Biosynthesis and characterization of rabbit tooth enamel extracellular-matrix proteins

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Tooth enamel biomineralization is mediated by enamel proteins synthesized by ameloblast cells. Two classes of proteins have been described: enamelines and amelogenins. In lower vertebrates the absence of amelogenins is believed to give rise to aprismatic enamel; however, rabbit teeth, which apparently do not synthesize amelogenins, form prismatic enamel. The present study was designed to characterize the enamel proteins present in rabbit tooth organs and to gain an insight into the process of biomineralization. Rabbit enamel extracellular-matrix proteins were isolated and characterized during sequential stages of rabbit tooth organogenesis. The biosynthesis of enamel proteins was analysed by metabolic ‘pulse–chase’ experiments as well as mRNA-translation studies in cell-free systems. Our results indicated that rabbit enamel extracellular matrix contains ‘amelogenin-like’ proteins. However, these proteins are not synthesized as typical amelogenins, as in other mammalian species, thus suggesting that they are the processing products of higher-molecular-mass precursors. An *N*-terminal amino acid sequence of 29 residues, considered characteristic of mammalian amelogenins, was present in the rabbit ‘amelogenin-like’ proteins. By using anti-peptide antibodies to this region, similar epitopes were detected in all nascent enamel proteins, including enamelines. These studies suggest that the *N*-terminal sequence might be characteristic of all enamel proteins, not only amelogenins.

INTRODUCTION

Tooth organogenesis is the result of reciprocal interactions between ectomesenchyme and epithelia. The cranial neural-crest-derived ectomesenchyme differentiates into odontoblast cells, which produce the dentine extracellular matrix (ECM) (dentinogenesis). In a reciprocal manner, the ectodermally derived epithelium appears to be instructed by the ectomesenchyme to differentiate into ameloblast cells, which synthesize and secrete the proteins that constitute the enamel ECM (amelogenesis) [1–4].

The ECM of developing mammalian dental enamel contains a mixture of proteins, representing 20–30 % of the dry weight of the forming enamel. During the process of maturation, the enamel becomes mineralized, with calcium hydroxyapatite crystals representing approx. 97 %, and only 1–3 % of organic material remains in the mature enamel [5].

The predominant proteins in developing enamel have been identified and characterized, according to conventions based on their physicochemical and compositional properties, into two major classes of proteins: amelogenins and enamelines [6–8]. By sequential extraction with guanidine hydrochloride followed by extraction with guanidine hydrochloride/EDTA, Termine *et al.* [8] found that the first extract was enriched with the amelogenin class of proteins. These proteins have relatively low molecular mass (5–40 kDa), and are enriched in the amino acids proline (20–30 %), glutamic acid, leucine and histidine [6,8–10]. In contrast, the

enamelin class of proteins require demineralization procedures (guanidine hydrochloride/EDTA) for their extraction. This extract was found to be enriched in acidic glycoproteins with molecular masses in the range 40–72 kDa, and enriched in the amino acids aspartic acid, serine, glutamic acid, glycine and alanine [8]. The enamelin proteins contain very few residues of methionine [11,12]. Immunological and biochemical methods have identified both enamelines and amelogenins in most of the mammalian species studied. Mouse, hamster, cow and human developing enamel matrices are highly enriched in the amelogenin class of proteins, as compared with enamelines [8,13–19]. In contrast, previous results from our laboratory, obtained by mRNA-translation studies, indicated that the fetal rabbit tooth organs only synthesized high-molecular-mass products, suggesting that they are enriched in the ‘enamelin’ class of proteins and apparently did not contain the ‘amelogenin’ class [11,20–22] of proteins.

Although it is accepted that the physiological activity of the enamel proteins is related to the process of biomineralization, the role that each of these proteins may play in this process is as yet unknown. It has been hypothesized that the enamelin class of proteins are synthesized first, and that they serve to initiate the process of nucleation [5,23–25]. The amelogenins, which are more hydrophobic, have been suggested to serve to displace the water during enamel maturation and thereby facilitate crystal growth and orientation, thus controlling the size and shape of the crystals [25,26]. This hypothesis is supported by data derived from *Chondrichthyes*

Abbreviations used: ECM, extracellular-matrix; EOE, enamel organ epithelia.

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(shark) teeth, which apparently do not contain the amelogenin class of proteins, yet show a mineralized enameloid with an aprismatic structure [27]. In contrast, the rabbit enamel, which also appears to be deficient in the amelogenin class of proteins, is characterized by crystals packed into ordered prism groups, as is seen in the enamel of other mammals [22,28].

In the present study, we wanted to determine if the rabbit ameloblast cells synthesize the amelogenin class of proteins, and, if not, how do rabbit teeth maintain the physico-chemical characteristics of prismatic enamel? Experiments were designed to characterize the rabbit enamel ECM by using antibodies raised against both classes of enamel proteins. The biosynthesis of these proteins was analysed by 'pulse-chase' experiments and by analysis of the enamel proteins' mRNA-translational activity. Our findings indicated that there are 'amelogenin-like' proteins present in the rabbit tooth enamel ECM. However, biosynthetic studies showed that the rabbit ameloblast phenotype is different in that it does not synthesize the lower-molecular-mass amelogenin class of proteins as other species (e.g. mouse) do. The 'amelogenin-like' proteins present in the rabbit ECM are suggested to originate from higher-molecular-mass precursors, thus illustrating a very different biosynthetic pathway from that in other mammalian species so far examined. An *N*-terminal sequence of 29 residues, considered characteristic for all amelogenins, was also present in the rabbit 'amelogenin-like' proteins. Moreover, this particular sequence appears to be present in all of the major enamel proteins synthesized by the rabbit ameloblast cells, as determined by using anti-peptide antibodies to a synthetic peptide containing this particular amino acid sequence.

MATERIALS AND METHODS

Extraction of tooth ECM proteins

Molars and incisors were dissected from New Zealand White rabbit fetuses and placed immediately in ice-cold phosphate-buffered saline (0.9% NaCl in 0.1 M-sodium phosphate buffer, pH 7.4). The dental papilla mesenchyme was mechanically removed, and the ECM, containing both enamel and dentine matrices, was washed several times with ice-cold phosphate-buffered saline to remove blood and other contaminating tissues. The tooth ECM proteins were extracted with 0.5 M-acetic acid by placing the tissue in a dialysis bag (3400 Da cut-off) filled with 0.5 M-acetic acid and dialysing them against 0.5 M-acetic acid for 7 days at 4 °C [20]. This method demineralizes the tissues and simultaneously solubilizes the dentine and enamel proteins (the inorganic ions pass through the dialysis bag while the proteins are retained). The extract was centrifuged at 13000 *g* for 10 min and the protein concentration was determined from the A_{280} by using purified bovine immunoglobulin (Bio-Rad, Richmond, CA, U.S.A.) as a standard. Mouse tooth ECM proteins were obtained in a similar manner from 2–5-day postnatal Swiss-Webster mice.

Isolation and characterization of enamel proteins

The enamel proteins were characterized by one- and two-dimensional gel electrophoresis. For one-dimensional gels, SDS/urea/12%-polyacrylamide slab gels were used. Gels were stained with Coomassie Blue or

silver stain [29]. Analysis by two-dimensional gel electrophoresis was done by the methods of O'Farrell [30,31] with some modifications. Proteins were resolved in the first dimension by isoelectric focusing in cylindrical gels with ampholytes in the pH range 5–8. Electrofocusing was carried out in 0.065 M- H_3PO_4 in the lower reservoir and 0.1 M-NaOH in the upper reservoir, at 400 V (constant) for 15 h, followed by 2 h at 800 V. Gels were removed from the glass, equilibrated in electrophoresis sample buffer (0.05 M-Tris/HCl, pH 6.8, containing 2% mercaptoethanol, 1% SDS and 0.001% Bromophenol Blue) for 10 min and then layered on the second-dimension gel, which was an SDS/urea 12% polyacrylamide slab gel. Gels were run at 6 mA/gel (constant current) overnight. Proteins were localized by staining with Coomassie Brilliant Blue R-250 (Bio-Rad) in 27% (v/v) propanol/10% (v/v) acetic acid/0.5% $CuSO_4$. The enamel-protein bands were excised from the gel with a razor blade, extracted in 100 mM-Tris/HCl, pH 8.0, containing 0.1% SDS and 1 mM-EDTA, dialysed for several days against water at 4 °C and then freeze-dried. Purity of the isolated bands was determined by electrophoresis of the isolated protein on one-dimensional gels, followed by silver staining.

Amino acid composition determination. The isolated proteins were hydrolysed overnight in 6 M-HCl at 110 °C, the HCl was removed by evaporation and the amino acid composition determined by ion-exchange chromatography (AAA Laboratories, Mercer Island, WA, U.S.A.).

Amino acid sequence determination. Proteins were isolated from gels as previously described, except that, instead of staining with Coomassie Blue, the gel was submerged in 4 M-sodium acetate. The high salt concentration precipitates the SDS not bound to proteins, and the proteins can be detected as clear bands on an opaque background [32]. The proteins were further purified by acetone precipitation [33] and recovered in trifluoroacetic acid. The amino acid sequence was determined in an Applied Biosystems model 470A gas-phase protein sequencer (USC Microchemical Core Facility, Los Angeles, CA, U.S.A.).

Immunodetection of enamel proteins

Polyclonal antibodies against rabbit enamelin were produced by intradermal injection of the homogenized rabbit ECM 70 kDa protein-gel bands into New Zealand White rabbits. The alloimmune response of rabbits to its own enamel has been previously documented [34]. The production and characterization of the rabbit enamelin antibody as well as the mouse amelogenin antibodies have been previously described [17,20,35]. The procedures for production of synthetic peptides to defined amino acid sequences, as well as the production and characterization of polyclonal antibodies against these peptides (anti-peptide antibodies), were devised by M. L. Snead, M. Zeichner-David, C. Bessem, A. Fincham, E. Lau, M. MacDougall, J. Vides, J. Termine & N. C. Slavkin (unpublished work).

'Western' blot analysis. Proteins fractionated by one- or two-dimensional gel electrophoresis were transferred to nitrocellulose filters [36]. Immediately after transfer, the nitrocellulose sheet was immersed in TBS buffer

[50 mM-Tris/HCl (pH 8.0)/150 mM-NaCl] containing 2% (w/v) gelatin and incubated for 30 min at room temperature. The nitrocellulose was washed in TBS and transferred to a fresh solution of TBS (containing 1% gelatin) containing a 1:500 or 1:1000 dilution of the antibody, and incubated at room temperature overnight [37]. The filter was washed with TBS and then incubated with a 1:3000 dilution of goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad) for 2 h. The filter was washed several times with TBS and then immersed in the colour-development solution (0.3 mg of Nitro Blue Tetrazolium/ml and 0.15 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M-NaHCO₃) (Bio-Rad). Colour development was stopped by immersing the filter in water.

Immunoprecipitation of enamel proteins. Radiolabelled proteins, obtained by mRNA translation in a cell-free system, were pre-absorbed to Protein A by dilution in SAC buffer [0.02 M-phosphate (pH 7.6), 0.15 M-NaCl, 0.25% Nonidet P-40, 10 mM-methionine] to 100 μ l, and 60 μ l of a 10% (v/v) suspension of Pansorbin (Calbiochem, La Jolla, CA, U.S.A.) in SAC buffer was added. The mixture was kept on ice for 15 min, centrifuged in a microfuge and the supernatant transferred to a clean tube. The antibody (50 μ g of IgG) was added, and the mixture incubated for 30 min on ice; then 50 μ l of 10% Pansorbin was added and the mixture incubated for an additional 15 min on ice. The samples were centrifuged (12000 *g* for 5 min), the supernatant was removed and the precipitate (antigen-antibody-Pansorbin) washed several times with SAC buffer. The samples were incubated with electrophoresis sample buffer at 60 °C for 20 min, centrifuged (12000 *g* for 5 min), and the supernatant was analysed by one- or two-dimensional gel electrophoresis, followed by fluorography [38].

Biosynthesis of enamel proteins

The biosynthesis of enamel proteins was analysed by metabolic labelling of the tooth organs in culture or by translational studies at the mRNA level.

Metabolic labelling. Rabbit tooth organs were dissected from 25-days-gestation fetuses. The dental papilla mesenchyme was mechanically separated with extreme caution not to disrupt the enamel organ epithelia (EOE). The EOE, with attached ECM, was preincubated at 37 °C in RPMI-1640 medium (GIBCO, Grand Island, NY, U.S.A.) for 15 min. The medium was removed and replaced with fresh medium containing 50 μ Ci of [³⁵S]methionine, [³H]proline or [³H]leucine/ml. The tissue was incubated for 30 min at 37 °C, the medium removed and replaced by fresh medium containing 10 mM of the corresponding non-radioactive amino acid used for radiolabelling, and the incubation continued for 30 min to 24 h. The tissue was sonicated, washed with cold phosphate-buffered saline and then extracted with acetic acid as described above. The proteins synthesized were analysed by one-dimensional SDS/urea/polyacrylamide-gel electrophoresis followed by fluorography, and then exposed to Kodak X-Omat AR film for 3–14 days at –90 °C.

mRNA translation. For mRNA preparations, developing tooth molar organs were dissected from rabbit

fetuses at sequential different developmental stages (20–28 days gestation). The intact molar or the micro-dissected EOE were frozen immediately on solid CO₂ and maintained at –90 °C until adequate sample sizes were collected. Total RNA was obtained by proteinase K (BRL, Rockville, MD, U.S.A.) digestion, followed by phenol extraction, and the polyadenylated-RNA fraction obtained by oligo(dT)-cellulose chromatography as previously described [20]. The mRNA-enriched fraction or the polyadenylated RNA were translated in a rabbit reticulocyte cell-free system (Amersham, Arlington Heights, IL, U.S.A.) by the method described by the suppliers, with [³⁵S]methionine, [³H]serine or [³H]leucine as amino acid precursor. The enamel mRNA translation products were characterized by immunoprecipitation with antibodies against enamel proteins, followed by gel electrophoresis and fluorography, as described above.

RESULTS AND DISCUSSION

Characterization of enamel proteins

In order to characterize the rabbit enamel ECM proteins, tooth organs were dissected from New Zealand White rabbit fetuses at 25–26 days of gestation. At this developmental stage, both incisors and molars are actively synthesizing and secreting enamel ECM proteins

Fig. 1. Fractionation of rabbit and mouse tooth ECM proteins

Tooth organ ECM proteins were extracted from 25-days-gestation New Zealand White rabbit fetuses and 2-days-postnatal Swiss Webster mice. The acetic acid-extracted proteins (200 μ g) were fractionated by electrophoresis in a SDS/urea/12%-polyacrylamide slab gel, and stained with Coomassie Blue. Lanes: 1, molecular-mass (kDa) markers; 2, mouse tooth ECM proteins; 3, rabbit tooth ECM proteins; 4, 70 kDa protein, cut, and extracted from a preparative gel, and re-electrophoresed to check its purity.

[20]. The dental papilla mesenchyme was removed and the ECM proteins were extracted in acetic acid. Total rabbit ECM proteins, containing both dentine and enamel proteins, were fractionated by one-dimensional SDS/urea/12%-polyacrylamide gel electrophoresis and compared with the well-characterized mouse enamel ECM proteins (Fig. 1). A major component of the rabbit ECM was a protein migrating at approx. 70 kDa. In contrast, the mouse ECM proteins contained a relatively lower proportion of this protein, but were highly enriched in proteins migrating between 18 and 26 kDa. These low-molecular-mass proteins have been characterized as amelogenins by their amino acid composition, and polyclonal antibodies against them have been produced [17,18]. The rabbit 70 kDa ECM protein was purified (Fig. 1, lane 4) and characterized by its amino acid composition as an enamelin (Table 1). Polyclonal antibodies against the rabbit 70 kDa enamelin have been raised in New Zealand White rabbits and characterized by several different methods [20,35]. The enamel ECM immunogens invoke an alloimmune response in the rabbit. It has been suggested that the ameloblast cells and the secreted enamel proteins do not come in contact with the vasculature, and therefore are not recognized by the rabbit immune system [34].

The rabbit anti-(70 kDa enamelin) antibody was used to search for other enamelins or related proteins present in the enamel ECM fractionated in a two-dimensional gel system (Fig. 2). Two identical gels were run: one was stained directly with Coomassie Blue (a), and the second gel was transferred to nitrocellulose paper for 'Western'-blot immunodetection (b). The results shown in Fig. 2 indicated that the 70 kDa rabbit enamelin, which migrates as one broad band in a one-dimensional gel, actually consists of several proteins with very close molecular masses. The 70 kDa 'complex' contained a 70 kDa protein, of pI approx. 6.6, three other proteins of

approx. 65–68 kDa with pI in the range 6.0–6.5, and one protein of approx. 60 kDa, of pI 5.3. In addition to these proteins, the antibody was cross-reactive with two proteins migrating in the 30 kDa region (pI 5.6 and 6.3 respectively) and two low-molecular-mass proteins, 18 and 20 kDa, with more neutral pI values (6.7 and 6.6 respectively). The rabbit ECM low-molecular-mass proteins showed similar isoelectric-focusing behaviour to that determined for mouse amelogenins [25].

Since the tooth ECM contains both enamel and dentine proteins, it is possible that some of the proteins present in the 70 kDa complex represent dentine-derived proteins. However, characterization of the anti-(70 kDa enamelin) antibody by indirect immunofluorescence localized the antigenic activity only in the ameloblast cells and enamel ECM [20,35]. No cross-reactivity was found in the odontoblast cell layer, predentine or dentine, thus indicating that all of the proteins detected by the antibody are ameloblast-derived proteins.

Proteins which were cross-reactive with the anti-(70 kDa protein) antibody were isolated from one-dimensional preparative gels, and their amino acid compositions determined (Table 1). The results indicated that, although the 70 kDa band contains several proteins, the overall amino acid composition resembled that of enamelin proteins. The 30 kDa protein also showed an enamelin-like amino acid composition. These proteins were enriched for the amino acids aspartic acid, serine, glutamic acid, glycine, alanine and leucine, but contained very few residues of methionine. Serine and glycine contents may be slightly elevated, owing to gel contamination, as described by Brown & Howard [39]. The 18 kDa and the 20 kDa proteins contained a very high proportion of proline residues (10–20%), thus resembling the amelogenin class of proteins. However, the overall amino acid composition of these low-molecular-mass proteins differed from that of typical amelogenins in

Table 1. Amino acid compositions of rabbit and mouse tooth enamel ECM proteins

Results are expressed as residues/1000 residues. Abbreviation: TR, trace.

Amino acid	Rabbit ECM proteins				ME*	MA†	RA‡
	70 kDa	30 kDa	20 kDa	18 kDa			
Asp	100	76	60	75	144	48	24
Thr	55	55	42	37	57	39	21
Ser	72	80	81	80	171	106	33
Glu	153	176	147	118	127	151	79
Pro	54	56	220	119	48	316	270
Gly	106	154	122	184	109	62	57
Ala	94	80	133	97	70	49	137
Val	55	52	52	35	55	42	30
Met	6	9	TR	TR	16	41	9
Ile	42	27	20	51	32	33	8
Leu	92	65	90	69	64	86	147
Tyr	23	17	TR	17	16	29	49
Phe	39	18	22	21	23	20	17
His	19	79	10	16	22	52	82
Lys	71	35	ND	49	44	16	15
Arg	30	13	TR	19	TR	13	21

* Mouse enamelins (Slavkin *et al.* [17]).

† Mouse amelogenins (Slavkin *et al.* [17]).

‡ Rabbit 'amelogenins' (22–25 kDa) (Fincham & Belcourt [40]).

Fig. 2. Western-blot immunodetection of rabbit enamel proteins

Rabbit tooth ECM proteins were extracted with acetic acid and fractionated by two-dimensional gel electrophoresis. Approx. 250 μ g of the extracted proteins was separated in the first dimension by isoelectric focusing (IEF) in a pH 5–8 gradient. The IEF gel was overlaid on an SDS/urea/12%-polyacrylamide slab gel for separation on the second dimension ('SDS') by molecular mass. Two gels were run simultaneously; one was stained with Coomassie Blue (a) and the other was electroblotted on to nitrocellulose paper and incubated overnight with a 1:500 dilution of the anti-(70 kDa rabbit enamel) antibody (b) as described in the Materials and methods section. Positions of molecular-mass (kDa) markers are shown to the left. Boxes enclose the positions of low-molecular-mass proteins similar to the amelogenin class of proteins.

being enriched in alanine residues, containing only traces of methionine, and showing a relatively low content of histidine residues as compared with typical amelogenins.

Fincham & Belcourt [40] reported the presence of 'amelogenins' of 22–25 kDa in rabbit enamel ECM. These investigators extracted the rabbit 'cheese-like'

enamel from continuously erupting adult rabbit incisors with guanidine hydrochloride, and isolated a fraction containing the 22–25 kDa proteins by chromatography on Bio-Rad P30 columns. The amino acid composition of these proteins (Table 1, RA), although differing from the amino acid composition obtained for the 18 and 20 kDa proteins reported in the present study, also had a higher content of alanine and a lower content of methionine residues as compared with mouse amelogenins (Table 1, MA) or other typical amelogenin compositions [14], thus suggesting that the rabbit low-molecular-mass proteins represent a hybrid between amelogenins and enamelines. It may be argued that the higher content of alanine residues is characteristic of the rabbit as a species [41,42]; however, the low methionine content appears to be a reflection of the enamelin class of proteins.

In order to characterize further the rabbit enamel ECM proteins, we isolated the 70 kDa, 30 kDa, 20 kDa and 18 kDa proteins from SDS/urea gels, purified them as described in the Materials and methods section, and used them to obtain a partial *N*-terminal amino acid sequence: 29 amino acids were determined from the 18 kDa, 22 amino acids from the 20 kDa and 11 residues were obtained from the 70 kDa (some contamination was found in the analysis of the 70 kDa protein, which was expected, since there is more than one protein in this area). No amino acid sequence was obtained for the 30 kDa protein, thus indicating a probably blocked *N*-terminal residue. The sequences obtained are shown in Fig. 3. All three proteins showed the same *N*-terminal amino acid sequence, except for the 70 kDa protein, which showed both leucine and methionine as *N*-terminal. This sequence is the same *N*-terminal sequence reported for the first 30 amino acids of mouse, pig, human and bovine amelogenins [43–45], the only exception being the *N*-terminal amino acid, which in all these species has been methionine and in the rabbit proteins could be either leucine or methionine.

The presence of the amelogenin *N*-terminal amino acid sequence in the 18 and 20 kDa rabbit proteins suggests that the rabbit enamel ECM does contain the amelogenin class of proteins. However, this same *N*-terminal sequence is also present in one of the components of the 70 kDa protein complex, which, according to their amino acid composition and pI values, may be characterized as 'enamelin' proteins. A possible explanation is that both amelogenins and enamelines share the same *N*-terminal amino acid sequence, thus explaining the observations by many investigators concerning the cross-reactivity between anti-enamelin and -amelogenin antibodies. Although the affinity of the antibody for its own immunogens has been stronger [12], antibodies produced against mouse or bovine amelogenins [8,17,46], or rabbit or human enamelines [12,19,35], are found to be cross-reactive with both enamelines and amelogenins. This cross-reactivity has been interpreted by many investigators as amelogenin contamination of the enamelin immunogen, or vice versa [46]. The *N*-terminal amino acid-sequence data reported in the present paper might indicate that there is at least one epitope shared by both enamelines and amelogenins, thus explaining the cross-reactivity between the antibodies. The fact that it has been possible to produce specific antibodies against amelogenins (which do not cross-react with the enamelin class of proteins) by means of enamelin absorption of the

	1	5	10	15
Am	Met	Pro-Leu-Pro-Pro-His-Pro-Gly-His-Pro-Gly-Tyr-Ile-Asn-Phe-		
18 kDa	Leu-Pro-Leu-Pro-Pro-His-Pro-Gly-His-Pro-Gly-Tyr-Ile-Asn-Phe-			
20 kDa	Leu-Pro-Leu-Pro-Pro-His-Pro-Gly-His-Pro-Gly-Tyr-Ile-Asn-Phe-			
70 kDa	Leu-Pro-Leu-Pro-Pro-His-Pro-Gly-His-Pro-Gly-			
	Met			
		20	25	
Am	Ser-Tyr-Glu-Val-Leu-Thr-Pro-Leu-Lys-Trp-Tyr-Gln-Ser-Ile-			
18 kDa	Xaa-Tyr-Glu-Val-Leu-Thr-Pro-Leu-Lys-Xaa-Tyr-Gln-Ser-Ile-			
20 kDa	Ser-Tyr-Glu-Val-Leu-Thr-Pro-			

Fig. 3. N-Terminal amino acid sequence of rabbit enamel ECM proteins

Rabbit enamel ECM proteins were fractionated by gel electrophoresis; the resolved bands of protein were cut and eluted from the gel as described in the Materials and methods section. A partial amino acid sequence was obtained for the 18, 20 and 70 kDa proteins with an Applied Biosystems model 470-A gas-phase protein sequenator. That of bovine, pig and human amelogenins is also shown (Am).

Fig. 4. Comparison of mouse and rabbit mRNA-translated proteins with ECM proteins

Polyadenylated mRNAs were obtained from rabbit and mouse tooth EOE and translated in a rabbit reticulocyte-lysate cell-free system in the presence of [35 S]methionine. The translation products were immunoprecipitated with 100 μ g of a mixture of equal parts of rabbit anti-(70 kDa enamelin) IgG and anti-(mouse amelogenin) IgG. The immunoprecipitated proteins (a, rabbit; c, mouse) were fractionated by two-dimensional gel electrophoresis (IEF, isoelectric focusing; SDS, SDS/polyacrylamide-gel), fluorographed, exposed to X-ray film and compared with tooth-ECM-extracted proteins from rabbit (b) and mouse (d) stained with Coomassie Blue. Positions of molecular-mass (kDa) markers are shown to the left.

polyclonal antisera or by production of monoclonal antibodies [46] supports this explanation. Another possible explanation is that the *N*-terminal sequence of the 70 kDa complex corresponds to a protein which is not an enamelin, but is actually a high-molecular-mass precursor of the rabbit 'amelogenin-like' proteins. The presence of amelogenin precursors in the 40 kDa range has been previously reported by Belcourt [47] in bovine tooth enamel.

Biosynthesis of enamel proteins

Analysis of the rabbit tooth enamel ECM proteins demonstrated the presence of at least two low-molecular-mass proteins with 'amelogenin-like' properties. However, when rabbit EOE mRNA was translated in a rabbit reticulocyte-lysate cell-free system, and the translation products were immunoprecipitated with a mixture of equal parts of rabbit anti-rabbit enamelin and rabbit anti-mouse amelogenin antibodies and then analysed by two-dimensional gel electrophoresis, only a family of high-molecular-mass proteins (six to eight proteins) was detected (Fig. 4a). All of these proteins had molecular masses in the same range (58–65 kDa) and pI values in the range 5.5–6.0, which are characteristic of the enamelin class of proteins. Small differences in molecular mass and pI between the ECM proteins (Fig. 4b) and the mRNA translation products are probably due to post-

translational modifications such as glycosylation, phosphorylation etc. of the ECM proteins. No low-molecular-mass proteins with 'amelogenin-like' characteristics were detected in the rabbit EOE-mRNA translation products. In contrast, when mouse EOE mRNA was translated in a cell-free system and analysed in a similar manner (Fig. 4), the results obtained were quite different. Only two proteins in the high-molecular-mass area were found, whereas most of the translated products were low-molecular-mass proteins with pI values in the range 6.2–7.0, similar to the amelogenin class of proteins present in the mouse enamel ECM (Fig. 4d).

The mRNA-translation experiments were done with [³⁵S]methionine as the amino acid precursor in the reticulocyte-lysate cell-free system. However, data on the amino acid composition of the rabbit ECM 'amelogenin-like' proteins indicated that these proteins contain very few residues of methionine. To be sure that the reason why we could not detect the presence of these proteins in the rabbit mRNA translation products was not due to the fact that we used [³⁵S]methionine as the radiolabelled precursor in the cell-free system, we also translated the rabbit EOE mRNA in the presence of [³H]serine or [³H]-

Fig. 5. Translation of mRNAs in a cell-free system

Mouse (1) and rabbit (2–4) EOE mRNAs were translated in the presence of [³⁵S]methionine (lanes 1 and 2), [³H]-serine (lane 3) or [³H]leucine (lane 4). The translation products were fractionated by SDS/urea/12% polyacrylamide-slab-gel electrophoresis, fluorographed and exposed to Kodak X-Omat AR film. Positions of molecular-mass (kDa) markers are shown to each side.

Fig. 6. Biosynthesis of rabbit tooth ECM proteins

Rabbit tooth EOE was incubated for 30 min at 37 °C with 50 μ Ci of [³⁵S]methionine (lane 1), [³H]proline (lane 2) or [³H]leucine (lane 3). The medium containing the labelled amino acid was removed and the incubation continued for 30 min in the presence of medium containing the same unlabelled amino acid as that used for radiolabelling. The ECM proteins were extracted with acetic acid, electrophoresed in SDS/urea/12%-polyacrylamide slab gels, fluorographed and exposed to Kodak X-Omat AR film. Positions of molecular-mass markers are shown to the left.

leucine (Fig. 5). No translation products similar in size to the mouse amelogenins were detected with either of these two amino acid precursors, thus suggesting that the rabbit EOE does not synthesize lower-molecular-mass amelogenins like those characterized in the mouse EOE.

The data indicate that the low-molecular-mass 'amelogenin-like' proteins present in the rabbit enamel ECM are not synthesized as original gene products, but are rather processing products from higher-molecular-mass precursors. In an attempt to determine the origin of the 'amelogenin-like' proteins, biosynthetic studies using metabolic 'pulse-chase' experiments were done. Enamel tooth organs were dissected from 25-days-gestation rabbit fetuses, and incubated in RPMI-1640 medium in the presence of [35 S]methionine, [3 H]proline or [3 H]leucine for 30 min at 37 °C. The medium was removed, and the tissue chased for an additional 30 min with unlabelled amino acid. After removal of the ameloblast cells by sonication, the ECM proteins were extracted and analysed by one-dimensional SDS/urea/polyacrylamide-gel electrophoresis (Fig. 6). The labelled protein profile obtained was very similar with all three amino acid precursors. The major proteins labelled were in the 30–70 kDa range, with practically no proteins detectable in the lower-molecular-mass range. When similar experiments have been performed with hamster tooth organs, the low-molecular-mass amelogenins are the predominant class of labelled proteins [19]. These results suggest that the processing of the rabbit higher-molecular-mass precursors to produce the 20 and 18 kDa proteins is not an intracellular process. It only takes 30 min for the enamel proteins to be synthesized and secreted to the extracellular matrix [48,49]. In an attempt to determine when we can first detect the presence of the low-molecular-mass proteins in the enamel ECM, similar

experiments were conducted with different 'chase' time periods ranging from 30 min to 24 h (results not shown). These experiments failed to detect the accumulation of labelled low-molecular-mass material in the ECM proteins, thus supporting the idea that the processing of the high-molecular-mass precursor is a slow event that takes place in the ECM.

Since the results suggest that the appearance of the low-molecular-weight 'amelogenin-like' proteins is a slow process, taking 24 h or more, we analysed rabbit ECM proteins obtained from molar tooth organs at different development stages. Our results indicated that, although the enamel proteins are initially synthesized at 23 days gestation [20], the low-molecular-mass proteins are not present in the extracellular matrix until 24 days of gestation in the rabbit molars (Fig. 7). A comparison of the ECM proteins and the mRNA-translation products obtained from rabbit tooth organs at different development stages indicated, once again, that no nascent low-molecular-mass amelogenins are synthesized in the rabbit EOE at any of the developmental stages sampled.

In search of the 'amelogenin-like' precursor protein(s)

We have established the fact that, although rabbit tooth organs do not synthesize the amelogenin class of proteins as do other species (e.g. mouse), there are 'amelogenin-like' proteins present in the rabbit enamel ECM, as suggested by Fincham & Belcourt [40]. These proteins (18 and 20 kDa) contain the same *N*-terminal amino acid sequence found in the amelogenins. This sequence is also present in a protein of the 70 kDa 'enamelin' complex. In an attempt to determine which protein of the 70 kDa complex contains this particular sequence, and if there are other proteins present in the rabbit enamel ECM that also share this sequence, we took advantage of the fact that our laboratory has

Fig. 7. Comparison of rabbit ECM and mRNA-translated proteins from different developmental stages

Molar tooth enamel ECM proteins and mRNAs were obtained from rabbit fetuses at different days of gestation. Lanes: 1, 22 days; 2, 23 days; 3, 24 days; 4, 25 days; 5, 26 days. The ECM proteins were detected by silver staining. The mRNA-translated products were immunoprecipitated with 100 µg of an equal mixture of anti-(rabbit 70 kDa enamelin) and anti-(mouse amelogenin) IgGs; gels were fluorographed and then exposed to Kodak X-Omat AR film. Positions of molecular-mass (kDa) markers are shown.

Fig. 8. Western-blot immunodetection of *N*-terminal amino acid sequences

Rabbit ECM proteins were fractionated in SDS/urea/12%-polyacrylamide slab gels, stained with Coomassie Blue or electroblotted on to nitrocellulose paper and incubated overnight with a 1:500 dilution of an anti-peptide antibody produced against residues 3–13 of the *N*-terminal amino acid sequence of mouse 26 kDa amelogenin. The antibody was removed, and the filter incubated with a 1:3000 dilution of a goat anti-rabbit IgG antiserum conjugated to alkaline phosphatase ('Immunostain'). Colour development was obtained as described in the Materials and methods section. The labelled arrows (1–9) indicate individual proteins referred to in the text. Key: IEF, isoelectric focusing; SDS, SDS/polyacrylamide-gel electrophoresis. Positions of molecular-mass (kDa) markers are shown.

produced monospecific polyclonal anti-peptide antibodies directed against synthetic peptides produced to well-defined amino acid sequences within the mouse 26 kDa amelogenin molecule. The experimental strategy was to use two-dimensional gel electrophoresis, followed by Western-blot immunoassay using an anti-peptide antibody produced against the *N*-terminal sequence from residues 3–13 of the conserved amelogenin sequence also found in the rabbit ECM proteins. The results in Fig. 8 show that there are several proteins recognized by the antibody; however, the degree of cross-reactivity was considerably different from one protein to another. The strongest activity was present in the low-molecular-mass proteins (7 and 8 in Fig. 8), and some of these proteins were not detected by the Coomassie Blue stain, e.g. protein 9. In addition to these proteins, several components of the 70 kDa complex (Fig. 8, proteins 1–4) were also detected by the anti-*N*-terminal antibody, although the cross-reactivity was considerably weaker as compared with the strong profile obtained for these proteins with the Coomassie Blue stain. The differences in cross-reactivity are probably due to the concentration and availability of the epitopes recognized by the antibody in each of the proteins. The protein of approx. 30 kDa (5; Fig. 8) was also recognized by this monospecific antibody, although the reaction was considerably weaker than with the other proteins.

These results suggest that several proteins in the rabbit enamel ECM contain an amino acid sequence similar to the known *N*-terminal sequence of mammalian amelogenins, and any of these proteins could be the precursor of the 'amelogenin-like' proteins. To determine which one of the mRNA-translated proteins present in the rabbit EOE contains this amino acid sequence, we immunoprecipitated the cell-free-system translation products with the anti-(*N*-terminal peptide) antibody and analysed them by two-dimensional gel electrophoresis (Fig. 9). The results indicated that apparently all the proteins (approx. six) that are immunoprecipitated by either anti-(mouse amelogenin) or -(rabbit enamelin)

Fig. 9. Immunoprecipitation of mRNA-translation products with an *N*-terminal-specific antibody

Rabbit EOE mRNA was translated in a rabbit reticulocyte cell-free system; the [35 S]methionine-labelled products were immunoprecipitated with 10 μ l of an antibody to the *N*-terminal peptide sequence of mouse amelogenins. Analysis was done by two-dimensional gel electrophoresis, followed by fluorography and exposure to Kodak X-Omat AR film for 3 weeks. The labelled arrows (a–f) indicate individual proteins referred to in the text. Key: IEF, isoelectric focusing; SDS/polyacrylamide-gel electrophoresis. Positions of molecular-mass (kDa) markers are shown.

antibodies are also precipitated by the anti-(*N*-terminal peptide) antibody. However, the affinity of the slightly more acidic proteins (c, d, e and f, Fig. 9) is considerably higher than the affinity for the anti-*N*-terminal antibody

shown by the slightly more basic proteins (a and b). These data suggest that the low-molecular-mass 'amelogenin-like' proteins might be processed from proteins d and c, although no direct evidence is yet available. In summary, it appears that an epitope contained in the *N*-terminal amino acid sequence is present in all nascent enamel proteins. This particular sequence appears to be preserved throughout the processing events that take place during enamel maturation, since it was also present in the *N*-terminal sequence of the 22–25 kDa proteins extracted from adult rabbit enamel (A. Fincham, unpublished work). The significance of this highly conserved, highly hydrophobic, sequence, and the fact that it appears to be present in all the enamel proteins, may be related to the function of these proteins, or it could represent a characteristic (like a fingerprint) of all enamel proteins. Indirect data suggest that the 30-amino-acid *N*-terminal sequence is the only sequence shared by amelogenins found in several mammalian species as well as in the rabbit enamel proteins. Northern-blot analysis of rabbit mRNA, using a mouse 26 kDa amelogenin cDNA clone [50], failed to detect amelogenin mRNA sequences in rabbit mRNA, yet recognized a homologous 13 S mRNA species present in mouse, rat, hamster and pig tooth organ mRNAs [22,50]. The 26 kDa mouse amelogenin cDNA clone showed striking homology to the amino acid sequences obtained for pig and bovine amelogenins [51]. However, this particular clone lacks 81 coding bases at the 5' end of the molecule, which include 26 amino acids from the *N*-terminal region of the protein [51].

Conclusions

In conclusion, our studies have shown that the rabbit ameloblast phenotype is different from that characteristic of other mammalian species such as mouse, hamster, rat, pig and bovine [46,51]. The rabbit tooth enamel organ epithelium does not synthesize the lower-molecular-mass amelogenin proteins, as do the other species. However, the rabbit ECM does contain low-molecular-mass proteins which resemble the amelogenin proteins found in other species. These proteins are highly enriched in the amino acid proline (10–20%). This enrichment confers a high degree of hydrophobicity to the molecule, a characteristic of the amelogenins which appears to be related to their role in controlling crystal growth by regulating water elimination. The main difference in the rabbit ameloblast phenotype is that it synthesizes these molecules via a higher-molecular-mass precursor, which is then processed to produce the 'amelogenin-like' molecules. The presence of these proteins in the ECM may explain why the rabbit enamel, in contrast with shark aprismatic enameloid, which does not have amelogenins [27], can produce prismatic enamel. These studies support the hypothesis that the role of the amelogenin class of proteins is to regulate the growth and orientation of the enamel crystals [25,26].

One possible explanation for the difference in the expression of enamel proteins between rabbit teeth and teeth from other mammalian species could be evolutionary. The 'amelogenin-like' proteins present in rabbit teeth might represent a primitive ancestor of the modern amelogenins present in other mammalian species which evolved after the lagomorphs.

Our studies indicate that the 70 kDa complex, which

shows characteristics of the enamelin class of proteins, also shares some sequences with the lower-molecular-mass amelogenins characterized in other species (thus explaining the antibody cross-reactivities). The fact that there are amino acid sequences conserved is suggestive of a structure–function relationship. That the *N*-terminal sequence is important for function is supported by the Northern-blot studies, which suggest that this may be the only sequence conserved in the rabbit primary structure. However, it could also be suggestive of the presence of specific sequences characteristic for all enamel proteins. The fact that the *N*-terminal-sequence-directed antibody cross-reacts with all of the high-molecular-mass nascent chains synthesized by the rabbit ameloblast cell (preliminary data indicate that mouse tooth ECM also contains high-molecular-mass proteins cross-reactive with this antibody) supports this hypothesis. Important information concerning the function and evolution of the enamel proteins will be available when more amino acid sequence data for enamel proteins are obtained.

We are extremely grateful to Ms. Lynn Williams, from the USC Microchemical Core Facility, for the amino acid sequence analysis, Mr. Pablo Bringas Jr. and Mr. Valentino Santos for the art work and photography, and Mr. Dwain Lewis for preparing this manuscript. These studies were supported by N.I.H. Grant DE06852.

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Received 24 August 1987/5 November 1987; accepted 9 December 1987