The nature and functional significance of dentin extracellular matrix proteins

WILLIAM T. BUTLER* and HELENA RITCHIE

Department of Basic Sciences, The University of Texas-Houston Health Sciences Center, Dental Branch, Houston, Texas, USA

ABSTRACT Odontoblasts are responsible for formation of predentin, which is transformed to dentin when apatite crystals are formed and the fibrillar matrix becomes mineralized. Odontoblasts are specialized cells that synthesize and secrete a unique set of non-collagenous proteins (NCPs), as well as the collagenous matrix largely comprised of type I collagen. The NCPs consist of dentin specific and mineralized tissue specific proteins, as well as other proteins that are found in a variety of tissues. Three dentin specific proteins have been recognized to date: dentin phosphoprotein (DPP), also called phosphophoryn, AG1 (dentin matrix protein 1, Dmp1) and dentin sialoprotein (DSP). DPP appears to be made by odontoblasts and appears at the mineralization front within a short time. It may be secreted via odontoblastic processes. DPP binds to collagen and potentially initiates formation of apatite crystals. A second DPP function appears to be to bind to the 100 face of growing apatite crystals and to inhibit or slow their growth; thus, DPP may play a dual role by initiating mineralization and then affecting the crystal growth and perhaps the habit of the crystals. Although no function has been ascribed to AG1 or DSP, they should prove to be important markers for the odontoblast phenotype. A recent unique finding is that two separate genes appear to code for more than one DSP mRNA; other transcripts may result from differential splicing. Examples of mineralized tissue specific proteins expressed by osteoblasts as well as odontoblasts are bone sialoprotein (BSP) and osteocalcin. Some NCPs expressed by osteoblasts, odontoblasts and several other tissues include osteopontin (OPN) and the chondroitin sulfate containing proteoglycans, decorin and biglycan. We propose that characterization of odontoblasts in tissues and cultures should rely upon utilization of sets of markers for the above NCPs and their mRNAs. Similar approaches are commonly used in investigations on osteoblasts. Finally, dentin (like bone) contains other molecules such as growth factors, and serum derived proteins, found within the matrix; no functional significance has yet been placed upon this finding. Future experiments should focus upon the elucidation of the three dimensional structures of the collagenous fibrillar network and of the NCPs to determine the relationships to mineralization. The role played by odontoblasts in controlling extracellular events, such as by selective secretory routes, will require careful exploration.

KEY WORDS: *dentin, predentin, collagen, phosphoproteins, sialoprotein, mineralization*

Odontoblasts

Odontoblasts are directly responsible for formation of the fibrillar ECM of dentin and for bringing about the deposition of carbonate apatite mineral within and around the collagenous fibers in a more indirect manner. These cells are aligned in a single layer at the margin of the dental pulp, and in their fully differentiated state, secrete ECM unidirectionally (Fig. 1). Mature odontoblasts originate from dental papilla mesenchyme, the differentiation process being characterized by specific morphological features and gene expression (Ruch, 1985; Thesleff, 1992). Whenever the odontoblastic precursor cells leave the cell cycle, they polarize such that the nucleus is removed from the apical, secretory portion. They develop characteristics of secretory cells as revealed by numerous rER, Golgi apparati and secretory granules (Weinstock and Leblond, 1974). The initial stages of secretion involve an uncalcified matrix, predentin, formed by young odontoblasts. As the odontoblasts continue to form additional ECM that calcifies to form dentin, they recede pulpally, leaving behind cell processes (odontoblastic processes) contained in numerous dentinal tubules, coursing through predentin and dentin (Fig. 1). Mature odontoblasts form several types of dentin (for a review see Linde and Goldberg, 1993). The initial thin layer, mantle dentin, located adjacent to

Abbreviations used in this paper: ALP, alkaline phosphatase; BAG-75, bone acidic glycoprotein 75; CNBr, cyanogen bromide; DPPs, dentin phosphoproteins; DSP, dentin sialoprotein; BSP, bone sialoprotein; ECM, extracellular matrix; HAP, hydroxyapatite; NCPs, non-collagenous proteins; PGs, proteoglycans; rER, rough endoplasmic reticulum.

***Address for reprints:** University of Texas, Houston Dental Branch, 6516 John Freeman Ave., Houston, Texas 77030, USA. FAX: 713-794.1854.

0214-6282/95/$03.00

Printed in Spain

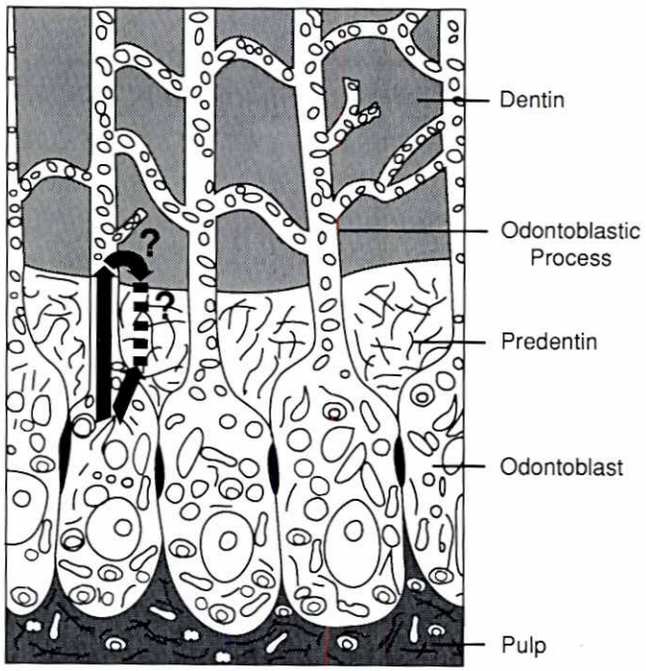

Fig. 1. Drawing depicting odontoblasts actively synthesizing predentin and dentin. *A single layer of mature odontoblasts line the pulp cavity and secrete an ECM apically. This matrix is initially unmineralized predentin, but it becomes mineralized as additional predentin is made by the cells. As the odontoblasts recede they form processes. There is evidence to indicate that some dentin NCPs (e.g. dentin phosphoproteins) are transported to the mineralization front (the predentin-dentin junction) more quickly than collagen. This finding opens the possibility that NCPs could be transported to dentin via odontoblastic processes (arrow through process). Another possibility is that they are secreted at the cell border and diffuse rapidly through predentin to the mineralization front (arrow adjacent to cell border).*

enamel, differs in morphological appearance from the major primary form, circumpulpal dentin. This fact may indicate differences in the mechanism of formation of mantle and circumpulpal dentin.

To understand the fundamental biochemical mechanisms involved in the process of dentinogenesis has been the goal of numerous scientists. At the heart of this search is the realization that we must develop a full understanding of the nature and biological dynamics of the mature, secretory odontoblast. In particular, much attention and research has been devoted to ECM macromolecules synthesized and secreted by odontoblasts, and to the role of these molecules in dentinogenesis and mineralization. In this review we will attempt to overview this information, focusing upon the nature of the ECM proteins and their genes and upon odontoblastic activities and secretory pathways. Since the number of published studies in this area is quite large, we were unable to include all of results from all of them in this review. We apologize for the necessity of our selectivity.

The fundamental *hypothesis* describing dentinogenesis can be summarized in the following manner. Odontoblasts secrete a fibrillar network, predentin, composed principally of type I collagen, that is unmineralized. The predentin layer is transformed to dentin 15-20 μm from the cell border, as odontoblasts continue to elaborate more predentin ECM material (Fig. 1). Thus, the cells recede pulpally, but are still connected to the tissue they are constructing via odontoblastic processes. The transformation of predentin to dentin involves several key regulatory events, that convert the collagenous fibers from a non-calcifying matrix to one in which plate-like apatite crystals are initiated and grow within and around collagen fibrils. The mechanisms involved in mineralization of predentin to form dentin are not understood, although several lines of evidence strongly suggest that the transformation involves the secretion of NCPs at the mineralization front, resulting in the initiation and control of mineralization by these NCPs. One hypothesis states that the secretory pathway (beginning as early as the rER) for collagen differs from that for other NCPs, such as dentin phosphoproteins (phosphophoryns; DPPs) this selectivity of secretion routes may thus be a key controlling factor in specifying the site of mineralization and the transformation of predentin to dentin. Other possible factors involved in the predentin-dentin conversion include enlargement and maturation of collagen fibrils, degradation and loss of proteoglycans and transport of calcium through the cellular layer. With this overview in mind, one can begin to comprehend how odontoblasts are involved in dentinogenesis in both an indirect and a direct manner.

Extracellular matrix proteins

The ECM of dentin consists of collagen fibers that form a matrix for the deposition of plate-like crystals of carbonate apatite. In addition, dentin contains a number of other proteins and proteoglycans, collectively referred to as non-collagenous proteins (NCPs). These macromolecules have been purified by extracting dentin while decalcifying the tissue, usually employing denaturing solvents (Butler, 1987). These approaches were originally used in studying bone NCPs (for review see Young *et al.*, 1992) and many of the isolation procedures for dentin were modeled after those originally used for bone (Linde *et al.*, 1980; Termine *et al.*, 1981). These harsh conditions are necessary to preserve structure because of the invariable presence of degrading enzymes that are co-extracted from the tissues. Therefore, it should be emphasized that the studies on characterization and predicted functions of bone and dentin ECM utilize denatured materials. Also, there is some evidence that these macromolecules may occur in the ECM as aggregates, or supramolecular complexes. Thus, one must be cautious when drawing firm conclusions about their functions, based upon what is observed with extracted, denatured components. Nevertheless, it is likely that experiments with individual bone and dentin NCPs will draw us closer to an understanding of the functional significance of these macromolecules and how to design future experiments for the elucidation of their roles in dentinogenesis and osteogenesis.

One category of dentin NCPs includes those that are found exclusively in dentin, and expressed only by odontoblasts, the *dentin-specific proteins*. To date, only three proteins are considered to be dentin-specific: dentin phosphoprotein, (DPP), AG1 (Dmp1) and dentin sialoprotein (DSP). Another group consists of ECM proteins found in bone, dentin and cementum and synthesized by the appropriate cells in these three tissues. Examples are bone sialoprotein (BSP) and osteocalcin (bone Gla protein, BGP). This category can be termed *mineralized tissue-specific proteins*. Dentin contains other macromolecules synthesized and secreted by odontoblasts which are also synthesized by other cell types and are found in the ECM of many or several other tissues, including soft tissues. Examples of this group include osteopontin and osteonectin. A fourth category of dentin proteins are those found in

the circulation after being synthesized primarily by the liver. These proteins are not synthesized by odontoblasts, but have a high affinity for dentin (and bone). The most abundant of these proteins is α_2HS-glycoprotein. Finally, dentin contains several growth factors, such as the bone morphogenetic proteins (BMP), insulin-like growth factors (IGFs) and TGFßs, entrapped within the matrix. The cellular origin of these growth factors has not been clearly demonstrated. For recent reviews concerning the NCPs of dentin see Linde and Goldberg (1993) and Butler (1995). One important consideration is that the odontoblastic phenotype should be assessed (for example, in passaged cells) by using dentin-specific and mineralized tissue-specific ECM probes. The approach of classifying bone cells (osteoblasts) on the basis of the expression of *sets* of ECM proteins or their mRNAs has become a standard practice in bone biology.

Collagen

The collagen fibrils which become mineralized in the transformation of predentin to dentin are primarily type I collagen, with smaller amounts of type V collagen, and perhaps some type I trimer. Early studies showed that the CNBr peptides from bovine and rat dentin were derived from type I collagen and, that, unlike soft tissues, no type III collagen CNBr peptides were observed (Butler, 1972; Volpin and Veis, 1973). Similarly, studies with odontoblasts in organ culture (Munksgaard *et al.*, 1978a) showed the synthesis of type I collagen; these results also demonstrated the synthesis and secretion of type I trimer (a heterotrimer consisting of three α1(I) chains) by odontoblasts. Biochemical and metabolic labeling studies with rat predentin confirmed the presence of type I collagen and type I trimer, and also indicated the presence of type V collagen and the absence of type III collagen (Sodek and Mandell, 1982). Synthesis of type V collagen by odontoblasts and its presence in dentin were subsequently shown by immunohistochemical studies (Bronckers *et al.*, 1986). More recently the presence of small amounts of type III-containing collagen fibrils in murine dentin was reported (Nagata *et al.*, 1992). Type III collagen was more frequently seen in the root than in the crown and the incidence of the type III-containing fibers was low. The expression of pro-α1(III) mRNA and protein by human and mouse odontoblasts has also been shown (Lukinmaa *et al.*, 1993). Nevertheless, the consensus of reports indicates that dentin collagen consists primarily of type I collagen (including type I trimer), with small amounts of type V. Unlike soft tissue collagens, type I and type III cofibrils are largely, or completely absent in mineralized tissues. This fact is considered significant because one might speculate that the presence of type III collagen may create a fibrillar matrix that would disallow or not promote mineral deposition.

The diameter and architecture of the fibrillar network in dentin collagen may be influenced by the presence of type V collagen. It is believed that type V molecules form an initial core and that they in some way dictate the diameter of the copolymeric type I/V collagen fibrils (Birk *et al.*, 1990). Recent studies show that about 15% of the type V molecules are covalently cross-linked to type I collagen in bone (Niyibizi and Eyre, 1994).

Consistent with these ideas is the observation that the cross-linking pattern and the molecular packing of dentin collagen is distinct (Yamauchi *et al.*, 1992). These studies indicate that mechanical forces brought about by mineralization of dentin collagen alter the fibrillar lattice. Thus, the collagen synthesized by odontoblasts exists in an architectural motif that is especially adapted for mineralization.

Dentin specific proteins

Dentin phosphoproteins (phosphophoryns)

Except for type I collagen, dentin phosphoprotein, (phosphophoryn, DPP) is the most plentiful ECM component in dentin. This protein is characterized by high contents of phosphoserine (45-50%) and aspartic acid (35-38%). Molecular masses of 155 kDa for bovine (Stetler-Stevenson and Veis, 1983), 72 kDa for mouse (MacDougall *et al.*, 1985) and 90-95 kDa for rat (Butler *et al.*, 1983; Sabsay *et al.*, 1991) have been reported. Despite these differences in size, the remarkable similarity of compositions suggests that related proteins are involved in dentin formation of these species. The high levels of aspartic acid and phosphoserine result in a polyanionic macromolecule with a pI estimated to be 1.1 (Jonsson *et al.*, 1978). DPP binds large amounts of calcium with a relatively high affinity (Zanetti *et al.*, 1981; Marsh, 1989b). It forms an insoluble aggregate in the presence of Mg^{++} and Ca^{++} (Kuboki *et al.*, 1979; Marsh, 1989a) and these observations have led to isolation schemes utilizing precipitations with $CaCl_2$ and $MgCl_2$ (Butler, 1987; Marsh, 1989; W.T. Butler and L.F. Riggan, unpublished data). This unusual and special affinity for calcium may also relate to the biological role played by DPP in mineral formation.

The complete sequence of DPP has not been reported. The NH_2-terminal sequences DDDN and DDPNDDDE, were found for two rat DPPs. Sequences with repeating residues of aspartic acid and of serine were detected in other areas of rat DPP (Sabsay *et al.*, 1991). DPP appears to form ß-sheetlike conformations in the presence of Ca^{++} (see Veis, 1993) and this structure would tend to create negatively charged surfaces that would be expected to interact with calcium in growing apatite crystals (Addadi, 1992). One of our present needs is to obtain cDNA and genomic clones for DPP, in order to determine the complete structure for the protein and its gene.

The DPP of rat consists of several variants. The highly phosphorylated forms HP1 and HP2, are clearly two distinct proteins, as revealed by amino acid composition and NH_2-terminal analyses (Butler *et al.*, 1983). More recently we have demonstrated that the MP (moderately phosphorylated) and LP (low phosphorylated) variants probably have the same protein backbone as HP2, but simply have lowered levels of phosphorylated serines (Butler, 1995; W.T. Butler and L.F. Riggan, unpublished data). Dentins of other species examined do not seem to display this heterogeneity of DPP types.

A number of *in vitro* experiments suggest that DPP is involved in initiation of the first mineral crystals of hydroxyapatite in dentin. DPP may bind to collagen fibrils in unmineralized predentin, near the middle of the collagen gap region where the initial mineral deposition is believed to begin. Because of its affinity for calcium, DPP may concentrate these ions and participate in formation of beginning apatite crystals. The relevant data supporting these conclusions are as follows. Binding studies with reconstituted collagen fibrils indicate that DPP has a high affinity for type I collagen (Stetler-Stevenson and Veis, 1986). Elegant studies by Traub *et al.* (1992) showed that DPP interacted with turkey tendon fibrillar collagen in the "e" band regions, corresponding to the gap regions. When immobilized on a stable support and incubated in physiological solutions of calcium and phosphate, DPP induced the formation of HAP (Linde *et al.*, 1989). DPP may also influence the crystal shape and size, by binding preferentially to the 100 face of growing crystals (Fujisawa and Kuboki, 1991). This dual role of

DPP as an initiator of HAP formation at low levels and an inhibitor at higher concentrations is supported by its influence on mineral formation studies, using an *in vitro* gelatin gel diffusion system (Boskey *et al.*, 1990). It also appears that a portion of DPP is firmly bound to the mineralized dentin collagenous matrix, and that the majority is readily extractable, consistent with the putative dual roles (Linde and Goldberg, 1993). In order to verify these conclusions, further experiments are obviously needed.

AG1 (Dmp1)

DPPs are the most abundant NCPs in dentin and are unique in that they have high contents of aspartic acid and phosphoserine. Because they were difficult to completely study by common biochemical methods, a molecular biology approach was recently utilized in attempts to characterize these proteins. A lambda gt 11 cDNA library, derived from odontoblasts/pulp cells, was first screened with polyclonal antibodies to rat incisor DPPs. Positive clones were then screened a second time with an anti-sense "wobble" poly-Asp nucleotide probe. The resulting DNA sequences from multiple clones obtained from this double selection procedure led to the identification of a unique, acidic, and potentially highly phosphorylated ECM protein, termed AG-1 (George *et al.*, 1993), later renamed dentin matrix protein 1 (Dmp1; George *et al.*, 1994a,b). AG1 cDNA codes for a 16 amino acid leader sequence and a 473 residue secreted protein with a predicted molecular weight of 53 kDa. The presence of 134 Asp and Glu residues as compared to 47 Arg, Lys, and His residues makes AG1 a very acidic protein, with a predicted net charge of -87 (before phosphorylation). Thus, the overall charge of AG1 is in between that of the sialoproteins (OPN, BSP and DSP) and DPPs. In addition, AG1 is serine-rich and has an overall composition between that of bone phosphoproteins and DPPs, with less aspartic acid and more glutamic acid than DPPs. That AG1 cDNA encodes an expressed protein was demonstrated by the finding that Western blots of dentin extracts probed with anti-recombinant AG1 antibodies yielded a single band (Mr~61 kDa). Furthermore, when incisor organ cultures were labeled with $^{32}PO_4$, a 61 kDa labeled protein was identified in the mineralized matrix extracts (George *et al.*, 1993). AG1 is believed to represent a new type of matrix protein in dentin.

A predominant feature of AG1 is that it has a high content of Ser residues (i.e., 107 Ser) which is also a hallmark for DPPs. It was reported that DPPs were substrates for phosphorylation by membrane-bound forms of casein kinases I and II, present in the rER and Golgi compartments of osteoblast-like cells (Wu *et al.*, 1992). Casein kinase II is known to referentially catalyze phosphorylation of Ser or Thr in the consensus sequence Ser/Thr-X-X-Asp/Glu/pSer on many acidic proteins. The consensus sequence for casein kinase I is Asp/Glu/pSer-X-X-Ser (George *et al.*, 1993). Given the interspersed distribution of serines in acidic patches of Asp and Glu, 55 of the 107 serine residues are likely candidates for phosphorylation by casein kinases I and II. If fully phosphorylated, the acidic nature of AG1 would be markedly enhanced, resulting in a net charge of -175/molecule at physiological pH. In general, bone matrix acidic proteins (i.e., OPN and BSP) are not as highly phosphorylated as predicted for AG1.

AG1 contains a single consensus sequence (Asn-X-Ser) for N-glycosylation and several Ser-X-Glu triplet sequences as potential O-glycosylation sites. Furthermore, AG1 has one RGD sequence, a potential integrin binding site (George *et al.*, 1993). The NH_2-terminus is identical to that of BAG-75, a bone derived phosphoprotein; however, the remainder of the sequence must differ from BAG-75 because the overall compositions are different.

By interspecific backcross analysis using full length AG1 cDNA as a probe, the of *AG1* gene was localized on mouse chromosome *5q21*, in tight linkage with *Fgf5* (George *et al.*, 1994). The middle region of mouse chromosome 5 shares a region of homology with human chromosome 4. In addition, *Fgf5* has been placed on human *4q21*. The tight linkage between *Fgf5* and *AG1* in mouse suggests that *AG1* will reside on *4q* as well, near human *4q21* (George *et al.*, 1994). Other mineralized tissue-related genes including *BMP3*, *TGFß*, and *OPN* have also been mapped to this region.

The long arm of human chromosome 4, *4q13-q21* has been identified as the locus for the Dentinogenesis imperfecta II. Because they were reportedly absent or markedly reduced in the teeth of patients with Dentinogenesis imperfecta II, DPPs have been considered as the candidate gene(s) for this disease. Since *AG*1 co-purifies with DPPs and shares properties of DPPs, *AG*1 could also be deficient in these patients and must be considered as a candidate gene for the genetic defect in Dentinogenesis imperfecta II (George *et al.*, 1994). A more recent report (MacDougall *et al.*,1994) disputes the claim that DPP is deficient in teeth of patients with this disease.

It is generally believed that hard-tissue forming cells (i.e., odontoblasts and osteoblasts) secrete a set of acidic regulatory macromolecules delivered to the pre-formed collagen type I matrix. These acidic macromolecules bind to the structure matrix and then initiate nucleation or facilitate the mineralization process through their very high calcium binding capacity. DPPs, the most prominent NCPs in dentin, are considered the prime candidates for regulatory roles in dentinogenesis. AG1, also a highly negatively charged molecule, could also be involved in the initiation of HAP formation and/or in regulating crystal growth processes in dentin. In contrast to the other two dentin-specific NCPs (DPPs and DSP), AG1 is present in dentin extracts in relatively low levels, which is compatible with some type of regulatory role (George, *et al.*, 1994). The availability of recombinant AG1 makes it feasible to study (1) calcium binding capacity *in vitro*, (2) the role of AG1 in the initiation of hydroxyapatite formation *in vitro*, and (3) the interaction of AG1 with other ECM molecules such as collagen type I.

Northern blot analysis of mRNA from various tissues showed that AG1 transcripts were only present in teeth, but not in skin, liver, brain, calvaria, and tibia (George *et al.*, 1993). *In situ* hybridization was used to determine the cellular origin of AG1 mRNA. *De novo AG*1 gene expression was readily detected in secretory stage odontoblasts of 2 day-old rat incisor, coincident with dentin matrix production (George *et al.*, 1995). No staining was seen in any of the cells from the cervical loop up to the point where the first polarized odontoblasts are present. No transcripts were detected in the pulp cells, Meckels cartilage or alveolar bone. Also no transcripts were detected in E16 or E20 (fetus) incisor tooth germs. However, a faint signal of AG1 mRNA was observed in the columnar ameloblasts in the presecretory stage, but not in preameloblasts, stratum intermedium and stellate reticulum of 2 day-old incisor tooth germs. These data show that AG1 expression is regulated developmentally and is essentially restricted to secretory odontoblasts.

Dentin sialoprotein (DSP)

Since NCPs of dentin may play crucial roles in dentinogenesis, much effort has been directed toward their isolation and characterization. Earlier studies on rat dentin NCPs revealed an unusual

glycoprotein with an Mr of 95 kDa (Butler *et al.*, 1981). This protein accounted for 5-8% of the weight of dentin NCPs and had a high carbohydrate (30%) and sialic acid (10%) content. This acidic protein was renamed DSP because of its overall resemblance to the bone sialoproteins (Butler, 1991). Like OPN and BAG-75, DSP is rich in Glu, Asp, Ser and Gly. Sedimentation equilibrium and amino acid analyses revealed a molecular weight of 53 kDa and approximately 350 amino acids. By Edman degradation the NH_2-terminal sequence was shown to be IPVPNLPL (Butler *et al.*, 1992).

Using a monospecific antibody to screen a rat incisor odontoblast/dental pulp cDNA lambda gt11 library, we isolated and sequenced a cDNA clone (Ritchie *et al.*, 1994). This clone (approximately 750 bp) contained a sequence coding for the NH_2-terminal amino acids of DSP. A second cDNA clone was isolated by using the first cDNA as a probe to rescreen the library. This second clone contained the full-length DSP coding region. From the sequence, we deduced that the rat DSP cDNA coded for a 366 amino acid protein comprised predominantly of Asp, Ser, Glu, and Gly. The amino acid composition calculated for this sequence was essentially identical to that for the purified DSP reported earlier; likewise the deduced molecular weight (53,045) was the same as that determined by sedimentation equilibrium. Six potential N-linked glycosylation and thirteen potential phosphorylation sites were present in the predicted DSP sequence. To date, we have not determined if DSP is a phosphoprotein. No Arg-Gly-Asp sequence was found, and the sequence for DSP was dissimilar to those of rat OPN and BSP (Ritchie *et al.*, 1994). However, the first three amino acids, IPV are like those for rat BAG-75, AG1 and OPN (LPV) and identical to that of human OPN (IPV) (Butler, 1991; Butler *et al.*, 1992).

Using immunohistochemical methods, DSP was localized to odontoblasts and pulp cells and was present in predentin and dentin but not in other tissues or cells such as in enamel, bone, muscle, or cartilage (Butler *et al.*, 1992; D'Souza *et al.*, 1992; Bronckers *et al.*, 1994). These data showed that DSP is initially expressed by young odontoblasts as they begin to secrete predentin, but not by preodontoblasts. Immunostaining was intense in secretory odontoblasts and within odontoblastic processes, suggesting that DSP may also be secreted at the mineralization front through these cell processes. During early stages of tooth development, preameloblasts (but not ameloblasts) were immunopositive for DSP. From these results it was concluded that DSP should be classified as a dentin-specific protein.

We examined various rat tissues for DSP mRNA expression using a ^{32}P-random primer-labeled DSP cDNA as a probe. Multiple DSP transcripts were detected near 4.6 kb and 1.5 kb in the incisors of 21 day-old rats (Fig. 2) and the tooth germs of 2 day-old rats. No DSP transcripts were detected in cells of other mineralized tissues such as tibia, calvaria, or osteoblast-like osteosarcoma (ROS 17/2.8) cells. Also no DSP transcripts were detected in brain, salivary gland, muscle, lung, kidney, heart, liver, spleen, intestine, or pancreas. As a positive control, these filters were reprobed with an OPN cDNA. As expected, OPN transcripts were detected in tibia, calvaria, ROS 17/2.8 cells and kidney. It is worth noting that OPN mRNA was also detected in both the incisors of 21 day-old rats and the tooth germs of 2 day-old rats (Ritchie *et al.*, 1994).

To identify the cells actively producing *DSP* transcripts, we utilized *in situ* hybridization using a digoxigenin-labeled DSP riboprobe and detected DSP mRNA in young polarized odontoblasts.

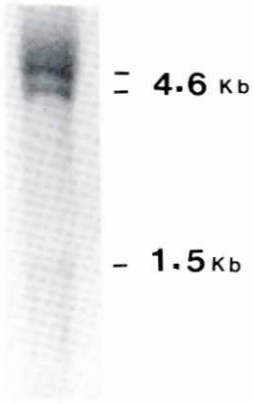

Fig. 2. Northern blot analysis of 21 day-old rat incisors. *Using rat DSP cDNA as a probe, 20 μg of total RNA was subjected to blot analysis. Multiple DSP transcripts were observed at 4.6 and 1.5 kb.*

These cells were obtained from 2 day-old tooth germs and were associated with early predentin matrix before the onset of mineralization, as well as in mature odontoblasts (Fig. 3). Apical to this region, pre-odontoblasts, non-polarized cells of the dental papilla that are in direct contact with the basement membrane, did not hybridize with the DSP riboprobe. No *DSP* transcripts were detected in other cells including the dental pulp and osteoblasts (not shown) of the 2 day-old rat jaw (Ritchie *et al.*, 1995). As noted earlier, previous studies showed that dental pulp was immunopositive for DSP protein in the mature zones of the neonatal rat incisor and in molars at day 5 of postnatal development (D'Souza *et al.*, 1992). Further *in situ* hybridization experiments are needed to clarify whether DSP transcripts are expressed in dental pulp cells of these specific developmental stages.

In order to understand the mechanisms which control the tissue specific expression of the rat *DSP* gene, we screened two rat genomic libraries using rat DSP cDNA as a probe. From a Sprague Dawley lambda dash genomic spleen library, a genomic clone containing approximately 15 kb of DNA insert was isolated. In addition, a 38 kb cosmic genomic clone from a Wistar rat pWE15 genomic library was also obtained. Southern blot analysis of *Eco*RI-digested lambda dash clone, using DSP cDNA or DSP specific oligonucleotides as probes, revealed that a 4 kb *Eco*RI fragment contained the *DSP* coding sequences (Fig. 4A). However, Southern blot analysis showed that a 6 kb instead of a 4 kb *Eco*RI fragment of the cosmic clone was recognized by DSP cDNA probe (Fig. 4B). The discrepancy in the sizes of EcoRI fragments from these two genomic libraries could be due to (1) the presence of more than one *DSP* gene in the rat genome or to (2) allelic polymorphism. These data and the observation of multiple *DSP* transcripts prompted us to determine if more than one *DSP* gene exists. To answer this question, rat genomic DNA, prepared from Sprague Dawley rat liver and digested with restriction enzyme *Eco*RI was subjected to Southern blot analysis. The genomic Southern blot indicated that both 6 kb and 4 kb EcoRI fragments were recognized by DSP cDNA probe under high stringency conditions (Fig. 4C). These data strongly suggest that two *DSP* genes exist in the rat genome (Ritchie *et al.*, 1995). Since we observed more than three transcripts with Northern blot analysis, it is likely that some *DSP* transcripts are generated by alternative splicing. We speculate that the *DSP* transcripts are derived from both two related genes and alternative splicing.

Recently we have re-examined the DSP protein fractions and have detected at least two distinct proteins reacting with DSP

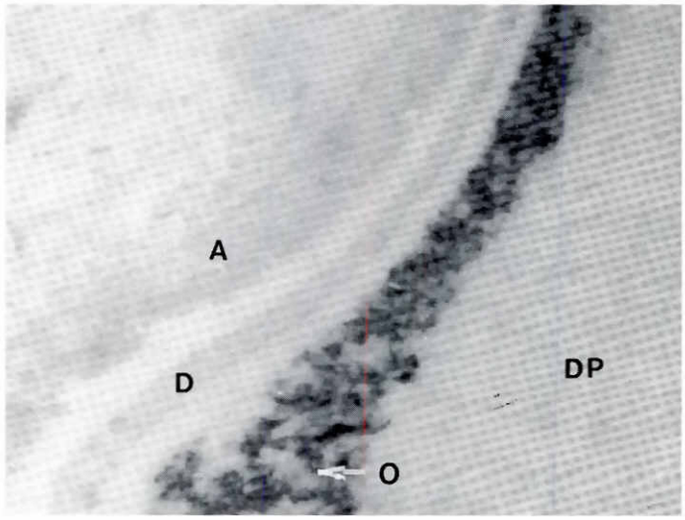

Fig. 3. *In situ* hybridization of 2-day old rat molar tooth germ. *A digoxigenin-labeled anti-sense DSP riboprobe was generated and used for in situ hybridization. A DIG (digoxigenin) nucleic acid detection kit (Boehringer Mannheim) was used to detect the hybridization reaction. DSP transcripts were localized specifically in polarized odontoblasts (O). No DSP transcripts were present in other cells of the newborn rat jaw, including the dental pulp (DP) and ameloblasts (A).*

antibodies on Western blots (Butler, 1995). These observations also support the conclusion that more than a single molecular species of DSP exists.

The DNA fragments of the rat lambda dash genomic clone which hybridized with the DSP cDNA were subcloned into plasmid vector pGEM7Z(+) for DNA sequencing using oligonucleotide primers corresponding to various regions of DSP cDNA. DNA sequencing demonstrated that this lambda dash genomic clone, the counterpart of the full length DSP cDNA clone, is comprised of five exons and four introns and spans approximately 6 kb (Ritchie, *et al.* 1994b). Exon 1 contains the 5' noncoding region. Exon 2 is 76 bp long and contains a 19 bp 5' noncoding sequence, followed by the sequence coding for the ATG start site and for the leader sequence and the NH_2-terminal two amino acids. Exons 3 and 5 encode 29 and 25 amino acid residues, respectively. Exon 5 also contains the stop codon and the 3' noncoding sequence. Exon 4 encodes 310 amino acids (Fig. 5).

DSP has an overall resemblance to the bone sialoproteins (i.e., OPN, BSP and BAG-75), being rich in Glu, Asp, Ser and Gly with a high carbohydrate content (Butler, 1991). DSP has a predicted net charge of -42 comparable to that of OPN. Similar to OPN, DSP has 13 potential phosphorylation sites for casein kinases I and II. These three acidic phosphoproteins from bone matrix have been considered as mineral regulatory molecules (Gorski, 1992). BSP may initiate mineral formation while OPN displays an inhibitory effect on mineral formation. Although we do not know its role, DSP might serve a similar role as a regulator of some facet of mineralization during dentinogenesis. Because of its tissue specific expression, DSP may prove to be a valuable marker for odontoblast lineage cells, for example in studying odontoblast differentiation.

Mineralized tissue-specific NCPs

At least two NCPs, osteocalcin and bone sialoprotein (BSP), can be termed mineralized tissue-specific. They were first recognized in bone extracts, but are also found in dentin and are expressed by odontoblasts. Osteocalcin and BSP should prove to be reliable markers for odontoblasts, particularly when used in conjunction with the dentin-specific proteins.

Osteocalcin

Osteocalcin (also called bone gla protein, BGP) is a small protein (ca. 50 amino acids) which contains three γ-carboxyglutamate (Gla) residues and one disulfide bond (reviewed in Hauschka *et al.*, 1989; Price, 1992). Osteocalcin is one of the most abundant proteins of bone matrix, from which it was originally isolated. In a Gla-dependent manner, osteocalcin binds tightly to hydroxyapatite and is an inhibitor of HAP formation *in vitro*. The high conservation of amino acids around and including the Gla residues implies that they are necessary for an important biological function. Osteocalcin may act as a chemoattractant for osteoclast precursors (Lian *et al.*, 1986) or may regulate the growth of apatite crystals in forming bone and dentin (Romberg *et al.*, 1986). Finally, the transcription of osteocalcin mRNA is regulated by 1,25-dihydroxyvitamin D_3, in a mechanism involving binding of the hormone to receptor and interaction of the receptor-ligand complex with specific elements in the promoter of the osteocalcin gene (Price and Baukol, 1980).

Osteocalcin is found in dentin ECM (Linde *et al.*, 1982) and is synthesized and secreted by odontoblasts *in vivo* (Bronckers *et al.*, 1985, 1987; Gorter de Vries *et al.*, 1987) and *in vitro* (DiMuzio *et al.*, 1983; Finkelman and Butler, 1984).

The rat prepro form of osteocalcin consists of 99 residues, a 49 amino acid prepro segment and a 50 residue processed protein (Hauschka *et al.*, 1989). The osteocalcin gene is located on human chromosome 1. It consists of four exons coding for a 26 amino acid signal peptide, a 49 residue pro segment and the secreted protein (Puchacz *et al.*, 1989). More recently, the existence of three osteocalcin genes within a 23-kilobase cluster in the mouse genome has been reported (Desbois *et al.*, 1994). Two of the genes *(OG1* and *OG2)* are expressed in bone while the third *(ORG)* is expressed in kidney but not bone. Experiments on the expression of these genes by odontoblasts have not been performed.

Bone sialoprotein (BSP)

BSP is a highly glycosylated and sulfated phosphoprotein originally isolated from bone (Fisher *et al.*, 1983a; Franzen and Heinegard, 1985). BSP consists of about 300 amino acids and is rich in Glu and Asp (Fisher *et al.*, 1990). It contains an Arg-Gly-Asp (RGD) sequence near its COOH-terminus and is capable of attaching osteoblasts and osteoclasts by interacting with the cell surface $\alpha_v\beta_3$ integrin. BSP also contains two poly-Glu sequences that are potential Ca^{++}/HAP binding sites. Several of the Tyr residues are in sequences and conformations that suggest that they are sulfated. With a rat osteosarcoma cell line, Midura *et al.* (1990) showed incorporation of [^{35}]sulfate into sulfate esters on N- and O-linked oligosaccharides and on tyrosine residues. The synthesis of BSP and its mRNA by odontoblasts was demonstrated in a series of elegant studies by Chen *et al.* (1992). In dentin, BSP was localized to odontoblasts and their processes and to peritubular dentin. The protein appears to be relatively restricted to mineralized tissues, being synthesized in bone, dentin and cementum, but also by hypertrophic chondrocytes and placental trophoblasts (Young *et al.*, 1992).

Although its function in mineralized tissues has not been clearly established, BSP has been shown to promote the formation of HAP crystals in a steady state agarose gel system, in the presence of low

calcium and phosphate concentrations (Hunter and Goldberg, 1993). Under these conditions, neither OPN nor controls induced HAP formation. Chemical modification of the carboxylate groups abolished the HAP inducing property of BSP, but dephosphorylation had no effect (Hunter and Goldberg, 1994). Curiously, poly(L- or D-glutamic acid) also induced the formation of HAP in this system, while poly (L- /or D-aspartic acid) was ineffective. It was proposed that the poly-Glu sequences of BSP might function to promote initiation and growth of HAP crystals in mineralizing tissues.

Other dentin ECM macromolecules

Proteoglycans (PGs)

Chondroitin sulfate containing PGs have been recognized as components of dentin and bone for a number of years. Two relatively small PGs found in bone, biglycan (also called PGI) and decorin (PGII) are comprised of core proteins of about 45 kDa (Fisher *et al.*, 1983a; Young *et al.*, 1992). Biglycan from bone has two chondroitin sulfate chains and decorin only one. While the two core proteins are distinctly different, they belong to a family of leucine rich proteins with several tandem repeats. Dentin may also contain decorin or biglycan or both.

Early studies were conducted to specifically characterize rat incisor dentin PGs by *in vivo* radiolabeling with [^{35}S]sulfate and separation of the PGs into predentin and dentin pools by differential extractions (Rahemtulla *et al.*, 1984). A major chondroitin sulfate-containing PG that was relatively small was seen in predentin and was rapidly incorporated into dentin, and a second larger PG in smaller amounts was only found in predentin. Most of the glycosaminoglycan chains were chondroitin-4-sulfate.

More recently Steinfort *et al.* (1994) utilized a similar labeling and extraction protocol, and then purified the PGs from the two compartments by sequential ion-exchange and gel chromatography, and subsequently by C18 HPLC. From rat incisor dentin they obtained five small PGs (PG 1-5) with core proteins of 25, 40, 115, 70 and 40-50 kDa, respectively. PG 4 appears to be BSP with attached chondroitin sulfate chain(s) and PG 5 is probably decorin and/or biglycan. PG 2 may be similar to a PG isolated from rat calvarial bone (Goldberg *et al.*, 1988) with a core protein of 37 kDa and called PGIII. Only significant levels of PG 5 were found in the nonmineralized (predentin) compartment. These investigators could not confirm the existence of the minor, high molecular weight PG in predentin seen by Rahemtulla *et al.* (1984).

It has been contended that PGs are secreted into predentin and in some manner prevent this tissue from mineralizing. Thus, a portion of the PGs would be metabolized or removed prior to mineralization of collagen fibrils and the conversion of predentin to dentin. To investigate the metabolism of PGs in bone and dentin, Prince *et al.* (1984) injected [^{35}S]sulfate into rats and sacrificed after various times up to 72 h The specific radioactivity of papain-resistant macromolecules (glycosaminoglycans) was determined for each time point. Incorporation of the label was maximal at 12 h and then declined to 50-75% of the value by 72 h. Thus, 25-50 % of the nascent PGs are lost from these tissues during a time in which dentin is formed. Steinfort *et al.* (1994) confirmed the rapid incorporation of a pool of PGs into dentin and that a portion of the PGs in the predentin are lost. Clearly, additional experiments are warranted to confirm the metabolism and functional significance of PGs in dentinogenesis and osteogenesis.

Osteopontin

Osteopontin (OPN) was initially discovered as a phosphoprotein secreted by transformed cells (Senger *et al.*, 1979, 1989; Chackalaparampil *et al.*, 1985) and was independently isolated from bone and characterized (Franzen and Heinegard, 1985; Fisher *et al.*, 1987; Prince *et al.*, 1987). From a ROS 17/2.8 cDNA library, the rat OPN cDNA was cloned and sequenced (Oldberg *et al.*, 1986). OPN, is a major bone NCP, expressed by preosteoblasts, osteoblasts and young osteocytes as well as chondrocytes (Butler, 1989). OPN has also been found in many nonmineralized tissues (for details see Butler, 1989; Denhardt and Guo, 1993). It is rich in Asp, Glu, and Ser and has a molecular weight of about 41.5 kDa. Rat OPN contains 12 phosphoserines, 1 phosphothreonine, 1 N-linked and several O-linked oligosaccharides (Butler, 1989). OPN cDNA from rat, mouse, pig, human, bovine and chicken have been sequenced. The conserved features of OPN among species include (1) an -RGD- sequence for mediating cell adhesion, (2) a poly Asp sequence for possible calcium binding, and (3) several potential casein kinase phosphorylation sites (Butler, 1989; Gorski, 1992; Denhardt and Guo, 1993).

The *OPN* gene was mapped to human chromosome 4 at 4q13 (Young *et al.*, 1990) and to ric^{r} on mouse chromosome 5 (Fet *et al.*, 1989). Mouse, pig, and rat *OPN* genes consist of 7 exons and extend over about 7 kb (Craig and Denhardt, 1991; Zhang *et al.*, 1992; A.R. Ridall, D.P. Dickinson, E.L. Daane and W.T. Butler, unpublished data). The expression of *OPN* in a variety of cell types is regulated by several hormones, growth factors, oncogenes and tumor promoters (Denhardt and Guo, 1993). OPN is a protein with diverse functions that include (1) cell attachment and cell signaling

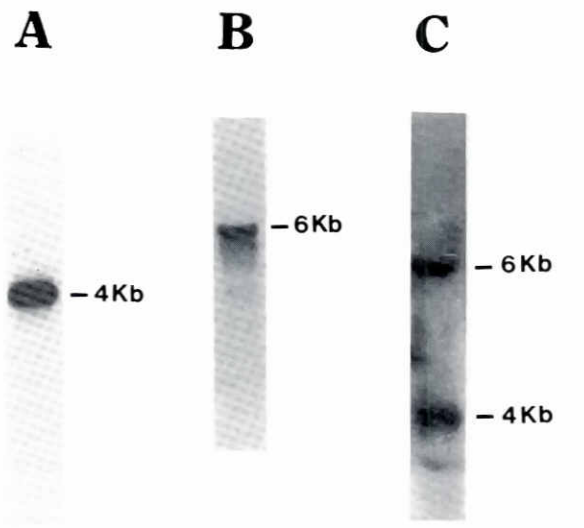

Fig. 4. Southern blot analysis. *A lambda dash rat genomic clone and a cosmid rat genomic clone were each digested with restriction enzyme EcoRI, then electrophoresed on 1% agarose gel. Rat genomic DNA obtained from Sprague Dawley rat liver was also digested with EcoRI, and electrophoresed on a 0.8% agarose gel. A random-primer labeled rat DSP cDNA was used for Southern analysis. As shown in panel A, this cDNA probe hybridized to a 4 kb fragment from the lambda dash rat genomic clone. However, as shown in B the same probe hybridized to a 6 kb EcoRI fragment (containing the DSP coding region) from the cosmid genomic clone. Following hybridization to rat genomic DNA, two DNA fragments (6 kb and 4 kb) were recognized (C).*

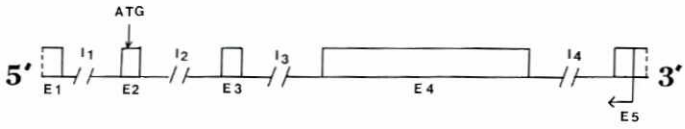

Fig. 5. Genomic organization of rat *DSP* gene. *The lambda dash genomic clone was subcloned and sequenced. The DNA sequence data indicated that the rat DSP gene consists of five exons (E1, E2, E3, E4 and E5) and four introns (I1, I2, I3, and I4). The arrow ponting downwards represents the ATG translation start site. The arrow ponting left represents the stop codon TAA. Exon 1 contains the 50 bp 5' noncoding region sequence. Exon 2 contains a 19 bp 5' noncoding sequence, the ATG start site, and the sequence codes for the leader sequence, and the NH_2-terminal two amino acids of DSP. Exons 3 and 5 encode 29 and 25 amino acid residues. Exon 5 also contains the stop codon and the 3' noncoding sequence. Exon 4 encodes 310 amino acids.*

via its RGD sequence; (2) regulation of the formation and remodeling of mineralized tissues, (3) interaction with Ca^{+2} in a way that influences OPN protein conformation and may be important in Ca^{+2} mediated or Ca^{+2} dependent processes, (4) inhibition of the growing apatite crystal lattices in bone and dentin and (5) cell migration (Denhardt and Guo, 1993).

Since OPN is synthesized by osteoblasts before the onset of mineralization, and alkaline phosphatase (ALP) is also expressed early in the differentiation stages of mineralized-forming cells including osteoblasts, these two proteins have been considered to be markers for the differentiated osteoblast. Similarly, we can ask whether OPN is present in odontoblasts and whether both OPN and ALP can be used as markers for odontoblast differentiation. Immunolocalization experiments showed weak signals for OPN in predentin and in the putative Golgi zone of some odontoblasts in rat incisors (Mark *et al.*, 1988). OPN immunoreactivity was also seen in the odontoblasts of 50-day-old porcine embryonic teeth (Chen *et al.*, 1993). OPN protein was isolated from dentin matrix (Fujisawa *et al.*, 1993). During root formation, OPN immunostaining was observed in the root odontoblast layer and in areas of Hertwig's epithelial root sheath (Somerman *et al.*, 1992). OPN immunostaining was present in predentin and dentin, as well as crown and root odontoblasts during the advanced stages (i.e., 15 day-old or older teeth) of dentinogenesis (Bronckers *et al.*, 1994). However, Helder *et al.*, using Northern blotting and *in situ* hybridization, detected no transcripts in the 1-3 day-old tooth tissues despite the weak OPN immunostaining in predentin and in some incisor odontoblasts (Helder *et al.*, 1993). They concluded that (1) OPN was not expressed in these developing rat tooth germs, (2) the previous immunostaining of predentin was due to OPN entrapment in the matrix from the blood stream, and (3) the weak OPN immunostaining in some odontoblasts was the result of the OPN resorption by odontoblasts from the predentin. In our laboratory, using rat OPN cDNA as a probe, we detected OPN mRNA in both the incisors of 21 day-old rats and the molar tooth germs of 2 day-old rats (Ritchie *et al.*, 1994a). These experiments do not show which type of cells (i.e., odontoblasts, preodontoblasts, or dental pulp cells, etc.) in tooth germs and incisors are responsible for producing OPN mRNA. The detection of OPN mRNA in 2 day-old molar tooth germs clearly indicates the expression of OPN in early tooth development and is consistent with the observations on immunolocalization of OPN in predentin and in some odontoblasts (Mark *et al.*, 1988; Bronckers *et al.*, 1993).

Expression of ECM proteins by odontoblasts: implications to the mechanisms involved in dentinogenesis

As stated earlier, our partial understanding about the role of ECM proteins in dentinogenesis is based upon their properties, as revealed by *in vitro* experiments. For example, both DPP and BSP are known to initiate formation of HAP crystals in *in vitro* models of mineralization. Other evidence for the odontoblastic synthesis and secretion of ECM proteins and their involvement in dentinogenesis and mineralization has been derived from several types of investigations. These studies include the following: 1) *in vivo* injection of radioisotopes which preferentially label one or more dentin ECM components, followed by autoradiography of histological sections or biochemical characterizations; 2) culture of odontoblasts as organ cultures (tooth germs or fragments of forming dentin) and radiolabeling of components, followed by immunochemical or biochemical techniques; 3) pulp cell cultures, which presumably mimic odontoblasts and 4) immunohistochemistry and *in situ* hybridization studies for detection of ECM components and their corresponding mRNAs within odontoblasts and surrounding tissues. Each type of approach has inherent limitations, but the data have provided a number of valuable insights.

Collectively, the results from these biosynthetic approaches indicate that odontoblasts synthesize and secrete predentin, consisting of predominantly type I collagen fibrils and PGs. Odontoblasts also synthesize a collection of NCPs that are somehow associated with the ECM at the mineralization front a short time after their synthesis. Concomitantly, there appears to be removal of some of the PGs present in predentin. Some NCPs of dentin are apparently secreted through odontoblastic processes; this selective secretion may involve a sorting mechanism within odontoblasts that begins in the rER, and which is distinct from the secretory route for procollagen (Rabie and Veis, 1991). These events combine to convert predentin to dentin. In this section, we will attempt to summarize some of the biosynthetic data which support these conclusions.

Weinstock and Leblond (1973) injected [^{3}H]serine and [^{3}H]proline into rats to demonstrate (autoradiography) that collagen is synthesized by odontoblasts and is secreted into predentin. Only after 24-30 h was the label found in dentin. In contrast, labeling of phosphoproteins with [^{33}P]phosphate showed that these proteins were secreted differently: the label was seen within a short time at the predentin-dentin junction. Experiments by DiMuzio and Veis (1978) utilized *in vivo* injections of [^{3}H]serine and the radiolabeling of DPP and type I collagen CNBr peptides was followed. Again, the routes of secretion for the two were different, as indicated by the times of labeling. Similar approaches were used by Maier *et al.* (1983) to show the formation of a collagen-DPP complex presumably at the mineralization front. Prince *et al.* (1984) studied the metabolism of PGs in bone and dentin following injections of [^{35}S]sulfate. Secretion into bone and dentin was maximal at 12 h and declined to 50-75% of the maximal levels by 72 h. The data showed that a portion of the PGs were degraded or turned over, within a window of time when predentin and osteoid are forming and become mineralized to form dentin and bone, respectively.

Several immunohistochemical investigations (MacDougall *et al.*, 1985; Nakamura *et al.*, 1985; Gorter de Vries *et al.*, 1986;

Rahima *et al.*, 1988) show that DPP is synthesized by odontoblasts (and not by other cell types) and is localized in odontoblastic processes and dentin, but not predentin. Using immunocytochemical techniques at the ultrastructural level, Rabie and Veis (1991) showed that secretory routes for collagen and DPP were different. Studies with organ cultures, (i.e. odontoblasts still attached to dentin or tooth slices) incubated with radiolabeled precursors), also demonstrated that DPP is a product of odontoblasts (Munksgaard *et al.*,1978b; DiMuzio *et al.*, 1985). In these studies, the cultures were labeled with [^{3}H]serine or [^{32}P]phosphate and then labeled phosphoproteins were isolated and characterized biochemically. Organ cultures were also used to demonstrate that odontoblasts synthesize type I collagen and type I trimer, but not type III collagen (Munksgaard *et al.*, 1978a). Cultures of rat tooth germs have been used to show the synthesis of osteocalcin and DSP by odontoblasts (Finkelman and Butler, 1985; Bronckers *et al.*, 1993).

Taken together, the studies on odontoblast function and biosynthetic capability reveal a cell type that is unique in the set of ECM proteins synthesized and the manner in which these macromolecules are secreted. These unique features tend to reveal how the process of dentinogenesis proceeds and is controlled. Further indepth understanding of this process is dependent on the detailed elucidation of the relationship of odontoblasts to ECM components.

Future directions

Much remains to be accomplished in order to better understand the process of dentinogenesis and the role played by the odontoblast. For example, we know that odontoblasts secrete a type I-rich collagenous matrix in predentin. Yet, we do not yet appreciate structural motifs that this complex protein may assume as it is being transformed to a mineralized matrix. Additional data are clearly needed (e.g., high resolution nuclear magnetic resonance spectroscopy) to elucidate forms and changes that transpire as mineralization proceeds. Likewise, the role of the NCPs in this process is hinted by several lines of experimentation, but no clear understanding of the supramolecular complex that occurs and how this complex regulates mineralization has been obtained. The three dimensional structures of the NCPs and especially the role of the large number of negatively charged phosphate and carboxylate groups must be elucidated. An understanding of the role played by odontoblasts in potentially directing the secretory pathways and affecting the site where the NCPs can interact with collagen and control mineralization is crucial. The process of removal of some of the PGs and the effect of this event on dentinogenesis needs to be studied.

We suggest that the characterization of odontoblasts and cells similar to odontoblasts (e.g., cultured pulp cells) should be undertaken by utilization of sets of markers for dentin specific and mineralized tissue specific proteins and their mRNAs. A similar approach is routinely used by cell biologists in bone research. One example of where such an approach would be useful is characterization/identification of odontoblast replacement cells responsible for formation of reparative dentin. Another example might be for characterization of cells involved in formation of certain oral cysts and tumors.

The observation that at least three proteins appear to be dentin specific (that is, they are found only in dentin and are expressed only by odontoblasts) suggests that they perform some unique function related to the process of dentinogenesis. Thus, DPP, AG1 and DSP may play crucial roles in the initiation and control of mineralization of dentin. To test this hypothesis and to perhaps uncover their function, one could use transgenic mice to explore the results of overexpression of a dentin specific protein. Alternatively, gene knockouts could be developed in order to generate mice devoid of expression of a dentin specific protein. Such approaches may be necessary to gather further clues concerning their functions and may provide important information about the overall process of dentinogenesis.

Acknowledgments

We thank Dr. Gerald Pinero for his help with the in situ hybridization experiments utilizing DSP riboprobes and Ms. Hui Hou for excellent technical assistance in our experiments on the DSP cDNA and genes. We are grateful for the excellent assistance of Ms. Laura Adams and Ms. Michele Thomas in preparation of this manuscript. The research reported here was sponsored by Grant DE-05092 from the National Institute of Dental Research.

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