crystallites with their *c*-axes co-aligned. Such crystalline arrangement is a hallmark of mineralized collagen fibrils of bone and dentin. Interestingly, in DMP1-rich PTD, which lacks collagen fibrils, the crystals are organized in a similar manner. Based on our findings we hypothesize, that *in vivo* DMP1 controls the mineral organization outside of the collagen fibrils and plays a major role in the mineralization of PTD.

1. Introduction

DMP1, also called AG1 in the early literature, is an acidic non collagenous phosphoprotein originally found in teeth (George et al., 1993), but later also detected in bones (Hirst et al., 1997; MacDougall et al., 1998), where it is primarily expressed by osteocytes (Toyosawa et al., 2001). DMP1 is a multifunctional protein involved in the biomineralization of bones and dentin (Ling et al., 2005; Lu et al., 2007; Qin et al., 2004), phosphate homeostasis (Feng et al., 2006), and differentiation of odonto- and osteoblasts (Almushayt et al., 2006; Narayanan et al., 2001). Mutations in this gene cause autosomal recessive hypophosphatemic rickets syndrome, manifested by rickets and osteomalacia with isolated renal phosphate-wasting (Feng et al., 2006; Lorenz-Depiereux et al., 2006). DMP1 belongs to the SIBLING (Small Intergrin Binding N-linked Glicoproteins) family, which are associated with mineralized tissues (Fisher and Fedarko, 2003), although they were found in other tissues as well (Fisher et al., 2004; Ogbureke and Fisher, 2005).

DMP1 is an acidic protein containing a large number of Ser (22%), Glu (15%) and Asp (13%) amino acids with a calculated pI = 4.15 in its non phosphorylated form (George et al., 1993). *In vivo* a high proportion of serines in DMP1 are phosphorylated; for example it is estimated that native mouse DMP1 contains 55 phosphates, suggesting that more than half of its serines are phosphorylated (George et al., 1993). In solution, as other SIBLING proteins (Fisher et al., 2001), DMP1 adopts an extended, unstructured conformation (George et al., 1993), while in the presence of calcium it undergoes self-assembly into filaments (He et al., 2005). DMP1 specifically binds to N-telopeptide sequence of collagen and is shown to affect collagen fibrogenesis (He and George, 2004). *In vivo*, secreted DMP1 is cleaved into two fragments, an acidic C-terminal 57 KDa and a 37 KDa N-terminal domain (Qin et al., 2003) which localize differently in the compartments of dentin and the growth plate of bone (Maciejewska et al., 2009). Both *in vitro* and *in vivo* studies suggest that the C-terminal 57 KDa fragment of DMP1 is primarily responsible for the function of this protein in biomineralization (Maciejewska et al., 2009; Tartaix et al., 2004).

There is a vast body of evidence indicating that DMP1 plays an important role in the biomineralization of dentin and bone. Mutant animals lacking DMP1 gene have severe bone and dentin defects, manifested by widened unmineralized predentin and osteoid and hypomineralized bone and dentin (Ling et al., 2005; Lu et al., 2007). A number of *in vitro* studies indicate that DMP1 strongly influences various aspects of calcium phosphate mineralization. Specifically, the studies of calcium phosphate mineralization in the gelatin gel assays revealed that depending on the phosphorylation level, DMP1 can induce crystal nucleation, inhibit mineralization, and affect crystal size in a concentration dependant manner (Tartaix et al., 2004). Furthermore, DMP1 supramolecular assemblies can control organization of mineral particles *in vitro* and transiently

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stabilize amorphous calcium phosphate (ACP) (He et al., 2003, 2005).

Despite numerous studies, the exact role of DMP1 in dentin mineralization is still unclear. Here we present the data from our immunohistochemical studies of fully mineralized rat incisors and the results of *in vitro* mineralization experiments suggesting that DMP1 might play an important role in the mineral formation and organization in the extafibrillar spaces in dentin and specifically in PTD and dentino-enamel boundary (DEB).

2. Materials and methods

2.1. Immunohistochemistry studies of rat incisors

Two-months-old Wistar rats were euthanized according to an approved protocol. The mandibles were extracted, immediately freeze-dried and mounted in epoxy resin (Epofix, EMS). The erupted portions of mandibular incisors were polished in the transverse plane using a Minimet 1000 polishing machine (Buehler, Lake Buff, IL) using 6, 1 and 0.25 μm Metadi diamond suspensions (Buehler, Lake Buff, IL). To seal the capillaries, i.e. dentinal tubules, the samples were infused with 3% gelatin. The polished samples were covered with 3% gelatin solution at 39 °C under vacuum for 1 h and then let set for an hour at room temperature. The samples were washed in PBS and re-polished with 0.25 μm diamond suspensions to expose the surface of the sample. The samples were etched for 5 min in 2% EDTA and 1% parafolmaldehyde aqueous solution to expose the antigen epitopes, followed by 5 rinses in PBS containing 0.5% BSA (PBS/BSA). The samples were blocked by 2% BSA and 0.15% glycine in PBS for 1 h at 4 °C followed by incubation with donkey Fab2 fragments and rinsed in PBS/BSA 5 times. The samples were incubated with primary rabbit anti mouse-DMP1 antibodies raised against its C-terminal portion (generous gift by Dr. Chunlin Qin, Baylor College of Dentistry, Dallas, TX) diluted 1:50, 1:100 and 1:1000 in PBS/BSA for 1 h. The samples were washed in PBS/BSA. The samples were incubated with secondary Alexa-Fluor 488 donkey anti-rabbit antibodies (Molecular Probes) diluted 1:100 in PBS/BSA for 1 h, followed by 5 rinses in PBS, mounted on dimpled glass slides and storied in a dark container at 4 °C before analysis. Samples were analyzed using Nikon TE2000-e Eclipse light microscope in the epifluorescence mode.

2.2. Generation of recombinant DMP1 (rDMP1)

Mouse DMP1 cDNA was inserted into the pGEX vector following its excision from pcDNA3 vector, (a generous gift from Dr. J. Feng (Baylor College of Dentistry, Dallas, TX)). The DMP1-pGEX was then transformed into the bacterial host BL21. Cells were cultured in LB + Amp media overnight at 37 °C. Protein expression was induced with 0.4 mM IPTG for 2–6 h. The bacterial lysate was cleared by centrifugation and applied directly to Glutathione Sepharose 4B (Amersham). After washing with PBS, GST-bound protein was eluted with thrombin. Thrombin was removed from eluates with *p*-Aminobenzamidine immobilized on Sepharose 4 Fast Flow matrix (Amersham). The purified protein was electrophoresed on a polyacrylamide gel to verify the molecular mass, and subsequent western blot analysis was carried out.

2.3. Generation of recombinant DMP-1 and APMP-1-adenovirus transfection

DMP1 adenovirus was added to MC3T3-E1 cells, which were then grown in serum-free medium for three days. The media were then collected and immediately frozen at −80 °C and lyophilized. Samples were resuspended in 6 M urea in 20 mM Tris–HCl. The cells and ECM were lysed in 4 M Guanidine-HCl pH 7.4 in the presence of a protease inhibitor cocktail (C# 118361450011), 1 tablet/50 ml medium and 10 mM NaF for several hours. Guanidine buffer was exchanged with 6 M Urea in 20 mM Tris–HCl using Amicon ultracentrifuge filter units (Millipore C#UFC901024). The samples collected from the media or the cells/matrix were purified by FPLC, using an anion exchange column – HiTrap Q HP ((Cat. no17–1154-01) from GE Healthcare). Proteins were eluted from the column by increasing salt concentration, using an elution buffer containing 6 M Urea in 20 mM Tris–HCl + 0.8 M NaCl (PH7.2) at a flow rate of 0.4 ml/min for 80 min. The quality of the protein preparation was assessed using SDS PAGE and western blot, using antibodies generously provided by Dr. J. Feng (Baylor College of Dentistry, Dallas, TX).

2.4. Mineralization procedure

Mineralization experiments were performed using a variation of a published "on grid" mineralization technique (Beniash et al., 2005; Deshpande and Beniash, 2008) High purity $CaCl_2{\cdot}2H_2O$, $Na_2HPO_4.7\ H_2O$ were obtained from Sigma–Aldrich. Stock solutions of $CaCl_2$ (6.68 mM) Na_2HPO_4 2 H_2O (4 mM) were prepared using deionized distilled water (DDW). A 10X PBS buffer with 100 mM sodium phosphate and 1550 mM NaCl was purchased from Fluka. The pH of 10X PBS solution was adjusted to give pH 7.8, when diluted 10 times. DMP1 in urea was dialyzed against DDW and concentrated to 2 mg/ml by SpeedVac. The solution was maintained at 4 °C for 48 h before the experiments.

For mineralization experiments, a 3.4X PBS solution was prepared from 10× PBS stock solution. Equal volumes of aqueous 4 mM $Na_2HPO_4\cdot2H_2O$, 3.4× PBS, 6.68 mM $CaCl_2\cdot2H_2O$ and 1 mg/ml protein solutions were mixed together to produce mineralization solution containing 9.5 mM phosphate, 1.67 mM Ca^{2+} and 250 μg/mL of the protein, with final pH = 7.8. Twenty μL droplets of the solutions were placed in a humidity chamber and carbon-coated TEM grids were placed on top of the droplets. The samples were incubated at 37 °C for 16 h at 100% humidity. After the incubation, the grids were dipped in DDW, blotted against filter paper and air-dried. Since at the conditions used in the study the mineral is fully mature after 16 h (Boskey and Posner, 1973; Termine et al., 1970), the effect of rinsing and drying on the samples was mininmal.

Some of the samples were demineralized using an aqueous solution containing 2% glutaraldehyde and 1% ethylenediaminetetraacetic acid (EDTA) at pH 8.0 and subsequently stained with 2% Uranyl Acetate in DDW. The samples were rinsed in DDW, air dried and subjected to transmission electron microscopy (TEM) analysis in the bright field mode.

2.5. TEM analysis

Transmission electron microscopy (TEM) and selected area electron diffraction (SAED) analysis of the products of the *in vitro* mineralization experiments were carried out using a JEOL 1210 TEM microscope operated at 100 kV and JEOL 2000EX operated at 200 kV. The micrographs were recorded using an AMT CCD camera (AMT, Danvers, MA). An aluminum film-coated TEM grid (EMS Hatfield, PA) was used as a standard to calibrate SAED patterns for *d*-spacing calculations. The micrographs were analyzed using ImageJ 1.38 × image processing software (Bethesda, MD).

Tomography tilt series of mineralized samples were acquired at nominal magnifications of 23,000 to 26,000, using Tecnai 12 transmission electron microscope (FEI, Hillsboro, OR) equipped with a LaB6 filament at 120 kV at the beam density of ~300 e^-/Å. The micrographs were recorded automatically using bottom mounted Gatan 2000 CCD camera (2048 × 2048 pix, with the physical pixel size of 14 μm. The micrographs were taken in a tilt range from −60° to 60° with of 1° increment from -45° to 45° and 0.5° increment from −60° to −45°and from 45° to 60°. Because of the strong contrast