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Role of matrix proteins in signalling and in dentin and enamel mineralisation

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Abstract

While the participation of the extracellular matrix protein in mineralisation processes has been extensively studied, a role for these proteins as signalling molecules has only been recently evoked. We report on the composition of enamel and dentin matrix, and discuss the multifunctionality of two molecules. Amelogenins are implicated in enamel three-dimensional organization and contribute to the initial mineralisation of the forming enamel. Some amelogenin spliced forms act as signalling molecules in odontoblasts. Dentin Matrix Protein-1 is also implicated in dentin mineralisation and in the differentiation of pulp embryonic cells into odontoblasts. This reveals a complex role of extracellular matrix molecules. ***To cite this article: M. Goldberg et al., C. R. Palevol 3 (2004).***

Résumé

Rôle de molécules matricielles dans la signalisation et la minéralisation de la dentine et de l'émail. Les recherches portant d'abord sur les molécules de la matrice extracellulaire en tant qu'initiateurs de minéralisation étudient maintenant leurs propriétés en tant que molécules de signalisation. Nous résumons la composition des matrices amélaire et dentinaire et prenons deux exemples de multifonctionnalité. Les amélogénines sont impliquées dans l'organisation tridimensionnelle et dans la minéralisation initiale de l'émail natif. Des formes épissées de la molécule agissent comme molécule de signalisation pour les odontoblastes. La protéine de la matrice dentinaire-1 intervient dans la minéralisation dentinaire et dans la différenciation de cellules pulpaires embryonnaires en odontoblastes. Ces données éclairent la complexité de molécules matricielles. ***Pour citer cet article : M. Goldberg et al., C. R. Palevol 3 (2004).***

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Mots clés : Dentine ; Émail ; Amélogénines ; Protéine de la matrice dentinaire-1 ; Biominéralisation

1. Introduction

The view that the molecules of the extracellular matrix are only implicated in the mineralisation of calcified tissues now appear simplistic in view of the number of articles also reporting signalling functions to some of these components. It is nowadays well recognized that the cells synthesize intracellular, pericellular and a complex extracellular matrix (ECM), especially dense and important in mineralised tissues. ECM molecules act as a three-dimensional template to promote nucleation, followed by crystal growth. ECM molecules also provide appropriate volume within which the mineral is formed. They are organized as complex hydrophobic spaces defined by glyco-lipoproteinic 'envelopes' (crystal ghosts in bone and dentin, matrix vesicles in bone and cartilage, tubules or nanospheres in enamel), which allow the accumulation of calcium and phosphate ions. These calcium and phosphate ions originate from the blood or interstitial fluids bypassing leaky junctional complexes together with their carrier molecules, such as albumin or lipids. A cascade of mineralisation reactions driven by thermodynamically unstable steps leads to the stabilization of the amorphous calcium phosphate, which is first transformed into calcium triphosphate, and then into calcium octophosphate. This forming mineral phase is further transformed into a thermodynamically more stable hydroxyapatite (HAP) form of lower energy level. After having induced the nucleation of the first calcium phosphate crystals, ECM components are then implicated in the control of crystal growth [4,11,13].

The global composition of enamel and dentin indicates substantial differences in composition of the two structures, which may interfere with their final biological and biomechanical properties (Table 1). The composition of the mineral and water phases in dental tissues is however more complex than it appears in Table 1. Firstly, there are different phases of water, bound or free, associated with the surface of crystals or with the organic matrix. The composition of the mineral phase is not uniform but includes a variety of HAP, which can be either rich or deficient in carbonates and magnesium. The HAP is associated with all the elements that are classified in the periodic table, except the lanthanide group. Yet, the organic matrix phase represent the most complex composition as shown in Tables 2 and 3.

2. Enamel matrix composition

Data related to the enamel matrix composition are summarized in Table 2. Despite it forms only 0.4% in weight and 2% in volume of the whole enamel, the list of molecules found in enamel is impressive. However, the functions of many of these molecules are still to be elucidated.

3. Dentin matrix proteins composition

The composition of dentin extracellular matrix proteins is similar to that of bone and cementum. However, proteins such as dentin sialoprotein (DSP), and also the dentin phosphoprotein (DPP), present both in dentin and bone, are much more abundant in the dentin (approximately 400 times). Other proteins are mostly expressed in bone (bone sialoprotein, osteopontin), but are also found in dentin, at least at early stages of tooth

Table 1
Global composition in weight and volume of two dental tissues: enamel and dentin
Composition globale en poids et volume de deux tissus dentaires : émail et dentine

	Enamel		Dentin	
	Weight	Volume	Weight	Volume
Mineral phase	96%	87–91%	70%	48%
Organic phase	0.4%	2%	20%	29%
Water	3.6%	7–11%	10–11%	23%

Table 2
Enamel matrix composition
Composition de la matrice de l'émail

Amelogenins	Spliced forms (28–25, 23, 20–21, 11, 9, 8.1, 6.9 kDa) One phosphate inserted in serine16 in the N-terminal TRAP domain	Degradation by MMPs, mostly by MMP 20. The peptides resulting from degradation disappear during maturation.
Enamelin	Highly glycosylated and phosphorylated molecule	Resident molecules
Ameloblastin, amelin, sheathlin		
Tuftelin	Sites of phosphorylation	Related to rod/inter-rod interface and initial mineralisation near the DEJ. Different from the tuft proteins (cytokeratin proteins?)
Proteinases	Metalloproteinases: MMP-9, MMP-20 (enamelysin) (present during enamel formation)	Serine-proteinases (present during enamel maturation)
Calcium-binding proteins	Annexins, calmodulin, calbindin, etc.	Implicated in intracellular calcium metabolism and biomineralisation of the transitory enamel
Sulphated glycoproteins	Rapid turnover	Unknown role
Transient expression of dentin matrix proteins (DSP, DPP, DMP-1)	In presecretory ameloblasts	In post-secretory ameloblasts?
Phospholipids	Membrane-bound phospholipids 66% (due to Tome's processes remnants)	Mineral-bound phospholipids 33% (taking origin in the serum? implicated in enamel mineralisation?)

formation. In contrast, other proteins, such as DMP-1, are present in equal amount in both bone and dentin.

Dual or multipotent biological properties of some of these molecules will be examined in the next parts of this review. It is obvious that these molecules are implicated in the formation of the organic scaffold and consequently in the mineralisation processes. However, it becomes clear nowadays that they are also involved in signalling functions of the cells that are responsible for their synthesis: the secretory odontoblasts and ameloblasts.

4. Multifunctionality of amelogenin

4.1. Role in amelogenesis

Amelogenins are expressed by ameloblasts during enamel formation. A heterogeneous mixture of amelogenins has been extracted from the forming enamel with large variations in MW from 28kDa to 5kDa. These multiple forms have been attributed to alternative splicing [9] and/or proteolytic cleavages [2,15]. Amelogenins are expressed by seven exons, and seven alternatively spliced amelogenin mRNAs have been isolated from the cDNA library, coding for amelogenins formed by 194, 180, 156, 141, 74, 59 and 44 amino acids residues in mouse [27]. In addition, the 25–28 kDa nascent proteins rapidly loose the telopeptides and the resultant amelogenin (23 kDa) then accumulates in the matrix after further cleavage (20–21 kDa). This is a crucial step in the mineralisation process, as the 21–20-kDa amelogenin can then bind minerals and form a solid-state aggregate. This follows by cleavage by either MMP20 or MMP-9; both metalloproteinases have been shown to degrade amelogernin in vitro ([2,8] and our unpublished data). A degradation cascade has been described [25], which results in small molecular species of 13 kDa and 11k Da, and then 5-, 4.5-, 4.3-kDa fragments and finally residual 3-kDa peptides, which are gradually eliminated from the forming tissue [8,18,25].

Three domains have been identified in the amelogenin molecule. The first is a highly conserved amino-terminal 'TRAP' (tyrosine-rich amelogenin peptide) sequence of 44/45 residues. This sequence is phosphorylated in serine-16, but not glycosylated. The TRAP segment has lectin-like properties and is similar to wheat germ agglutinin as it binds *N*-acetyl-D-glucosamine [23]. The second domain is a central hydrophobic core of 100–130 residues, extremely rich in proline (one residue on four). Finally, the carboxyl-terminal domain is formed by a small acidic hydrophilic sequence of 13 amino acids [8].

Table 3
Dentin matrix composition
Composition de la matrice de la dentine

Group	Molecule	Structure	Potential role
Collagens (90%)	Type-I collagen	2(αI)1, 1(αI)2	>Scaffold
	Type-I trimer	3(αI)1 (11%)	>Elasticity of the tissue
	Types III, IV, V, VI	Minor collagens 1–3%	>Increase in culture and pathologic dentins
Non-collagenic proteins (10%)			
Phosphorylated proteins: the SIBLINGs family (Small Integrin-Binding Ligand N-linked Glycoprotein)	☞DSPP , cleaved into ★DPP and ★DSP ☞DMP-1 (or BAG-75?) ☞BSP ☞OPN + one related molecule: ☞MEPE/OF45	Properties: *Casein-kinase phosphorylation sites *RGD sequence *Polyacid stretches	*Mineralisation: initial nucleation dependent on the concentration, and crystal growth *Signalling molecules *Inhibition of the complement-mediated attack
Non phosphorylated matrix proteins	☞Matrix GLA- protein ☞Osteocalcin ☞Osteonectin		Inhibitor or promoter of mineralisation?
Proteoglycans and glycosamino-glycans	☞CS/DS containing SLPRs: Decorin–biglycan ☞KS containing SLRPs: Lumican–fibromodulin–osteoadherin		>Inhibitors? Or promoter of mineralisation? >Collagen fibrillogenesis
Proteins also expressed in ameloblasts	Amelogenin 5–7 kDa	Spliced forms A+4 & A-4	Chondrogenic inducing agent- Signalling effects
Growth factors	☞TGF-β ☞ILGF-I and II ☞FGF-2 ☞VEGF, PDGF		Active or latent form? May be activated? Angiogenic ?
Metallo-proteinases	MMP-1 MMP-2 and -9 MMP-3 MMP-20 MT1-MMP TIMP –1 to -3	Collagenase Gelatinase A & B Stromelysin 1 Enamelysin Activation of proforms Forming complexes with MMPs	☞Native collagen cut into 1/4–3/4 ☞Degradation of collagen peptides ☞Proteoglycans ☞Amelogenin expressed by odonto-blasts ☞Inhibition of MMPs
Other enzymes	* Alkaline phosphatase		*Phosphate release
Serine-proteinases	* Cathepsins		*Degradation
Serum-derived proteins	α_2HS-glycoprotein * Albumin * Immunoglobulins Lipoproteins: HDL & LDL		Detected mostly in mineralised tissues ☞Lipid carrier ☞involved in dentin mineralisation?
Lipids	Phospholipids	☞Membrane phospholipids and ☞Extracellular matrix components	Initial nucleation and crystal growth

Although amelogenins do not possess in vitro the specific crystal-modulating properties characteristic of certain acidic mineralised tissue proteins [17], examination of the forming enamel at the ultrastructural level supports the existence of tubules and stippled (granular) materials where the first crystal may be formed (see [11] for a review). The significance of the stippled materials has been disputed as it was suggested to result from defective fixation methods, or to constitute residual biological material before intracellular re-internalisation of excesses or/and degraded peptides during enamel formation [12,24]. However, rapid freezing and freeze substitution that preserve the actual native structure of the forming enamel, and radioautography, suggest that this stippled material is a real structure and represents an enamel precursor. These granules result from self-association properties between the N- and C- termini of amelogenin, which lead to the formation of nanospheres where initial mineralisation may be triggered [7]. The role of amelogenin in enamel formation and mineralisation is substantiated by the following in vivo findings:

- the supra-molecular control of HAP crystal growth is under the control of amelogenin [5];
- amelogenin-deficient mice display an amelogenesis imperfecta phenotype and disorganized hypoplastic enamel (this suggests that amelogenins are not required for the initiation of mineral crystal formation, but rather for the organization of crystal pattern and for the regulation of enamel thickness [10]);
- finally, N-terminal-deleted amelogenin has been seen to induce in vivo severe enamel defects in mouse, whereas C-terminal-deleted amelogenin does not alter enamel formation [6] (Figs. 1 and 2).

4.2. Amelogenins and MMP-20 (enamelysin) in odontoblasts, dentin and periapical tissues

The importance of amelogenins in enamel formation has been clearly demonstrated. The molecules are

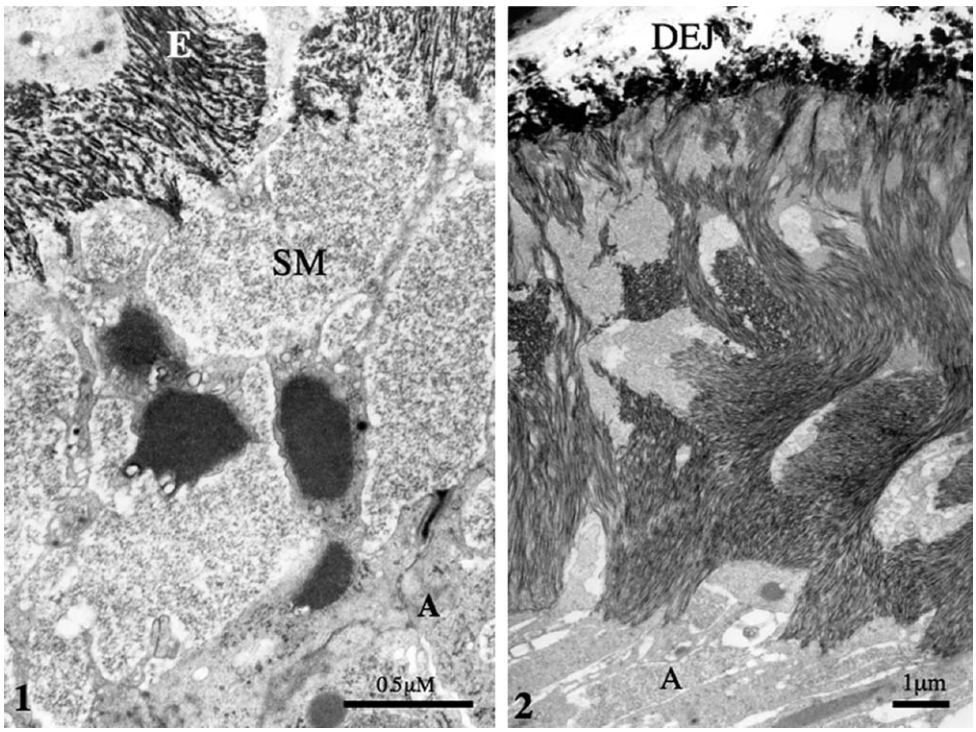


Fig. 1. Alteration of the apposition zone of the forming enamel of a N-terminal-deleted amelogenin mouse. Stippled material (SM) is abundant. Electron-dense apoptotic nuclei are seen. **A** = ameloblasts.
Fig. 2. Structural alterations of the forming enamel of a N-terminal deleted mouse. DEJ: dentino-enamel junction.
Fig. 1. Altération de la zone d'apposition de l'émail en formation chez une souris à amélogénine ayant une délétion N-terminale. Le matériel punctiforme (SM) est abondant. On observe des noyaux en apoptose denses aux électrons. **A** = améloblastes.
Fig. 2. Altération structurale de l'émail en formation chez une souris portant une délétion N-terminale. DEJ = fonction amélo-dentinaire.

already expressed by presecretory ameloblasts, but no enamel is formed at the early stages, as the secreted enamel proteins presumably diffuse through the porous predentin and the unmineralized mantle dentin. At least some of these proteins, and in particular the different low-molecular-weight species of amelogenin are thought to be internalised into odontoblasts, thus preventing their accumulation in the dentin. The internalised amelogenins are then presumably destroyed in the lysosomes of the odontoblasts. Amelogenin diffusion is gradually diminished by dentin mineralisation, causing amelogenins and the other enamel proteins, to be retained at the dentin–enamel junction (DEJ), initiating enamel formation.

Two series of data have demonstrated that the situation is in fact more complex and suggest that the amelogenin peptides may actually serve as signalling molecule in the secretory odontoblasts. Firstly, the demonstration of amelogenin splice products in the odontoblast gene library [21,30], and secondly, the identification of amelogenin degrading enzyme, enamelysin or MMP-20, in odontoblasts.

The group of Veis [1] identified in dentin extract a chondrogenic inducing agent, which was later revealed to be an amelogenin [21]. Veis et al. [30] have demonstrated that it was a spliced-gene product. Two specific cDNAs were found in the odontoblast gene library, the first one being expressed by exons 2 to 7, and the second one by exons 2, 3, 5, 6d and 7, in other words, all exons except 4. The first corresponding recombinant molecule was designated as A+4 and was shown to have a molecular weight of 8.1 kDa. The second, named A–4, was smaller (6.9 kDa). They both enhanced the in vitro incorporation of sulphate into proteoglycans and increased type-II collagen synthesis. The two molecules also stimulated Sox9 and Cbfa1 mRNA expression. In vivo, the expression of BSP and BAG-75 (DMP-1) was enhanced in implant assays. Along this line, it was also recently demonstrated that amelogenins are transiently expressed by odontoblasts at early stages of dentin formation [22]. Furthermore, results from our laboratory, using agarose beads soaked in A+4 or A–4 solutions and implanted in the pulp of rat's molar, have shown that amelogenin molecules stimulated the recruitment of stem cells or the dedifferentiation of resident adult cells. The recruited cells were shown to divide both in the area of implantation and in the central part of the pulp. The daughter cells developed two phenotypes: an osteoblast-like phenotype for the cells regrouped around agarose beads, with osteopontin labelling, and an odontoblast-like cells, as suggested by the positive DSP immunostaining, for the cells located at the surface of the wounded pulp and involved in the construction of a reparative dentinal bridge. However, final conclusions for these experiments await confirmation of the phenotype of these cells by more specific markers.

Taken together, these data suggest that amelogenins are not only involved in enamel formation, but may also function as signalling molecules. This property of amelogenin is currently exploited therapeutically in periodontal and bone regeneration (Emdogain). Our experiments open gates on the healing of pulp damages.

During crown formation, the enamel organ plays a role in regulating enamel formation and consequently in the secretion and diffusion of amelogenins in dentin. During root formation, the epithelial Hertwig's root sheath is instrumental in the recruitment and differentiation of root odontoblasts. For a long time, differences between the root and crown dentin have been recognized. It is also the case for the coronal and root pulp [28], shown to respond in a different way to BMP-7. It was suggested that the environment was different during the formation of the tooth, and this may be related to the presence or absence of amelogenin. Bosshardt and Nanci [3] denied the presence of

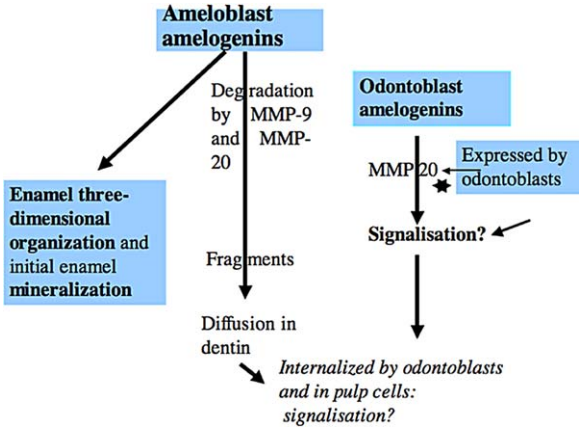
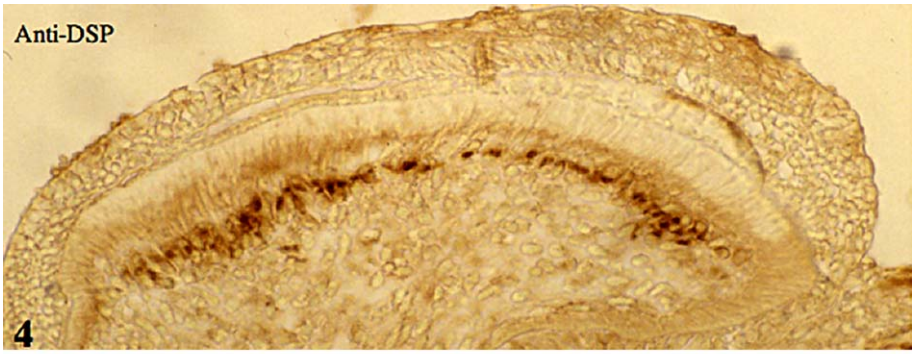
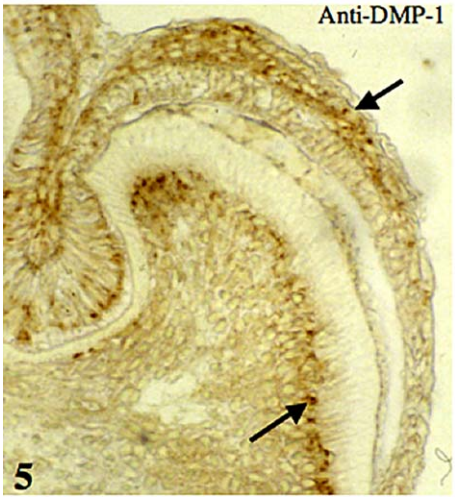


Fig. 3. Schematic representation of ameloblastic and odontoblastic amelogenins, pathways of degradation and signalisation.
Fig. 3. Représentation schématique des voies de dégradation et de signalisation des amélogénines amélo- et odontoblastiques.

amelogenin within Hertwig's sheath. As a conflicting view, two alternate splice forms of amelogenins (M180 and LRAP) were identified in the periodontal region (bone, ligament and cementum) of mouse tooth roots [16]. Altogether, and taking into account the different methods used, the different stages of formation and the tissues that were examined, it may be stated that the absence of amelogenin in the Hertwig's root sheath seems to interfere with the root dentin formation, but is not crucial, may be because some other molecule(s) are over-expressed and rescue partially the deficiency. It comes out clearly from the study that was carried out on the amelogenin null mice [16] that the lack of M180 and LRAP expression is correlated with cementum defects. The defects are associated with an increase expression of nuclear factor kB ligant (RANKL), a regulator of osteoclastogenesis. This provides another evidence for the signalling functions of amelogenins.

Our recent immunohistochemical and immunogold electron microscopic results confirmed the presence of MMP-20 protein in both ameloblasts and odontoblasts of rat incisor and showed that MMP-20 protein colocalizes with amelogenin, suggesting that in odontoblasts too, amelogenin may be the target substrate. This co-localization of amelogenin and MMP-20 would argue for local synthesis of both enzyme and substrate.





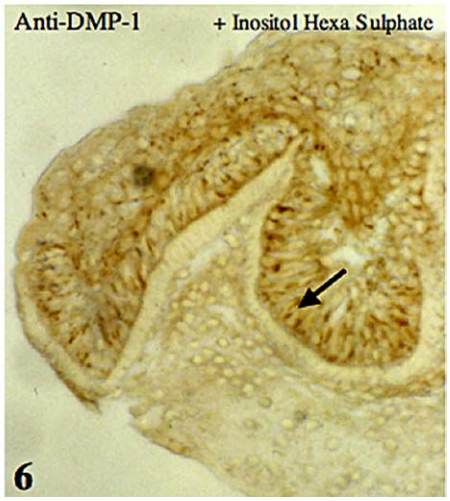


Fig. 4. Immunohistochemical staining of DSP. The odontoblasts and mineralisation front are positively stained.
Fig. 5. Anti DMP-1 staining. Positive reaction is seen in odontoblasts and in a few enamel organ cells.
Fig. 6. After inhibition of phosphorylation by inositol hexasulphate, immunostaining for DMP-1 is seen in ameloblasts and in the enamel organ.
Fig. 4. Coloration immunohistochimique de sialoprotéine dentinaire (DSP). Les odontoblastes et le front de minéralisation sont positivement colorés.
Fig. 5. Coloration dirigée contre le DMP-1. Une réaction positive s'observe dans les odontoblastes et dans quelques cellules de l'organe de l'émail.
Fig. 6. Après inhibition de la phosphorylation par l'hexasulfate d'inositol, l'immunocoloration pour la DMP-1 s'observe dans les améloblastes et dans l'organe de l'émail.

Unlike ameloblasts, where MMP-20 is found to be expressed in the cells and secreted into the matrix, suggesting a role in the processing of enamel proteins leading to enamel maturation, MMP-20 expressed in odontoblasts was not detected in the corresponding predentin/dentin matrix. This suggests that the local degradation of amelogenin by MMP-20 in odontoblasts would produce low-molecular-mass peptides, which may serve as signalling molecules. A schematic representation of the possible various pathways and hypothesis is shown in Fig. 3.

5. Multifunctional role of dentin matrix protein-1

The distribution of dentin sialoprotein (DSP) or the staining of dentin phosphoprotein (DPP) with the 'stains all' or with the phosphotungstic acid/chromic acid, both revealing phosphorylated proteins, stained the predentin–dentin junction but not the predentin. These observations provided evidence that these two daughter molecules resulting from the dissociation of the native DSPP are involved in dentin mineralisation. Autoradiographic data by Weinstock and Leblond [31], using either radiolabelled phosphate or tritiated serine, have shown rapid labelling of the mineralisation front as early as 1–2 h after injection. Despite the fact that such radiolabelling does not appear in in vitro experiments [29], it is assumed that phosphorylated molecules are transported by odontoblast processes and secreted near the transition between predentin and dentin. Immunostaining with a DSP antibody (Fig. 4) was positive along a border 0.5–2.5 μm thick, designated as metadentin by Goldberg and Septier [14]. Inhibition of the phosphorylation of the molecule abolished the staining. Clearly, DSP plays a role in dentin mineralisation.

In contrast, the immunolocalization of dentin matrix protein-1 (DMP-1), another phosphorylated protein member of the SIBLING family, was positive in odontoblasts and, when the staining was missing in odontoblasts, was shifted to presecretory and secretory ameloblasts (Fig. 5). When the phosphorylation was inhibited by the casein kinase inhibitor (inositol hexasulphate), all the labelling was seen in the enamel organ and none in the odontoblasts (Fig. 6) [26]. This suggests that DMP-1, identified originally as a extracellular matrix protein implicated in dentin mineralisation, is also acting as a signalling molecule, especially when the molecule is dephosphorylated. Along this line, it has now been demonstrated that overexpression of DMP-1 induces the differentiation of embryonic mesenchymal cells into odontoblast-like cells [19], and the gene transcription by activation of intracellular Ca^{2+} store [20].

To conclude, the two examples given here provide evidence of the dual functions of the molecules of the extracellular matrix, which now appear more complex than previously thought. The multifunctionality of extracellular matrix molecules is now under investigation in many laboratories.

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