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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 enamelin and amelogenin by confocal microscopy and using two antibodies, one raised against a sequence in the porcine 32 kDa enamelin region and the other raised against full-length recombinant mouse amelogenin. We further investigated the interaction of the porcine 32 kDa enamelin and recombinant amelogenin using immuno-gold labeling. This study reports the quantitative co-localization results for postnatal days 1–8 mandibular mouse molars. We show that amelogenin and enamelin 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 enamelin 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 enamelin 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.

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), enamelins (Hu et al., 2001a,b; Hu and Yamakoshi, 2003) and ameloblastins (Krebsbach et al., 1996), as well as proteinases [MMP-20 and KLK-4 (Bartlett and Simmer, 1999)] into 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, 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

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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 enamelin 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 enamelin 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 enamelin 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 enamelin *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 (RP-HPLC), 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 enamelin

The 32 kDa enamelin 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 Sørenson 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-enamelin antibody raised against the sequence EQDFEKPKEKDPPK located in the middle of the 32 kDa enamelin region (courtesy of Dr. Jan Hu, University of Michigan) was used. The antibody was found to be highly specific to enamelin 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 enamelin only (32 μg/mL), A 10:1 ratio of rP148: enamelin, (200 μg/mL: 32 μg/mL) in 1 mM sodium phosphate, 9% NaCl pH 7.4 (PBS), were incubated at 37 °C for 4 h on 300 mesh carbon-coated grids. The grids were incubated with primary antibody for 2 h at 37 °C and then secondary antibody (1:100 anti-rabbit colloidal gold 6 nm, Electron Microscopy Sciences) for 2 h 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 1–8 day mouse were dissected and fixed in 10% neutral buffered formalin solution for at least 24 h. 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 7 μm 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 10 mM sodium citrate, 0.05% Tween 20, pH 6.0 in a 60 °C water bath overnight. The sections were allowed to cool in dH_2O before being rinsed in TBS and then incubated with 0.3% H_2O_2 for 15 min. After washing with TBS sections were blocked with 1% bovine serum albumin (BSA) for 15 min 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