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Conservation and Variation in Enamel Protein Distribution During Vertebrate Tooth Development

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ABSTRACT Vertebrate enamel formation is a unique synthesis of the function of highly specialized enamel proteins and their effect on the growth and organization of apatite crystals. Among tetrapods, the physical structure of enamel is highly conserved, while there is a greater variety of enameloid tooth coverings in fish. In the present study, we postulated that in enamel microstructures of similar organization, the principle components of the enamel protein matrix would have to be highly conserved. In order to identify the enamel proteins that might be most highly conserved and thus potentially most essential to the process of mammalian enamel formation, we used immunoscreening with enamel protein antibodies as a means to assay for degrees of homology to mammalian enamel proteins. Enamel preparations from mouse, gecko, frog, lungfish, and shark were screened with mammalian enamel protein antibodies, including amelogenin, enamelin, tuftelin, MMP20, and EMSP1. Our results demonstrated that amelogenin was the most highly conserved enamel protein associated with the enamel organ, enamelin featured a distinct presence in shark enameloid but was also present in the enamel organ of other species, while the other enamel proteins, tuftelin, MMP20, and EMSP1, were detected in both in the enamel organ and in other tissues of all species investigated. We thus conclude that the investigated enamel proteins, amelogenin, enamelin, tuftelin, MMP20, and EMSP1, were highly conserved in a variety of vertebrate species. We speculate that there might be a unique correlation between amelogenin-rich tetrapod and lungfish enamel with long and parallel crystals and enamelin-rich basal vertebrate enameloid with diverse patterns of crystal organization. *J. Exp. Zool. (Mol. Dev. Evol.) 294:91–106, 2002.*

Many biominerals such as enamel, dentin, or bone grow within a preformed protein matrix that determines the dimension and shape of the inorganic mineral component (Lowenstam, '81; Heuer et al., '92). This protein matrix organizes into subunit compartments, which determine the dimension and orientation of organizing mineral

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crystals (Heuer et al., '92). Among vertebrate biominerals, there are fundamental differences between the collagenous protein matrix that contributes to the assembly of bone, cementum, and dentin crystallites and the noncollagenous protein matrix that is associated with the formation of enamel crystals (Lowenstam, '81). In tooth enamel, a highly organized matrix of enamel proteins is closely associated with the formation of enamel hydroxyapatite crystals (Diekwisch et al., '93).

In many vertebrates, the basic organization of the tooth enamel mineral phase is remarkably similar and includes long and parallel-organized hydroxyapatite crystals organized into enamel prisms (Slavkin and Diekwisch, '96). Even though one would expect similarities in crystal organization to go along with similarities in protein composition and organization, as of yet there is no proof for this assumption. With the exception of isolated amelogenin sequences from reptilian and amphibian teeth, little is known about the enamel protein composition of nonmammalian vertebrates (Ishiyama et al., '98; Toyosawa et al., '98; Sire, in press). In mammals, however, a number of novel tooth enamel proteins have been discovered recently, including ameloblastin, enamelin, tuftelin, and enamel proteases (Deutsch, '89; Krebsbach et al., '96; Bartlett et al., '96; Hu et al., '97; Simmer et al., '98). Although some of these novel enamel proteins have also been localized in tissues outside of teeth, they might be of relevance to the mechanisms of enamel formation (Deutsch et al., '91; Zeichner-David et al., '95, '97; MacDougall et al., '98). While the nonamelogenin enamel proteins amount to 10% of the enamel protein matrix, the major protein component (90%) of the mammalian enamel protein matrix is amelogenin (Termine et al., '80a, b), a protein that is believed to be of significant functional relevance for all stages of enamel formation (Simmer and Fincham, '95; Diekwisch, '98).

A number of studies have shown amelogenin protein localization in ameloblasts and in the enamel layer of all vertebrate classes (Herold et al., '80; Slavkin et al., '82, '83; Slavkin and Diekwisch, '96, '97). Subsequently, several authors have published on the immunohistochemical localization of amelogenins in agnathans, fish, and urodeles, including hagfish agnathan teeth (Slavkin and Diekwisch, '96, '97), Calamoichthys actinopterygian scales (Zylberberg et al., '97), Lepisosteus actinopterygian teeth (Ishiyama et al., '99), lungfish sarcopterygian teeth (Satchell et al., 2000), and Triturus urodelian teeth (Kogaya, '99). A related finding of amelogenin exon 4 conservation in hagfish agnathan vertebrates based on RT-PCR amplification (Slavkin and Diekwisch, '96, '97) might be considered questionable at this point because exon 4 is the least conserved of all amelogenin epitopes (Girondot et al., '98). However, recent advances in the cloning and sequencing of nonmammalian enamel genes (Ishiyama et al., '98; Toyosawa et al., '98) have strongly supported the case of a wide evolutionary conservation of amelogenin proteins.

The similarity of enamel crystal structure in many vertebrates and the conservation of amelogenins in many vertebrates might suggest that the protein matrix composition and organization would be similar and consequently enamel proteins were highly conserved between vertebrate classes. In order to test this hypothesis, we have decided to determine the presence and localization of the enamel proteins, amelogenin, enamelin and tuftelin, and the enamel proteases, MMP20 and EMSP1, in vertebrates from all four vertebrate classes via peroxidase immunohistochemistry. In order to screen a small but representative variety of vertebrates, the following species were chosen: a mammal (mouse, *Mus musculus*), a reptile (gecko, *Hemidactylus turcicus*), an anuran amphibian (green tree frog, *Hyla cinerea*), a sarcopterygian fish (lungfish, *Neoceratodus forsteri*), and a chondrychthian fish (shark, *Heterodontus francisci*). Following immunohistochemical analysis, we determined that enamel protein epitopes were distributed within all species investigated and thus highly conserved among vertebrates. Though mainly distributed in the enamel layer and enamel organ, enamel proteins other than amelogenin were also found in tissues surrounding the tooth organ and in layers outside of the enamel organ. Enamelin featured a distinct association with shark ameloblasts and enameloid. In contrast, amelogenin was more or less exclusively distributed in the enamel/ameloblast complex of the species investigated in this study.

MATERIALS AND METHODS

Tissue preparation

The following experimental animals were used in the present study: a 6-day and a 12-day postnatal mouse (*Mus musculus*), a gecko (*Hemidactylus turcia*; 43mm total length), a juvenile green tree frog (*Hyla cinerea*), a larval

lung-fish (*Neoceratodus forsteri*; stage 46; Kemp, '81), and a young hornshark (*Heterodontus franciscus*; 22cm total length). Animals were sacrificed by decapitation according to Baylor College of Dentistry animal care regulations. Mandibles were dissected and fixed immediately. For immunohistochemistry, tissues were fixed with 10% buffered formalin, decalcified in 4% EDTA and dehydrated in a graded series of ethanols. Specimen were embedded in paraffin and cut at 5μm thickness. Sections were mounted on coated glass slides.

Immunohistochemistry

Immunoreactions were performed following the instructions of the Zymed Histostain SP kit (San Francisco, CA). All reactions were carried out in a humidified chamber at room temperature. Briefly, sections were treated against endogenous peroxidase using methanol and 3% hydrogen peroxide and then blocked using in 10% goat serum for 10 min. Sections were incubated with primary antibody for two hours. Primary antibodies were diluted in phosphate buffered saline (PBS). The dilution of the primary antibody was determined in preliminary experiments. As a methodological control, the primary antibody was replaced with normal serum. Sections were washed three times in PBS and subsequently incubated for 10 min with biotinylated IgGs (either anti-chicken (MMP20) or anti-rabbit) as secondary antibodies. After washing in PBS (three times), sections were exposed to the streptavidin-peroxidase conjugate for 10 min and then washed again in PBS (three times). Signals were detected using the AEC Substrate-Chromogen mixture of the Zymed Histostain kit. Sections were counterstained using hematoxylin and mounted with GVA-mount.

List of primary antibodies and dilutions.

The following five primary antibodies were used in this study. Western blotting analyses of the antibodies used in this study have established specificity to a single protein epitope; reference publications are quoted below.

A. Polyclonal antibody against a recombinant mouse amelogenin (M179), IgG preparation (Simmer et al., '94). Dilution 1:100.

B. Polyclonal antibody against the full-length MMP20 (enamelysin) amino acid sequence generated in chicken. Dilution 1:100.

C. Polyclonal rabbit antibody generated against a recombinant pig EMSP1 from *E. coli* that was excised from SDS-PAGE gels (Hu et al., 2000). Dilution 1:50.

D. Polyclonal peptide antibody against the N-terminal enamelin portion. The antigen was a modified hexadecapeptide (MPMQMPRM PGFSSKSE) corresponding to the N-terminal enamelin amino acids 1–16 (Fukae et al., '96; Hu et al., '97; Dohi et al., '98). Dilution 1:100.

E. Polyclonal rabbit antibody against a synthetic polypeptide derived from the tuftelin sequence (QSKDTTIQELKEKIA) (Diekwisch et al., '97). Dilution 1:50.

Controls

The following controls were performed to test for antibody specificity: tissue controls—the specificity of the antibody was evaluated in nondental tissues; antibody controls by using a dilution series; controls with pre-adsorbed antibody to exclude unspecific binding; controls with pre-immune serum to control for binding to serum components; and omission of primary antibody as a systematic control.

RESULTS

Mouse, gecko, frog, lungfish, and shark featured characteristic tooth organs at the onset of tooth development.

In all five species investigated, the developing tooth enamel/enameloid was immediately surrounded by one or more distinct layers of epithelial cells. Mice teeth featured a fully developed enamel organ with highly polarized and prismatic ameloblasts immediately adjacent to the developing enamel (Figs. 1A–1F). The ameloblast cell layer was lined by a thin layer of perpendicular oriented stratum intermedium cells (Figs. 1A–F). The coronal center of the enamel organ was filled with a seemingly irregular network of star-shaped cells, the stellate reticulum (Figs. 1A–1F). The enamel organ was enclosed by an outer enamel epithelium (Figs. 1A–1F). In the case of the gecko (Figs. 1G–1L) and frog (Figs. 1M–1R) enamel organ, the ameloblast cell layer appeared to have gained prominence while the other three cell layers were less distinct. Nevertheless, also in gecko and frog stratum intermedium, stellate reticulum, and outer enamel

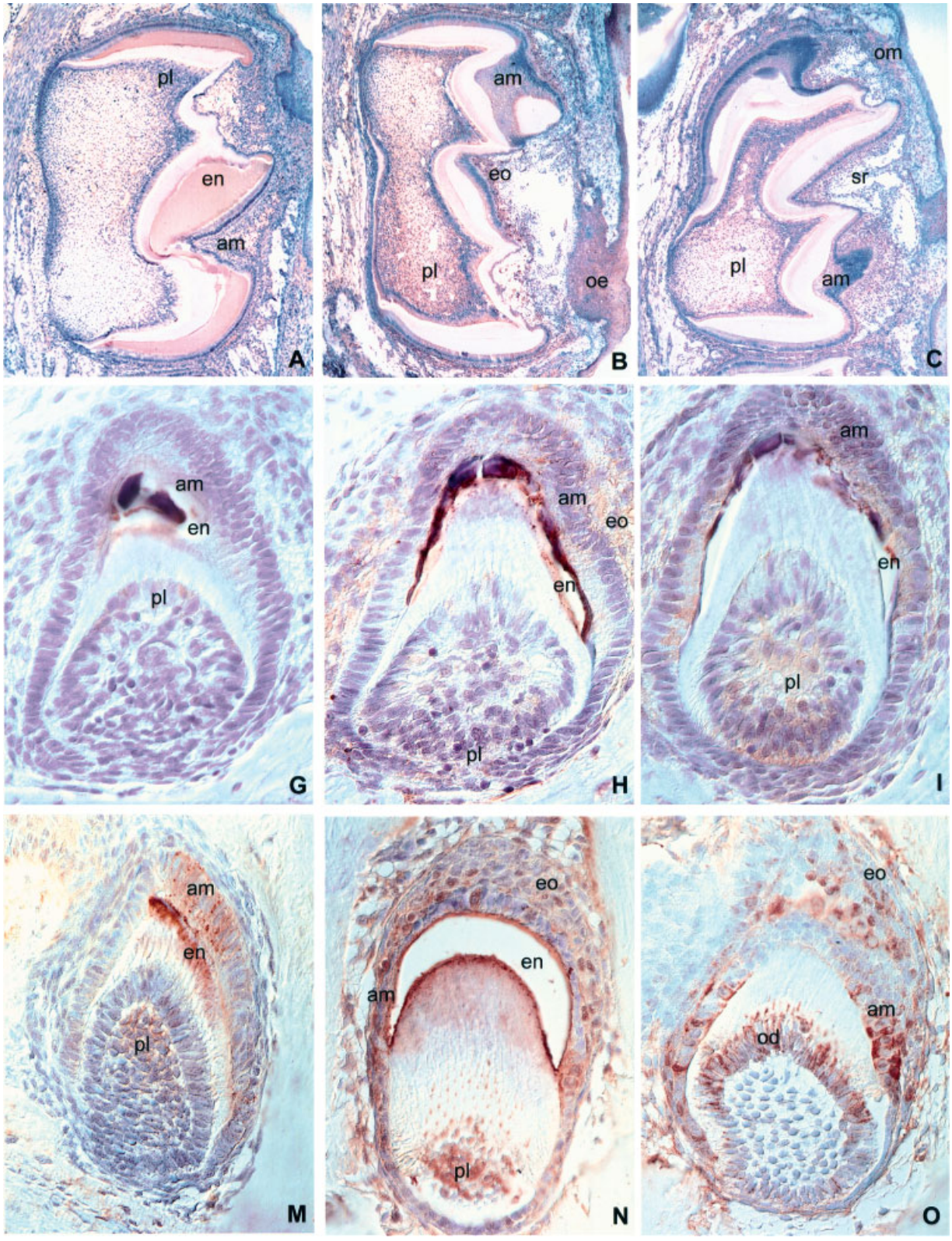
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Fig. 1 (left)

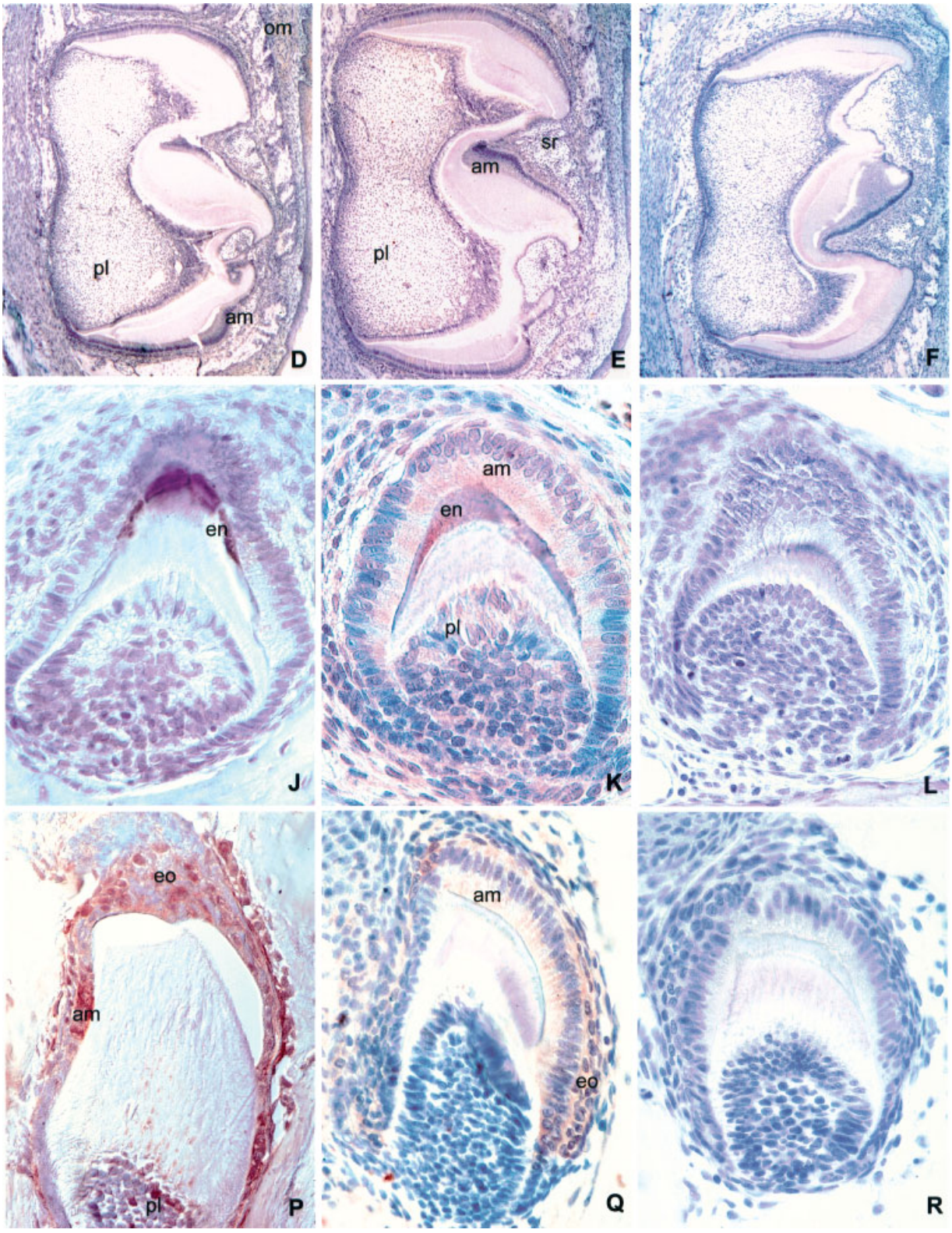


Fig. 1 (right)

epithelium were distinguished following morphological criteria (Figs. 1G–1L and 1M–1R). In the case of the lungfish, the enamel organ was limited to a single layer of cuboidal ameloblasts with prominent nuclei (Figs. 2A– 2F). Lastly, in the hornshark, the crown enameloid was lined by a dense layer of ameloblast-type cuboidal cells which formed the innermost component of a complex enameloid-organ (Figs. 2G–2L). This shark enameloid organ was differentiated into layers, including an outer line of cuboidal cells resembling an outer enamel epithelium, an intermediate layer of stellate reticulum-type loosely arranged cells, and the aforementioned inner layer of ameloblast-type enameloid epithelium (Figs. 2G–2L).

The recombinant amelogenin antibody reacted predominantly with the ameloblast cell layer and the enamel layer

In order to detect amelogenin distribution and conservation in a number of vertebrate species, we used an antibody against a mouse-recombinant M179 that was generated in *E. coli* (Simmer et al. '94). The M179 amelogenin antibody reacted with the ameloblast and the enamel layer in mouse, gecko, frog, and lungfish (Figs. 1A,1G,1M, 2A). In the shark tooth, the anti-amelogenin antibody labeled the shaft of the enameloid, the enamel organ, and the ameloblast-like cells (Fig. 2G). There was a distinct difference between amelogenin distribution in the secretory and in the maturation stage of mouse enamel development (Figs. 3A, 3B). While in both stages, the amelogenin antibody reacted with the entire enamel layer. An intense reaction with the ameloblast secretory vesicles was only observed during the secretory stage (Fig. 3A). During the maturation stage, there was only a weak and diffuse reaction throughout the ameloblast layer (Fig. 3B). Also odontoblasts demonstrated changes in reactivity for amelogenin antiserum: during the secretory stage, only a few isolated odontoblasts were labeled heavily (Fig. 3A) while during the enamel maturation stage, the entire odontoblasts layer exhibited weak labeling (Fig. 3B).

Fig. 1 left and right is on p. 94 and 95.

Fig. 1. Distribution of enamel proteins, amelogenin, MMP20, EMSP1, enamelin, and tuftelin, in developing mammalian, reptilian, and amphibian teeth. **A–F** are from a six-day postnatal mouse (*Mus musculus*) first mandibular molar. **G–L** are from a mid-mandibular gecko tooth organ (*Hemidactylus turcicus*). **M–R** are from a mid-maxillary tree frog tooth (*Hyla cinerea*). The first vertical column (A,G,M) features immunoreactions using an antibody against a recombinant mouse M179 amelogenin. The second vertical column (B,H,N) contains immunoreactions using an antibody against the enamel metalloproteinase MMP20 (enamelysin). The third vertical column (C,I,O) consists of micrographs of immunoreactions using an antibody against the enamel serin proteinase EMSP1. The fourth column (D,J,P) features immunoreactions using an antibody against the enamelin C-terminus. The fifth column (E,K,Q) contains immunoreactions using an antibody against one of the first enamel proteins described, tuftelin. And, the sixth column (F,L,R) consists of control photomicrographs that were obtained from immunostained sections in which the first antibody was replaced by phosphate buffer. **A,G,M**: The antibody against the recombinant M179 amelogenin reacted with the ameloblast layer (am) and the enamel layer (en) in all three species—mouse, gecko, and frog. Some anti-amelogenin immunoreactivity was also detected in the pulp (pl) of all three species, most noticeable in the frog pulp. **B,H,N**: In all three species—mouse, gecko, and frog—the antibody against MMP20 (enamelysin) reacted with the ameloblast layer (am) and other layers of the enamel organ (eo; stratum intermedium, stellate reticulum, and outer enamel epithelium), and the pulp (pl). The enamel layer (en) was clearly marked in the gecko and in the frog (1H and 1N). In the mouse section, the labeling of the oral epithelium (oe) was also recorded (1B). **C,I,O**: On the mouse sections, the anti-EMSP1 antibody reacted with the ameloblast layer (am), the stellate reticulum (sr), the pulp (pl), and the oral mucosa (om)(1C). In the gecko section, the anti-EMSP1 antibody stained the ameloblast layer (am), the enamel layer (en), and the pulp (pl)(1I). In the frog section, the anti-EMSP1 antibody labeled the ameloblast layer (am), other layers of the enamel organ (eo)(here: stellate reticulum and parts of the outer enamel epithelium), and the odontoblasts (od) and their processes (1O). **D,J,P**: In the six-day postnatal mouse molar, the anti-enamelin antibody demonstrated a distinct reaction in the ameloblast cell layers (1D). A weak reaction with the anti-enamelin antibody was found in the pulp (pl) and the oral mucosa (om)(1D). In the gecko tooth, the anti-enamelin reaction was exclusively limited to the enamel layer (en)(1J). In the frog tooth, the anti-enamelin antibody stained the pulp (pl) and all layers of the enamel organ (eo), including ameloblasts (am), stratum intermedium, stellate reticulum and outer enamel epithelium (1P). **E,K,Q**: In the six-day postnatal mouse molar, the anti-tuftelin antibody reacted with the ameloblast layer (am), the stellate reticulum (sr) and the pulp (pl)(1E). In the gecko, the anti-tuftelin antibody labeled the ameloblast layer (am) and the enamel layer (en)(1K) while in the frog, the anti-tuftelin antibody stained the ameloblast layer (am) and adjacent layers of the enamel organ (eo)(1Q). **F,L,R:** In all three species, mouse, gecko, and frog, the control reaction did not yield any labeling. Magnification: 30 × (mouse), 500 × (gecko, frog).

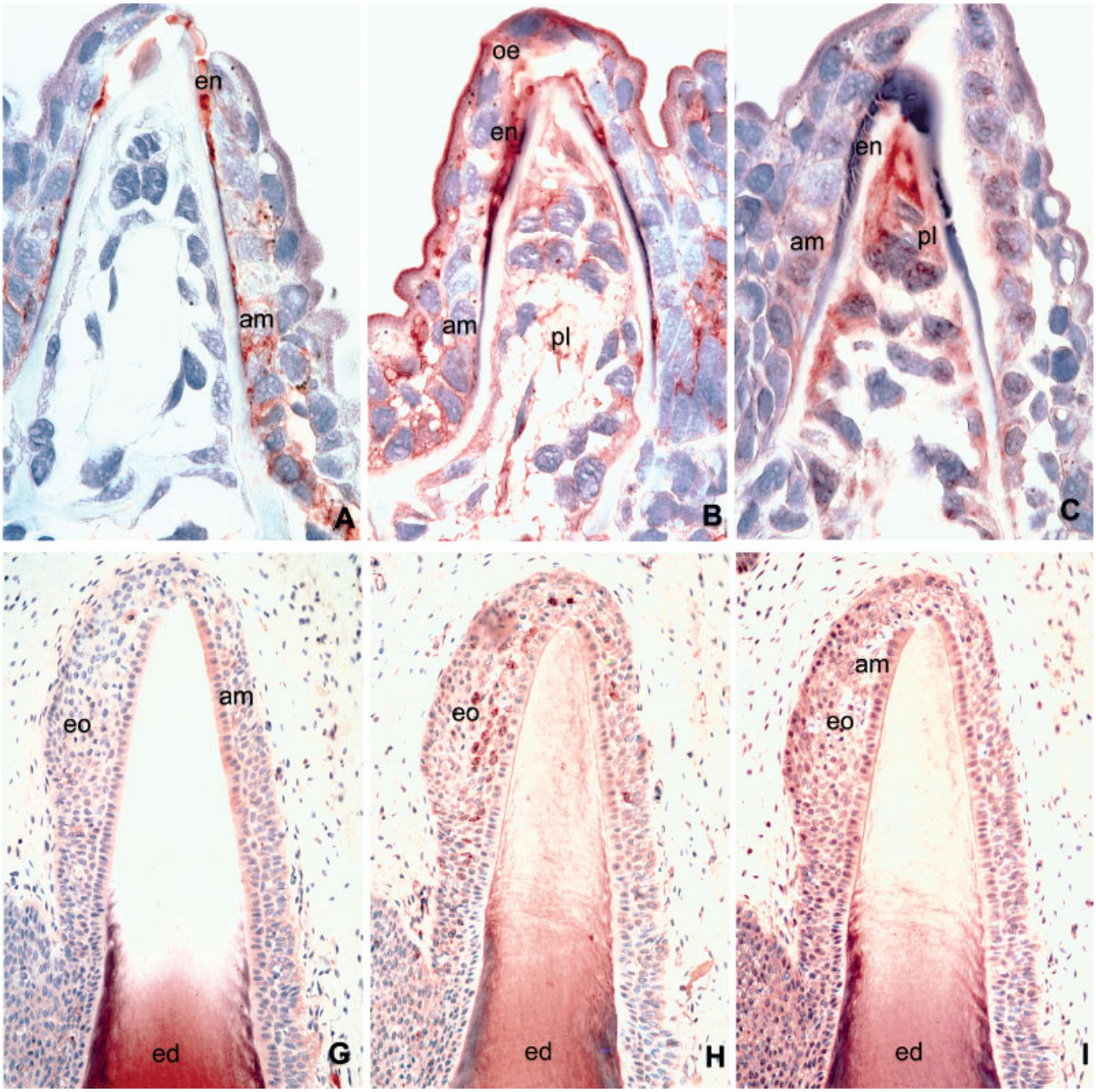
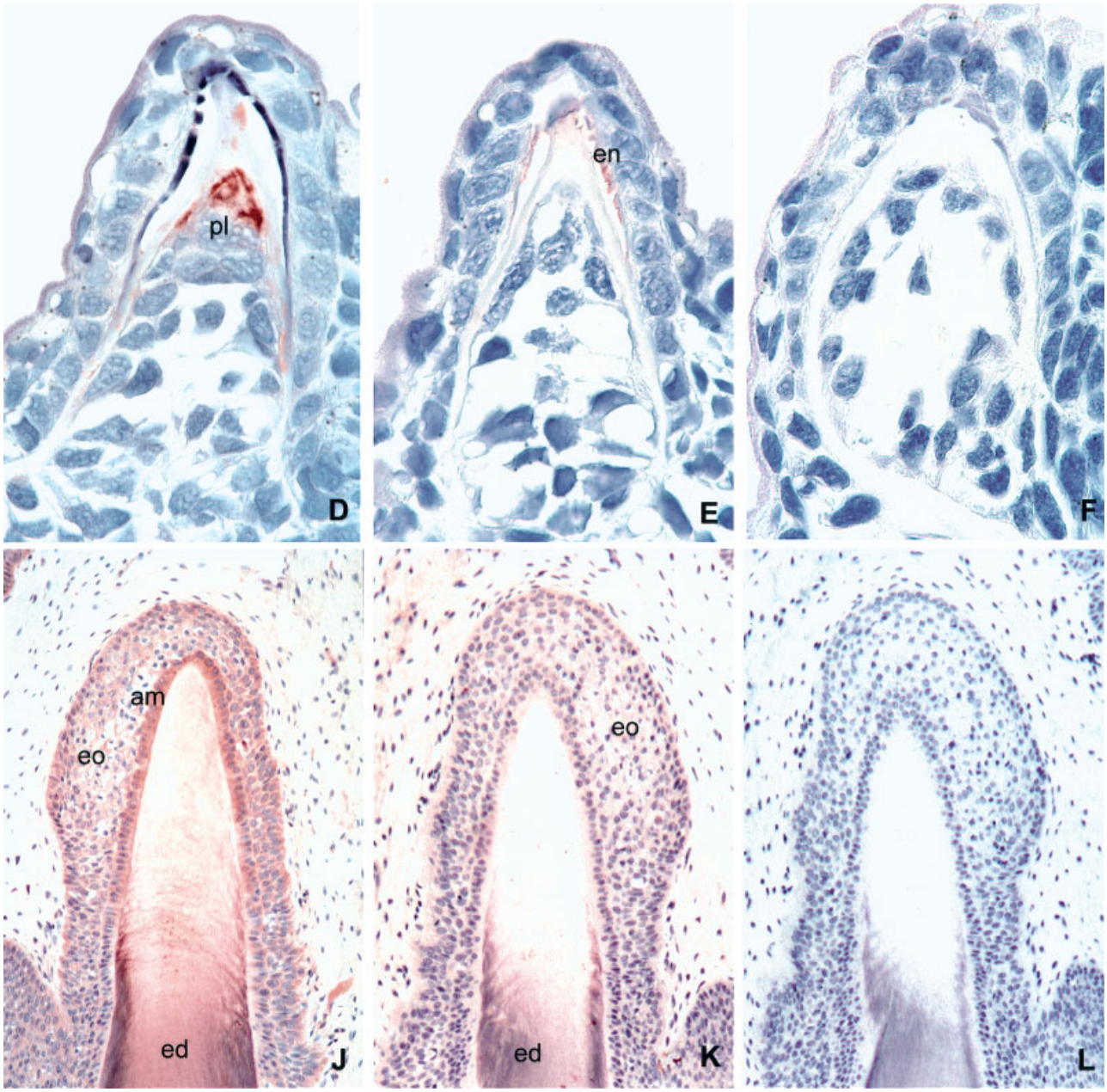


Fig. 2. Distribution of enamel proteins, amelogenin, MMP20, EMSP1, enamelin, and tuftelin, in developing fish teeth. **A–F** are from a lungfish (*Neoceratodus forsteri*) larval tooth, and **G–L** are from a pre-eruption hornshark (*Heterodontus francisci*) tooth. The first vertical column (A,G) features immunoreactions using an antibody against a recombinant mouse M179 amelogenin. The second vertical column (B,H) contains immunoreactions using an antibody against the enamel metalloproteinase MMP20 (enamelysin). The third vertical column (C,I) consists of micrographs of immunoreactions using an antibody against the enamel serin proteinase EMSP1. The fourth column (D,J) features immunoreactions using an antibody against the enamelin C-terminus. The fifth column (E,K) ontains immunoreactions using an antibody against one of the first enamel proteins described, tuftelin. And, the sixth column (F,L) consists of control photomicrographs that were obtained from immunostained sections in which the first antibody was replaced by phosphate buffer. **A-F:** Immunoreactions in the lungfish tooth (A). The anti-amelogenin antibody reacted with the enamel layer (en) and the ameloblast layer (am) (B). The anti-MMP20 (enamelysin) antibody labeled the oral epithelium (oe), enamel (en), and pulp (pl) (C). The anti-EMSP1 antibody reacted with the ameloblast cell layer (am), the enamel layer (en), and the pulp (pl) (D). The anti-enamelin antibody only stained the coronal tip of the pulp (pl) (E). The anti-tuftelin antibody labeled the enamel layer (en) exclusively (F). There were no reaction products in the control tooth. **Figs. 2G-L:** Immunoreactions in the shark tooth. In all five cases using the antibodies against amelogenin, MMP20, EMSP1, enamelin,



and tuftelin, the antibodies reacted with the enameloid (ed)-containing shaft of the shark tooth (G–K). In the case of amelogenin and tuftelin, the tip of the tooth enameloid was free of reaction products, while the other three antibodies, MMP20, EMSP1, and enamelin, also exhibited a week reaction at the tip of the tooth. All five antibodies—amelogenin, MMP20, EMSP1, enamelin, and tuftelin—reacted with the shark enamel organ (eo) (G–K) with enamelin (J), demonstrating the strongest reaction in the shark ameloblast layer (am). Also in the shark, the control was without reaction products (L). Magnification: 1,000 × (lungfish), 200 × (shark).

The MMP20 (enamelysin) enamel proteinase was detected predominantly in the enamel organ and in the pulp

For MMP20 (enamelysin) detection, we used a polyclonal antibody against the full-length enamelysin (Uchida et al., '91; Bartlett et al., '96). In mouse, gecko, frog, and lungfish, the anti-enamelysin antibody recognized the enamel organ including the ameloblast layer, the enamel layer, and the pulp (Figs. 1B,H,N; 2B). Higher magnification images revealed discrete staining of the

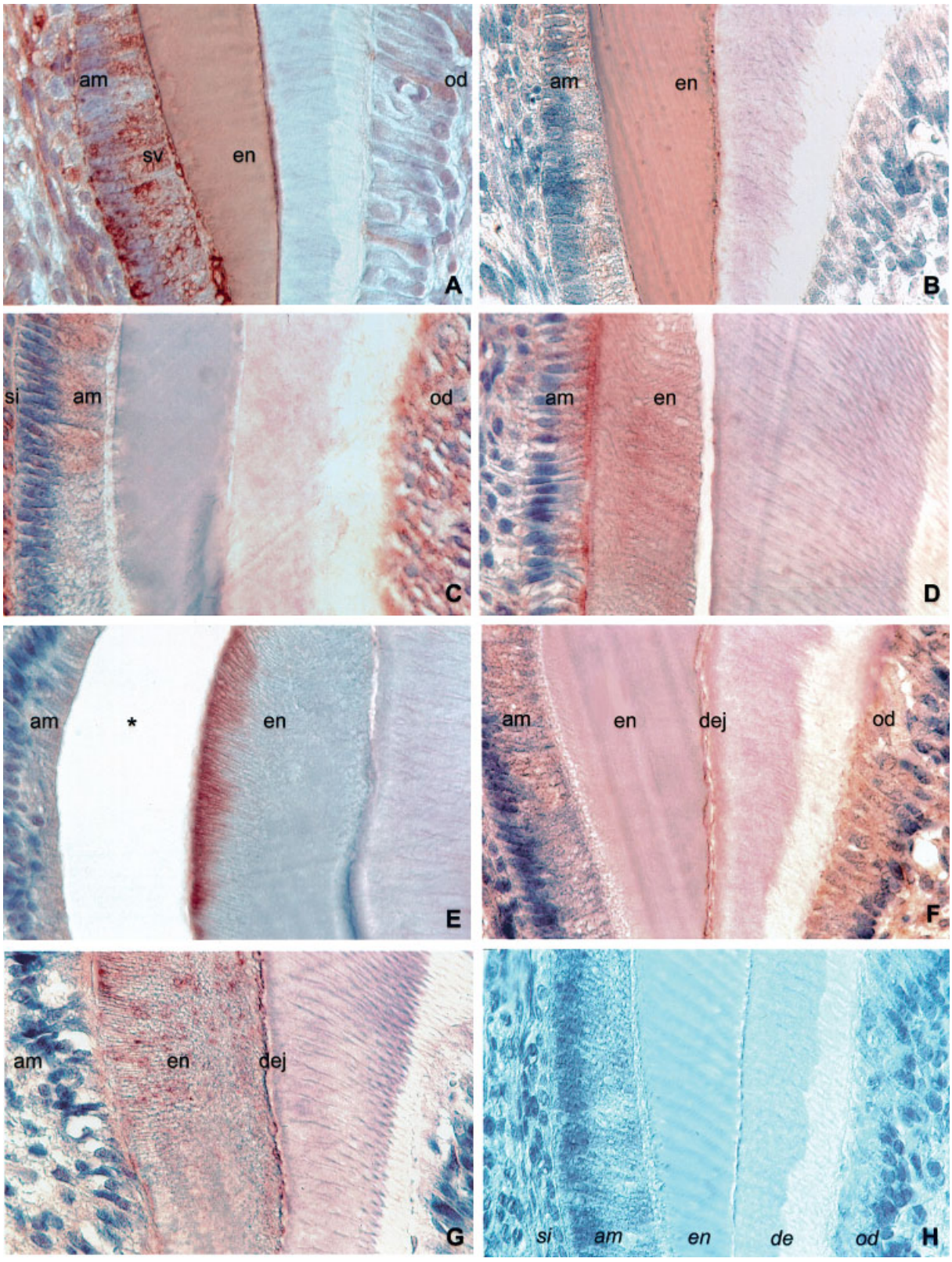

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Fig. 3

ameloblast secretory pole, dentin-enamel junction, and odontoblasts in the mouse (Fig. 3F). On the mouse section, a labeling of the oral epithelium was also recorded (Fig. 1B). In the shark tooth, the anti-MMP20 antibody reacted with the enameloid of the mineralizing shark tooth and the shark enamel organ (Fig. 1H).

The EMSP1 enamel serin proteinase was detected predominantly in the enamel organ and in the pulp and exhibited stage-dependent changes in mouse enamel

The antibody used for EMSP1 detection was a polyclonal rabbit antibody generated against a recombinant pig EMSP1 from *E. coli* that was excised from SDS-PAGE gels (Hu et al., 2000). The anti-EMSP1 antibody reacted with the enamel/enameloid layer in the gecko, in the lungfish, and in the shark (Figs. 1I; 2C,I); with the ameloblast cell layer in mouse, gecko, frog, lungfish, and shark (Figs. 1C, 1I, 1O, 2C, 2I); with other cell layers of the enamel organ including stellate reticulum in mouse, frog, and shark (Figs. 1C, 1O, 2I); and with the pulp in mouse, gecko, frog (restricted to odontoblasts), and lungfish (Figs. 1C, 1I, 1O, 2C). On mouse molar sections, we detected a stage-dependent distinct difference in protein distribution using the anti-EMSP1 antibody (Figs. 3C, 3D). During the secretory stage, the EMSP1 antibody reacted with stratum intermedium, ameloblasts, and odontoblasts, but not in the enamel layer (Fig. 3C), while during the maturation stage, the EMSP1 antibody caused an intense stain in the enamel layer and at the ameloblast secretory pole (Fig. 3D).

The enamelin antibody featured a distinct localization pattern in the ameloblasts and enameloid of the developing shark enameloid organ

For preparing N-terminal specific anti-enamelin antibodies, a modified hexadecapeptide (MPMQMPRMPGFSSKSE) corresponding to the N-terminal enamelin amino acids 1–16 was synthesized and used for anti-peptide antibody production in rabbit (Fukae et al., '96; Hu et al., '97; Dohi et al., '98). In six-day postnatal mouse molars, the anti-enamelin antibody reacted distinctly with the ameloblast cell layer, but also demonstrated staining in the pulp and the oral mucosa (Fig. 1D). In 12-day postnatal molars, labeling in the superficial enamel layer and at the ameloblast secretory pole was detected (Fig. 3E). In the gecko, the anti-enamelin antibody labeling was restricted to the enamel layer (Fig. 1J). In the frog tooth, all four layers of the enamel organ, ameloblasts, outer enamel epithelium, stellate reticulum, and stratum intermedium were stained using the anti-enamelin immunoreaction (Fig. 1P). A positive signal was also detected in the odontoblasts (Fig. 1P). In the lungfish, only the tip of the pulp reacted with the anti-enamelin antibody (Fig. 2D). Lastly, in the shark, the anti-enamelin antibody recognized the entire enamel

Fig. 3. High magnification images of enamel protein localization in mouse molars at selected stages of development. Figs. 3D and 3E were from 12-day postnatal mouse molars; all other figures from six-day postnatal mouse molars. **3A and 3B:** Amelogenin localization in secretory stage ameloblasts (A) and maturation stage ameloblasts (B) using an antibody against a recombinant M179 amelogenin. (A) Positive amelogenin immunoreactions were in ameloblast secretory vesicles (sv), at the proximal ameloblast pole (am), in the enamel layer (en), and in isolated odontoblasts (od). (B) The amelogenin antibody demonstrated a distinct reaction in the enamel layer (en). A less intense reaction was also recorded in the ameloblast layer (am) and, even less intense, in the odontoblasts layer (od). **3C and 3D:** EMSP1 immunolocalization in secretory stage ameloblasts (1C, six-day postnatal) and maturation stage ameloblasts (1D, 12-day postnatal). During the secretory stage (C), EMSP1 epitopes were in ameloblasts (am), in the stratum intermedium (si) and in odontoblasts (od). During the maturation stage (D), EMSP1 epitopes were concentrated at the ameloblast secretory pole (am) and in the enamel layer (en). **3E:** Immunolabeling of the superficial enamel layer (en) and of the ameloblast secretory pole (am) using an antibody against the enamelin C-terminus on 12-day postnatal mouse molar sections. The asterisk (*) indicates a preparation artifact between enamel layer and ameloblasts. **3F:** MMP20 immunohistochemistry demonstrating a color reaction at the ameloblast secretory pole (am), the dentin-enamel junction (dej) and in odontoblasts (od) as well as a weak reaction in the enamel layer (en). **3G:** Tuftelin localization in ameloblasts (am) and at the dentin-enamel junction (dej). Tuftelin epitopes were also detected in the interprismatic framework of the enamel layer (en). **3H:** The control section indicates the localization of the stratum intermedium (si), the ameloblast layer (am), the enamel layer (en), the dentin layer (de), and the odontoblasts layer (od). Magnification: 500 ×.

organ including a distinctly stained ameloblast layer as well as the mineralizing portion of the enameloid (Fig. 2J).

The tuftelin antibody had preferred reaction sites in the frog, gecko, and lungfish ameloblast and enamel layers but was also detected in other parts of the developing tooth organ

The anti-tuftelin antibody was a polyclonal rabbit antibody against a synthetic polypeptide derived from the tuftelin sequence (QSKDTTIQELKEKIA)(Zeichner-David et al., '97). The anti-tuftelin antibody reacted with the ameloblast cell layer in mouse, gecko, and frog (Figs. 1E, 1K, 1Q, 3G). Other parts of the enamel organ, including stellate reticulum, were labeled in mouse, frog, and shark (Figs. 1E, 1Q, 2K, 3G). The enamel/enameloid layer was detected with the anti-tuftelin antibody in mouse, gecko, lungfish, and shark (Figs. 1E, 1K, 2E, 2K, 3G).

Using the indirect immunoperoxidase method, the control sections did not exhibit any labeling

Controls were performed in a number of ways as described in the Materials and Methods section. We photographed the controls in which the primary antibody was replaced with normal serum. All control sections from mouse, gecko, frog, lungfish, and shark did not show any labeling (Figs. 1F, 1L, 1R, 2F, 2L, 3H).

DISCUSSION

In the present study, immunohistochemical methods were applied to ask the question whether enamel proteins were widely and specifically distributed throughout the vertebrate subphylum. To address this question, we assayed the localization and distribution of five proteins that have been classified at some point as enamel proteins, amelogenin, enamelin, and tuftelin and the proteinases MMP20 (enamelysin) and EMSP1. In order to screen a small but representative variety of vertebrates, we selected a mammal (mouse, *Mus musculus*), a reptile (gecko, *Hemidactylus turcicus*), an anuran amphibian (*Hyla cinerea*), a sarcopterygian fish (lungfish, *Neoceratodus forsteri*), and a chondrichthyan fish (shark, *Heterodontus francisci*). Our immunohistochemical data indicated that enamel proteins were widely distributed within the vertebrate subphylum. While often associated with the enamel layer or the enamel-forming cells (ameloblasts), four of the five proteins investigated were also found in other tissues of the tooth organ and beyond. Among the five enamel proteins investigated, amelogenin was the only protein that was more or less exclusively distributed in the enamel/ameloblast complex in the species investigated in this study.

In the present study, we have applied immunoreactions using mammalian enamel protein antibodies as a strategy to detect whether and to what degree these enamel proteins were conserved in other vertebrate classes. Basically, we have been using these antibodies as a bait to detect whether proteins with a similar epitope constellation were present in nonmammalian species. While this strategy has been used in previous studies as well (Herold et al., '80, '89; Slavkin et al., '82, '83; Zylberberg et al., '97; Ishiyama et al., '99; Kogaya, '99), it is not free from systematic errors. As the principle antigen-antibody reaction is based on the reaction between an antibody and prominent epitopes of an antigenic protein, only slight changes in amino acid composition at the crucial binding site might cause the antibody not to bind with the antigen. In this situation, the detection of evolutionary conservation of a given protein by immunological similarity might yield negative results even in case of an overall sequence homology. False-positive results in which a specific site of a nonenamel protein would yield a reaction with the enamel protein antibody are also possible but less likely to occur. Like many other proteins, enamel protein antibodies have a high affinity to the hydroxyapatite present in enamel and dentin, but our control sections indicated that the indirect immunoperoxidase strategy was free from this type of unspecific reaction. Another indicator for the validity of our strategy was the consistency in distribution pattern that we found within each single species.

The ultimate strategy to determine homologies and presence of enamel proteins in several vertebrate species, the cloning and sequencing of non-mammalian enamel genes, has significantly advanced in the case of amelogenin (Ishiyama et al., '98; Toyosawa et al., '98). Even though this strategy does not provide any quantitative data on the prevalence of specific enamel proteins, the presence of a particular gene product can be determined with a higher degree of certainty. Meanwhile, sequences of snake amelogenin (Ishiyama et al., '98), caiman amelogenin (Toyosawa et al., '98), and Xenopus amelogenin (Toyosawa et al., '98) have been identified.

Nonmammalian sequences of enamel proteins other than amelogenin have not yet been identified because this strategy is time-consuming and rather costly. As a consequence, our screening procedure using homology to mammalian antibodies as a detection method for the presence of enamel proteins was the strategy of choice at this point.

Using our immunohistochemical screening procedure and M179 recombinant amelogenin antibodies, we detected a distinct and intense amelogenin immunolabeling in the enamel and the ameloblast layer of all species investigated. This finding confirms previous reports on a high conservation of the amelogenin gene in many vertebrates (Herold et al., '80; Slavkin et al., '82, '83; Slavkin and Diekwisch, '96, '97; Kogaya, '99). Changes in mouse amelogenin distribution from the secretory stage to the maturation stage, as demonstrated in this study, are reported for the first time but have been observed in previous studies already (Diekwisch et al., '97). Moreover, in the present study, we clearly document discrete amelogenin labeling in isolated odontoblasts, confirming earlier reports on amelogenin splice products in odontoblasts and pulp (Nebgen et al., '99; Veis et al., 2000). In the past, the presence of amelogenin-related products in mesenchymal tissues such as pulp and odontoblasts has often been refuted (e.g., Karg et al., '98), but our discrete localization data as well as recent in situ hybridization data (Veis, personal communication) indicate that odontoblasts do express and contain amelogenins. While the function of these mesenchymal amelogenins is not well understood, a role in epithelial-mesenchymal signaling or during mineral induction has been proposed (Nebgen et al., '99; Veis et al., 2000).

Apart from amelogenin, which with a few exceptions was exclusively limited to the ameloblast/enamel region, all other ''enamel'' proteins were also found in other parts of the tooth organ and the head as well. Thus, these proteins cannot be termed as ''enamel-specific'' proteins in a strict sense. On the other hand, most of them feature a distinct and conserved expression pattern in enamel, suggesting they play some defined role during enamel formation. This discussion has become most applicable in the case of tuftelin, one of the first nonamelogenin enamel proteins described (Deutsch, '89). The term ''tuftelin'' was created after its presumed localization in the enamel tufts (Robinson et al., '89), a protein-rich structure at the dentin-enamel junction resembling grass tufts in their visual appearance. Following its sequence determination (Deutsch et al., '91), tuftelin has been defined as an independent gene product and most recently localized in a number of nontooth-specific tissues including kidney, lung, liver, and testis (MacDougall et al., '98). In the present study, we found tuftelin to be conserved in tooth-related tissues—notably enamel, ameloblasts, and other cells of the enamel organ in all species investigated. In high-magnification images of mouse sections, tuftelin was distinctly localized at the dentin-enamel junction and in the enamel prism sheaths, indicating that tuftelin may have a significant role during enamel formation. Besides in its tooth-specific localization, tuftelin was also found in other organs (data not shown) and in the oral mucosa. Thus, our findings suggest a distinct function of tuftelin in enamel formation and in the development of other tissues as well.

As in the case of tuftelin, the nomenclature of nonamelogenin enamel proteins has been nonstraightforward at best. One of the nonamelogenin enamel proteins has been synchronously termed ameloblastin, amelin, and sheathlin, causing an enamel biology logodaedaly or at least an editorial thereof (Snead, '96). The case of enamelin, one of the other nonamelogenin enamel proteins, has not been less confusing. Initially used as a term to describe all nonamelogenin enamel proteins (Termine et al., '80a, b), the word ''enamelin'' has only more recently become associated with a specific gene product found in enamel and elsewhere (Fukae and Tanabe, '87; Uchida et al., '91; Hu et al., '97; Dohi et al., '98). Previous investigations in porcine tooth germs (Hu et al., '97; Dohi et al., '98) have localized enamelins in the outermost enamel layer, a finding that was confirmed in the present study using mouse as an experimental model. As such, enamelin might function during the spacing and nucleation of initial enamel crystals. We further detected enamelin to be highly conserved in the enamel layer and in cells of the enamel organ including ameloblasts in all species investigated, suggesting a defined and important function of enamelins during enamel formation in all vertebrates.

Several authors have postulated the presence of specific proteases in the developing enamel matrix (Suga, '70; Crenshaw and Bawden, '84; DenBesten and Heffernan, '89; Smith et al., '89). Recently, two distinct enamel proteinases have been cloned and sequenced: enamelysin or MMP20, a metalloproteinase (Bartlett et al., '96) and EMSP1, an

enamel matrix serine proteinase (Simmer et al., '98). In previous studies, MMP20 has been associated with ameloblasts and odontoblasts by means of in situ hybridization (Fukae et al., '98), while EMSP1 was detected in transition and early maturation stage ameloblasts (Simmer et al., '98; Hu et al., 2000). In the present study, we have confirmed these previous findings using our mouse model system. In addition, we have documented a distinct change in EMSP1 localization between secretory-stage tooth organs and early maturation stage tooth organs. While EMSP1 was localized in secretory-stage ameloblasts and stratum intermedium throughout the entire cell layer, EMSP1 was heavily concentrated at the ameloblast secretory pole and in the enamel layer during the early maturation stage. This finding corroborates a presumed and significant role of EMSP1 during maturation stage enamel matrix degradation (Simmer et al., '98). Furthermore, both enamel proteases, MMP20 and EMSP1, were localized in the enamel organ including enamel and ameloblasts as well as in the dental papilla/pulp in all species investigated, suggesting that both enzymes might play significant roles in several tissues during tooth development, including enamel formation, and that its role in enamel development might be highly conserved. Both enzymes also reacted in other tissues (data not shown) indicating that these are ubiquitous enzymes with important functions during enamel formation. Based on their known function related to enamel matrix processing and degradation we speculate that the enamel proteases might have been gradually recruited to the process of enamel development throughout the evolution of vertebrate enamel formation.

While all five proteins were associated with the enamel organ in all species investigated, there were also significant differences in the localization and distribution of these proteins in various species. As mentioned, amelogenin was the one protein most specifically associated with the ameloblast layer and the enamel organ. However, in frog, gecko, and mouse, distinct amelogenin epitopes were also localized in the dental papilla and pulp and in odontoblasts. The implications of these findings have been discussed. Tuftelins were quite specifically associated with the ameloblast layer in frog as well as gecko and lungfish enamel. They might play a role during ameloblast differentiation and enamel formation of these species, but our observations provide no further clues toward a general evolutionary trend. In contrast to the extremely confined amelogenin reaction products, the two enamel proteases EMSP1 and MMP20 were almost ubiquitous in all species investigated. While playing a distinct role during enamel maturation, they appear to have many other functions in other tissues as well.

Interestingly, enamelin featured a highly confined distribution in shark and frog ameloblasts, while in other species it either exhibited a weak localization pattern or was distributed in other tissues than the enamel organ. This finding suggests that enamelin might play a distinct role during shark enameloid formation while this role might have become rudimentary or lost in mammalian teeth. Our speculation of an amelogenin/enamel and enamelin/enameloid relationship is further supported by our amelogenin immunoreactions, which were rather less specific in shark, compared to the highly distinct distribution pattern in all other species investigated. Together, these observations lend themselves to the hypothesis that enamelins might play a major role in shark enameloid formation while only playing a rudimentary role in more derived vertebrates. In contrast, functional and morphological studies have clearly established amelogenin as a major player during mouse enamel formation (Diekwisch et al., '93; Diekwisch, '98), while its function in sharks and other basal vertebrates might be less important as the less than distinct immunoreactions might suggest.

Our findings of a predominance of amelogenin in amphibian, reptilian, and mouse enamel in tandem with a predominance of enamelin in sharks are confirmed by previous observations by Herold et al. ('89) using monoclonal antibodies. Herold and his colleagues isolated a 27kDa protein fraction to generate a monoclonal amelogenin antibody and an acidic 60kDa–70kDa protein fraction for a monoclonal enamelin antibody. They concluded from their studies that enamelins appeared prior to amelogenins in evolution because enamelins were found in all species including fish and larval amphibians, while amelogenins were restricted to vertebrates with true enamel such as adult amphibians, reptiles, and mammals. Our studies concur with these results but suggest that lesser amounts of amelogenin-related epitopes are present in enamelin-bearing chondrichthyan teeth and that the difference between amelogenin/enamelin content in enamel and enameloid is gradual rather than absolute. Herold et al. ('89) were using monoclonal antibodies, which are sensitive for minute changes in epitope

composition or configuration, and which therefore may have resulted in a lack of amelogenin-reactivity in chondrichthyans. While Herold's antibodies provided a significant advantage over previous polyclonal antibodies generated by crude gel extract immunization, we used second-generation polyclonal antibodies either derived from polypeptides based on unique sequence information or on recombinant proteins. However, whatever the level of amelogenins in enameloid might be, current data suggest that during the progression of vertebrates, enamelin-rich enameloid mineralized tissues lost their status as vertebrate tooth coverings in favor of amelogenin-rich enamel.

With our studies confirming the possibility of such a trend in vertebrate evolution, i.e., the replacement of enamelin-based enameloid with amelogenin-based enamel, the question arises whether this trend corresponds to a change in protein function related to enamel/enamelin mineral organization. In numerous publications we have established a tight relationship between organic enamel matrix subunit organization and enamel hydroxyapatite crystal growth and habit (Diekwisch et al., '93, '95; Diekwisch, '98) and, in fact, it was our publication that was the first to correlate organic enamel matrix subunit organization with inorganic crystal dimensions (Diekwisch et al., '93). Herold's speculation that enamelins might be associated with the presence of large hydroxyapatite crystals appears almost visionary from today's perspective, especially considering recently published immunogold studies (Hu et al., '97). However, large apatite crystals are not common for all enamelin coverings, especially because many fish enameloid tissues only contain many small crystals (Diekwisch et al., in press), and chondrichthyans contain fluorapatite instead of hydroxyapatite. Nevertheless, there is an evolutionary trend that coincides with the occurrence of the tetrapods and the predominance of amelogenins in vertebrate tooth coverings as described in this study and Herold et al. ('89): the emergence of ''true'' vertebrate enamel characterized by highly parallel oriented crystals and extremely long c-axis dimensions. Now, after assembling the puzzle of pieces of evidence related to the function of enamel proteins in vertebrate evolution, it appears fair to speculate that these features of tetrapod enamel, namely the growth of extremely long and parallel-oriented apatite crystals, might be an achievement of the amelogenins entering the portfolio of enamel proteins during the evolution of vertebrate outer tooth coverings. It remains unclear exactly as to how amelogenins might contribute to the extremely organized and oriented enamel crystal growth and whether enamelins, which are only present in lesser amounts in tetrapod enamel (less than 10%), still contribute to the presence of large crystals per se. Nevertheless, the changes of enamel protein composition as presented in this study are highly suggestive and provide further evidence for the importance of the organic enamel protein matrix for enamel crystal growth and habit.

In summary, we have localized five proteins associated with mammalian enamel formation in a wide variety of vertebrate species. Among these five proteins, amelogenin was the one protein that was most consistently associated with the enamel organ. The predominant localization pattern of enamelin in the shark enameloid compared to the confined localization pattern of amelogenin in lungfish, frog, gecko, and mouse enamel provides further support for our hypothesis that amelogenin is the major protein involved in vertebrate enamel formation (Diekwisch et al., '93, '95; Diekwisch, '98) while enamelin, in comparison, might play an important role during the development of chondrichthyan enameloid. The other proteins—tuftelin, EMSP1, and MMP20—were detected in the developing teeth of all species investigated; however, their localization pattern was less closely associated with the enamel organ than that of amelogenin. As to their specific function related to enamel formation, amelogenin demonstrated a most distinct association with tetrapod and lungfish enamel formation, and enamelin was most prominently associated with developing shark enameloid, while the other three proteins might have functions that go beyond enamel formation in non-mammalian vertebrates.

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