Production and characterization of antibodies against murine dentine phosphoprotein

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Experiments were designed to produce and characterize a polyclonal antibody directed against mouse dentine phosphoprotein, the major non-collagenous protein of the dentine extracellular matrix. Dental extracellular matrix proteins from 2-day-postnatal Swiss-Webster-mouse tooth organs were extracted with 0.5 M-acetic acid, followed by 4 M-guanidinium chloride/0.5 M-EDTA. Mouse dentine phosphoprotein yields were further increased by precipitation with 1 M-CaCl₂. Final purification was achieved by excising and eluting dentine phosphoprotein polypeptide bands from preparative sodium dodecyl sulphate/urea/polyacrylamide gels. Mouse dentine phosphoprotein is a single component of approx. 72 kDa and has a characteristic amino acid composition of 33% aspartic acid and 55% serine/phosphoserine. A polyclonal antibody was raised in rabbits against purified mouse dentine phosphoprotein and was shown to be monospecific by enzyme-linked immunoabsorbent, dot-immunobinding and 'Western transfer' assays. This antibody was used to detect the expression and localization of dentine phosphoprotein in 1-day-postnatal mouse tooth organs. This antigen was localized intracellularly within the monolayer of odontoblasts, which line the perimeter of the dental papilla mesenchyme, and within the odontoblastic cell processes, which traverse the predentine matrix. Newly forming mineralized dentine matrix was also cross-reactive with the dentine phosphoprotein specific antibody. The non-mineralized predentine matrix did not contain any detectable cross-reactive antigens.

INTRODUCTION

Studies on the mechanisms of biomineralization have shown two fundamental modes of mineral formation: (1) 'organic-matrix-mediated', involving an extracellular matrix (ECM), and (2) 'biologically induced', without the production of an organic ECM (for a review, see Lowenstam, 1981). In those systems where biomineralization is associated with ECMs (e.g. cartilage, bone, dentine and enamel), the specific structure and organization of the organic ECM components have been implicated in directing the inorganic crystal phase formed. Constituents of the ECM have been suggested to function in the inhibition, nucleation, and regulation of the size, shape and orientation of the crystals formed (for a review, see Weiner, 1984).

One biological system well-suited for investigation of matrix-mediated biomineralization is the developing vertebrate tooth organ. During tooth formation (odontogenesis) the ectomesenchyme-derived odontoblasts and inner enamel epithelial-derived ameloblasts each synthesize and secrete distinct ECMs (dentine and enamel), which mineralize, expressing discretely orientated, shaped, and sized hydroxyapatite crystals (for a review, see Slavkin, 1974). The specific ECM proteins of dentine and enamel appear to regulate the processes of biomineralization, although individual proteins have not been causally associated with particular steps in hydroxyapatite crystal formation.

During dentinogenesis, the cranial-neural-crest-derived ectomesenchymal cells differentiate into odontoblasts,

which produce the dentine ECM consisting of approx. 86% Type I and Type I trimer collagens and several non-collagenous proteins. The non-collagenous proteins identified in the dentine ECM include proteoglycans, glycoproteins, glycosaminoglycans, γ -carboxyglutamate-containing proteins (Gla-proteins) and dentine phosphoproteins (DPPs) [for reviews see Veis (1978) and Linde (1984)]. Most of these proteins are also present in the ECM of bone, a collagen-associated mineralizing system closely related to the highly mineralized dentine ECM (Leaver *et al.*, 1975; Glimcher, 1976). Although the exact biological function of these proteins is not known, a role in mineralization has been suggested. To date, the only class of non-collagenous proteins which appear to be found exclusively in the dentine ECM are the highly phosphorylated DPPs.

Of the non-collagenous proteins, DPP is the major constituent, representing as much as 50% of this fraction. DPP from several species has been characterized, such as that from rat (Jontell *et al.*, 1980; Butler *et al.*, 1983), bovine (Veis *et al.*, 1972; Termine *et al.*, 1980), hamster (Lyyruu *et al.*, 1982) and rabbit (Richardson *et al.*, 1977). Although major differences in the molecular mass and number of phosphoproteins have been reported, several unique physical-chemical characteristics are shared: (1) aspartic acid and serine comprise approx. 70–85% of the total amino acid residues (Linde, 1984); (2) these proteins have a high degree of phosphorylation (400 phosphate or more residues/1000), usually present as phosphoserine residues (Linde *et al.*, 1980; Richardson *et al.*, 1978); (3) DPPs are extremely anionic (Veis & Perry, 1967), with a

Abbreviations used: ECM, extracellular matrix; Gla-proteins, γ -carboxyglutamate-containing proteins; DPP, dentine phosphoprotein; GdmCl, guanidinium chloride; e.l.i.s.a., enzyme-linked immunosorbent assay; SDS, sodium dodecyl sulphate.

reported isoelectric point for rat DPP of about 1.1 (Johnsson *et al.*, 1978); and (4) DPPs have a strong affinity for Ca^{2+} (Lee *et al.*, 1977; Zanetti *et al.*, 1981) in that DPPs are preferentially precipitated by Ca^{2+} (Kuboki *et al.*, 1979). Whereas the biological function of DPP is not as yet known, physico-chemical properties suggest a function in the biomineralization of the dentine ECM. Most theories have centred on DPP acting as a nucleator (Posner & Betts, 1981) or modulator of hydroxyapatite crystal formation (Termine, 1980).

Here we describe the identification of mouse DPP and the production of a polyclonal monospecific antibody against purified DPP. The expression and localization of DPP *in situ* was determined by indirect immunofluorescence microscopy. DPP was localized within odontoblast cells, cell processes that transverse the predentine matrix and extracellularly within the mineralized dentine matrix. DPP was not found within the non-mineralized predentine ECM layer. Our results indicate that DPP is a specific phenotypic marker for odontoblast cytodifferentiation, and supports its possible role in the biomineralization of the dentine ECM.

METHODS AND MATERIALS

Protein extraction

Tooth organs (incisors and molars) from 2-day-postnatal Swiss Webster mice (Simonson, Gilroy, CA, U.S.A.) were dissected and placed in cold phosphate-buffered saline (0.01 M-phosphate/0.9% NaCl, pH 7.4 (Gibco, Grand Island, NY, U.S.A.). The dental pulp mesenchyme were removed, and the ECMs rinsed in several changes of cold phosphate-buffered saline. The ECMs, containing both enamel and dentine matrices, were demineralized with 0.5 M-acetic acid (Fukae & Shimizu, 1974). The extract was centrifuged, the ECM residues were further extracted with 4.0 M-GdmCl/0.5 M-EDTA for 1 day at 25 °C, dialysed against distilled water for several days to remove the GdmCl, and then freeze-dried.

Purification and characterization of DPP

Once the identification of DPP was established in the GdmCl/EDTA extract, the DPP yield was further increased by precipitation with 1.0 M- CaCl_2 (Kuboki *et al.*, 1979). The CaCl_2 precipitate extract was then fractionated by electrophoresis on SDS/urea/10% -polyacrylamide slab gels (Ornstein, 1964; Davis, 1964). The DPP band was identified by its characteristic staining profile with Coomassie Brilliant Blue (poor stain) and Stains-All (intense blue stain) (Green *et al.*, 1973; Termine *et al.*, 1980). A single band (~ 72 kDa) exhibiting intense staining with Stains-All was detected, excised and eluted from the gel in 0.1 M-Tris/HCl (pH 8.0)/0.1% SDS/1 mM-EDTA, by shaking for 24 h at room temperature. The extracted protein was dialysed against 1 mM-Tris/HCl, pH 7.4, for 2 days at 4 °C, followed by distilled water for 1 day, and then freeze-dried. The purity of isolated DPP was determined by re-electrophoresis. A portion of purified DPP (100 μg) was hydrolysed in 6 M-HCl for 24 h at 110 °C, and the amino acid composition was determined (AAA Laboratory, Mercer Island, WA, U.S.A.).

Preparation of DPP antibodies

The proteins precipitated with CaCl_2 were separated by gel electrophoresis on SDS/urea/10% -polyacrylamide gels. The DPP bands were excised, and prepared for immunization by the method of Gomer & Lazarides (1981).

Control sera were collected from New Zealand White rabbits before immunization. Antibodies were raised by intradermal injection of approx. 100 μg of purified DPP in homogenized gel. After 4 weeks, two boosters of approx. 50 μg of protein in Freund's incomplete adjuvant were administered. Blood was collected 10–14 days after each injection by cardiac puncture. Serum was prepared by centrifugation and stored at -20 °C. The IgG fraction was obtained by chromatography on DEAE-Affigel Blue (Bio-Rad, Richmond, CA, U.S.A.) as described by the suppliers.

Characterization of mouse DPP antibodies

The presence, specificity and sensitivity of DPP antibodies were assayed by the following techniques.

(a) **E.I.I.s.a.** Serially diluted DPP-specific and preimmune antibodies were tested against 30 μg of each extract (GdmCl/EDTA, CaCl_2 precipitate and purified DPP) by using the method of Engvall & Perlmann (1972) as modified by Wolters *et al.* (1976). The plates were read at 405 nm with a microELISA Minireader MR 590 (Dynatech Instruments, Santa Monica, CA, U.S.A.).

(b) **Dot-immunobinding assay.** Aliquots (2 μl) of serially diluted antigens (GdmCl/EDTA extract, CaCl_2 precipitate and purified DPP) were spotted on nitrocellulose and treated by the method of Hawkes *et al.* (1982).

(c) **'Western transfer'.** The GdmCl/EDTA extract, CaCl_2 precipitate and purified DPP were each separated on a SDS/urea/5–20% -polyacrylamide gradient gel. Proteins were electrophoretically transferred to nitrocellulose membranes by using a modified version of the method of Towbin *et al.* (1972) for different time periods at maximum voltage (100 V) and 1.2 mA. Dilutions of DPP antibody were incubated overnight at room temperature (MacDougall *et al.*, 1984).

(d) **Indirect immunofluorescence microscopy.** Half-mandible specimens of 1-day-postnatal Swiss Webster mice were dissected, fixed in ethanol/acetic acid (99:1, v/v) (Silver *et al.*, 1981), and embedded in paraffin (Sainte-Marie, 1962). Previous studies have shown that, at this developmental stage, mouse mandibular tooth organs synthesize and secrete dentine ECM (Gaunt & Miles, 1967).

The tissue sections were incubated with various dilutions of DPP-specific antibody or control preimmune antibody containing 0.5% Tween 20 for 1 h at room temperature. Sections were then washed three times, each for 10 min, each incubated with a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-(rabbit heavy and light chain) serum (Cappel Laboratories, Cochranville, PA, U.S.A.) for 30 min at room temperature, and washed again three times (10 min each time). Slides were mounted in Aquamount (Lerner Labs, New Haven, CT, U.S.A.) and examined with a Zeiss epifluorescence microscope.

After documentation of the indirect-immunofluorescence-microscopy results, the identical sections were then counterstained with haematoxylin and eosin.

RESULTS

Isolation, purification and partial characterization of mouse DPP

To isolate murine DPP, incisor and molar tooth organs from 2-day-postnatal Swiss Webster mice were dissected and mechanically depulped. Previous histological data has shown that, at this developmental stage, both tooth organs are actively producing dentine and enamel ECM proteins (Gaunt & Miles, 1967). The proteins contained in the ECM were sequentially extracted with acetic acid followed by GdmCl/EDTA. The gel profiles of these extracts stained with Coomassie Brilliant Blue and Stains-All are shown in Fig. 1 (lanes 2 and 3 respectively). The acetic acid extract was shown to contain several proteins, several of which have been characterized as collagens, enamelines and amelogenins (Slavkin *et al.*, 1981, 1983). Specific staining for phosphoproteins with Stains-All (Fig. 1, lane 3) indicated that DPP was not detected in the acetic acid extract. The ECM residues remaining from the acetic acid extraction were re-extracted with GdmCl/EDTA. In this second extract, fewer proteins were detected with Coomassie Blue (Fig. 1, lane 4), and a major protein of approx. molecular mass 72 kDa was intensely stained with Stains All (Fig. 1, lane 5), indicating the presence of DPP.

Further purification was achieved by precipitation of DPP in the presence of 1 M-CaCl₂ (Kuboki *et al.*, 1979). This procedure decreased the number of other protein components determined by Coomassie Blue staining (Fig.

Fig. 1. Purification of murine DPP from 2-day-postnatal Swiss-Webster-mouse tooth organs

Stages of protein purification were analysed by SDS/urea/10%-polyacrylamide-gel electrophoresis. Lanes 1, 2, 4, 6, and 8 were stained with Coomassie Brilliant Blue. Lanes 3, 5, 7 and 9 were stained with Stains-All. 1, Molecular-mass markers; 2 and 3, tooth polypeptides extracted with 0.5 M-acetic acid; 4 and 5, dental ECM proteins extracted with GdmCl/EDTA; 6 and 7, dental proteins precipitated with 1 M-CaCl₂; 8 and 9, purified mouse DPP (72 kDa).

Table 1. Comparison between mouse, hamster and rat DPP amino acid compositions

Amino acid	DPP composition (residues/1000 residues)		
	Mouse	Hamster*	Rat†
Aspartic acid	339	326	374
Threonine	10	23	9
Serine‡	551	420	534
Glutamic acid	38	59	24
Proline	6	7	2
Glycine	29	68	25
Alanine	10	22	12
Cysteine	—	—	—
Valine	Trace	9	—
Methionine	Trace	4	—
Isoleucine	2	6	2
Leucine	4	9	—
Tyrosine	2	6	2
Phenylalanine	2	3	—
Histidine	Trace	11	7
Lysine	3	15	9
Arginine	3	13	—

* From Lyaruu *et al.* (1982).

† Highly phosphorylated DPP, from Butler *et al.* (1983).

‡ Includes phosphoserine residues.

1, lane 6) and enriched the extract with respect to the 72 kDa DPP (Fig. 1, lane 7). Final purification was achieved by excising the 72 kDa band region present in the CaCl₂-precipitated extract from preparative SDS/urea/10%-polyacrylamide gels. The DPP was extracted from the gels and re-electrophoresed. No other proteins were detected in this extract, as shown by staining with Coomassie Brilliant Blue (Fig. 1, lane 8), and only a single 72 kDa band was detected with Stains All (Fig. 1, lane 9). The purified 72 kDa DPP has exactly the same electrophoretic mobility on SDS/urea/5–20%-polyacrylamide gradient gels as the DPP component present in the initial GdmCl/EDTA extract, indicating that no degradation had taken place through the different purification steps representing our experimental protocol.

Conclusive characterization of the 72 kDa protein as murine DPP was obtained by amino acid composition. Table 1 shows the amino acid composition of this protein compared with that reported for hamster and rat DPP. The 72 kDa protein is enriched in both aspartic acid (33%) and serine/phosphoserine (55%) residues, with these two amino acid residues representing 88% of the total amino acid residues.

Production and characterization of monospecific polyclonal antibodies directed against mouse DPP

To determine whether DPP can be considered as a specific phenotypic marker for odontoblast cytodifferentiation, and to establish the distribution of this protein in mouse molar tooth organs *in situ*, monospecific polyclonal antibodies to electrophoretically purified DPP were raised in rabbits. The CaCl₂-precipitated proteins from 2-day-postnatal Swiss Webster mice were electrophoresed on preparative SDS/urea/10%-polyacrylamide gels. The DPP bands (72 kDa) were excised, homogenized

Fig. 2. Determination of the presence of antibodies directed against purified mouse DPP by an e.i.s.a.

Continuous lines represent values obtained with anti-(mouse DPP) IgG, whereas broken lines represent values for control preimmune IgG. ■, Purified mouse DPP (72 kDa); ▲, CaCl₂-precipitated dental proteins; ●, GdmCl/EDTA-extracted dental ECM proteins.

and used to immunize rabbits. Re-electrophoresis of the antigen showed no detectable contaminants of different molecular masses with either Coomassie Brilliant Blue or Stains-Allstaining. Antibodies were produced as described in the Methods and materials section; the rabbits were bled, whole serum was prepared and the IgG fraction isolated by chromatography on DEAE-Affigel Blue.

The specificity and sensitivity of the mouse DPP antibodies was characterized by an e.i.s.a. Results indicated specific DPP antibodies with dilutions of 1:500 capable of reaction 10-fold greater than that of control preimmune serum (Fig. 2). The relative enrichment of DPP through the purification was reflected in higher A_{490} values obtained with purified protein (the same concentration of DPP-specific antibody being maintained).

The specificity of DPP antibodies was also demonstrated by a dot-immunobinding assay using mouse tooth GdmCl/EDTA extract, CaCl₂-precipitated proteins and purified DPP. Controls included Type I collagen (the major component of the dentine ECM), and GdmCl/EDTA extract of mouse long-bone ECM (which contains several low-phosphorylated phosphoproteins). Mouse tooth GdmCl/EDTA extract, CaCl₂-precipitated proteins and the purified DPP at levels from 0.25 to 5.0 µg were positive with dilutions up to 1:2000 of the rabbit anti-(mouse DPP) IgG fraction (Fig. 3). Type I collagen and mouse long-bone GdmCl/EDTA extract were negative, showing no cross-reactivity with the DPP antibody at any concentration tested. No extracts showed immunostaining when tested against preimmune IgG fractions over the same protein concentration range.

The monospecificity of the DPP antibody for the 72 kDa DPP band was demonstrated by 'Western transfer'. The three samples (GdmCl/EDTA extract, CaCl₂-precipitated proteins and purified DPP) were fractionated on a SDS/urea/5–20%-polyacrylamide gradient gel and electrophoretically transferred to a

Fig. 3. Dot-immunobinding assay of mouse DPP antibody

Serial dilutions of extracts were dotted on to nitrocellulose paper and incubated with a 1:200 dilution of mouse DPP IgG. Samples were then incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG, and the colour was developed with 4-chloro-1-naphthol and H₂O₂. (a) Tooth ECM GdmCl/EDTA extract; (b) CaCl₂-precipitated dental proteins; (c) purified mouse DPP (72 kDa); (d) Type I collagen; (e) mouse long-bone ECM GdmCl/EDTA extract.

nitrocellulose paper for 30 min. We determined that, under the conditions used for the transfer (maximum voltage, 1.2 mA), the time interval was extremely critical. Transfers for periods greater than 30 min resulted in the DPP polypeptide being transferred out of the gel and through the nitrocellulose membrane into the buffer.

Fig. 4. 'Western-transfer' assay using mouse DPP antibody

Mouse tooth ECM GdmCl/EDTA extract (lane 1), CaCl_2 -precipitated dental proteins (lane 2) and purified mouse DPP (lane 3) were fractionated on a SDS/urea/5–20% polyacrylamide gradient gel. After electrophoretic transfer of the proteins to a nitrocellulose filter, the membrane was incubated with a 1:100 dilution of DPP antibody overnight. The filter was washed, incubated with a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG and the colour developed as described for Fig. 3.

Once the optimal conditions for the transfer of DPP were determined, the IgG fraction at a 1:100 dilution showed a positive reaction only with the 72 kDa band present in each of the extracts (Fig. 4).

Expression *in situ* and localization of mouse DPP

Having shown the polyclonal antibody raised against mouse DPP to be monospecific, the localization and distribution of mouse DPP antigens in 1-day-postnatal Swiss-Webster-mice molar tooth organs was analysed by indirect immunofluorescence microscopy. A solution of DPP antibody (1:40 dilution) containing 0.5% Tween 20 was incubated with the tissue sections for 1 h at room temperature and washed with phosphate-buffered saline, pH 7.4, containing 0.025% Triton X-100, for 30 min. After incubation with a 1:20 dilution of fluorescein-isothiocyanate-conjugated goat anti-rabbit IgG, the sections were washed again for 30 min and examined by Zeiss epifluorescence microscopy. As can be seen in a lateral section (Fig. 5a), the localization of the antigen was confined to the single layer of polarized columnar odontoblast cells which lay adjacent to the predentine matrix along the periphery of the dental papilla mesenchyme. At higher magnification the intracellular

immunostaining was shown to be localized centrally over the supranuclear cytoplasm. The positive-staining reaction had a circular appearance, which defined the Golgi-apparatus region adjacent to the nucleus of the odontoblasts (Fig. 5c). Orientation of the localization of odontoblast nuclei and the region of the Golgi apparatus are shown in the haematoxylin and eosin staining of the same section (Fig. 5b). Analysis of tooth sections from the most mature mesial-buccal-cusp region showed the presence of DPP extracellularly. Immunostaining was detected over the forming mineralized dentine layer and within odontoblastic cell processes which transverse the predentine into the dentine mineralization front (Fig. 6a). No reaction was detected within the non-mineralized predentine ECM, dental papilla mesenchyme cells, blood vessels, epithelial-derived stellate reticulum, stellate intermedium or ameloblast cells. Control preimmune antibody showed no immunostaining with any tooth organ tissues (Fig. 6b).

DISCUSSION

Extraction of mouse tooth-organ ECM with GdmCl/EDTA and further analysis of the extract by SDS/urea/polyacrylamide-gel electrophoresis has led to the identification of a 72 kDa polypeptide as DPP by its characteristic staining profile with Stains-All. Further characterization of this protein as DPP was obtained by its unique amino acid composition: 88% of the molecule comprises serine and aspartic acid residues. The presence of a single DPP component in mouse tooth organs is in agreement with results of other studies using hamster (Lyaru *et al.*, 1982), bovine (Stetler-Stevenson & Veis, 1983) and rabbit (Richardson *et al.*, 1977) tooth organs, which also contain a single DPP. To date, only the rat tooth organ has been reported to contain more than one DPP component (Butler *et al.*, 1983).

The reported molecular masses for DPPs greatly vary, not only between species, but within species. Some studies have suggested that these differences were due to degradation of the protein during the extraction procedures (Termine *et al.*, 1980). We found that the mouse DPP isolated with our extraction scheme is stable, showing no changes in molecular mass throughout the different steps of purification. It has also been suggested that molecular-mass differences are due to the unique amino acid composition of DPP; a very high negative charge could result in abnormal behaviour of the protein when determining molecular mass by several methods (Jontell *et al.*, 1982). The molecular mass of mouse DPP reported in the present study is close to values reported for other gnawing animals such as hamster (75–80 kDa; Lyaru *et al.*, 1982) and rabbit (80 kDa; Richardson *et al.*, 1977). The molecular mass of DPP for these animals was determined under electrophoretic conditions similar to those used in the present study.

Biosynthetic and localization studies of DPP *in situ* have progressed rather slowly, owing to lack of specific probes for this protein. Prior to the present study, only indirect evidence for the localization of DPP had been established by using labelled amino acid precursors followed by optical-microscopic autoradiographic examination (Weinstock & LeBlond, 1973), or by microdissection of the dentine extracellular-matrix layers followed by biochemical analysis (Carmichael *et al.*,

Fig. 5. Localization of mouse DPP *in situ* by direct immunofluorescence microscopy

Paraffin sections of 1-day-postnatal Swiss-Webster-mice molar tooth organs were incubated with a 1:40 dilution of the mouse DPP antibody, followed by incubation with a 1:20 dilution of fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. (a) Intense immunostaining shows the DPP antigens localized within the odontoblast cell monolayer lining the perimeter of the entire dental papilla mesenchyme (DPM). No staining was detected in the enamel organ epithelia (EOE). (b) Haematoxylin-and-eosin-counterstained section showing region of odontoblast nuclei (N) and the Golgi apparatus (G). Abbreviations used: PD, predentine extracellular matrix; Am, ameloblasts. (c) Intracellular localization of DPP antigens within the odontoblasts at the level of the Golgi apparatus.

Fig. 6. Extracellular localization of DPP using indirect immunofluorescence microscopy

A high-magnification ($\times 346$) fluorescence micrograph of the mesial-buccal-cusp region of a 1-day-postnatal mouse molar tooth organ is shown. (a) Immunostaining with mouse DPP antibody showing the DPP antigens localized exclusively within odontoblasts (O), odontoblastic cell processes (OCP) which transverse the predentine matrix (Pd), and within the forming mineralized dentine matrix (D). No immunostaining is seen within the ameloblasts (Am). (b) Control showing no immunostaining with preimmune IgG within any tooth tissues. Further abbreviation used: DPM, dental papilla mesenchyme.

1975). In order to develop a specific probe for DPP, a polyclonal antibody was produced in rabbits against isolated mouse DPP (72 kDa). This antibody was shown to be monospecific, as determined by an e.l.i.s.a. (Fig. 2), dot-immunobinding assay (Fig. 3) and 'Western transfer' (Fig. 4). It is important to note that this antibody was not cross-reactive with other components of the dentine ECM, such as Type I collagen (Fig. 3d). The antibody did not cross-react with mouse long-bone ECM GdmCl/EDTA extract, which has been shown to contain several low-molecular-mass phosphoproteins with a lower degree of phosphorylation than DPP (Fig. 3e). These results clearly indicate the uniqueness of DPP and its specificity as a phenotypic marker for odontoblast cell differentiation. By using the mouse DPP-specific antibody, DPP antigen can be identified and localized *in situ* both within secretory odontoblasts and as a component of the forming mineralized dentine ECM. The mouse DPP antibody immunostained intracellular as well as extracellular antigens.

Weinstock & Leblond (1973) used [^3H]serine and [^{32}P]phosphate in autoradiographic studies to localize rat DPP, and suggested that DPP was synthesized and secreted by odontoblast cells and passed between the predentine-layer collagen fibres, accumulating at the predentine/dentine junction. The localization of DPP *in situ* by indirect immunofluorescence microscopy reported here shows direct evidence that odontoblasts synthesize and secrete DPP in mouse molar tooth organs. Moreover, our results demonstrate the previously suggested concept that odontoblasts have two levels of secretion, one level at the distal end of the cell body and the second level

through the odontoblastic cell process in close approximation to the mineralization front (Linde, 1984). Our data shows conclusively that DPP is transported *through* the predentine layer via the odontoblastic cell processes and is secreted *distally* at the level of the mineralization front. These results clearly illustrate why DPP has not been found to be a component of the non-mineralized predentine ECM (Jontell & Linde, 1983). The localization of DPP in these studies suggests a biological function related to the biomineralization of the dentine ECM, possibly as a nucleator to initiate hydroxyapatite crystal formation. The DPP specific antibody produced and characterized in our studies could serve as a useful probe in future studies related to the basic mechanisms of ECM-mediated biomineralization.

Another important aspect of the immunological-localization data obtained in these studies is the ability of this antibody probe to identify a particular biochemical phenotype during dentinogenesis. The high degree of odontoblastic specificity demonstrated in our studies suggests that the expression of DPP can be used as a tissue-specific marker for the cytodifferentiation of cranial-neural-crest-derived ectomesenchyme into secretory odontoblasts. The mouse DPP antibody could serve as a tissue-specific probe in studies related to the fundamental mechanism of epithelial-mesenchyme interactions during odontogenesis. It may now be possible to investigate and identify specific epigenetic signals from the epithelially derived dental basement membrane which result in the differentiation of ectomesenchyme into odontoblasts and the mechanisms of DPP expression *de novo*.

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