Histochem Cell Biol (2005) 124: 197–205
DOI 10.1007/s00418-005-0009-9

ORIGINAL PAPER

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Developmental appearance of dentin matrix protein 1 during the early dentinogenesis in rat molars as identified by high-resolution immunocytochemistry

Accepted: 14 April 2005 / Published online: 28 July 2005
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Abstract Dentin matrix protein 1 (DMP 1) is an acidic phosphoprotein that has been postulated to play an important role in mineralized tissue formation. We have examined rat molar tooth germs by applying a high-resolution immunocytochemical approach with the purpose to identify the temporal and spatial localization of DMP 1 at the onset of dentinogenesis. Upper molar tooth germs of 2- to 3-day-old Wistar rats were fixed in a cacodylate-buffered 0.1% glutaraldehyde + 4% formaldehyde fixative, left unosmicated and embedded in LR White resin. The sections were incubated with a polyclonal DMP 1 antibody for postembedding colloidal gold immunolabeling and examined in a Jeol 1010 transmission electron microscope. The earliest localization of DMP 1 was in the Golgi region as well as in the nucleus of differentiating odontoblasts. When mineralization spread from matrix vesicles to the surrounding matrix, DMP 1 was extracellularly detected around the mineralizing globules. In the regions of fully mineralized mantle dentin, it was present in the mineralized regions, mainly around the peritubular dentin. The appearance of DMP 1 during early dentinogenesis implies a direct role for this protein in both odontoblast differentiation and matrix mineralization.

Keywords Dentin matrix protein 1 · Dentinogenesis · Mineralization · Noncollagenous proteins · Odontoblasts · Immunocytochemistry

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Introduction

Odontoblasts orchestrate a series of complex mechanisms that result in mineralization of dentin. Once odontoblasts embark on their differentiation program from ectomesenchymal neural crest-derived cells, they establish a single layer, secrete a collagen-based matrix, and release numerous membrane-bound matrix vesicles (Arana-Chavez and Massa 2004). Although there is consensus that matrix vesicles initiate the mineral deposition in dentin (Ten Cate and Nanci 2003), the mechanism by which mineralization spreads from the vesicles to the surrounding organic matrix remains a subject of controversy.

The extracellular dentin matrix is 90% composed of collagen type I fibrils, which provide the structural framework that define compartments for ordered mineral deposition (Traub et al. 1992). However, it is well established that collagen alone does not have the capacity to induce matrix-specific mineral formation from metastable calcium phosphate solutions that do not spontaneously precipitate (Saito et al. 1997). Thus, the attention has been drawn to the noncollagenous proteins that compound the remaining 10% of the organic dentin matrix. The noncollagenous matrix components comprise a number of highly phosphorylated proteins that possess calcium-binding properties and function either as promoters or as inhibitors of mineral deposition in in vitro experiments (Hunter et al. 1996). They include dentin sialophosphoprotein (DSPP)—a large parental protein that subsequently suffers cleavage to originate two products, dentin sialoprotein (DSP) and dentin phosphoprotein (DPP), dentin matrix protein 1, 2, and 3 (DMP 1, DMP 2, and DMP 3, respectively), and small amounts of osteopontin, bone sialoprotein, osteonectin, and osteocalcin (Butler and Ritchie 1995; Papagerakis et al. 2002; He et al. 2003a, b).

Dentin matrix protein 1 is a major acidic noncollagenous matrix protein that was first cloned from the

mineralized dentin matrix (George et al. 1993) and later from bone matrix (MacDougall et al. 1998). Special attention has been drawn to the potential capability of this protein for binding to the collagen fibrils in mineralized tissues. It is a large 66 kDa molecule that contains two peptide clusters at the carboxyl end, ^{349}DSESSEEDR^{357} and ^{424}SEENRDSDSQDSSR^{437}, which have demonstrated high binding affinity to collagen in in vitro experiments (He and George 2004) and therefore they would be responsible for binding of DMP 1 to collagen monomers. In addition, based on its high negative charge, DMP 1 has been postulated to play an important role in mineralization tissue formation by initiating and modulating the deposition of the mineral phase (George et al. 1993). This assumption was further confirmed by findings showing that recombinant DMP 1 can initiate apatite nucleation in vivo (He et al. 2003a, b). Indeed, recent results verified that the DMP 1-collagen complex initiates the mineral deposition on the collagen surface when incubated in a pseudo-physiological buffer, whereas no deposition of calcium phosphate occurred when DMP 1 was absent (He and George 2004). These studies suggest that cooperative interactions between DMP 1 and collagen type I might be an essential step in the biomineralization process in matrix-mediated mineralization.

In osteoblasts, DMP 1 appears to act as an activator of certain transcriptional pathways leading to the expression of alkaline phosphatase. When in conjunction with other osteoblast transcriptional factors, this protein may regulate the expression of the osteocalcin gene (Narayanan et al. 2001, 2003). It has been reported that undifferentiated osteoblasts synthesize DMP 1 that is first transported into their nucleus by binding its active NLS3 domain to a soluble transport factors such as a importin, providing a regulated, unidirectional import of DMP 1 into the nucleus of osteoblasts (Narayanan et al. 2001). In the nucleus, DMP 1 is responsible for regulating the transcription of matrix genes involved in matrix mineralization. When osteoblasts are polarized, DMP 1 undergoes some conformational changes facilitating phosphorylation by casein kinase II. The overall conformational change leads to the exposure of the nuclear export signal (NES) peptide sequence and the rapid export of DMP 1–Ca^{2+} complex to the extracellular matrix where it binds specifically to the collagen fibrils, and initiates the nucleation of hydroxyapatite (He and George 2004).

It has been showed that overexpression of DMP 1 in MC3T3-E1 and C3H10T1/2 cells specifically induced gene expression of DSP and DMP 2, two odontoblast marker genes that encode secretory stage-specific phenotype, responsible for building dentin extracellular matrix deposition and for its mineralization (Narayanan et al. 2001). DMP 1 null mice express 25% less DSPP mRNA than wild-type animals (Ye et al. 2004). Thus, it is possible that DMP 1 could exhibit a similar dual functional role during odontoblast differentiation. Therefore, the first step would be the immunolocalization of DMP 1 inside the nucleus of odontoblasts.

Immunocytochemistry clearly offers a suitable method for the identification of DMP 1 and for mapping its distribution within the mantle dentine extracellular matrix and in the nuclei of differentiating and fully differentiated odontoblasts in vivo. In order to test the hypothesis that DMP 1 may be present during the early stages of dentin mineralization, we have applied a high-resolution immunocytochemical approach to examine the labeling of DMP 1 at the odontoblast nuclei and cytoplasm as well as within the mantle dentin matrix in rat molars. The rat molar tooth germ is a fascinating biological model for temporal studies, since at the bell stage of odontogenesis its cusp slopes display a progressive developmental sequence from the cervical loop to the future cusp tips.

Material and methods

Principles of laboratory animal care (NIH publication 85-23, 1985) and national laws on animal use were observed for the present study, which was authorized by the Ethical Committee for Animal Research of the University of São Paulo, Brazil.

Tissue processing for transmission electron microscopy

Upper molar tooth germs of 2- to 3-day-old Wistar rats were removed and quickly placed in a fixative containing 0.1% glutaraldehyde + 4% formaldehyde (freshly prepared from paraformaldehyde) buffered at pH 7.4 with 0.1 M sodium cacodylate (Arana-Chavez and Nanci 2001). Specimens were immersed in a beaker containing 40 ml of fixative at room temperature, which was subsequently placed in a 20 cm×20 cm glass recipient filled with ice and immediately taken inside a Pelco 3440 laboratory microwave oven (Ted Pella, Redding, CA, USA). The temperature probe of the oven was submerged into the fixative and the specimens were exposed to microwave irradiation at a 100% setting for three periods of 5 min, with the temperature programmed to a maximum of 37°C (Massa and Arana-Chavez 2000). After microwave irradiation, specimens were transferred into fresh fixative and were left overnight in it at 4°C. They were then washed in 0.1 M sodium cacodylate buffer, pH 7.4 for 1 h, left undemineralized and unosmicated, and subsequently dehydrated in graded concentrations of ethanol and embedded in LR White resin. Some specimens were post-fixed with 1% osmium tetroxide for 1 h, dehydrated as described above, and embedded in Spurr resin. Toluidine blue-stained 1-μm-thick sections were examined in a light microscope, and regions containing the sequence of initial stages of odontogenesis were trimmed for ultrathin sectioning. Sections of 80-nm thickness were cut with a diamond knife on a Leica Ultracut R ultramicrotome, collected

onto 200-mesh nickel grids coated with 2% parlodion in amyl acetate, stained with uranyl acetate and lead citrate, and examined in a Jeol 1010 transmission electron microscope operated at 80 kV.

Additional specimens, fixed and washed as described above, were dehydrated in graded concentrations of ethanol and embedded in JB4 historesin (Polysciences, Warrington, PA, USA) into BEEM capsules. Sections of 1-nm thickness were cut using a glass knife in a Microm HM360 microtome, collected onto glass slides, and stained with hematoxylin and eosin. Coverslips were mounted with Entellan (Merck, Germany) before examining the slides in a Nikon Optiphot 2 light microscope.

Antibody purification and postembedding colloidal gold immunolabeling

Polyclonal DMP 1 antibody was affinity-purified as described earlier, using rDMP1 coupled to CNBr-activated Sepharose column (Srinivasan et al. 1999). The grids were blocked with 1% ovalbumin in phosphate buffer saline (PBS) for 15 min before incubating with the polyclonal DMP 1 antibody diluted 1:10 for 3 h. Then, the grids were washed with PBS, blocked with PBS-1% ovalbumin for 15 min and incubated with a protein A-gold complex (Electron Microscopy Sciences, USA) for 1 h for revealing the sites of antibody-antigen binding. After the protein A-gold, the grids were jet-washed with PBS followed by distilled water. All steps were carried out at room temperature (Arana-Chavez and Nanci 2001). Sections were then stained with uranyl acetate and lead citrate, and examined in a Jeol 1010 transmission electron microscope operated at 80 kV. Controls for the specificity of the labeling consisted of incubating the sections with pre-immune serum or with protein A-gold alone.

Results

The molar tooth germs examined in the present study displayed sequential stages of odontoblast differentiation, as dentinogenesis begins at the future cusp tips and spreads down through the cusp slopes to the cervical loop (Fig. 1). It is owing to the fact that the ultrathin sections for the postembedding immunodetection of DMP 1 had necessarily to be unosmicated, post-fixed samples were used as a reference for the identification of some matrix components, especially for matrix vesicles (Fig. 2a, b). A basal lamina was evident at the epithelial–ectomesenchymal interface at the early stages of odontoblast differentiation. Round bodies almost 100 nm in diameter were identified as matrix vesicles. They were observed close to the odontoblast processes and to the collagen fibrils at certain distance from the basal lamina and showed a content with variable electron opacity. Peripheral extracellular matrix of dental

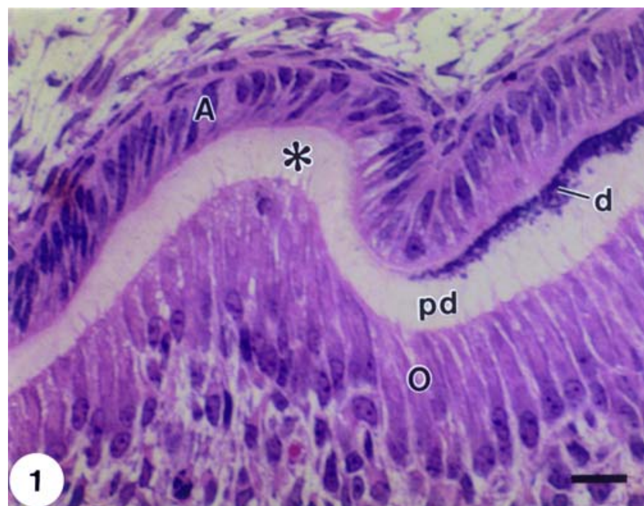

Fig. 1 Light micrograph from a rat molar tooth germ at the early stages of dentinogenesis examined in the present study. The dentin deposition is gradually spreading down through a cusp slope from the future cusp tip (at the *right side* of the micrograph) to the cervical loop (at the *left side*). Thus, the earlier stages of dentinogenesis are observed at the left side, where the forming matrix is unmineralized (*asterisk*). When the matrix becomes mineralized (*d*), a layer of newly formed matrix (the predentin *pd*) is always interposed between the odontoblasts (*O*) and the mineralizing front. *A* differentiating ameloblasts. Hematoxylin-eosin staining. *Bar* = 10 μm

papilla at this stage was composed of fine fibrils oriented perpendicular to the basal lamina. The differentiating odontoblasts became gradually columnar and numerous cell processes appeared in the matrix (Fig. 2a). The unosmicated sections processing for immunocytochemistry revealed that the early unmineralized mantle dentin matrix was free of gold particles at this time point (Fig. 2b).

With an increase in the deposition of the matrix, the intracellular presence of DMP 1 was highly evident at the Golgi region (Fig. 3a), over several cisterns of rough endoplasmic reticulum, as well as dispersed at the supranuclear region of the cytosol (Fig. 3b). Some intracellular particles were immunodetected inside the nucleus of differentiating odontoblasts (Fig. 4), besides the Golgi region and the supranuclear cytosol, despite the fact that the intracellular labeling was always weak. Although numerous matrix vesicles appeared at this stage, the developing mantle dentin was unlabeled (data not shown).

Once the matrix vesicles became mineralized, which exhibited a somewhat irregular contour and a mineral content with a high electron opacity, the first extracellular gold particles localizing DMP 1 were detected around them (Fig. 5). Subsequently, when the mineral phase spread from the mineralized matrix vesicles, the immunoreactivity for DMP 1 was more evident around the mineralizing globules (Fig. 6). In the later stages of mantle dentin mineralization, when the mineralized matrix formed a continuous band, small patches of gold particles were present in the mantle dentin and at the

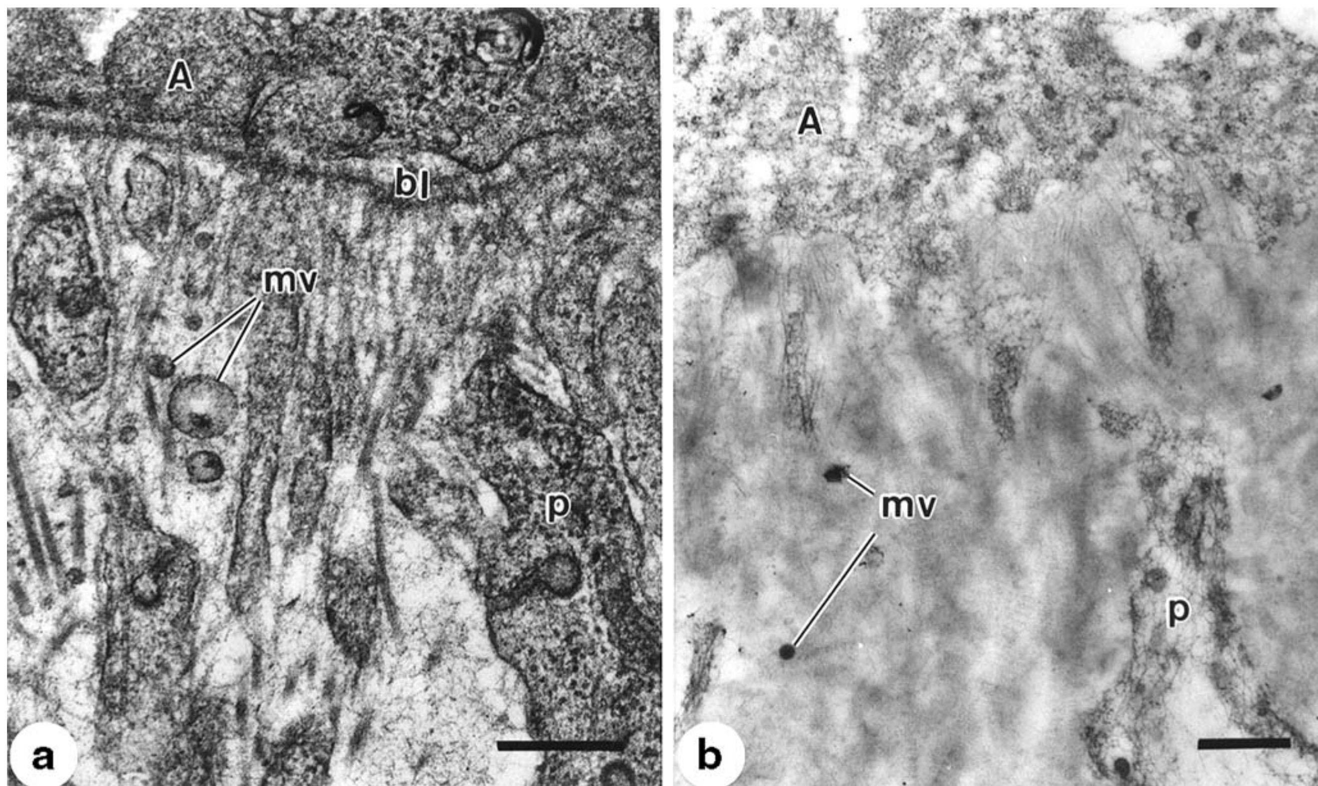

Fig. 2 Electron micrographs showing two regions of early mantle dentin at the same stage of development from an osmicated specimen (**a**) and from an unosmicated sample incubated with the anti DMP 1 antibody (**b**). In **a**, some matrix vesicles (*mv*) appear as rounded membrane-bound bodies interspersed among the early unmineralized mantle dentine and close to an odontoblast process (*P*), which are typical unmineralized matrix vesicles. In **b**, two unmineralized matrix vesicles (*mv*) appear as rounded bodies at a similar place than the ones showed in **a**. Note the absence of gold particles around the matrix vesicles. *A* ameloblasts; *P* odontoblast process; *bl* basal lamina. *Bars* = 0.5 μm

boundary between the mineralized dentin and the predentin, the mineralization front, whereas the predentin matrix was unlabeled (Fig. 7). In some mineralized areas, the labeling for this antibody appeared to be localized over the peritubular dentin (Fig. 8). Intracellularly, the gold particles were always detected at the Golgi region, indicating the continuous synthesis of DMP 1 by fully differentiated odontoblasts (Fig. 9).

In control specimens for the immunocytochemical assays, i.e., those incubated with pre-immune serum or with the protein A-gold complex alone, all the odontoblasts—including their nuclei—as well as the developing dentin matrix were free of labeling (Fig. 10).

Discussion

The present ultrastructural study showed the presence of DMP 1 at early stages of dentinogenesis. The earliest localization was in the Golgi region as well as in the nucleus of differentiating odontoblasts. At later stages, when mineralization spread from matrix vesicles to the surrounding matrix, DMP 1 was extracellularly detected around the mineralizing globules. In regions of fully mineralized mantle dentin, it was present in the mineralized regions, mainly at the peritubular dentin and at the mineralization front between dentin and predentin.

A number of studies focusing several aspects of the DMP 1 function have been previously carried out, majority of them showing the expression of this non-collagenous protein by in situ hybridization or by employing in vitro systems with the intent to elucidate several biochemical aspects (D'Souza et al. 1997; Narayanan et al. 2001; George et al. 2003; He et al. 2003a, b). However, whereas in situ hybridization approaches detect gene transcripts, they do not necessarily indicate that a given protein will be really processed for secretion. The suitable methods for detecting the complete molecule are immunological assays by which specific antibodies localize the protein in its final secreted form, intra

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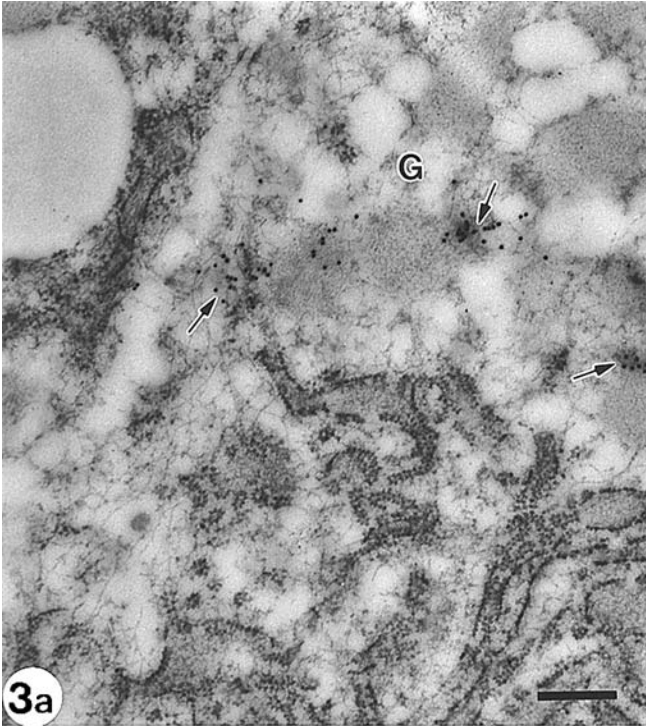
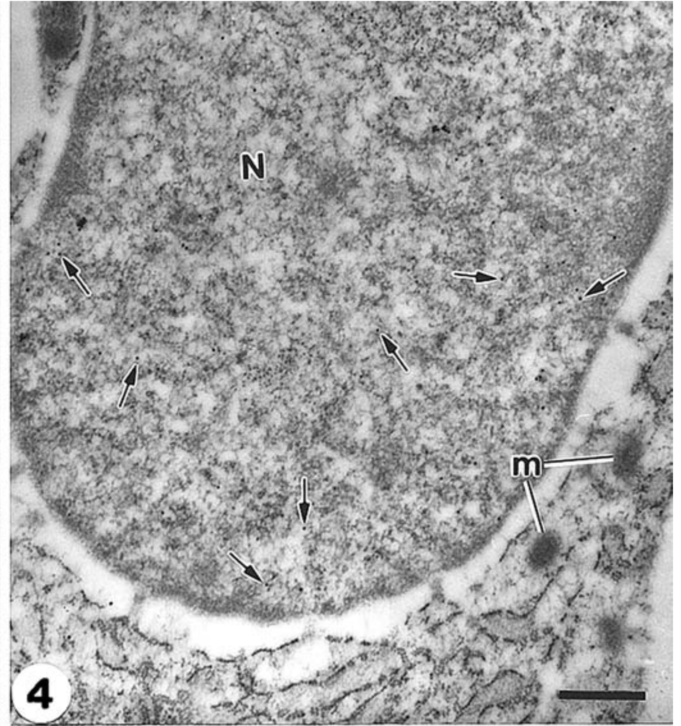
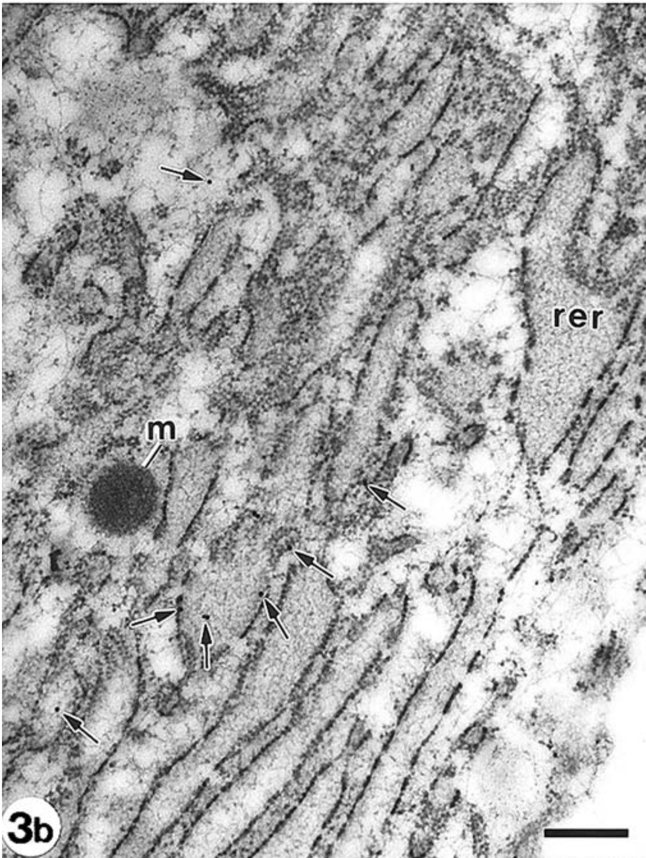
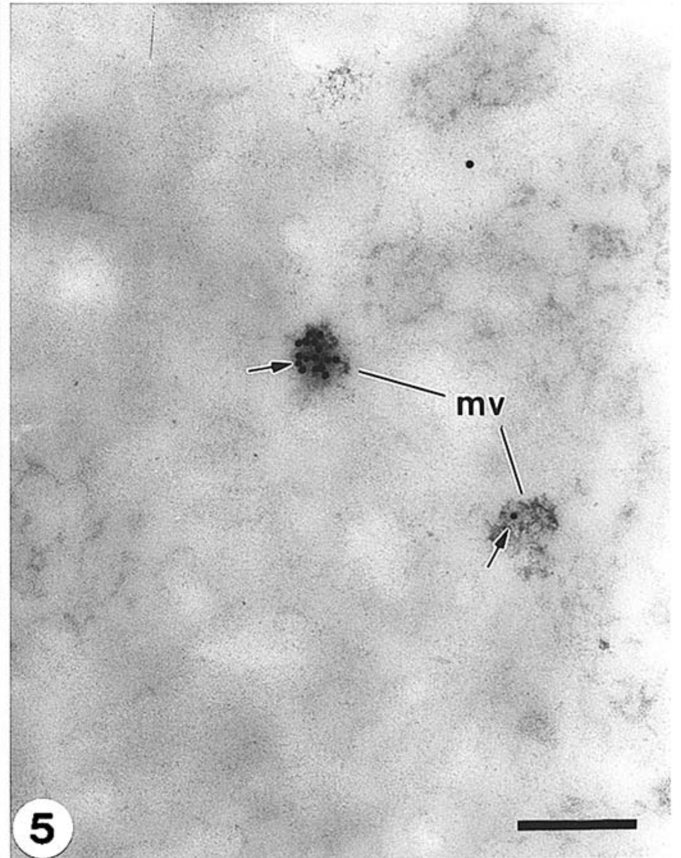
Fig. 3 Electron micrographs illustrating, in **a**, the Golgi region (*G*) of a differentiating odontoblast that exhibits patches of gold particles immunoreactive for DMP 1 (*arrows*). In **b**, the supranuclear region from the same cell of **a**, in which few particles (*arrows*) appear dispersed over the cytosol and in relation to the cisterns of the rough endoplasmic reticulum (*rer*). Note a mitochondrion (*m*) free of gold particles. *Bars* = 0.25 μm. **Fig. 4** Electron micrograph showing labeling for DMP 1 (*arrows*) within the nucleus (*N*) of a differentiating odontoblast. Note some adjacent mitochondria (*m*) which are free of gold particles. *Bar* = 0.5 μm. **Fig. 5** Electron micrograph showing a region of the developing mantle dentin in which two mineralizing matrix vesicles (*mv*) exhibit some associated gold particles immunoreactive for DMP 1 (*arrows*). *Bar* = 0.25 μm

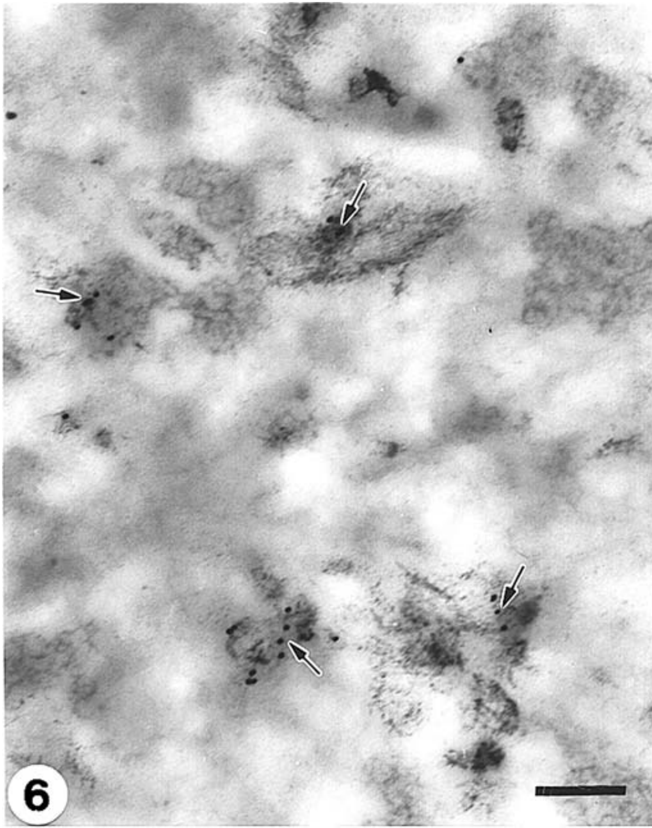
and/or extracellularly. Indeed, colloidal gold immunocytochemistry is a powerful tool that correlates biochemical composition with structure by examining specimens at higher magnifications. To the best of our knowledge, this is the first time that the temporal and spatial localization of DMP 1 has been investigated during the early dentinogenesis by applying high-resolution immunocytochemistry.

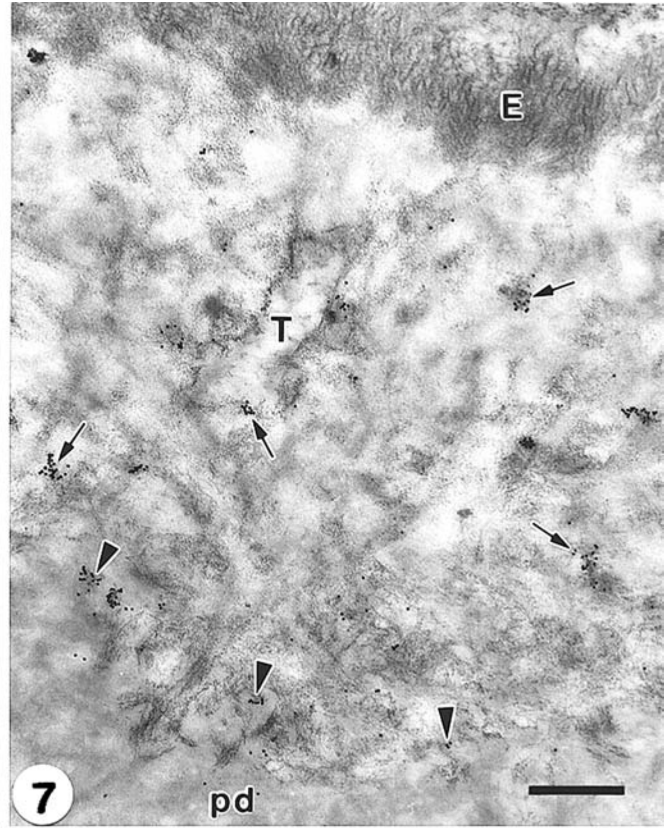


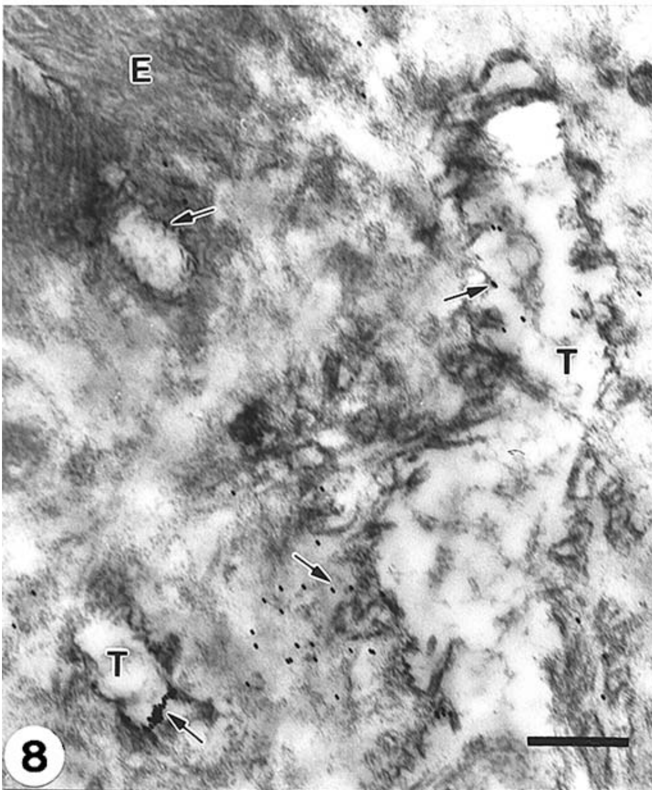


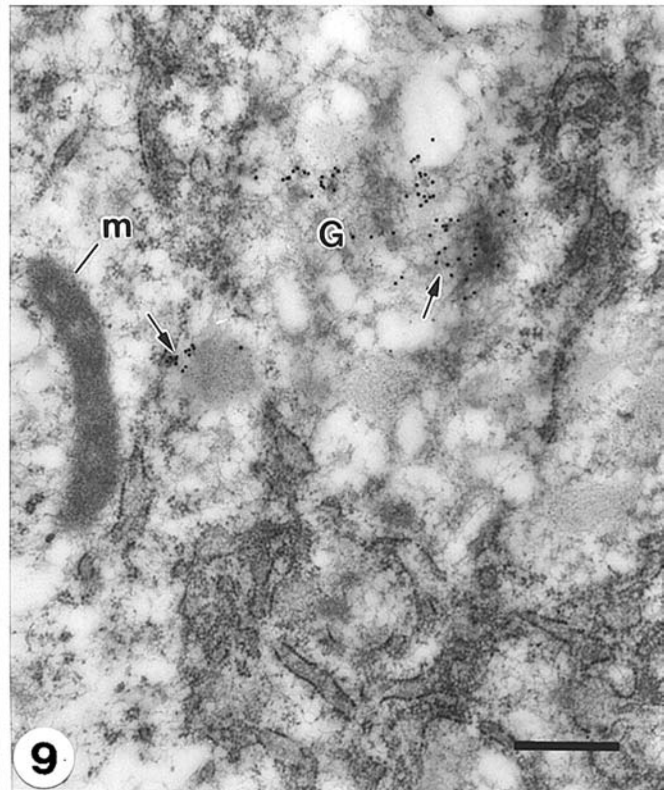












Although it has been shown that DMP 1 is a key noncollagenous protein of dentin, it is present in small amount in dentin matrix and odontoblasts (Butler et al. 2003). Therefore, its immunodetection at the ultrastructural level is undoubtedly influenced by the degree of tissue preservation. Thus, processing procedures were

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Fig. 6 Electron micrograph illustrating a stage in which the mineralization is spreading from matrix vesicles to the surrounding mantle dentin matrix. Note the immunoreactivity for DMP 1 (*arrows*) in close relation to the electron opaque mineralizing globules. *Bar* = 0.25 µm, **Fig. 7** Electron micrograph showing a stage in which a band of fully mineralized mantle dentin is observed. Note the labeling for DMP 1 that appears as patches of gold particles (*arrows*) within the mineralized matrix and at the mineralization front (*arrowheads*). At the top, the first layer of enamel (*E*) that contains fine mineral crystals can be also observed. *pd* predentin; *T* dentinal tubule. *Bar* = 0.5 µm, **Fig. 8** Electron micrograph showing a higher magnification view of the fully mineralized mantle dentin. Labeling for DMP 1 that appears as patches of gold particles can be observed on the peritubular dentin region (*arrows*). *T* dentinal tubules, *E* developing enamel. *Bar* = 0.5 µm, **Fig. 9** Electron micrograph showing the Golgi region (*G*) of a fully differentiated odontoblast. The presence of gold particles (*arrows*) indicates that the synthesis of DMP 1 is kept even after the odontoblasts reach their fully differentiation stage. A long unlabeled mitochondrion (*m*) can be observed. *Bar* = 0.5 µm

carefully standardized in the present study by which tissues were rapidly fixed in aldehydes under microwave irradiation, a procedure that has been shown to improve immunodetection of noncollagenous proteins in mineralized tissues (Arana-Chavez and Nanci 2001), moreover post-fixation with osmium tetroxide had to be avoided (Bendayan 1995). These careful procedures yielded an adequate balance between retention of antigenicity and ultrastructural preservation of the tooth germs. In addition, since the purpose of this study was also to correlate the presence of DMP 1 with the onset of dentin mineralization, the specimens were left undecalcified. Although the mineral deposits may be less evident after immunocytochemical reactions, their typical electron opacity clearly revealed their presence in undecalcified hard specimens (McKee and Nanci 1995).

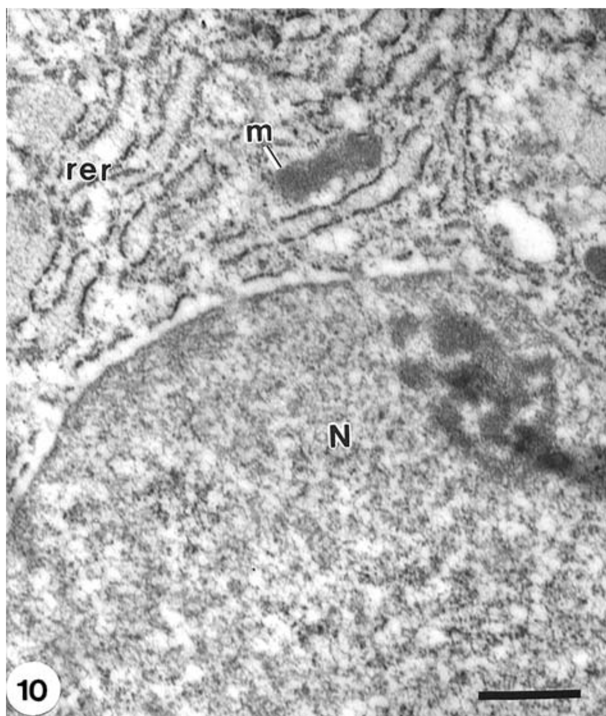

Fig. 10 Electron micrograph from an ultrathin section that was incubated with the protein A-gold complex, i.e., avoiding the DMP 1 antibody (control group). Observe the absence of gold particles at the nucleus (*N*) and other cell organelles such as the mitochondrion (*m*) and the rough endoplasmic reticulum cisterns (*rer*). *Bar* = 0.5 µm

Our results show the earliest immunolocalization of DMP 1 in the Golgi region of differentiating odontoblasts; slightly further up on the cusp slopes, it was also detected inside their nucleus. These results correlate well with the intracellular localization of DMP 1 reported by Narayanan et al. (2001). The initial intracellular immunolocalization of DMP 1 at the Golgi region of differentiating odontoblasts coincides with findings from in situ hybridization studies, which have been previously reported that the expression of this protein starts when odontoblasts differentiate (George et al. 1995; D'Souza et al. 1997). The present results additionally revealed a few gold particles dispersed in the supranuclear region of the cytosol and inside the nucleus. Although nuclear labeling for noncollagenous proteins is generally considered to be indicative of background, the non-related organelles contained in the same incubated sections, e.g., mitochondria, appeared free of gold particles. In addition, the odontoblast nuclei from control specimens for immunocytochemical assays, i.e., those incubated with pre-immune serum or avoiding the DMP 1 antibody and subsequently with protein A-gold were free of labeling. Thus, it is likely that the observed labeling over the nucleus of odontoblasts is the result of a specific immunoreaction. This finding is consistent with the fluorescent nuclear labeling for DMP 1 in cultured osteoblasts found by Narayanan et al. (2003). They hypothesized that DMP 1 is imported into the nucleus for playing a role in the regulation of specific genes that control the osteoblast differentiation before its export into the extracellular matrix. It has been shown that the overexpression of DMP 1 in cultured pluripotent and mesenchyme-derived cells induces the expression of DSP and DMP 2 genes, which are considered to be odontoblast-specific markers (Narayanan et al. 2001).

When ectomesenchymal cells differentiate into polarized odontoblasts, they secrete a collagen type I-rich matrix and release matrix vesicles. As the early unmineralized dentin matrix does not yet contain DMP 1, it is likely that the onset of mineral deposition into matrix vesicles takes place without the participation of this acidic phosphorylated protein. This is according to the idea that the early mantle dentin matrix formed by differentiating odontoblasts is devoid of noncollagenous proteins (Arana-Chavez and Katchburian 1998). The first appearance of DMP 1 into the matrix occurs when the majority of matrix vesicles are mineralized. Although unosmicated samples do not offer a clear trilaminar appearance of plasma membranes, and therefore we were not able to observe the matrix vesicles as membrane-bounded bodies, the correlated examination of osmicated specimens at the same stages permitted us

to identify unmineralized matrix vesicles with a homogeneous content. The mineralizing matrix vesicles are identified by their altered contour that appears significantly irregular, as it has been well established earlier (Katchburian 1973).

When mineralization spreads from matrix vesicles to the surrounding matrix, DMP 1 was extracellularly detected around the mineralizing globules, where it may participate on the matrix-mediated nucleation of the hydroxyapatite (He and George 2004). The crystals, which are released through the membrane of matrix vesicles, expose the preformed hydroxyapatite to the extracellular fluid. Although the extracellular fluid normally contains sufficient Ca^{2+} and $PO_4^{(3-)}$ to support continuous crystal multiplication and growth, some local conditions must be present in the matrix surrounding the vesicles for the formation of new crystals (Anderson 2003). The collagen scaffold that bound to the matrix vesicles surfaces may serve as a bridge for crystal propagation out into the extravesicular matrix (Wu et al. 1991) in association with acidic noncollagenous proteins, the highly phosphorylated proteins that possess calcium-binding capability (Butler and Ritchie 1995; Papagerakis et al. 2002; He et al. 2003a, b). DMP 1 has abundant acidic domains, which are negatively charged after phosphorylation. In addition, DMP 1 has a specific binding capacity to the *N*-telopeptide, which is located at the edge of gap region of the collagen fibril. Thus, it has been postulated to play an important role in mineralization tissue formation by initiating and modulating the deposition of the mineral phase (George et al. 1993). Cooperative interactions between DMP 1 and collagen fibrils may be essential for the matrix-mediated mineralization, once the mineral particle deposits at the collagen surface adsorbed with DMP 1 (He and George 2004; He et al. 2003a, b).

In the regions of fully mineralized mantle dentin, DMP 1 was present in the mineralized regions and at the mineralization front, between the mineralized dentin and the predentin, where mineral deposition is ongoing. Recent studies with DMP 1 null mice had shown that the thickness of mineralized dentin is reduced in DMP 1 null mice compared with that of wild-type animals, while the predentin is expanded. It was assumed that the DMP 1 null predentin tends to fail to mature into dentin, remaining hypomineralized (Ye et al. 2004). These findings appear to confirm the present considerations regarding the role of this protein in dentin mineralization; thus, it is crucial to note the association between collagen fibrils and DMP 1 leading to the progress of mineralization. In addition, immunoreactivity for DMP 1 in mineralized dentin was observed at the peritubular dentin that is poor in collagen (Weiner et al. 1999). It is possible that due to its calcium binding capacity, DMP 1 may play a role in the high degree of mineralization that takes place in peritubular dentin. Indeed, the DMP 1 null specimens displayed a irregular wall of peritubular dentin that yields wider dentinal tubules than those of wild-type specimens (Ye et al. 2004).

In summary, our findings reinforce the idea that mineralized tissue formation is a cell-mediated process. After the initial rapid mineralization mediated through matrix vesicles, the mineralization that subsequently occurs within specific regions of the collagen fibrils might be initiated by acidic phosphoproteins such as DMP 1, which are initially immobilized onto collagen. These collagen-acidic phosphoprotein complexes can then induce apatite formation and along with other noncollagenous proteins regulate the assembly of mineralized dentin.

Acknowledgements The authors thank Dr. Kevin Tompkins for his help. They also would like to thank Gaspar F. de Lima for ultrathin sectioning, and Edson Oliveira for his help with the electron micrograph printing. This work was supported by grants from Fapesp (01/13782-0) and CNPq (Brazil), and NIH-DE 11657 (USA).

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