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Localization and Quantitative Co-localization of Enamelin with Amelogenin

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Abstract

Enamelin and amelogenin are vital proteins in enamel formation. The cooperative function of these two proteins controls crystal nucleation and morphology in vitro. We quantitatively analyzed the co-localization between enamel and amelogenin by confocal microscopy and using two antibodies, one raised against a sequence in the porcine 32 kDa enamel region and the other raised against full-length recombinant mouse amelogenin. We further investigated the interaction of the porcine 32 kDa enamel and recombinant amelogenin using immuno-gold labeling. This study reports the quantitative co-localization results for postnatal days 1, 2, 3, 4, 5, 6, 7 and 8 mandibular mouse molars. We show that amelogenin and enamel are secreted into the extracellular matrix on the cuspal slopes of the molars at day 1 and that secretion continues to at least day 8. Quantitative co-localization analysis (QCA) was performed in several different configurations using large (45 μm height, 33 μm width) and small (7 μm diameter) regions of interest to elucidate any patterns. Co-localization patterns in day 8 samples revealed that enamel and amelogenin co-localize near the secretory face of the ameloblasts and appear to be secreted approximately in a 1:1 ratio. The degree of co-localization decreases as the enamel matures, both along the secretory face of ameloblasts and throughout the entire thickness of the enamel. Immuno-reactivity against enamel is concentrated along the secretory face of ameloblasts, supporting the theory that this protein together with amelogenin is intimately involved in mineral induction at the beginning of enamel formation.

Keywords

enamelin; amelogenin; protein-protein interaction; confocal microscopy; quantitative co-localization analysis

1. INTRODUCTION

The formation of dental enamel is a complex process where ameloblasts regulate the secretion of essential proteins and proteinases in a well-timed and regulated manner. Ameloblasts secrete structural proteins such as amelogenins (Snead et al., 1985), enamelines (Hu et al., 2001b, Hu and Yamakoshi, 2003, Hu et al., 2001a) and ameloblastins (Krebsbach et al., 1996), as well as proteinases [MMP-20 and KLK-4 (Bartlett and Simmer, 1999)] into

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the extracellular matrix where they are critical for the normal development of enamel. It is believed that interactions between these proteins may be essential for controlling enamel crystal formation (Bouropoulos and Moradian-Oldak, 2004, Fan et al., 2009, Fan et al., 2011, Hu et al., 2001a, Iijima et al., 2010, Yang et al., 2011)

Amelogenin is regarded as the major structural protein as it comprises more than 90% of the extracellular matrix protein content. Studies using genetically engineered *amelogenin-null* mice have demonstrated that amelogenin is needed for the formation of organized prisms in normal enamel (Gibson et al., 2001). Numerous investigators therefore have developed strategies to elucidate the structure and function of this important protein, both *in vitro* (Bromley et al., 2011, Delak et al., 2009, Du et al., 2005, Fincham et al., 1998, Lakshminarayanan et al., 2009, He et al., 2008, Beniash et al., 2012, Zhang et al., 2011) and *in vivo* (Fincham et al., 1995, Paine et al., 2003, Gibson et al., 2001.). These studies have supported the theory that amelogenin controls the organization and the oriented growth of enamel crystals. In addition it has been shown that deletion of the highly conserved N- and C- terminal domains forms ill-defined crystals, thus indicating that these domains are essential in protein-protein or protein-mineral interactions (Paine et al., 2003, Sire et al., 2005). Much of the *in vitro* studies on amelogenin has concentrated on the self-assembly of amelogenin into “nanospheres” (Fincham et al., 1995, Moradian-Oldak et al., 2002). An investigation into amelogenin-amelogenin interactions has also been performed using *in vivo* sources (Brookes et al., 2000). Published data also considers the interaction of amelogenin with ameloblastin (Ravindranath et al., 2004), biglycan (Wang et al., 2005) as well as enamelin (Yamakoshi et al., 2003).

Enamelin is the largest enamel protein, however constitutes approximately less than 5% of the crude extracellular matrix extracted by biochemical means. Enamelin is vital to normal enamel development since a true enamel layer is not formed in *enamelin-null* mice (Hu et al., 2008). In contrast to the largely hydrophobic amelogenin, enamelin is generally hydrophilic. While throughout enamel secretory stage in mice enamelin mRNA expression is seen together with that of amelogenin, its expression is terminated prior to amelogenin in the maturation stage (Hu et al., 2001a). Like amelogenin, once enamelin is secreted it is rapidly degraded into a number of proteolytic products. In the case of porcine enamel, the 32 kDa enamelin is stable and regularly isolated for use in structural and functional studies (Fan et al., 2008, Yamakoshi, 1995, Yamakoshi et al., 1998). Although evidence for the presence of the 32 kDa enamelin as an isolated fragment in rodents is lacking (Brookes et al., 2011), there is a remarkably high conservation pattern in the region of the 32 kDa enamelin fragment. This high level of homogeneity among species was suggestive of a critical function of enamelin around the 32 kDa region (Al-Hashimi et al., 2009).

The cooperative function of amelogenin and enamelin was first proposed following the observations that when combined, enamelin promoted the kinetics of nucleation of apatite crystals in a dose-dependent manner (Bouropoulos and Moradian-Oldak, 2004). A previous study involving the immunoprecipitation of isolated porcine 32 kDa enamelin and recombinant porcine amelogenin showed that they interact (Fan et al., 2009). Spectroscopic studies have further indicated that amelogenin and enamelin interact since it has been shown that amelogenin self-association is affected by enamelin addition (Yang et al., 2011). However, all these studies have been executed *in vitro* and an *in vivo* study is needed to verify these interactions. In contrast to well-defined temporal and spatial patterns of mRNA expression for amelogenin and enamelin, data on patterns of protein expression for these two proteins are limited (Hu et al., 2001a, Uchida et al., 1991).

Our present study therefore focuses on confocal microscopy as well as quantitative co-localization analysis to support the hypothesis that amelogenin and enamelin interact *in vivo*

and to give insight as to when these proteins are secreted. Spatial co-localization between two fluorescently labeled proteins is a common approach in microscopy. However, most co-localization techniques rely on visually based interpretation, and therefore are prone to random error and bias (Costes et al., 2004). By using quantitative co-localization analyses significantly more information can be obtained that removes the bias and errors of visual interpretation.

We propose that by utilizing confocal microscopy and quantitative co-localization one can determine whether enamel and amelogenin are spatially related. Our investigation is based on the assumption that if two molecules are within the same area, there is a potential for them to interact. By using mouse mandibular 1st molars at differing postnatal ages (P1 – P8, inclusive) and two antibodies, it is possible to ascertain when both enamel and amelogenin are secreted into the extracellular matrix of enamel, as well as whether they are co-localized, which would support the possibility of their interaction *in vivo*. One antibody was raised against a sequence in the center of the porcine 32 kDa enamel fragment (Hu et al., 2008) and the other antibody raised against full-length recombinant mouse amelogenin (rM179) (Simmer et al., 1994). Direct visualization by TEM of amelogenin assemblies interacting with enamel *in vitro* is further provided by using an immuno-gold labeling technique.

2. MATERIALS AND METHODS

2.1 Expression and purification of recombinant amelogenin

An engineered mutant of full length recombinant amelogenin (rP172) lacking the hydrophilic C-terminal 24 amino acids (rP148) was expressed in *E. coli* and purified as previously described (Ryu et al., 1999, Sun et al., 2006). The rP148 form has amino acids 2-149 of porcine amelogenin (P173) (Yamakoshi et al., 1994, Sun et al., 2006). Briefly, rP148 was purified by ammonium sulfate precipitation and reverse phase high performance liquid chromatography (RPHPLC), using buffer A (0.1% trifluoroacetic acid: TFA) and buffer B (0.1% TFA, 60% acetonitrile). The protein solutions were lyophilized and stored at –20°C until required.

2.2 Extraction and Purification of the 32 kDa enamel

The 32 kDa enamel fragment was extracted following the method previously described (Yamakoshi, 1995, Fan et al., 2008). Briefly, enamel scraped from unerupted 2nd and 3rd unerupted molars of freshly dissected 6 month old pig mandibles (Farmers John Clougherty Co., Los Angeles, CA, USA) through Sierra for Medical Sciences (Santa Fe Springs, CA, USA) were pooled and homogenized in 50 mM Sørensen buffer, pH 7.4 with proteinase and phosphatase inhibitors. The resulting supernatant was treated with ammonium sulfate to first make a 40% saturated solution and then a 65% saturated solution. The resulting pellet was resuspended in 0.1% TFA and purified by RP-HPLC, firstly using a C4 column (250 × 10 mm, Phenomenex) followed by a C18 column (250 × 10 mm, Phenomenex). The protein concentration was determined using the method described by Bradford (Bradford, 1976).

2.3 Immunogold labeling and Transmission Electron Microscopy (TEM)

A 1:100 anti-enamel antibody raised against the sequence EQDFEKPKEKDPPK located in the middle of the 32 kDa enamel region (courtesy of Dr. Jan Hu, University of Michigan) was used. The antibody was found to be highly specific to enamel and did not cross react with other enamel proteins (Fan et al., 2008, Hu et al., 2008). Samples including amelogenin rP148 only (200 µg/mL), 32 kDa enamel only (32 µg/mL), A 10:1 ratio of rP148: enamel, (200 µg/mL: 32 µg/mL) in 1mM sodium phosphate, 9% NaCl pH 7.4 (PBS), were incubated at 37°C for 4 hours on 300 mesh carbon-coated grids. The grids were incubated with primary antibody for 2 hours at 37°C and then secondary antibody (1:100

anti-rabbit colloidal gold 6 nm, Electron Microscopy Sciences) for 2 hours at 37°C. The grids were then examined under a Jeol 1400 TEM with a voltage of 100 kV.

2.4 Tissue Preparation

Mandibular processes of postnatal days 1, 2, 3, 4, 5, 6, 7 and 8 day mouse were dissected and fixed in 10% neutral buffered formalin solution for at least 24 hours. Samples of day 5 and above were subjected to decalcification with 10% EDTA for approximately 1 – 2 weeks. The tissues were then processed for histology and embedded in paraffin. Tissue sections of 7µm thickness were cut from the wax blocks and mounted onto glass slides. To ascertain which sections to use for immunofluorescence some sections were stained with hematoxylin and eosin (data not shown).

2.5 Simultaneous Double Immunofluorescence staining

Tissue sections were subjected to an antigen retrieval step by incubation in 10mM sodium citrate, 0.05% Tween 20, pH 6.0 in a 60°C water bath overnight. The sections were allowed to cool in dH₂O before being rinsed in TBS and then incubated with 0.3% H₂O₂ for 15 minutes. After washing with TBS sections were blocked with 1% bovine serum albumin (BSA) for 15 minutes before incubation overnight at room temperature with a mixture of the primary antibodies (1:1000 chicken anti-amelogenin and 1:500 rabbit anti-enamelin). After washing with TBS, sections were incubated with secondary antibody for amelogenin (1:100 goat anti-chicken-FITC) and for enamel (1:100 goat anti-rabbit-Texas Red) for 3 hours at room temperature, before mounting with Vectashield® Hard Set™ mounting medium with DAPI. The degree of homology between mouse and porcine enamel in the region where the antibody was raised was such that the antibody recognized enamel from both species (Brookes et al., 2011, Fan et al., 2008, Hu et al., 2008). Several controls were performed to ascertain the specificity of the antibodies which included incubating each primary antibody with both secondary antibodies, as well as each secondary antibody with both primary antibodies. The sections were visualized using a Leica TCS SP5 confocal microscope.

2.6 Quantitative Co-localization Analysis

Using the Leica Application Suite Advanced Fluorescence software, version 2.5.2.6939 confocal images were analyzed with a threshold of each channel at 30% and a background correction of 20%, and scatter grams were generated (Figures S1 and S2, Supplementary Information). A scatter gram demonstrates the images unique co-localization profile. Co-localization statistics were generated for Pearson's correlation and to determine an overlap coefficient. The co-localization coefficients M1 and M2 for amelogenin and enamel, respectively, were calculated using the following equations (Manders et al., 1993):

$$M1 = \frac{\sum_i S1_{i,coloc}}{\sum_i S1_i} \quad M2 = \frac{\sum_i S2_{i,coloc}}{\sum_i S2_i}$$

M1 and M2 describe the contribution of each component from two selected channels to the region of interest (ROI) using scatter gram. S1 and S2 are signal intensities of pixels in channels 1 and 2 respectively. $S1_{i,coloc} = S1_i$ if $S2_i > 0$ and $S2_{i,coloc} = S2_i$ if $S1_i > 0$. For example, if the red-green pair of channels is selected and M1 and M2 are 1.0 and 0.5 respectively, this means that all red pixels co-localize with green pixels, but only half of green pixels co-localize with red ones. The value of 1.0 for each channel indicates perfect co-localization. For amelogenin and enamel co-localization patterns were analyzed utilizing smaller regions of interest. These included a large (45µm in height, 33µm width) and a small (7µm diameter) area. Images were analyzed in a variety of different approaches, which included

traversing from the root to the tip of the molar and from the ameloblasts to the dentin-enamel junction, thereby covering the entire thickness of the enamel.

3. RESULTS

3.1 Immunogold labeling of the 32 kDa enamelin interacting with amelogenin

Based on previous immunohistochemistry data in porcine enamel showing a prominent presence of the 32 kDa enamelin fragment (Uchida et al., 1991), where the truncated amelogenin “20K” is also present, we hypothesized that enamelin and amelogenin associate in the extracellular matrix and exert a cooperative effect in controlling mineralization. We have previously used the truncated recombinant porcine amelogenin rP148 and the native 32 kDa enamelin fragment isolated from developing porcine enamel as models to study their interactions *in vitro*. Here we used the antipeptide antibody against enamelin to directly visualize their interactions *in vitro* by TEM (Fig 1). Following labeling with the secondary antibody conjugated with gold nanoparticles, the enamelin solution exhibited the presence of many gold particles (Fig 1A). The typical nano-chains formed by truncated rP148 amelogenin self-assembly are seen as electrolucent structures in Fig 1B, but no gold particles are present. Note the presence of gold particles in a row representing the enamelin molecules co-assembled with amelogenin in Fig 1C. The localization of enamelin with respect to amelogenin nanospheres or oligomers observed in Fig 2C is interpreted as direct association and possible co-assembly of the two proteins.

3.2 Enamelin and amelogenin localization and co-localization

From a visual comparison of confocal images at different ages (Fig. 2) it is possible to see where enamelin (red) and amelogenin (green) localize as well as where these proteins co-localize (yellow/orange color). This visual comparison shows that secretion of enamelin and amelogenin is first observed on the cuspal slopes of the molars, where enamel is first formed, as early in development as postnatal day 1 (Fig. 2A). It can also be seen that at this early stage enamelin and amelogenin co-localize where a yellow or orange color appears because the combined light emissions from the red and green fluorophores are seen by the eye as a yellow/orange color. Samples from postnatal day 2 (Fig. 2B) shows that the pattern of secretion of both proteins follows enamel formation, extending towards the cusp and the cervical loop of the molar, co-localization is also seen throughout the thickness of the developing enamel. As development of the enamel proceeds on days 3 & 4 (Fig 2C & 2D), it can be seen that secretion continues along the portion of the molar where enamel will mature and that co-localization is still visible along the secretory face of the ameloblasts. As the enamel layer thickens, day 5 and 6 (Fig's 2E & 2F) amelogenin can be seen throughout the thickness of the enamel, whereas the majority of the enamelin, and co-localization, can be seen at the secretory face of the ameloblasts. This trend continues in samples from days 7 & 8 (Fig. 2G & 2H), where enamel at the cervical loop of the molar is the last to be formed.

To quantify the percentage of co-localization in the confocal images, QCA was performed on each image to obtain the co-localization coefficients M1 and M2. Large regions of interest (33–45 μm) were used to generate values for the signal intensity of either the green or red channel and the co-localization intensity ($S_{i,\text{coloc}}$), which is the number of pixels that have a co-localizing signal (See M&M for details). The first region of interest analyzed is indicated by the white arrow in each image (Fig. 2), subsequent regions of interest were analyzed, moving away from the cervical loop, in a manner that ensured the entire signal area was utilized with the final region of interest analyzed indicated by the purple arrow. The signal intensity was divided by the co-localization intensity to obtain the co-localization coefficients which describe the contribution of each color channel, red or green, to the co-localization signal. Numerous regions of interest were analyzed on the image wherever a red

or green signal was seen (between the white and purple arrows) and the co-localization coefficients generated were plotted (Fig.3). As can be seen from these co-localization coefficient graphs in early development, day 1, 2 & 3 (Figs. 3A, 3B & 3C) the percentage of amelogenin (light gray) that co-localizes with enamel (dark gray), is higher than the percentage of enamel co-localizing with amelogenin. As development of the enamel progresses on days 4 and 5 (Fig's 3D & 3E) there are similar percentages of both amelogenin co-localizing with enamel and enamel co-localizing with amelogenin. On days 6, 7 and 8 (Fig's 3F, 3G & 3H), when the enamel is more mature, there is a higher percentage of enamel co-localizing with amelogenin than the percentage of amelogenin co-localizing with enamel.

This trend can be clearly seen when the average of the co-localization coefficients for each age is calculated and plotted (Fig. 4). In the early ages (days 1 – 3) more amelogenin (light gray) is co-localizing with enamel (dark gray) than enamel co-localizing with amelogenin. This is shown by the difference between the average co-localization coefficient percentages. From day 1 to day 8 the percentage of co-localized amelogenin begins relatively high at 50%, 57%, and 47% in the first three days, then decreases to 23%, on day 4, and remains relatively low in the range of 13% – 17% with a dip to 8% on day 7. Over the same time period, the percentage of co-localizing enamel starts at 40%, and 39% on the first two days, then decreases from 32% to 24% to 12% from days 3–5, followed by a rebound to 25% and 24% on days 6 and 7, and ending at 35% on day 8.

3.3 Enamelin is secreted into the extracellular matrix before amelogenin

As our findings indicate that enamel was secreted before amelogenin (Figs 2–4), we took a closer look at the quantitative co-localization results analyzed from postnatal day 1 (Fig. 5A). A visual inspection of the confocal image (Fig. 5A) shows that there is only a red color visible at the edges of the visible signal area (ROI1) (protein secretion), indicating more enamel present at the edge. As previously described a higher percentage of amelogenin co-localizes with enamel; however at the edges of the region of protein secretion, no co-localization between the proteins can be seen (Fig. 5B). To determine whether this was due to absence of amelogenin a graph was generated that plotted the signal intensity of each region of interest (Fig. 5C). By comparing the signal intensities it is seen that both proteins are present, however, there is less amelogenin signal than enamel signal, especially at the edges of the region of protein secretion, indicating that at day 1 enamel is secreted into the extracellular matrix before amelogenin.

3.4 Co-localization pattern in 8 day mouse molar

In order to ascertain the pattern of enamel and amelogenin protein secretion QCA was performed on a postnatal day 8 confocal image (Fig. 6) in a series of different configurations A, B, and C. A study revealed that enamel expression terminated on postnatal day 9 of mouse molars (Hu et al., 2001a), thus using postnatal day 8 ensured that both proteins are present so that a pattern can be deduced. Firstly, small regions of interest (ROI=7 μ m) were taken along the secretory face of the ameloblasts (Fig. 6, Configuration A, red), and then through the thickness of the enamel near the cervical loop (Fig. 6, configuration B, yellow) and further towards the cusp (Fig. 6, configuration C, blue) where the enamel is more mature. Along the secretory face of ameloblasts (Fig. 7A1) co-localization decreases as you move from the cervical loop towards the tip of the molar (ROI 1 – 35). The average percentage of amelogenin co-localizing with enamel was 40.9%, and the average percentage of enamel co-localizing with amelogenin was 38.8%. These percentages were remarkably very similar and to support this similarity the signal intensity of each region of interest was plotted (Fig. 7A2). As well as showing the similarity in signal intensity, this graph also shows that there is greater signal intensity for enamel between ROI 10 to 20.

Within the same region on the co-localization coefficient graph (Fig. 7A1) it can be seen that more amelogenin co-localizes with enamel than enamel co-localizing with amelogenin, indicating there is more 'free' enamel. This could be due to a slight delay in protein degradation by MMP-20.

To support the observation that enamel and amelogenin are spatially present in similar ratios, at the secretory face of the ameloblasts, we performed the analysis from the ameloblasts taken across the thickness of the enamel (Fig's 7B & 7C). Moving across the enamel layer, from the ameloblasts, near the cervical loop (Fig. 7B1), the co-localization of enamel and amelogenin first decreases sharply before leveling out. The co-localization coefficients are again similar, indicating that equal amounts of enamel and amelogenin are present at the cervical loop. As the enamel matures (Fig. 7C1), again the co-localization of enamel and amelogenin decreases sharply as you move away from the ameloblasts, however, at a certain distance (approximately 10 μ m) from the ameloblasts, no co-localization between enamel and amelogenin can be seen. Even though both proteins are present, as can be seen by the signal intensity graphs (Fig's 7B2 & 7C2), only amelogenin is clearly visible (Fig 6, Configurations B and C).

DISCUSSION

Significant advances have been made in identifying the critical players in protein-mediated enamel biomineralization. Studies using *Amel* and *Enam* knock-out mice have proved that amelogenin and enamel are both absolutely essential for normal enamel development (Gibson et al., 2001, Hu et al., 2008) while the lack of the latter has the more severe effect since it results in no true enamel being formed. Our present investigation was based on the hypothesis that the two proteins have a cooperative function during the early stage of enamel crystallite formation through direct protein-protein co-assembly (Bouropoulos and Moradian-Oldak, 2004). We have recently described interactions between enamel and amelogenin, but the majority of these data were based on *in vitro* observations (Bouropoulos and Moradian-Oldak, 2004, Fan et al., 2009, Fan et al., 2011, Iijima et al., 2010, Yang et al., 2011). Because of its relative abundance and stability we have used the porcine 32 kDa enamel for our *in vitro* structural-functional studies. In the case of rodent enamel however the 32 kDa does not accumulate as a stable intermediate product and larger products containing this highly glycosylated and phosphorylated motifs may well be the functional segment (Brookes et al., 2011). While protein-protein interactions are believed to play an important role in the process of enamel biomineralization, (Fincham et al., 1999), other than amelogenin self-assembly, *in vivo* data on amelogenin-non amelogenin interactions is limited.

In order to give further insight into the putative cooperative function of amelogenin and enamel we took advantage of quantitative co-localization techniques and performed a systematic analysis of the localization of enamel with respect to amelogenin in mouse mandibular molars. We performed an additional *in vitro* study using TEM immuno-gold and anti-enamel antibodies to show that the gold particles lined up in rows where amelogenin nanochains were formed.

We concentrated on confocal images from mandibular 1st molars from mice between the ages of postnatal day 1 to 8. Previous research into the expression of enamel and amelogenin by *in situ* hybridization (Hu et al., 2001a) has indicated that mRNA expression of these two proteins in maxillary mouse molars was first observed in pre-secretory ameloblasts at postnatal day 2 and that expression of both proteins was seen until enamel expression terminated in maturation stage ameloblasts at postnatal day 9. Thus, using samples up to postnatal day 8 ensures that both amelogenin and enamel will be present.

This study showed that secretion of amelogenin and enamelins starts at the cuspal slopes of molars, as seen on postnatal day 1 and 2 (Fig's 2A and 2B). We observed that secretion continues until at least postnatal day 8 and that secretion is last seen in the cervical loop of molars (Fig. 2H). This is in agreement with the study of mRNA expression of these two proteins (Hu et al., 2001a) except that the latter study reports that mRNA expression of both amelogenin and enamelins is not seen until postnatal day 2. This difference could be explained by developmental differences between maxillary and mandibular molars (McCollum and Sharpe, 2001) or by individual difference between mice and their strains.

Confocal microscopy has been used to determine co-localization between two fluorescently labeled proteins for a number of years. Co-localization is an important tool in many biological studies, as spatially related proteins have a high probability of interacting. However, most co-localization analyses rely on visual interpretation. Using quantitative co-localization analysis, significantly more data can be generated that removes bias and errors of visual interpretation (Costes et al., 2004). Reliable quantitative co-localization analysis is only limited by the quality and suitability of the confocal image which might be dependent on the antibodies used, and the resolution of the pixels. This study utilized the co-localization coefficients M1 and M2 as these coefficients are not dependent upon the intensities of the signals and can compare signal intensities for different fluorophores when their signal intensities differ (Manders et al., 1993). These coefficients give a measure of the amount of fluorescence of the co-localizing objects of the image relative to the total fluorescence. By plotting the percentages of co-localization using the M1 and M2 of amelogenin or enamelins against the regions of interest, patterns of co-localization can be revealed and comparisons can be made even if the epitope recognized on enamelins is shorter (i.e. one epitope of 14 amino acids) than amelogenin (the entire full-length). The use of an anti-peptide antibody against enamelins may allow us to detect the full-length protein but following enamelins proteolysis we are limited to detection of those products that contain that epitope. Such products may include the recently reported rat enamelins proteolytic products; the 60–70, 37, and 50 kDa products detected by Western blot analysis. While the pattern of proteolysis of porcine enamelins is well documented (Hu and Yamakoshi, 2003) information for rodent proteolytic pattern is limited. In the present study it is not possible to follow migration pattern of proteolytic products from the C-terminal (Yamakoshi, 1995). It is notable that signals for amelogenin and/or enamelins were not detected within the ameloblasts. The lack of intracellular signals may be due to the lack of exposure of the epitopes inside the cells. Following their secretion and processing by MMP-20 epitopes on both amelogenin and enamelins can be more available for interaction with the antibody.

As seen from the graphs generated, the percentage of co-localization between enamelins and amelogenin varies with the postnatal age of the mouse (Fig 4). At early ages, when enamelins is initially developing, there is a greater percentage of amelogenin co-localizing with enamelins than enamelins co-localizing with amelogenin, therefore there is more 'free' enamelins than 'free' amelogenin. As the enamel matures this pattern becomes reversed. For a time, on days 4 and 5, the percentages of co-localization of enamelins and amelogenin are similar, and then the percentage of co-localizing enamelins becomes greater than the percentage of co-localizing amelogenin (days 6–8), indicating there is now more 'free' amelogenin than 'free' enamelins. This also indicates that there is more amelogenin present in the extracellular matrix than enamelins at later stages, which is in accordance with the recent report that the extracellular matrix at maturation stage is devoid of enamelins (Brookes et al., 2011).

Since we observe more 'free' enamelins at day 1 we interpret the data to suggest that enamelins is secreted before amelogenin. Our analysis of the confocal images of postnatal day 1 (Fig.5) revealed that only the red enamelins fluorophore is visible at the edges of an

area of discernible signal. The pattern of co-localization, as determined by the co-localization coefficients, shows that there is no co-localization present at these edges, and that co-localization begins further from the edge of the visible signal (Fig. 5B). To ascertain whether the lack of co-localization at the edge is due to absence of one of the proteins, the fluorescence intensity signal at the edge was plotted (Fig. 5C) for both amelogenin and enamel. Although both proteins are present, the fluorescence signal for amelogenin is much lower than that for enamel. This greater fluorescence signal of enamel indicates that more of this protein has accumulated at the edge, suggesting that secretion of enamel may have begun before secretion of amelogenin.

Analysis of postnatal day 8 (Fig. 6) revealed that amelogenin and enamel co-localize at the secretory face of ameloblasts and that co-localization decreases as the enamel matures. This process occurs both throughout the thickness of the enamel layer (Fig's 7B & 7C) as well as along the secretory face of ameloblasts moving towards the cusp of the molar (Fig. 7A). Unfortunately, this study cannot determine conclusively that secretion happens within the secretory, transition, or maturation stages as these stages are determined by the morphology of the ameloblasts, which cannot be clearly seen on confocal images. However, as the enamel matures, secretion of both amelogenin and enamel ceases thereby no co-localization is seen in mature enamel. The co-localization percentages for amelogenin and enamel seen along the secretory face of the ameloblasts are very similar, suggesting that amelogenin and enamel are spatially present in similar quantities. As this is very close to where secretion of these proteins occurs, it is possible to infer that secretion rates of these proteins are similar and that they may be present in a 1:1 ratio before they begin being processed by MMP-20. This interpretation is consistent with the theory that amelogenin and enamel cooperate to control crystal nucleation and morphology (Bouropoulos and Moradian-Oldak, 2004, Fan et al., 2011, Iijima et al., 2010, Iijima and Moradian-Oldak, 2004).

Although only one image of each postnatal day is represented we have performed this experiment multiple times to ensure the accuracy of these findings. We use quantitative co-localization analysis as a tool to elucidate the trend that cannot be clearly seen by a visual analysis of the images, thereby the percentages that we have used within this text is a guide to highlight and clearly describe the trend and not an accurate measurement of co-localization at that particular stage as there will be differences between samples.

It is impossible to determine the interaction site for each protein using confocal microscopy, however it has been suggested that enamel interacts with amelogenin within the 32 kDa fragment (Fan et al., 2009), most likely with the N-terminal region of amelogenin (Ravindranath et al., 1999). Since the antibody used in this study is raised from a sequence (EQDFEKPKEKDPPK) within the 32 kDa fragment, and protein-protein interactions can obscure antibody epitope sites, it is possible to infer that this area on the 32 kDa enamel is not where the interaction occurs. Due to the fact that the amelogenin antibody was raised against full-length protein, there are many possible epitope sites and so it cannot be determined where the interaction occurs. As amelogenin has many epitope sites it is possible to get a stronger signal when using this antibody. To counteract differing signal intensities between amelogenin and enamel, co-localization coefficients M1 and M2 were used, as these are not dependent upon the intensities of the signals.

Development of quantitative co-localization technique opens up many possibilities for future studies. Using antibodies raised to different epitopes on the enamel sequence, as well as specific epitopes on the amelogenin sequence one can elucidate where specific MMP-20 cleavage products are located within the developing enamel and study which parts of enamel and amelogenin are important in enamel formation. It is also possible to elucidate

the localization of ameloblastin, or biglycan, in relation to enamelin or amelogenin and gain information as to whether these proteins interact. One could also determine when and where MMP-20 or KLK4 are secreted into the extracellular matrix. This technique could be used in *MMP-20* knockout mice to see whether enamelin and amelogenin, as well as other proteins, are found in significant quantities though out the entire thickness of the enamel in the absence of cleavage by the proteases.

5. CONCLUSIONS

We used dual color confocal microscopy to report quantitative co-localization fractions of amelogenin and enamelin in postnatal days 1, 2, 3, 4, 5, 6, 7 and 8 mandibular mouse molars. We show that amelogenin and enamelin are secreted into the extracellular matrix at day 1 and that secretion continues to at least day 8. At day 8 enamelin and amelogenin co-localize near the secretory face of the ameloblasts and appear to be secreted approximately in a 1:1 ratio. Co-localization decreases as the enamel matures, both along the secretory face of ameloblasts and throughout the entire thickness of the enamel. Enamelin is concentrated along the secretory face of ameloblasts; the finding that enamelin and amelogenin co-localize in vivo suggests that they cooperate to control crystal nucleation and morphology. Confocal microscopy, in conjunction with quantitative co-localization analysis, can give invaluable data to increase our knowledge of enamel formation. These approaches can tell us which proteins are involved and at what times and places they are secreted or removed from the extracellular matrix. This information, in turn, tells us which proteins are in a position to interact with one another.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

TEM images of immunogold labeling of enamelin, amelogenin and their complexes using the anti 32 kDa enamelin antibody and secondary antibody conjugated with 6nm gold particles. A) Solution of the 32 kDa enamelin fragment in PBS. B) Solution of the rP148 recombinant amelogenin in PBS. C: Mixture of enamelin-amelogenin in PBS (1:10).

Figure 2.

Confocal images of mouse mandibular molars showing immunofluorescence of amelogenin (green) and enamelin (red) at different ages, day 1 – 8 (A – H respectively). Co-localization of amelogenin and enamelin is revealed by overlapping signals resulting in yellow staining. The white arrow represents region of interest 1 for QCA analysis and the purple arrow is the last region of interest to be analyzed. Nuclei are stained with DAPI (blue).

Figure 3.

Co-localization coefficient graphs obtained from confocal images of mouse mandibular molars in Figure 2. Each graph represents differing ages of mouse used, days 1 – 8 (A – H, respectively). Each image was analyzed using a large region of interest to incorporate the entire area of the signal seen and starts from the white arrow through to the purple arrow. Graphs were generated by plotting the co-localization coefficients against the region of interest. The co-localization coefficients of amelogenin are shown in light gray and the co-localization coefficients of enamel are shown in dark gray.

Figure 4.

Comparison of the differences in the average co-localization coefficients of each region of interest obtained from each confocal image of mouse mandibular molars. The average co-localization coefficient of amelogenin is in light gray and the average co-localization coefficient of enamel is in dark gray.

Figure 5.

Confocal image of postnatal day 1 mouse mandibular molar (A) showing the first and last regions of interest (white circles) analyzed as well as direction of quantitative co-localization analysis (white arrow). See scatter gram in Figures S1. B) Graph of co-localization coefficients obtained from the regions of interest shown in A. C) Graph of the signal intensity of each region of interest. Amelogenin is represented in light gray and enamel in dark gray. ROI1 and ROI32 are the first and last regions of interest corresponding to 5B and 5C.

Figure 6.

Confocal image of postnatal day 8 mouse mandibular molar showing analysis of three different configurations (A, B and C). See scatter gram in Figures S2. A) Regions of interest analyzed (red circles) along the secretory face of the ameloblasts and direction of analysis (red arrow). ROI1 and ROI35 are the first and last regions of interest corresponding to figures 7A1 and 7A2 respectively. B) Direction of analysis through the enamel layer at the cervical loop (yellow arrow). ROI1 and ROI19 are the first and last regions of interest corresponding to figures 7B1 and 7B2 respectively C) Direction of the regions of interest analyzed (blue arrow) through the enamel layer. ROI1 and ROI20 are the first and last regions of interest corresponding to figures 7C1 and 7C2.

Figure 7.

A) Graph of the co-localization coefficients (A1) and the signal intensity (A2) obtained from the regions of interest analyzed in configuration A from Fig 6. B) Graph of the co-localization coefficients (B1) and signal intensity (B2) found at specific distances from the ameloblasts analyzed from regions of interest obtained in configuration B (Fig. 6). C) Graph of co-localization coefficients (C1) and the signal intensity (C2) found at specific distances from the ameloblasts analyzed from regions of interest obtained in configuration C (Fig. 6). Amelogenin is represented in light gray and enamel in dark gray.