The enamel protein amelogenin binds to GlcNAc (Ravindranath, R. M. H., Moradian-Oldak, R., and Fincham, A. G. (1999) J. Biol. Chem. 274, 2464–2471) and to the GlcNAc-mimicking peptide (GMP) (Ravindranath, R. M. H., Tam, W., Nguyen, P., and Fincham, A. G. (2000) J. Biol. Chem. 275, 39654–39661). The GMP motif in the N-terminal region of the cytokeratin 14 of ameloblasts binds to trityrosine motif peptide (ATMP) of amelogenin (Ravindranath, R. M. H., Tam, W., Bringas, P., Santos, V., and Fincham, A. G. (2001) J. Biol. Chem. 276, 36586–36597). K14 (Type I) pairs with K5 (Type II) in basal epithelial cells; GlcNAc-acylated K5 is identified in ameloblasts. Dosimetric analysis showed the binding affinity of amelogenin to K5 and to GlcNAc-acylated-positive control, ovalbumin. The specific binding of [3H]ATMP with K5 or ovalbumin was confirmed by Scatchard analysis. [3H]ATMP failed to bind to K5 after removal of GlcNAc. Blocking K5 with ATMP abrogates the K5-amebogenin interaction. K5 failed to bind to ATMP when the third proline was substituted with threonine, as in some cases of human X-linked amelogenesis imperfecta or when tyrosyl residues were substituted with phenylalanine. Confocal laser scan microscopic observations on ameloblasts during postnatal (PN) growth of the teeth showed that the K5-amebogenin complex migrated from the cytoplasm to the periphery (on PN day 1) and accumulated at the apical region on day 3. Secretion of amelogenin commences from day 1. K5, similar to K14, may play a role of chaperone during secretion of amelogenin. Upon secretion of amelogenin, K5 pairs with K14. Pairing of K5 and K14 commences on day 3 and ends on day 9. The pairing of K5 and K14 marks the end of secretion of amelogenin.

Ameloblasts synthesize and secrete the proteins involved in the microstructure and biomineralization of the enamel. Amelogenin constitutes 90% of the total enamel proteins secreted by ameloblasts. It is a non-glycosylated single polypeptide of about 180 amino acids (1–3). The N-terminal 45 amino acid residues are referred to as tyrosine-rich amelogenin polypeptide (TRAP). The C-terminal 13-amino acid sequence

**Amelogenin Interacts with Cytokeratin-5 in Ameloblasts during Enamel Growth**

Ameloblasts synthesize and secrete the proteins involved in the microstructure and biomineralization of the enamel. Amelogenin constitutes 90% of the total enamel proteins secreted by ameloblasts. It is a non-glycosylated single polypeptide of about 180 amino acids (1–3). The N-terminal 45 amino acid residues are referred to as tyrosine-rich amelogenin polypeptide (TRAP). The C-terminal 13-amino acid sequence

in the TRAP region called “amelogenin tyrosyl motif peptide” (ATMP: FYPSYGYEPMGGW) possesses unique ligand-binding properties, in that it binds specifically to N-acetylglycosamine (GlcNAc) (4) and GlcNAc-mimicking peptides (GMPs) (5). Mutations in the ATMP sequences in the human X-linked amelogenesis imperfecta (AI) (6). The mutated forms of ATMP failed to bind with GlcNAc or the GMP (4, 5). Loss-of-function “mutations” of ATMP correlated well with the loss of amelogenin-ligand interaction.

A GMP motif is found in the N-terminal region of Type I cytokeratin 14 (K14), a differentiation marker for ameloblasts prior to amelogenin synthesis (7). The purified or recombinant amelogenin and TRAP bind to K14 in vitro dosimetrically (8). Scatchard analysis confirms the specific interaction between K14 and ATMP in vitro. GlcNAc and GMP blocked binding of rM179 or ATMP with K14. Mutated ATMP failed to bind to K14 when the third proline was substituted with threonine, as in some cases of human X-linked AI (6) or when tyrosyl residues were substituted with phenylalanine. Amelogenin co-assembled with K14 in the perinuclear region of ameloblasts on day 0. The K14-amebogenin complex migrated to the apical region of the ameloblasts on day 1 and accumulated there between days 3 and 5 and collapsed on day 9. Autoradiography with [3H]ATMP and [3H]GMP corroborated the dissociation of amelogenin and K14 at the Tomes’ process of the ameloblast, suggesting that K14 plays a chaperone role for nascent amelogenin polypeptide during secretion of amelogenin (8).

K14 (Type I) pairs with Type II K5 in basal epithelial cells. Such pairing of Type I and Type II cytokeratins are known to occur during epithelial cell differentiation (9). K5 is also present in ameloblasts (10, 11). The pairing of K5 with K14 requires N-terminal regions of both cytokeratins (12). We hypothesize that, if the N-terminal region (GMP motif) of K14 is bound to the ATMP motif of amelogenin, it may not pair with K5 until amelogenin is disassociated from it. Furthermore, we hypothesize that K5, per se, might also bind to amelogenin, because it possesses GlcNAc residues in the N-terminal region similar to other Type II keratins (K8) (13). If K5 were to bind to amelogenin, it might define the relative role of Type I and II cytokeratins in secretion of amelogenin. If pairing of K14 and K5 were to occur in ameloblasts, it would be important to define the event in the context of amelogenin secretion and enamel formation.

We present evidence to show that K5 binds to amelogenin, leading to the secretion of amelogenin and pairing with K14. The in vitro and in situ observations, during different stages of

**mercaptopethanol; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; HSA, human serum albumin; BSA, bovine serum albumin; FITC, fluorescein isothiocyanate; TRITC, tetramethylrhodamine isothiocyanate; GD2, diisialoganglioside 2; GD3, diisialoganglioside 3; GM2, monosialoganglioside 2; GD1b, diisialoganglioside 1b.**
enamel formation, define the interactions among amelogenin and cytokeratins. The pairing of K14 with K5 does occur after secretion of amelogenin and just prior to the disintegration of cytokeratins. These findings are novel and critical, and they define the relative role of cytokeratins in the secretion of amelogenin during enamel formation. The results are important for understanding the events taking place during secretion of amelogenin, enamel formation, and the formulation of corrective measures to prevent abnormal enamel development in genetic disorders like AI and different kinds of epidermolysis bullosa caused by mutations in K14 or K5 (14–18).

EXPERIMENTAL PROCEDURES

Mice—All observations were made on Swiss Webster mice at different developmental stages ranging from newborn day “0” through postnatal days (PN) 1, 3, 5, 7, and 9. A total of 25 normal, healthy, female pregnant Swiss Webster mice (Charles River Breeding) were used to obtain a sufficient number of litters as was carried out previously (8). The Institutional Animal Care and Use Committee (Los Angeles, CA) approved all protocols involving mice.

Isolation and Purification of K5—We have isolated and purified K5 from HeLa cells (19). We have obtained HeLa cells (CCL-2 obtained from ATCC) and have grown them in Eagle’s minimum essential medium (MEM) supplemented with fetal bovine serum (10%) and antibiotics at 37 °C until further use. Equivalent amounts of HeLa cells were used for each experiment. The membrane proteins were isolated using a Microcon concentrator with a cut-off size of 10,000 Da. The proteins from the supernatants were collected. The proteins from the supernatants from cells that were homogenized with approximately three volumes of ice-cold Tris-Triton buffer (25 mM Tris–HCl, pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonil fluoride). The keratin fraction was obtained from cells that were homogenized in equal volumes of ice-cold Tris-Triton buffer (25 mM Tris–HCl, pH 7.4, 0.5% Triton X-100, 1 mM EDTA, 1 mM phenylmethylsulfonil fluoride). The extract was centrifuged at 10,000 × g for 30 min. The final pellet was solubilized in about 1.5 volumes of buffered urea-mercaptoethanol (BME) solution (25 mM Tris–HCl, pH 7.4, 9 mM urea, 1 mM EDTA, 100 mM BME, 1 mM phenylmethylsulfonil fluoride). The extracted proteins were purified by the USC Microchemical Core Laboratory, using a reversed-phase high performance liquid chromatograph (C18 analytical column with a gradient of 0–100% B in 45 min; buffer B contained 70% (v/v) aqueous acetonitrile, 0.08% trifluoroacetic acid; buffer A contained 0.1% trifluoroacetic acid). Protein concentrations were determined by Lowry et al. (23), and purity and homogeneity were assessed by affinity-purified monoclonal antibody for K5. The antibodies used included mouse anti-K5 (1:1000) (Chemicon International, Temecula, CA) and sheep monoclonal K5 antibody (1:100) (Binding Site, Birmingham, UK). The availability of a source of purified K5 allowed us to study the binding properties of this molecule with amelogenins.

Protein Preparations from Ameloblasts—We have isolated ameloblasts from mouse postnatal mandibular first molars at different developmental stages ranging from newborn day “0” through postnatal days (PN) 1, 3, 5, 7, and 9. Isolated ameloblasts were pooled, frozen, and thawed (four cycles) to extract the proteins as described with modifications mentioned previously (4, 8).

Preparation of Synthetic Peptides—All the polypeptides (ATMP, T-ATMP, and F-ATMP) used in this study were synthesized by the University of Southern California Microchemical Core Laboratory using an Applied Biosystems model 430A single-column peptide synthesizer with the modified Merrifield procedure (24). Peptides were purified as mentioned previously (4, 8).

1H Labeling of ATMP—The 13-residue ATMP [P1]HYPYSGYE-

PMGGW was prepared and purified by Amersham Biosciences as mentioned previously (8).

Western Blot Analysis—K5 purified from HeLa cells isolated from ameloblast extracts during different developmental days (newborn to PN day 9) were resolved by gradient SDS-PAGE (4–12%) and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P Transfer Membrane, Millipore Corp.) at 100 mA for 35 min using a semi-dry transblot apparatus (Bio-Rad Scientific Instruments) (5, 8). Protein transfer was assessed by staining the strips with 0.1% Fast Green (Sigma) in 40% methanol and 10% acetic acid (5, 8). The membranes were washed (five times) and blocked with PBS (pH 7.2) containing ~1% HSA, and were overlaid with appropriate antibodies. The antibodies used included mouse anti-K5 (1:1000, Chemicon International) and sheep monoclonal K5 antibody (1:100). The Western blots also were treated with monocular antibodies common for both N-linked and O-linked (IgM antibody, 1:100, Calbiochem) GlcNAc and with monoclonal antibody specific for O-linked GlcNAc (IgG1, Alexis Biochemicals, 1:1000). Enzyme-linked secondary antibody from Roche Diagnostics (BM Chemiluminescence Western-blotting kit, 1:2000) was used. The use of GlcNAc in proteins was also confirmed by staining the Western blots with Datura stramonium lectin (8).

Binding of Amelogenin to Varying Concentrations of K5 by Enzyme-linked Immunosorbent Assay—enzyme-linked immunosorbent assay was performed using the purified K5 as an antigen following the protocol described previously (8). Antigen coating was done by adding 100 μl of a serial dilution of proteins at 37 °C. The coated antigen-coated plates were blocked (50°C) to micorteret plates (Falcon 3915, Fisher Scientific) and incubating the plates at 4 °C overnight. Wells were blocked with PBS containing 1% HSA for 90 min at 37 °C. One-hundred microliters of a known amount of amelogenin protein (rM179, 5 pmol/100 μl) was added to wells, and the mixture was incubated for 1 h at 37 °C. After washing the plates five times, the primary antibody against the rM179 protein (8) (at a dilution of 1:6000) (5) was added, and the mixture was incubated for 1 h at 37 °C and then incubated with a secondary antibody (1:5000, goat anti-rabbit IgG, Jackson Immunoresearch, West Grove, PA) for 1 h. After washing, a substrate (1,2-O-phenylenediamine dihydrochloride, In-vitrogen) in citrate-phosphate buffer and hydrogen peroxidase was added. The strips were developed manually. To assess whether [3H]ATMP binds GlcNAc residues of K5, we treated blots of K5 on PVDF membranes, after blocking with PBS, 1% HSA for 1 h at 37 °C, N-acetylglucosaminidase recombiant from Escherichia coli (A8605, Sigma). 100 μl (5 units) of the enzyme suspension (in PBS, pH 7.0) was overlaid on two strips of membranes. Control strips were overlaid with blocking buffer. A thin strip of PVDF was placed over the beginning of the control and experimental strips. The strips were washed four times with PBS, and were overlaid with appropriate antibod-

Expression and Purification of Recombinant Proteins—Preparation and purification of recombinant mouse amelogenin rM179 were carried out as previously described (4, 22).

Preparation and Purification of Synthetic Peptides—All the polypeptides (ATMP, T-ATMP, and F-ATMP) used in this study were synthesized by the University of Southern California Microchemical Core Laboratory using an Applied Biosystems model 430A single-column peptide synthesizer with the modified Merrifield procedure (24). Peptides were purified as mentioned previously (4, 8).

1H Labeling of ATMP—The 13-residue ATMP [P1]HYPYSGYE-

PMGGW was prepared and purified by Amersham Biosciences as mentioned previously (8).

Dosimetric Binding of K5 to [3H]ATMP—Atmospheric K5 is approximately 10 μl of [3H]ATMP (30 × 105 dpm in Tris-buffered saline, pH 7.2) was added to 1.5-mL polypropylene microcentrifuge tubes containing increasing amounts of K5 in 0.1 M glycine–NaOH, pH 10. The mixture was gently shaken at 37 °C as described previously (5, 8). Ovalbumin was used as positive control and BSA as a negative control.

Specific Binding of K5 to [3H]ATMP as a Function of Increasing Concentration of ATMP—The total binding of labeled ATMP to K5 (333
pmol) was determined using increasing concentrations of [3H]ATMP (2–200 pmol). The nonspecific binding of labeled ATMP was determined in the presence of 40 nmol of unlabeled ATMP at 37 °C for 2 h and was subtracted from the total binding to obtain the specific binding. Ovalbumin was used as positive control. The total binding of [3H]ATMP to ovalbumin (700 pmol) was determined using increasing concentration of the labeled ATMP (0.5–250 pmol). The Scatchard plot further analyzed the specific binding as described previously (8). Slope ($y$), $B_{max}$, $r$, or $r^2$, and the levels of significance of the slopes are presented.

**Loss-of-function Mutations of ATMP Result in Loss of Binding to K5 as Assessed by Western Blot Analysis**—rM179 on SDS-PAGE were electrotransferred to PVDF membranes at 100 mA for 35 min using a semi-dry transblot apparatus. Protein transfer was assessed as described previously. After blocking the membrane with 1% HSA in PBS for 1 h at 37 °C and washing, the membranes were overlayed with K5 alone or K5 preincubated (for 1 h) with ATMP or K5 preincubated with T-ATMP or F-ATMP. The strips were immunostained with affinity-purified murine monoclonal antibody. [3H]ATMP bound to K5 as well as to ovalbumin, showing the respective positions of the proteins.

**Morphometry of Enamel and Ameloblast during Development**—The serial sections (6/H9262 M) were obtained from paraffin-embedded mouse mandibular incisors on different postnatal days and stained with Mallory. The width of enamel and length of ameloblasts were measured at the 20%, 40%, 60%, and 80% levels from the base of the incisor using the software program Image-Pro Plus 4.0 (Media Cybernetics). At each level, the mean width and length were obtained after measuring nine sections (three incisors from three mice).

**Immunochemical Localization of K5 in Ameloblasts**—For immunochemical analyses, sagittal sections of mandibular molar tissues (6 μm)
were mounted on Histostik-coated slides (Accurate Chemical and Scientific Corp., Westbury, NY). Details of immunolocalization methodology are the same as described previously (8). Tissue sections were stained with primary sheep monoclonal antibody for K5 (IgG specific for the first 16 sequences at N-terminal (Binding Site) diluted in phosphate-buffered saline (PBS, pH 7.2) (1:50) 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 peroxidase-conjugated secondary antibody (donkey anti-sheep IgG) diluted in PBS pH 7.2 (1:1000). IgG isotype 1 was used as a negative control. The sections were counterstained with hematoxylin, and images were examined under digital microscopy.

**Colocalization of Amelogenins, K5 and K14, with Laser Scanning Confocal Fluorescence Microscopy in Ameloblasts during Enamel Formation**—To examine the spatial distribution and colocalization of K14, K5, and amelogenin, sagittal sections of mouse postnatal mandibular

**FIG. 3.** Loss-of-function mutations of ATMP results in loss of binding to K5 as assessed by Western blot analysis. Lane 1, standard; lane 2 reveals the gel showing the position of rM179 as stained with Coomassie Blue; lanes 3–5 were immunostained with affinity-purified murine monoclonal antibody for K5 specific for the N-terminal, first 16 amino acids. Lane 3, K5 preincubated with ATMP did not stain; lane 4, K5 preincubated with T-ATMP reacted with rM179 and stained; lane 5, K5 preincubated with F-ATMP reacted with rM179 and stained with anti-K5 monoclonal antibody.

**FIG. 4.** Dynamics of growth of enamel and ameloblast during different postnatal days. The serial cross-sections (6 μm) from the whole length of mouse mandibular incisors (postnatal days 0, 1, 3, 5, 7, and 9) were stained with Mallory, and the width of the enamel and the height of ameloblasts were measured at 20%, 40%, 60%, and 80% levels from the posterior (base or cervical) to incisal (tip) of the incisor using the software program Image-Pro Plus 4.0 (Media Cybernetics). Observations were made on nine sections/percent level obtained from three mice for each time point of development. The distance between the base of the tooth or (cervical) and the tip of the tooth (incisal) was divided into 100 units. The x-axis shows the percentage of the distance. The y-axis refers to the height of ameloblasts or the width of enamel (microns). Vertical and horizontal arrows refer to the direction of growth. The inverted arrows refer to condensation.
molars at different developmental stages were prepared as described previously (8). The sections were deparaffinized and rehydrated, and endogenous peroxidase activity was blocked with 3% H2O2. The sections were stained with primary antibody against K14 (affinity-purified murine monoclonal antibody specifically recognizing the 14 amino acid sequences of C-terminal sequences of K14). The sections were incubated with fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat anti-mouse IgG, Jackson ImmunoResearch), 1:40 dilution, for 30 min at room temperature. The sections were sequentially stained with the primary antibody against recombinant mouse amelogenin (1:500) for 1 h and then incubated with the secondary antibody coupled with tetramethylrhodamine isothiocyanate (TRITC) to goat anti-rabbit IgG (Jackson ImmunoResearch), 1:40 dilution for 30 min at room temperature. The sections also were stained sequentially with the primary affinity purified human anti-sheep K5 antibody at 1:100 dilution (Binding Site) for 1 h at 37 °C. Rabbit anti sheep IgG coupled with Cy5 (1:500 dilution) (Jackson ImmunoResearch) were used as a secondary antibody for 30 min at room temperature. Replacing the primary antibody with TBS and IgG isotypes performed negative controls. Sections then were washed and mounted immediately with glycerol (95% glycerol with 5% phosphate buffer as mounting media). The sections also were stained sequentially with the primary antibody against recombinant mouse amelogenin (1:500) for 1 h and then incubated with the secondary antibody coupled with tetramethylrhodamine isothiocyanate (TRITC) to goat anti-rabbit IgG (Jackson ImmunoResearch), 1:40 dilution for 30 min at room temperature. Replacing the primary antibody with TBS and IgG isotypes performed negative controls. Sections then were washed and mounted immediately with glycerol (95% glycerol with 5% phosphate buffer as mounting media).

RESULTS

Native amelogenin (M180, protein with 180 amino acids) differs from recombinant amelogenin (rM179, 20.16 kDa) in the presence of the N-terminal methionine and phosphoserine-16. Earlier, we observed that the Amelogenin Trityrosyl Motif Peptide (ATMP: PYPSYGYEPMGGW), localized in the C-terminal region of TRAP, binds to GlcNAc, ovalbumin, a glycoprotein containing terminal GlcNAc (4), GlcNAc-mimicking peptides (GMP) (5) and K14, a GMP-carrier protein found in ameloblasts (8). K14 (Type I cytokeratin) is known to pair with K5 (Type II) in basal epithelial cells (9). K5 is also present in ameloblasts (10, 11).

rM179 Interacts with K5 More Avidly Than with Ovalbumin—Solid matrix immunoassay analysis of the interactions between rM179 and K5 or ovalbumin showed dosimetric binding of rM179 with K5 and ovalbumin (Fig. 1A), but no dose-dependent binding was observed with BSA. Although 0.75 pmol of K5 showed 50% binding with rM179, ovalbumin required 2.1 pmol for 50% binding with rM179 (Fig. 1A).

Specific Binding of [3H]ATMP to K5—The purity and homogeneity of ATMP was determined by high performance liquid
chromatography, and a typical profile of the purified fraction was illustrated previously (8). The dosimetry of K5 and ovalbumin binding to \[^{3}H\]ATMP was compared (Fig. 1B). The radio-labeled ATMP bound to Western blots of K5 and ovalbumin (inset, Fig. 1B); however, the binding is abrogated if Western blots of K5 or ovalbumin were pretreated with \(N\)-acetyl-\(D\)-glucosaminidase indicating that the ATMP motif of amelogenin specifically recognizes GlcNAc residues on K5. The specific binding of \[^{3}H\]ATMP to K5 and ovalbumin as a function of increasing concentration of ATMP was evaluated (Fig. 2, A and B). The nonspecific binding was measured with unlabeled ATMP and subtracted from the total binding to obtain the specific ATMP-K5 interaction. A Scatchard analysis of the binding was carried out. The results indicated that the peptide-binding site is homogenous with respect to the association constant (inset, Fig. 2, A and B). The slope \((y)\) differed between K5 \((0.00401 - 0.01432x)\) and ovalbumin \((0.00928 - 0.04928x)\). \(B_{\text{max}}\) of K5 is 0.280 and that of ovalbumin is 0.188.

ATMP, But Not Mutations of ATMP, Bind to K5—Direct binding of \[^{3}H\]ATMP on a purified fraction of K5 and ovalbumin are documented by Western blot analysis in Fig. 1B (inset). The binding of K5 to rM179 was assessed in Western blots before and after preincubating K5 with ATMP or loss-of-function mutations of ATMP. In one of the two mutant ATMP peptides (T-ATMP), the third proline is substituted with threonine (the T-ATMP mutation has been found in some cases of human X-linked AI, and in the other (F-ATMP), all three tyrosyl residues are replaced by phenylalanine. The binding of K5 to rM179 is abrogated after pretreatment with ATMP but not after pretreatment of K5 with T-ATMP or F-ATMP, suggesting that mutated ATMP is not capable of binding to K5 as does ATMP (Fig. 3).

K5 Distribution in Ameloblasts during Enamel Growth—To determine K5-amelogenin interaction in situ during development, morphometry of ameloblasts, enamel growth, and the distribution and migration of K5 in ameloblasts were studied. Enamel growth was quantified by measuring the width of enamel in cross-sections of mouse incisors from day 0 to day 9. The height of the tooth is distinguished into four zones from the base of the tooth as 20%, 40%, 60%, and 80%. Fig. 4 shows that maximum enamel growth occurs between day 3 and day 5 (solid circles). On day 3 the length of enamel at incisal (60%) is maximum enamel growth occurs between day 3 and day 5 (open circles). The length of the ameloblast (Fig. 4, open circles) increases from cervical to incisal on days 0 and 1. It tends to decline at the incisal end but increases at the cervical end (base) from 45 \(\mu\)m (day 1) to almost 100 \(\mu\)m (day 9). The ameloblast morphometry revealed that the growth of ameloblasts is maximal in the cervical region (10–30%) from days 5 to 9, whereas the growth is maximum in the middle region (40–60%) from days 3 to 5. These findings define the critical site and temporal sequence of phases of development of mice incisor to study the interaction between amelogenin with K5, the GlcNAc-carrier protein.

Immunolocalization of K5 during Enamel Growth—During the corresponding postnatal days of development of teeth, immunolocalization of K5 in ameloblasts of molars was carried out using an affinity-purified monoclonal antibody specific for the N-terminal, first 16 amino acid residues, of K5. Fig. 5 shows distribution of K5 in the cytoplasm at the cervical (Fig. 5, panel 1) and mid-regions (Fig. 5, panel 2) on day 0. The immunostaining was weak at the cervical region, and the positive immunostaining was scattered. However, the increase in the staining intensity at the mid-regions indicates commencement of accumulation, and the immunostaining for K5 increased in intensity, indicative of accumulation, at the apical region of the cytoplasm adjacent to the extracellular matrix on day 1 (Fig. 5, panel 3). Cytoplasmic distribution of K5 and accumulation of K5 at the apical end is evident (Fig. 5, panel 4). K5 accumulated at the apical end and, particularly, at the Tomes’ process on day 5 (Fig. 5, panel 5) and day 7 (Fig. 5, panels 6 and 7). K5 staining reached maximum at the Tomes’ process on day 7, when the enamel has commenced shrinkage. On day 9 (Fig. 5, panel 8), K5 immunostaining is low and poor, when ameloblasts commenced losing their integrity.

Immunolocalization of K5 in the Western Blots of the Ameloblast Extracts during Enamel Growth—Using equivalent amounts of proteins isolated from ameloblasts, we have analyzed the profile of the K5 expression. The Western blot analysis showed a distinct band stained with K5 affinity-purified monoclonal antibody. The intensity of staining increased gradually until day 7 and then decreased on day 9. The presence of GlcNAc residues in K5 was detected in the Western blots of the protein isolated from ameloblasts obtained from day 0 through day 7 PN. Two different monoclonal antibodies, specific for N- and O-linked GlcNAc (107914 IgM, Calbiochem) (I) and specific for O-linked GlcNAc (mAb RL2, IgG1, Alexis Co.) (II), were used. Note the protein with molecular mass of 58 kDa (K5) is O-GlcNAc-acetylated. There are other proteins (see I) that show reactivity with the monoclonal staining for both N- and O-linked GlcNAc.

**Fig. 6. Immunolocalization of K5 in the Western blots of the ameloblast extracts during enamel growth.** A, using equivalent amount of proteins isolated from ameloblasts on different postnatal days of development of teeth. The Western blot analysis showed a distinct band stained with K5 affinity-purified monoclonal antibody. The intensity of staining increased gradually until day 7 and then decreased on day 9. B, the presence of GlcNAc residues in K5 was detected in the Western blots of the protein isolated from ameloblasts obtained from day 0 through day 7 PN. Two different monoclonal antibodies, specific for N- and O-linked GlcNAc (107914 IgM, Calbiochem) (I) and specific for O-linked GlcNAc (mAb RL2, IgG1, Alexis Co.) (II), were used. Note the protein with molecular mass of 58 kDa (K5) is O-GlcNAc-acetylated. There are other proteins (see I) that show reactivity with the monoclonal staining for both N- and O-linked GlcNAc. Fractions other than 58 kDa do not stain with mAb RL2 specific for O-GlcNAc. In addition, the staining of the 58-kDa band is reduced on day 7, although the intensity of anti-K5 staining increased on day 7 (A).
codes are indicated in Fig. 7. Prior to secretion of enamel (day 0), K5 co-localized with amelogenin (purple signal) in the cytoplasm (data not shown). On day 1, the purple granules migrated toward the periphery (Fig. 8D). The granules of varying size suggested aggregation. Yellow signals (K14+/amelogenin+) were also observed at the periphery. For further details on yellow signals, see previous publication (5). On day 3, triple signals, purple (K5+/amelogenin+), turquoise blue (K5+/K14+ pairs), and white (K14+/K5+/amelogenin+) were observed at the periphery (Fig. 8F). Two distinct layers of signals, an upper layer of dense purple and a lower layer of intermittent white and turquoise blue, were observed at the incisal region (60%) (Fig. 8H). On day 5, turquoise blue signals were noted in the Tomes’ processes of ameloblasts at the incisal region (60%), the terminal of which showed the purple signal (Fig. 9D). The turquoise blue signal indicates the pairing of K14 and K5 (Fig. 9D). The red signals are seen in the extracellular matrix adjacent to Tomes’ process. During shrinkage of the enamel on days 7 and 9 at the incisal region (60%), the turquoise blue signal faded (Fig. 9H). A layer of pink vesicles was seen on day 9 (Fig. 9H) in the apical region of the ameloblasts indicative of disintegration. These co-localization studies, together with our earlier confocal observation (5), suggest that both K5 and K14 occur in ameloblasts (from day 0 to 7), interact with amelogenin, transport amelogenin independently to the Tomes’ process, pair (turquoise blue signal) after releasing amelogenins, and may disintegrate by day 9. The presence of membrane vesicles (Fig. 9H) in the ameloblasts (light pink upward arrows) suggests the commencement of cell death.

DISCUSSION

Properties of the N-terminal “Head” Region of K5—Expression of cytokeratin is specific for each epithelial cell type and its state of differentiation (26). Earlier, we reported the dynamics of K14 during ameloblast growth and differentiation during different postnatal days of mice (8). In ameloblasts, K14, a member of the family of acidic-Type I cytokeratin (51.6 kDa, isoelectric pH 5.3), co-exists with Type II keratin K5 (58 kDa, isoelectric pH 7.4). Similar to K14, K5 consists of a conserved rod domain with four α-helical regions separated by short nonhelical globular sequences commonly referred to as head (N-terminal) and tail (C-terminal) domains (27). Similar to K8, another Type II keratin, K5, expresses GlcNAc at the N-terminal region. Our observations with specific murine monoclonal antibodies for O- and N-linked GlcNAc residues reveal that GlcNAc acylation of K5 may involve Ser/Thr residues as in K8. These residues at the N-terminal region are proximal to proline and/or valine (27, 28). Removal of GlcNAc residues from purified K5 abrogates binding of the [3H]ATMP motif of amelogenin.

Amelogenin Binds to K5 and Ovalbumin, a Glycoprotein with Terminal GlcNAc—Recombinant amelogenin (rM179) binds to both K5 and ovalbumin, another glycoprotein containing two residues of N-linked GlcNAc and one residue of O-linked GlcNAc (25). The dosimetric interaction between K5 and amelogenin differs from that of ovalbumin and amelogenin. Although ovalbumin required 2.1 pmol for 50% binding with rM179, about 0.75 pmol of K5 is sufficient to obtain 50% binding with rM179 (Fig. 1A), suggesting that amelogenin may have at least a 3-fold higher binding affinity for K5. A comparison of the affinity constants (slope) and $B_{max}$ values of [3H]ATMP-bound K5 (0.280 pmol) and ovalbumin (0.188 pmol) (Fig. 2, A and B) indicate that the binding constant of ATMP to K5 is 1.5-fold greater than to ovalbumin. The $B_{max}$ of ATMP to K14 (10.180 pmol) (based on Ref. 8) is about 36-fold greater than that to K5. Our observations of the specific affinity and binding of ATMP with ovalbumin caution against treating sections with ovalbumin prior to immunostaining for amelogenin. In a number of ultrastructural immunostaining studies involving amelogenin in ameloblasts (29, 30), the thin sections were pretreated with 1–4% ovalbumin prior to immunostaining with anti-amelogenin polyclonal antibodies. Because ovalbumin is a GlcNAc carrier with a molecular size twice that of amelogenin, binding...
of ovalbumin to amelogenin in sections may create a stearic hindrance to antibody binding, even if the epitope recognized by the antibodies is found at the C-terminal end of amelogenin. Therefore, the electron microscopic observations of the presence of electron dense granules in the vesicles with anti-ame
genin polyclonal antibody on the ovalbumin-treated section may not indicate the vesicular localization of amelogenin. It is possible that these granules reacting to the anti-ame
genin polyclonal antibodies could be due to other enamel proteins, such as enamelin, for it is known that the antibodies produced against amelogenin cross-react with other enamel proteins such as enamelin (29, 31–33). In support, the poly
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The growth of ameloblasts also varies between the cervical and incisal ends. The length of ameloblasts, which may signify the pronounced synthetic activity between mid-region and the incisal region is longer than that of the cervical region during the early days of postnatal development. During the later half, the length of the ameloblasts in the cervical regions is longer than that of the incisal region. Concomitant with the growth of ameloblasts from the cervical to the incisal end (Fig. 5, panels 1 and 2), K5 moves from cytoplasm to the apical end of the ameloblast. Co-localization of K5 and amelogenin was evident by the purple signal or pixels in confocal microscopy (Figs. 8 and 9). The absence of red signals at dual wavelengths on days 0 and 1 in the cytoplasm suggests either the absence of free amelogenin or the masking of amelogenin by cytokeratins. Migration of purple signals on day 1 to the periphery and the presence of both yellow (green plus red) and purple (blue plus red) signals at the apical region of the ameloblasts suggest co-migration of amelogenin and K5 and amelogenin and K14 independently from the sites of synthesis to the Tomes’ process. Different colors of pixels point out the possible events that may occur at the Tomes’ process. Red pixels are seen outside the Tomes’ process, suggesting release of amelogenin. Earlier (8), we observed specific accumulation of [3H]GMp in the apical region of ameloblasts, suggesting that the ATMP site of amelogenin is free (unoccupied by K5 or K14) at the site of secretion in the Tomes’ process. Furthermore, when purple pixels accumulate at the apical end of the ameloblasts, white and turquoise blue pixels appear indicative of co-assembly of all three proteins (amelogenin, K5, and K14) and the pairing of K5 and K14, respectively. The temporal sequence of pairing may differ with different epithelial tissues. In ameloblasts, the pairing (turquoise blue pixels) was first observed on day 3 and increased on day 7 and disappeared thereafter. Because pairing occurred after secretion of amelogenin, we infer that the pairing of K5 and K14 signals the end of a major function of ameloblasts, namely completion of secretion of amelogenin. Because pairing of K5 and K14 requires N-terminal regions of both cytokeratins (12), it is not surprising that it occurred after the release of amelogenin. Following pairings of K5 and K14 on day 9, cytoplasmic vacuolization and formation of membrane vesicles were observed in ameloblasts suggestive of its degradation or programmed cell death.

**Summary of the Events Occurring at the Tomes’ Process**—Present findings on K5 and amelogenin should be viewed in the light of the previous observations made on co-localization of K14 and amelogenin. Amelogenin has a GlcNAc-binding motif. The motif is localized in the N-terminal region of the amelogenin, namely in tyrosine-rich amelogenin polypeptide. The ATMP motif binds to GlcNAc and to a peptide-mimicking GlcNAc (GMp). Although the GMp motif is seen in Type I cytokeratin K14, GlcNAc residues are found in K5, a Type II cytokeratin. The significance of the presence of GlcNAc in K5 and the GMp motif in K14 would not have come to light but for their interaction with the ATMP domain of amelogenin. Interaction of cytokeratins with other proteins is not uncommon (see Table II in Ref. 8). The presence of GMp and GlcNAc in the N-terminal region of K14 and K5, respectively, and the presence of ATMP in the N-terminal region of amelogenin, suggest that there is an N-terminal-N-terminal interaction between cytokeratins and amelogenins. There may be several GlcNAc-containing proteins within the cytoplasm. O-GlcNAc-acylated proteins similar to K5 also are found within cytoplasm, which may include transcription factors, kinases and phosphatases, nuclear proteins, and oncogene...
and tumor-suppressor products (34). Recently, a unique O-GlcNAc-transferase, which is localized in the cytosol and nucleus and is involved in the glycosylation of the above-mentioned proteins, was cloned independently (35, 36). Amelogenin may or may not bind to these and other GlcNAc-acylated proteins in the cytoplasm for the following reasons: 1) A membrane may physically separate the GlcNAc-acylated protein from amelogenin; 2) The binding motif on amelogenin may not be accessible due to its location and orientation; 3) The orientation of the GlcNAc may not be accessible due to differences in the linkages of GlcNAc (O- or N-linked) or the presence of other sugars in the glycoconjugates. For example, the monospecific monoclonal antibodies that bind to the disialyl residues of gangliosides GD3 (Mel-1) or GD2 (14.2Ga) do not recognize a closely related chain of sugars in the other gangliosides, such as GM2, GM3, or GD1b (37). Therefore, there are several ways by which amelogenin may be prevented from binding to GlcNAc in the microenvironment within ameloblasts. However, co-assembly of amelogenin with K5 (purple pixels) and K14 (yellow pixels) suggests the interaction between amelogenin and cytokeratins. Probably, cytokeratin binding to a nascent amelogenin polypeptide could be a mechanism to prevent interaction of amelogenin with other cytosolic and nuclear proteins carrying O-GlcNAc. The co-migration of yellow pixels (5) and purple pixels (this study) toward the Tomes’ process defines the role of the cytokeratin as chaperones, similar to heat-shock proteins (38). The role of K5 during amelogenin secretion is similar to that of K14, as presented earlier (8). Most importantly, cytokeratin pairing only in the Tomes’ process marks the release of amelogenin. Because the N-terminal regions of both K5 and K14 are free after release of amelogenin, pairing is feasible. The pairing event commences on day 3 and ends by day 9. The factors promoting dissociation of K5 or K14 from amelogenin and disintegration of the cytokeratin pairs on day 9 deserves investigation. It is known that some of the native amelogenins are phosphorylated at serine 16. Studies made on other cytokeratin-associated proteins indicate that phosphorylation may dissociate proteins from their cytokeratins. It remains unclear whether amelogenin phosphorylation contributes to dissociation of amelogenin-K5 or amelogenin-K14 couples prior to amelogenin secretion. A study of the enzymes involved in serine phosphorylation in the Tomes’ process requires attention.

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Amelogenin Interacts with Cytokeratin-5 in Ameloblasts during Enamel Growth
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