Spatiotemporal Expression of Ameloblastin Isoforms during Murine Tooth Development*

Received for publication, June 8, 2007, and in revised form, October 4, 2007 Published, JBC Papers in Press, October 5, 2007, DOI 10.1074/jbc.M704731200

Rajeswari M. H. Ravindranath 1, Asokan Devarajan §, and Takashi Uchida 5

From the 1 Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California Los Angeles, California 90033 and the 5 Department of Oral Biology, Division of Molecular Medicinal Science, Hiroshima University Graduate School of Biomedical Sciences, Kasumi 1-2-3, Hiroshima 734, Japan

Ameloblasts synthesize and secrete the enamel matrix proteins (amelogenin, ameloblastin, and enamelin). This investigation examined the profiles of ameloblastin in the ameloblasts and in the enamel matrix during different postnatal (PN) days (days 0–9) of development of mouse molar, using an antibody specific for C-terminal sequence of ameloblastin (CT; GNK VHQPQVHNAWRF). Ameloblastin is found in three different molecular sizes (37, 55, and 66 kDa) in both ameloblasts and enamel matrix during PN development. In the ameloblasts, the sequence of expression of these fractions varied. The 37-kDa fraction was observed (even before the appearances of mRNA of the proteases, enamelysin and kallikrein-4) on days 0 and 1, persisted until day 3, and was not found thereafter. Other isoforms (55 and 66 kDa) distinctly appeared in ameloblasts after day 1, reached a peak on day 5, and remained thereafter. The Ct-positive granules appeared beaded in the ameloblasts on day 3. In the extracellular matrix, a 37-kDa (but not 66- or 55-kDa) fraction was detected on days 0 and 1 and remained in the matrix throughout the PN days. The larger isoforms (55 and 66 kDa) appeared in the enamel matrix from day 3 onward. On days 0–3, but not later, the 37-kDa isoform co-localizes with amelogenin in Tomes’ process and formative enamel, as revealed by laser scan confocal microscopy. Autoradiography confirmed accumulation of [3H]-labeled ameloblastin trityrosyl motif peptide in the regions of Tomes’ process and formative enamel from day 0 to 3. These observations suggest that the 37-kDa isoform interacts with amelogenin during early tooth development.

During tooth development, enamel matrix is formed by ameloblasts, which synthesize and secrete two major classes of structural proteins, glycosylated (ameloblastin and enamelin) and nonglycosylated (amelogenin) (1). In amelogenin and ameloblastin-null mice, the enamel is defective, disorganized, and hypoplastic (2, 3), suggesting that amelogenin and other matrix proteins (enamelin and ameloblastin) may not facilitate enamel formation independent of one another. Although the role of amelogenin in enamel maturation has been studied extensively (1), there is a paucity of information on the role of ameloblastin in the enamel growth.

Yamakoshi et al. (4) have estimated that “the calcium-binding association constants for different enamel matrix proteins ranged from a high of $1.2 \times 10^{4} M^{-1}$ to a low of $4.4 \times 10^{3} M^{-1}$” and found that the Ca$^{2+}$-binding constant decreased in the following order: ameloblastin > enamelin > amelogenin. The most compelling evidence emerges from recently developed ameloblastin-null mice (3). Severe enamel hypoplasia and hypomineralization and detachment of ameloblasts from the matrix are observed in ameloblastin-null mice. Although amelogenin is attached to the dentin surface in ameloblastin-null mice, it failed to progress into enamel, suggesting that “ameloblastin may provide a scaffolding to organize enamel matrix protein structures to initiate crystal formation and growth” (3). A recent report on the binding interaction between amelogenin and ameloblastin in both native and recombinant ameloblastin (5) favors the speculation of a cooperative heteromolecular interaction between ameloblastin and amelogenin during enamel formation.

In order to understand the organization, integrity, and stabilization of enamel, there is a need to elucidate the spatiotemporal expression of ameloblastin during enamel development. In this investigation, we have identified unique, hitherto unknown and stage-specific isoforms of ameloblastin during postnatal developmental stages of mouse molars, using a unique antibody specific for the C-terminal sequence of ameloblastin (GNK VHQPQVHNAWRF) (6). The findings emanating from this investigation are important for understanding the assembly of matrix proteins leading to maturation of enamel.

**EXPERIMENTAL PROCEDURES**

**Mice**—We have used 50 normal, healthy, female Swiss Webster pregnant mice (Charles River Breeding) to obtain 350 litters, required for this study. All protocols involving mice were approved by the Institutional Animal Care and Use Committee (University of Southern California, Los Angeles, CA). Molars were obtained from mice at different developmental stages, ranging from newborn (NB) 2 day 0 through postnatal (PN) days 1, 3, 5, 7, and 9. Sagittal sections (6 µm) from the first mandibular molars of day “0” (NB) and days 1, 3, 5, 7, and 9 (PN) were

---

1 To whom correspondence should be addressed: Center for Craniofacial Molecular Biology, School of Dentistry, University of Southern California, 2250 Alcazar St., Los Angeles, CA 90033. Tel.: 323-442-3171; Fax: 323-442-2981; E-mail: ravindr@usc.edu.

2 The abbreviations used are: NB, newborn; PN, postnatal; LSCM, laser scan confocal microscopy; PBS, phosphate-buffered saline; HSA, human serum albumin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; ATMP, amelogenin trityrosyl motif peptide; Ct, C-terminal sequence of ameloblastin.

3 This work was supported by NIDCR, National Institutes of Health Grant RO1 DE-13204-05. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
Expression of Ameloblastin Isoforms during Tooth Development

To study the stage-specific secretion of ameloblastin during different PN days 0, 1, 3, 5, 7, and 9, we have isolated and pooled both ameloblasts and enamel strip from the first mandibular molars (100 molars for each postnatal day). After removing the first molars from mandibles, they were placed in dispase II solution at 4 °C for 30 min (days 0 and 1) or 1 h (other PN days). This treatment facilitates separation of ameloblasts from the extracellular enamel matrix and further cleaning under the dissection microscope. Ameloblast cell layer and the underlying matrix can easily be separated from the tooth organ for all of the PN days except for newborn (day 0), in which the matrix alone is broken into two or three pieces but not the ameloblasts.

**Protein Preparations from Ameloblasts**—Isolated ameloblasts, after assessing purity and homogeneity by examining sections under microscope, were frozen, thawed (four cycles) to extract the proteins, and the extraction procedure was modified from an earlier protocol (7). The protein extracts were homogenized with approximately 3 volumes of ice-cold Tris-HCl (25 mM Tris-HCl, pH 6.0, with 1 mM urea, 100 μM EDTA, 10 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride). The homogenates were incubated for about 30 min at 37 °C, centrifuged for 5 min at 8000 × g in a Beckman Microfuge 12, and the supernatant was collected. The proteins from the supernatants were isolated using a Microcon concentrator with a cut-off size of 10 kDa and centrifuged at 2,000 rpm at 37 °C for 5 min at 80 °C until further use. Protein concentration was estimated (8), and equivalent amounts of isolated proteins (80 μg) were used for electrophoresis and Western blot analysis.

**Protein Preparations from Developing Extracellular Matrix**—Postsecretory stage extracellular matrix samples, which were primarily dentino-enamel, were suspended in Tris-HCl (Tris-HCl, pH 7.4) containing 250 mM EDTA, protease, and phosphatase inhibitors and 10 mM β-mercaptoethanol as described (7). The final pH was adjusted to 8.0. The suspended matrix was homogenized and incubated at 4 °C overnight. The pooled extract was clarified by centrifugation, and the supernatant of soluble proteins was collected after three washes and further isolated using a Microcon concentrator with a cut-off size of 10 kDa and centrifuged at 2,000 × g for 12 min. The protein fraction was collected and stored at −80 °C until further use. Protein concentration was estimated (8), and equivalent amounts of isolated proteins (80 μg) were used for electrophoresis and Western blot analysis.

**Assessment of Stage-specific Expression of Ameloblastin Isoforms**—Protein isolated from ameloblasts and extracellular matrix extracts during different developmental days (NB to PN day 9) were resolved by 10% SDS-PAGE and electrotransferred to polyvinylidene difluoride membranes at 100 mA for 35 min using a semidyblot apparatus (Bio-Rad). Protein transfer was assessed by staining the polyvinylidene difluoride strips with 0.1% Fast Green as mentioned previously (5). Replicas were treated with ameloblastin antibody (Ct antibody, recognizes the C-terminal residues 406–420, GNKβHVQPQVH-NAWRF of ameloblastin) and resuspended in PBS (pH 7.2) for 18 h at 4 °C after blocking the membrane with PBS containing 1% HSA for 1 h at 37 °C. The membranes were washed five times with PBS containing 0.1% HSA. Enzyme-linked secondary antibody from Roche Applied Science (BM Chemiluminescence Western blotting kit, 1:2000) was used, as mentioned previously (5).

**Immunochemical Localization of Ameloblastin**—For immunochemical analyses, sections of mouse mandibular molar (6 μm) obtained on postnatal days 0, 1, 3, 5, 7, and 9 were mounted on Histostik-coated slides (Accurate Chemical and Scientific Corp., Westbury, NY). Details of the immunolocalization methodology were described in our previous publications (7, 9). Tissue sections were stained with the primary antibodies for ameloblastin. The IgGs were diluted 1:50 in PBS (pH 7.2) and incubated at 37 °C for 1 h. After washing with PBS containing 0.1% HSA and 0.1% Tween 20, the sections were incubated with fluorescein isothiocyanate (FITC)-labeled secondary antibody (goat anti-rabbit IgG) diluted in PBS, pH 7.2 (1:1000). IgG isotype 1 were used as a negative control. The images were examined under fluorescence microscopy.

**Colocalization of Ameloblastin and Amelogenins with LSCM**—To examine the spatiotemporal distribution and colocalization of ameloblastin and amelogenin, sections of mouse postnatal mandibular molars at different postnatal days were prepared as described previously (7, 9). In this experiment, we used rabbit primary antibody for both amelogenin and ameloblastin. Consequently, we have modified the protocol of immunostaining as recommended earlier (10, 11). The sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 3% H2O2. The sections were blocked with 1% human serum albumin and were stained with primary antibody against amelogenin (rabbit antibody, 1:500) for 1 h at 37 °C. The sections were incubated with TRITC (goat anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA), 1:40 dilution, for 30 min at room temperature. After washing three times (10 min each), the sections were sequentially stained with the rabbit primary anti-ameloblastin (Ct) antibody (6) (1:50) at 37 °C for 1 h. After washing, the slides were incubated with the secondary antibody coupled with FITC-goat anti-rabbit IgG (Jackson ImmunoResearch), 1:40 dilution, for 30 min at room temperature in the dark. Replacements of the primary antibody with Tris-buffered saline and also IgG isotypes were performed as negative controls. Sections were then washed immediately with glycerol (95% glycerol + 5% PBS) and examined with a Zeiss LSCM (model 510; Carl Zeiss, Oberkochen, Germany) equipped with a 514 Å argon laser and a 543 Å helium-neon laser.

**Binding of [3H]ATMP to Newly Secreted Ameloblastin at Tomes’ Process**—Amelogenin trityrosyl motif peptide is localized in the N-terminal region of amelogenin, which has ligand binding properties with GlcNAc, NeuAc (12), and GlcNAcmimicking peptide (Gmp) (13), cytokeratin 14 and 5 (7, 9), and ameloblastin (5). The Gmp domain is present intermittently in the polypeptide sequence of ameloblastin and has affinity for ATMP. Therefore, the following autoradiographic study was undertaken to see whether the 13-residue ATMP (P[3H]YPYSGYEP MGGW) binds to ameloblastin secreted during different postnatal days at the Tomes’ process and extracellularly. Radiolabeled ATMP was prepared from tritium gas by Amersham Biosciences, and after labeling, the material co-
Expression of Ameloblastin Isoforms during Tooth Development

chromatographed with the ATMP was synthesized by the University of Southern California Microchemical Core Laboratory. A mass spectrum is consistent with the proposed structure. The material was supplied as a water/ethanol (1:1, v/v) solution in a silanized borosilicate multidose vial with additional screw cap under nitrogen. Polypeptides were purified by high pressure liquid chromatography on a Vydac C18 300-Å (protein or peptide; 250 × 4.6 mm) column with a gradient of solution A (0.01 M aqueous trifluoroacetic acid) and solution B (0.01 M trifluoroacetic acid in acetonitrile), 0–100% B over 30 min, at a flow rate of 1.0 ml/min. The peptide was supplied in an aqueous solution. The peptides were stored in the absence of light and air at −20 or 4 °C, respectively.

Sections from mouse mandibular molar (sagittal sections, 6 μm) were used to assess the localization of [3H]ATMP during enamel formation. Sections were treated with 50 milliunits of N-acetylgalactosaminidase (Sigma) (14) to ascertain specific binding to GlcNAc-mimicking peptide domain. The sections were blocked with PBS (pH 6.0) with 1.0% HSA at 37 °C for 1 h and then incubated with [3H]ATMP for 2 h at 37 °C. After washing the slides three times with PBS (0.1% HSA, pH 6.0), the slides were dried for 30 min at room temperature. Each slide was dipped separately in emulsion fluid diluted in water at 1:1 dilution (Autoradiography Emulsions, Type NTB2 for β emitters; International Biotech Inc., Eastman Kodak Co.) in the dark for 1 min, dried, and stored at 4 °C. The autoradiographs were developed in Kodak DEKTOL Developer according to the manufacturer’s recommendations and counterstained with hematoxylin. Digital and phase-contrast microscopy was used to identify the grains on sections.

RESULTS

Immunolocalization of Ct Antibody during Different Days of Postnatal Growth of Enamel—To localize and assess the distribution of Ct-reactive fraction of ameloblastin in ameloblasts and enamel, histological sections of tooth during different postnatal days of development were immunostained with Ct antibody and FITC-conjugated secondary antibody. Sections stained with FITC-conjugated anti-rabbit IgG secondary antibody served as control. Distinct FITC fluorescence of granular structures was observed in the ameloblast cell layer and in newly formed enamel (days 0 and 1), suggesting that synthesis and secretion of ameloblastin has already commenced. The ameloblastin granules are distinctly aggregated on the walls of the ameloblasts on day 3 (Fig. 1). The clustering of the ameloblastin granules or vesicles is unique and consistent. These fluorescent granular structures are released into the enamel, as evidenced by accumulation of dense aggregates on the formative enamel. The beaded appearance of fluorescent ameloblastin is seen both in ameloblasts and in enamel on day 5 (data not shown). The specific distribution of the dense fluorescent deposits in the formative enamel is unique. The density of the granules is very high in enamel. On day 7 and, particularly, on day 9, ameloblasts showed signs of disruption and shrinkage (see also Ref. 9).

Stage-specific Expression of Ameloblastin Isoforms: Ameloblasts during Postnatal Development—One of the most critical requirements for assessing the ameloblastin profile during postnatal development was to carefully isolate the ameloblast cell layers from the newborn (day 0) through day 9 from the mouse mandibular molars, which involves removal of adherent tissues under a dissection microscope. Fig. 2A shows the isolated ameloblast layer on day 0. The dentino-enamel matrix was also isolated intact as a thin membranous matrix cup and cleaned of adherent tissues on different PN days of tooth organ (Fig. 2B).

The proteins isolated from the purified ameloblast layer on different days of postnatal development were resolved electrophoretically, Western blotted, and immunostained with affini-
Expression of Ameloblastin Isoforms during Tooth Development

Stage-specific Variation of Ameloblastin Isoforms: Extracellular Matrix at Different Postnatal Development—The proteins were extracted from the extracellular matrix of the first mandibular molars at different PN stages of development, after extracting and purifying the extracellular matrix. Fig. 4A showed the Western blots (lane 2, Fast green; lane 3, immunostaining) of the entire sequence of the recombinant ameloblastin (used as control). Fig. 4B showed the stage-specific expression of ameloblastin isoforms in enamel matrix during different postnatal stages of development. As in ameloblasts, the Ct antibody detected two intensely staining bands at 66 and 55 kDa. Day 0 (37 kDa); day 1 (37 kDa/faint 55 and 66 kDa), day 3 (faint 37 kDa/55 kDa/66 kDa), day 5 to day 9 (55 kDa/66 kDa).

Amelogenin Co-localizes with the Ameloblastin Granules in Ameloblasts, Tomes’ Process, and Enamel—Co-localization of amelogenin with ameloblastin (yellow fluorescence) can be observed in sections of molars on days 0 and 3 of the postnatal growth. The sections were stained sequentially with the fluorescent dye-conjugated antibodies (green, ameloblastin (FITC); red, amelogenin (Rhodamine Red)) and examined under laser-scanning confocal fluorescence microscopy (Fig. 5, A and B). A and B showed that the stratum intermedium, the ameloblasts, the interface between ameloblasts and odontoblasts, and the odontoblasts all stained with both anti-amelogenin and anti-ameloblastin antibodies. Since both primary antibodies were rabbit antibodies, it may be construed that the staining is due to background coloration. But we have specifically avoided non-specific staining as described under “Experimental Procedures.” The staining could be due to presence of both the proteins in other regions of the tooth organ, which is well supported in the literature (15–17). Our observations on co-localization are restricted to the yellow signals observed at the formative regions of enamel. Prior to the formation of enamel (day 0), amelogenin co-localized with ameloblastin (yellow signal) in the proximal region of the cytoplasm (Fig. 5C). Since the Western blot in Fig. 4 showed only the 37-kDa fraction on day 0 and 1, it is suggestive that the co-localization of amelogenin with ameloblastin may involve the 37-kDa fraction. On day 1, the yellow granules are distinctly observed toward the periphery at Tomes’ process. At Tomes’ process, the region of ameloblasts which the matrix proteins are released, co-localization of amelogenin and ameloblastin was observed as distinct yellow fluorescence (Fig. 5D), suggestive of their co-assembly in vivo, and justifies investigating their interaction during the develop-
Ameloblastin May Signify Inter-action—Whereas confocal microscopy indicated co-distribution and co-migration of ameloblastin and amelogenin in the formative enamel, autoradiographic studies with [3H]ATMP on GlcNAcase-treated sections provided information on localization of ATMP not only at the Tomes’ processes (Fig. 6, A and B) but also in the formative enamel (Fig. 6C), suggesting the possibility of binding of labeled ATMP with extracellular matrix proteins carrying the GMP sequence. Ameloblastin does contain GMP sequence (5). Accumulation of ameloblastin at the apical region of ameloblasts (Tomes’ process) commenced on day 1 and increased on day 3 (Fig. 6).

DISCUSSION

Production of enamel, a calcium phosphate bioceramic, on the outer surface of the tooth involves “time- and position-dependent intra- and intermolecular interactions between different gene products” (amelogenin, ameloblastin, enamelin, and tuftelin) and “the formation of an extracellular matrix microenvironment that initiates, nucleates, and then mediates growth of calcium hydroxyapatite crystals” (18). Although the enamel investigators recognize that a multistep sequence is required to produce the bioceramic and may involve time-dependent secretion of different proteins and homo- and heteromolecular assemblies to create the microenvironment for biomineralization, the molecular mechanisms that regulate the microenvironment of enamel bioceramic have not been clarified. The sequential expression of ameloblastin-isofoms adds new dimension to this aspect of enamel formation.

Fig. 7 shows the amino acid sequence of recombinant ameloblastin. A variety of anti-ameloblastin antibodies were developed against the full-length of the recombinant protein (19), or to recombinant polypeptide sequence with selected amino acid residues, 27-47 (Nt), 98-107 (M-1), 224-232 (M-2), 386-399 (M-3), 406-419 (Ct) (6), 206-378 (18), or 175-348 (20). Lee et al. (18) have extracted proteins from six regions (apical to incisal regions) of the entire tooth and immunostained with rabbit antibodies formed after immunization with the entire ameloblastin sequence 206-378 (designated as “common ameloblastin antibody”) (see Fig. 7) and with an N-terminal peptide sequence (residues 105-119 (YEYSLPVHPPLPSQ)) called “E3a antibody”). Although more than eight protein frac-

The yellow granules of varying size suggested aggregation. Fig. 5E (day 3) provides a unique profile of enamel due to a histological aberration. This enamel layer appears to have peeled off from the ameloblasts. The enamel matrix is spread as a layer showing granulation. This region uniquely exhibits granular yellow signals. In addition, the yellow granules (ameloblastin/amelogenin) are also accumulated at some regions of the periphery of the ameloblasts (Fig. 5E). On days 5, 7, and 9 at the incisal region (60%), the yellow florescent granules could not be observed (data not shown). These co-localization findings, together with Western blot studies on ameloblastin isoforms during different PN days in ameloblasts (Fig. 3) and enamel (Fig. 4B), suggest that both amelogenin and ameloblastin may be secreted simultaneously by the ameloblasts (from day 0 to 3).
Expression of Ameloblastin Isoforms during Tooth Development

The results of the present investigation are unique in that, using a C-terminal antibody, we establish that ameloblastin may not be a single entity during the course of development and implied that there may be various transcripts of ameloblastin that are developmentally expressed. We report synthesis and secretion of one particular isoform (37 kDa) devoid of the N-terminal sequence of full-length ameloblastin. The 37-kDa fraction has stained intensely in the isolated proteins of ameloblasts on days 0 and 1 and faintly on day 3 but not after day 3. This is a remarkable finding, since it qualifies the 37-kDa isoform as “early postnatal ameloblastin.” A fraction somewhat similar to that position is also observed only in the extracts of the most apical region (region 1) of the rat incisor (18). It may be argued that this fraction may be a result of protease activity that normally processes enamel proteins. However, the 37-kDa fraction is present on day 0 of PN development. The proteases were first detected on PN day 2 (enamelysin) or on PN day 3 (KLK4) of the first molar of mice (21), after the appearance of the 37-kDa fraction. Furthermore, the other isoforms (55 and 66 kDa) appear long after the appearance of 37 kDa, suggesting that the 37-kDa fraction may not be a degradation product of the full-length ameloblastin. Isolation and sequencing of the encoding cDNA may provide additional support to the above contention.

The 37-kDa isoform persists in the extracts of the extracellular matrix on all days (from PN day 0 to 9) of development, further confirming that it is incorporated as such from the ameloblasts. In extracellular matrix, it remains highly intense on day 0 and 1 but remained less intense or diffused on all other postnatal days, suggesting that what is secreted by ameloblasts as the 37-kDa fraction is retained in the enamel until day 9. The changes in the intensity could be due to the decrease in the relative proportion of the 37-kDa fraction in relation to other proteins or an effect mineralization of enamel matrix.

Very similar to the 37-kDa fraction, there are two other major isoforms (55 and 66 kDa) reacting to Ct antibody in both ameloblasts and enamel during different postnatal days of development of tooth. These two fractions are strongly comparable with 54- and 66-kDa isoforms reacting to E3a antibody (18). The major isoforms with 66 kDa have been observed by several investigators (18, 20). We were able to observe their secretion in ameloblast on day 3 onward, although the secretion would have commenced on PN day 1, since a faint Ct-reactive 66 kDa fraction is discernible in the Western blots. The intensity of both 55- and 66-kDa fractions intensified from day 3, indicating that they could be the “late postnatal ameloblastin” isoforms. They were not found in extracellular matrix on PN day 0 and 1 but observed from day 3 onward, confirming that the ameloblastin isoforms are secreted later during PN days of development. Thus, Ct antibody staining establishes two distinct species of isoforms: 1) early PN 37-kDa isoforms secreted on days 0 and 1 and 2) late PN 66- and 55-kDa isoforms secreted from day 3 onward. This observation suggests a multistep sequence in the formation of enamel. The significance of demarcation in relation to different PN days is far from clear; however, one can construe that a time-dependent secretion of ameloblastin may be related to secretion of amelogenin during formation of the enamel.

The functional capabilities of ameloblastin to interact with amelogenin were elucidated by the ligand binding domains in the peptide sequence (5). The peptide sequence also contains a putative Src homology 3 binding domain (DPVPPPLPS) (18) and an α2-integrin binding domain (DGEA) (22) in addition to fragment amelogenin-binding peptide sequence or a GlcNAc-mimicking peptide sequence (5). Our focus is to assess the possible interaction between ameloblastin isoforms and amelogenin during postnatal days, since amelogenin is expressed exclusively in secretory ameloblasts (23). LSCM revealed co-localization of amelogenin and ameloblastin in the Tomes’ process as well as in the extracellular enamel matrix on days 1 and 3. Interestingly, no co-localization was observed after day 3, when two other isoforms (55 and 66 kDa) are secreted. Co-localization with LSCM indicates a closer affinity between amelogenin and ameloblastin between days 0 and 3, suggesting a possible interaction between amelogenin and the 37-kDa fraction of ameloblastin.

Autoradiographic observations with [3H]ATMP on days 0, 1, and 3 reveal that the [3H]ATMP is localized in the Tomes’ processes as well as in the formative extracellular matrix (Fig. 6C). The presence of [3H]ATMP in cytoplasm of ameloblasts could be due to binding of ATMP with proteins located intracellularly. It may also include CK14 in addition to ameloblastin. However, the presence of [3H]ATMP at the formative enamel confirm that the co-localization may signify specific interaction between amelogenin and the ameloblastin isoforms secreted on day 0 until day 3. It may imply two steps of events in amelogenin secretion: step 1, on days 0 – 3, when amelogenin is bound to 37-kDa ameloblastin isoforms, as evidenced by autoradiographic observations; step 2, a day after day 3, when amelogenin and ameloblastin may secrete independently of one another. Peak accumulation of radiolabeled ATMP on day 3 instead of day 0 suggests that on day 0 amelogenin may have another function to perform, namely to anchor to dentin surface at the dentin-enamel junction by bonding with contents released from dentinal tubules. We have reported that the surface of dentin has a layer of keratan sulfate rich in sulfated sialic acids and sulfated GlcNAc, emanating from the dentinal tubules, and a potent ligand for amelogenin (24). A stoichiometric relationship between amelogenin and sulfated sialic acids in NMSO3 (a sulfated sialyl lipid (molecular weight: 1456.7) sodium [2,2-bis(docosyl-oxymethyl)propyl-5-acetoamido-3,5-dideoxy-4,7,8,9-tetra-O-(sodium-oxy sulfonyl)-n-glycero-n-galacto-2-nonulopyranosidosidonate]) (25) has been identified.

Interaction between ameloblastin and one or more of the ameloblastin isoforms between days 0 and 3 may be the first step in a heteromolecular interaction between two enamel proteins. Earlier, evidence was presented for heteromolecular interaction between amelogenin and ameloblastin using their recombinant versions and in vitro experiments (5). These findings add further support to the observation that the co-localization of amelogenin with ameloblastin may involve specific binding between the two proteins.
Expression of Ameloblastin Isoforms during Tooth Development

Although in vitro observations indicated that amelogenin is capable of interacting with 66-kDa ameloblastin, the presence of the 37-kDa isoform in ameloblasts and in enamel matrix during early stages of development, co-localization of the amelogenin and 37-kDa ameloblastin, and [³H]ATMP binding at the junction of ameloblasts and formative enamel on days 0–3 favor the contention that in situ, amelogenin may be associated with the 37-kDa isoform of ameloblastin. The association may involve ATMP site of amelogenin with a fragmented GMP site of ameloblastin in the 37-kDa embryonic isoform. This study establishes that heteromolecular interaction between amelogenin and other enamel proteins may commence during early postnatal stage of the development of tooth.

Subsequent secretion of amelogenin and other ameloblastin isoforms could be independent of one another to perform alternate and complementary functions for the formation of calcium phosphate bioceramics. Support for the above contention emanates from the identification of multiple consensus phosphorylation sites for various kinases (casein kinase II, protein kinase C, and cAMP-dependent protein kinase) in ameloblastin (18), suggesting that amelogenin can function as a phosphate donor for the incremental growth of calcium hydroxyapatite crystals. In contrast, amelogenin contains one covalently bound phosphate group per molecule (serine 16) (26), and there is no evidence to suggest that enamelin or tuftelins are phosphorylated (27). We concur with the view (18) that the different isoforms and other enamel proteins may commence during early postnatal stage of the development of tooth.

Acknowledgments—We are indebted to Dr. Charles F. Shuler (Chairman, Center for Craniofacial Molecular Biology, University of Southern California) for support and encouragement, to Dr. Mepru H. Ravindranath (John Wayne Cancer Institute, Santa Monica, CA) for valuable discussions, and to Pablo Bringas, Jr., and Valentino Santos for technical assistance.

REFERENCES

1. Fincham, A. G., Moradian-Oldak, J., and Simmer, J. P. (1999) J. Struct. Biol. 126, 270–299
2. Gibson, C. W., Yuan, Z. A., Hall, B., Longenecker, G., Chen, E., Thayagarajan, T., Sreenath, T., Wright, J. T., Decker, S., Piddington, R., Harrison, G., and Kulkarni, A. B. (2001) J. Biol. Chem. 276, 31871–31875
3. Fukumoto, S., Kiba, T., Hall, B., Ishara, N., Nakamura, T., Longenecker, G., and Krebsbach, P. H. (2004) J. Cell Biol. 167, 973–983
4. Yamakoshi, Y., Tanabe, T., Oida, S., Hu, C. C., Simmer, J. P., and Fukae, M. (2001) Arch. Oral. Biol. 46, 1005–1014
5. Ravindranath, H. H., Chen, L., Zeichner-David, M., Ishima, R., and Ravindranath, R. M. H. (2004) Biochem. Biophys. Res. Commun. 323, 1075–1083
6. Uchida, T., Murakami, C., Dohi, N., Wakida, K., Satoda, T., and Takahashi, O. (1997) J. Histochem. Cytochem. 45, 1329–1340
7. Ravindranath, R. M. H., Basilrose, R. R., Sr, R. M., Ravindranath, N. H., and Vaitheswaran, B. (2003) J. Biol. Chem. 278, 20293–20302
8. Lowry, O. H., Rosebrough, N. H., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
9. Ravindranath, R. M. H., Tam, W., Bringas, P., Jr., Santos, V., and Fincham, A. G. (2001) J. Biol. Chem. 276, 36586–36597
10. Negoeescu, A., Labat-Moleur, F., Lorimier, P., Lamarcq, L., Guillermot, C., Chambaz, E., and Brambilla, E. (1994) J. Histochem. Cytochem. 42, 433–437
11. Lewis Carl, S. A., Gillette-Ferguson, I., and Ferguson, D. G. (1993) J. Histochem. Cytochem. 41, 1273–1278
12. Ravindranath, R. M. H., Moradian-Oldak, J., and Fincham, A. G. (1999) J. Biol. Chem. 274, 2464–2741
13. Ravindranath, R. M. H., Tam, W., Nguyen, P., and Fincham, A. G. (2000) J. Biol. Chem. 275, 39654–39661
14. Ravindranath, R. M. H., and Graves, M. C. (1990) J. Virol. 64, 5430–5440
15. Oida, S., Nagano, T., Yamakoshi, Y., Ando, H., Yamada, M., and Fukae, M. (2002) Dent. Res. 81, 103–108
16. Iacob, S., and Veis, A. (2006) Eur. J. Oral Sci. 114, 194–200
17. Nagano, T., Oida, S., Ando, H., Gomi, K., Arai, T., and Fukae, M. (2003) J. Dent. Res. 82, 982–986
18. Lee, S. K., Kim, S. M., Lee, Y. I., Yamada, K. M., Yamada, Y., and Chi, J. G. (2003) Mol. Cells 15, 216–225
19. Fong, C. D., Hammerstrom, L., Lundmark, C., Wurtz, T., and Slaby, I. (1996) Adv. Dent. Res. 10, 195–200
20. Krebsbach, P. H., Lee, S. K., Matsuki, Y., Kozac, C., Yamada, K. M., and Yamada, Y. (1996) J. Biol. Chem. 271, 4431–4435
21. Hu, J. C., Sun, X., Zhang, C., Liu, S., Bartlett, J. D., and Simmer, J. P. (2002) Eur. J. Oral Sci. 110, 307–315
22. Xiao, G., Wang, D., Benson, M. D., Karsenty, G., and Franceschi, R. T. (1998) J. Biol. Chem. 273, 32988–32994
23. Nanci, A., Bendayan, M., and Slavkin, H. C. (1985) J. Histochem. Cytochem. 33, 1153–1160
24. Ravindranath, R. M. H., and Basilrose, R. M. (2005) Acta Histochem. 107, 43–56
25. Ravindranath, R. M. H. (2005) J. Glycoconj. J. 22, 129
26. Fincham, A. G., Moradian-Oldak, J., and Sarte, P. E. (1994) Calcif. Tissue Int. 55, 398–400
27. Deutsch, D., Palmyn, A., Fisher, L. W., Kolodny, N., Termine, J. D., and Young, M. F. (1991) J. Biol. Chem. 266, 16021–16028