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XBP1 May Determine the Size of the Ameloblast Endoplasmic Reticulum

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Abstract

Ameloblasts progress through defined stages of development as enamel forms on teeth. Pre-secretory ameloblasts give rise to tall columnar secretory ameloblasts that direct the enamel to achieve its full thickness. During the maturation stage, the ameloblasts shorten and direct the enamel to achieve its final hardened form. Here we ask how the volume of selected ameloblast organelles changes (percent volume *per* ameloblast) as ameloblasts progress through six defined developmental stages. We demonstrate that mitochondria volume peaks during late maturation, indicating that maturation-stage ameloblasts maintain a high level of metabolic activity. Also, the endoplasmic reticulum (ER) volume changes significantly as a function of developmental stage. This prompted us to ask if X-box-binding protein-1 (XBP1) plays a role in regulating ameloblast ER volume, as has been previously demonstrated for secretory acinar cells and for plasma cell differentiation. We demonstrate that *Xbp1* expression correlates positively with percent volume of ameloblast ER.

Keywords

X-box-binding-protein-1; enamel development; organelle volume

INTRODUCTION

Ameloblasts progress through defined stages of development as dental enamel matures from a soft cheese-like substance into its final hardened form. Pre-secretory ameloblasts stop dividing and enlarge into tall columnar secretory ameloblasts that align more or less vertically to the adjacent enamel and are responsible for secreting enamel matrix proteins. During the maturation stage, the ameloblasts become shorter and assist in the removal of proteins from the matrix as the enamel hardens (Smith, 1998; Bartlett and Simmer, 1999). Once the enamel is fully mature, ameloblasts regress and become part of the reduced enamel organ that covers and protects the completed enamel surface until the tooth erupts (Kallenbach, 1970). In this study, we quantify the percent volume of various organelles as rat incisor ameloblasts progress through six defined morphological stages of enamel development. The endoplasmic reticulum (ER) displayed one of the most dramatic differences in percent volume as a function of developmental stage. The secretory ameloblasts had dramatically more ER as a percent of total

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cell volume than did prior or subsequent stages. This prompted us to inquire how ER abundance is regulated within the ameloblast.

The ER is the site of synthesis and folding of secretory pathway proteins. Previously, it was demonstrated that the transcription factor XBP1 is required for B-cells to differentiate into antibody-producing plasma cells (Reimold *et al.*, 2001). XBP1 was also demonstrated as being responsible for expansion of the plasma cell ER and secretory apparatus (Shaffer *et al.*, 2004). A similar role for XBP1 was demonstrated in exocrine glands, such as in pancreatic and salivary acinar cells (Lee *et al.*, 2005). Interestingly, the active form of XBP1 is derived by a spliceosome-independent endoribonuclease-mediated reaction by the ER transmembrane protein kinase (IRE1). IRE1 removes 26 bp of the *Xbp1* transcript, creating a frame shift that encodes the active transcription factor XBP1(S) (Yoshida *et al.*, 2001; Calton *et al.*, 2002; Lee *et al.*, 2002). It was subsequently demonstrated that IRE1 is required for plasma cell secretory function, thereby demonstrating that XBP1(S) is responsible for expansion of the ER and secretory apparatus (Iwakoshi *et al.*, 2003).

Like exocrine gland cells, ameloblasts present in the secretory stage of development have an expanded ER and secretory apparatus. We therefore asked if enamel organ cells express *Xbp1*, and if so, whether *Xbp1* expression levels correlate positively with the quantity of ER present within the ameloblast. Since ameloblast ER percent volume differs dramatically depending on the developmental stage, we also asked if *Xbp1* expression levels are dictated by developmental stage during dental enamel formation.

MATERIALS & METHODS

All handling, care, and usage of animals were approved by McGill University and The Forsyth Institute.

Stereology of Ameloblast Organelles at Defined Stages of Development

Sprague-Dawley rats were perfused with 5% glutaraldehyde in 0.5 M sodium cacodylate buffer (pH 7.4). Mandibular jaws were removed and decalcified in EDTA, and enamel organ segments were dissected from each incisor at 6 locations along the labial aspect. The segments were embedded in Epon from which thin sections were cut, and a series of 4-8 non-overlapping micrographs was taken across the length of each ameloblast on a Philips 400 electron microscope. Four different microscope fields were sampled *per* tooth at each of the 6 locations. Micrographs were projected through an enlarger at x30,000 final magnification. A double square lattice test system mounted on a transparent acetate sheet was superimposed over each micrograph. The numbers of points hitting ameloblast organelles (Table) were counted across all micrographs. Point count data were pooled to yield a mean volume fraction of organelles for a given unit sample.

Immunohistochemical Identification of Active IRE1 in Ameloblasts

Mouse incisors were formalin-fixed, decalcified in a solution of 20% sodium citrate 4% formic acid, paraffin-embedded, and sectioned. Sections were incubated in blocking agent (goat serum) for 20 min, overnight in anti-phospho IRE1-specific antisera (1:100) (Lipson *et al.*, 2006), in peroxidase-conjugated antibody (Vectastain Elite Reagent, Vector Laboratories, Burlingame, CA, USA), and Sigma Fast DAB substrate, and counterstained with 0.1% FastGreen. Sections were examined by light microscopy for the presence of active IRE1. The negative control section was not treated with the IRE1-specific antisera.

Analysis of Splice Form Abundance by *Pst*I Digestion of *Xbp1* cDNA

Total RNA was extracted from first molar enamel organs by the use of Trizol reagent from 4-day-old mice (secretory stage) or 11-day-old mice (maturation stage). cDNA was prepared with the SuperScript II first-strand synthesis system (Invitrogen, Carlsbad, CA, USA). Mouse *Xbp1* primers were: F5'-AAACAGAGTAGCAGCGCAGACTGC-3' and R5'-TCCTTCTGGGTAGACCTCTGGGAG-3'. The primer annealing temperature was 66°C, and reactions containing 100 ng cDNA proceeded for 35 cycles. The PCR product was digested with *Pst*I and run on a 2% agarose gel to identify the presence of spliced *Xbp1* mRNA.

Quantitative Real-time PCR (qPCR) Analysis of Secretory- and Maturation-stage Enamel Organ

The PCR temperature profile was 3 min 95°C initial melt, then 20 sec 95°C, 30 sec 65°C for 45 cycles, then 30 sec 95°C, for 1 cycle, and 1 min 55°C, followed by stepwise temperature increases from 55°C to 95°C to generate the melt curve. Standard curves were generated with each primer set by use of untreated control cDNA preparations and a 10-fold dilution series ranging from 1000 ng/mL to 100 pg/mL. PCR efficiencies and relative expression levels of total *Xbp1*, *Xbp1*(S), *Enam*, and *D3Ucla1* (RAMP4), as a function of the stably expressed internal reference control gene eukaryotic translation elongation factor 1 alpha 1 (*EF1a1*), were calculated as previously described (Pfaffl, 2001; Kubota *et al.*, 2005).

Primer sequence for expression analyses were: (*Xbp1*) F5' - TCCGCAGCACTCAGACTATGT-3' and R5' - ATGCCCAAAGGATATCAGACTC-3'; (*Xbp1*) (S), F5' - GAGTCCGCAGCAGGTG-3' and R5' - GTGTCAGAGTCCATGGGA-3' (Back *et al.*, 2005); (*Enam*) F5'-CTTTGGGGGTCGTCCTTATTACTC-3' and R5'-CCTCTGGGGGTGGGTCATC-3'; and (*D3Ucla1*) F5'-CACAGCAAGAACAATACTCAGCG-3' and R5'-CTACCGACGCCTTTTCTTCG-3'. Internal control primers for *EF1a1* were: F5'-ATTCCGGCAAGTCCACCACAA-3' and R5'-CATCTCAGCAGCCTCCTTCTCAAA C-3'.

Statistical Analysis

Statistical significance was evaluated by a non-parametric analysis of variance (ANOVA) with Bonferroni's post-test.

RESULTS

Ameloblast Organelle Volume as a Function of Developmental Stage

The percent volume of selected rat incisor ameloblast organelles at 6 different stages of enamel development was quantified by stereology. Each percent volume represents data generated from approximately 4 ameloblasts *per* incisor from 2 incisors, performed on 10 different animals *per* stage. Shown are the averaged percent volumes obtained for each organelle and the average number of points counted *per* ameloblast at each developmental stage (Table). We calculated percent volume by dividing the number of points over an organelle by the total number of points counted *per* ameloblast. The developmental stages examined were the pre-secretory stage (Pre-SEC), the start of the secretory stage (Start-SEC), the end of the secretory stage (End-SEC), the early maturation stage (Early-MAT), the late maturation stage (Late-Mat), and the regression stage (Regress). In general, the nucleus occupied a smaller proportionate volume of ameloblasts as development progressed, while the cytosol remained relatively constant. In contrast, the mitochondria, granules, dense bodies/vacuoles, and filaments tended to occupy a proportionately larger volume of ameloblasts as development progressed.

The ER, Golgi, and mitochondria percent volumes as a function of developmental stage are presented in graph form (Fig. 1). Note that as a percentage of cell volume, both the ER and Golgi rose to a peak at the late secretory stage and declined thereafter. Interestingly, with the exception of the regression stage, the percent volume of the mitochondria steadily rose as enamel development progressed.

Identification and Localization of Active Phosphorylated IRE1 in the Mouse Enamel Organ

Because activated IRE1 splices the *Xbp1* transcript to create *Xbp1(S)*, we asked if secretory-stage ameloblasts with peak ER volume express phosphorylated (active) IRE1. Furthermore, we asked if IRE1 expression is down-regulated during the maturation stage, when the ameloblasts become shorter and the percent volume of ER *per* ameloblast progressively reduces (Fig. 1). Sections of continuously erupting maxillary incisors were stained by immunohistochemical methods with antisera that detect phosphorylated IRE1. Diffuse staining was observed throughout the enamel organ tissues (Fig. 2A). However, the secretory-stage ameloblasts stained strongly, indicating high levels of activated IRE1 (Fig. 2B). The staining was less intense in maturation-stage ameloblasts (Fig. 2C).

Quantification of Total *Xbp1* and *Xbp1(S)* Expression in the Developing Enamel Organ

The immunohistochemical results indicated that IRE1 was activated at greater levels in secretory-stage ameloblasts as compared with maturation-stage ameloblasts. We therefore asked if the expression of spliced *Xbp1* also differed between these two major stages of amelogenesis. First molar enamel organs from 4-day-old mice (secretory stage) and first molar enamel organs from 11-day-old mice (maturation stage) were assessed for total and spliced (active) *Xbp1* expression. The 26-bp *Xbp1* splice region contains a PstI site (Fig. 3A) that is removed upon splicing and is useful in determining the relative amount of *Xbp1(S)* vs. unspliced transcripts. Relatively more *Xbp1(S)* mRNA was present in first molar enamel organs from the secretory stage than was present at the maturation stage of development (Fig. 3A). To quantify this difference more precisely, we designed qPCR primer sets that recognize total *Xbp1* message or that recognize only the active *Xbp1(S)* form. Overall, approximately twice as much *Xbp1* message was expressed in the secretory-stage (Sec) vs. the maturation-stage (Mat) enamel organ ($P < 0.01$). However, this difference increased approximately five-fold ($P < 0.01$) when the expression levels of *Xbp1(S)* were assessed (Fig. 3B). The kidney served as an epithelium-derived negative control, and enamelin, which is expressed at greatly reduced levels in the maturation stage, served as a positive control to confirm detection of reduced maturation-stage expression. XBP1(S) was previously demonstrated to induce the expression of ribosome-associated membrane protein 4 (RAMP4; *D3Ucla1*) (Lee *et al.*, 2003; Acosta-Alvear *et al.*, 2007). Therefore, RAMP4 also served as a positive control.

DISCUSSION

Significantly, the percent volume of the ameloblast mitochondria peaked at late maturation, when proteins are removed from the enamel matrix (Robinson *et al.*, 1988) and when most of the enamel mineral (hydroxyapatite) precipitates. Precipitation of a single unit cell of hydroxyapatite releases between 8 and 14 hydrogen ions. The exact number depends primarily on the source of the inorganic phosphate (Simmer and Fincham, 1995). Thus, the ameloblasts likely expend considerable amounts of energy to buffer or flush out the substantial quantities of hydrogen ions produced when mineral precipitates (Smith *et al.*, 2005). Additionally, substantial energy expenditure may be required to remove protein from maturation-stage enamel. Therefore, although maturation-stage ameloblasts do not secrete large quantities of protein into the enamel matrix, they are still highly active, and this is when the percent volume of mitochondria *per* ameloblast reaches its peak.

Also significant was that the ER and Golgi occupy the greatest percent volume during the secretory stage. This result was expected, since secretory cells, such as plasma cells or exocrine cells, have an abundance of ER into which signal-peptide-containing proteins are translocated prior to their secretion (Shaffer *et al.*, 2004; Lee *et al.*, 2005). Once ameloblasts progressed to the maturation stage, when the majority of protein secretion ends, the ER percent volume *per* ameloblast decreased significantly. This confirms a prior stereology study demonstrating that percent volume of the ameloblast ER decreased in maturation, while the volume of mitochondria increased (de Assis *et al.*, 2003). Analysis of these data prompted us to characterize the molecular mechanisms that regulate the size of the ER during amelogenesis.

Previously, it was demonstrated that the differentiation of a B-cell into an antibody-producing plasma cell requires the transcription factor XBP1 (Reimold *et al.*, 2001), and it was also shown that XBP1 expression is necessary to expand the ER and increase protein synthesis necessary for plasma cell differentiation (Shaffer *et al.*, 2004). Furthermore, expansion of the ER by XBP1 is not restricted to plasma cell differentiation. XBP1 is also essential for the well-developed ER in pancreatic and salivary gland acinar cells (Lee *et al.*, 2005), and over-expression of XBP1 in NIH 3T3 fibroblasts significantly increases the surface area and volume of the fibroblast ER. We therefore investigated if XBP1 plays a role in regulating the volume of the ameloblast ER during enamel development. Although a 25-35% reduction in ameloblast number occurs when ameloblasts progress from the secretory through the maturation stage (Smith and Warshawsky, 1977), we found an approximately two-fold higher overall *Xbp1* expression and a five-fold greater active *Xbp1*(S) expression in the secretory stage compared with the maturation stage. These differences are significant even if the reduction in ameloblast number is taken into account.

Our experimental qPCR procedures used whole enamel organs that included ameloblasts and companion cell layers of the enamel organ (stratum intermedium, stellate reticulum, and outer dental epithelium). Thus, the observed differences in *Xbp1* expression may have been greater if we had been able to assay just the ameloblasts. Previously, using laser capture microdissection, we have tried to isolate ameloblasts for PCR procedures. However, these attempts proved unsuccessful, presumably because of mRNA degradation during the prolonged demineralization procedures necessary for generating tooth sections.

Here we demonstrated that a decrease in *Xbp1* expression is associated with a decrease in ameloblast ER volume. In contrast, analysis of a preponderance of data in other experimental systems demonstrated that an increase in *Xbp1* expression is associated with an increase in ER volume (Reimold *et al.*, 2001; Iwakoshi *et al.*, 2003; Shaffer *et al.*, 2004; Sriburi *et al.*, 2004, 2007; Lee *et al.*, 2005; Fagone *et al.*, 2007). Although the difference may at first seem subtle, our findings suggest that sustained levels of *Xbp1* expression are necessary to maintain an expanded ER *in vivo*, and that if *Xbp1* expression is reduced, the ER percent volume will become correspondingly reduced. We conclude that ameloblast ER volume positively correlates to the level of *Xbp1* expression, and that *Xbp1* expression levels are dictated by developmental stage during enamel formation.

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Figure 1.

Percent volume *per* ameloblast of the ER, mitochondria, and Golgi at 6 defined developmental stages. Selected values presented in the Table were graphed so that the reader may better observe the magnitude of the differences among the various stages. Each percent volume represents data generated from approximately 4 ameloblasts *per* incisor from 2 incisors, performed on 10 different animals *per* stage. Error bars represent the standard error of the mean. Abbreviations: Sec, secretory stage; Mat, maturation stage; Regress, regression stage of enamel development.

Figure 2. Identification of active IRE1 in ameloblast cells of the enamel organ as determined by immunohistochemical methods. **(A)** Staining for the active phosphorylated form of IRE1 in an adult mouse mandibular incisor [20X magnification]. **(B)** Enlargement of the indicated secretory-stage ameloblasts from panel A [40X magnification]. **(C)** Enlargement of the indicated maturation-stage ameloblasts from panel A [40X magnification]. **(D)** Negative control section treated with the secondary, but not primary, antisera [20X magnification]. Note that staining for active IRE1 was reduced in the maturation stage as compared with the secretory stage of enamel development. Scale bar in panel A represents 100 μm for panels A and D. Scale bar in panel C represents 50 μm for panels B and C. am, ameloblasts.

Figure 3.

Quantification of total *Xbp1* and spliced *Xbp1*(S) expression in mouse enamel organ during the secretory and maturation stages of enamel development. (A) Top: the 26-bp region spliced out of the *Xbp1* RNA. This results in a reading frame shift that encodes the active form of XBP1(S). Also shown is the PstI site present within the spliced region. Bottom: RT-PCR of the *Xbp1* mRNA. Primers for PCR spanned the splice region so that, after digestion with PstI, the spliced *Xbp1*(S) and unspliced *Xbp1*(U) were identified. Note that more *Xbp1*(S) was present in the secretory stage compared with the maturation stage of enamel development. (B) Top: Total *Xbp1* and *Xbp1*(S) expression as assessed by qPCR. Primers for qPCR were designed to amplify total *Xbp1* or were designed such that elimination of the splice region was necessary for amplification of only *Xbp1*(S). The results of the secretory stage (first molar enamel organ from 4-day-old mice) are expressed relative to the results of the maturation-stage (first molar enamel organ from 11-day-old mice) expression levels. Note that the secretory stage expressed approximately two-fold more total *Xbp1* than the maturation stage. However, this difference increased to approximately five-fold for *Xbp1*(S). The kidney served as an epithelium-derived negative control. Bottom: Enamelin served as a positive control, because

its expression drops dramatically during the maturation stage, and RAMP-4 also served as a positive control, because its expression is induced by *Xbp1*(S). All qPCR results were as a function of the stably expressed internal reference control gene *EF1a1* (Kubota *et al.*, 2005) and were calculated as previously described (Pfaffl, 2001; Kubota *et al.*, 2005). Six samples were assessed for each developmental stage, and the results were repeated three times. Error bars represent the standard error of the mean. Abbreviations: Sec, secretory stage; Mat, maturation stage.

Table
Stereology Data-Organelle Percent Volume/Ameloblast $\pm$ SEM^a

Ameloblast	Pre-SEC ^b	Start-SEC	End-SEC	Early-MAT ^c	Late-MAT	Regress ^d
Nucleus	41.197 $\pm$ 0.896	26.965 $\pm$ 0.418	17.240 $\pm$ 0.301	19.957 $\pm$ 0.894	17.774 $\pm$ 0.444	20.463 $\pm$ 1.388
Cytosol	41.172 $\pm$ 0.374	42.648 $\pm$ 0.609	37.021 $\pm$ 1.064	45.980 $\pm$ 0.586	44.887 $\pm$ 1.063	45.997 $\pm$ 1.688
Endoplasmic reticulum	8.447 $\pm$ 0.193	20.172 $\pm$ 0.474	28.191 $\pm$ 0.881	18.220 $\pm$ 0.535	7.028 $\pm$ 0.315	4.463 $\pm$ 0.373
Mitochondria	5.196 $\pm$ 0.1930	4.799 $\pm$ 0.139	6.862 $\pm$ 0.290	8.532 $\pm$ 0.341	8.590 $\pm$ 0.302	7.289 $\pm$ 0.732
Golgi	2.064 $\pm$ 0.163	4.324 $\pm$ 0.158	6.331 $\pm$ 0.440	4.270 $\pm$ 0.410	3.372 $\pm$ 0.217	1.351 $\pm$ 0.228
Granules	0.005 ^e $\pm$ 0.003	0.320 ^e $\pm$ 0.014	0.496 ^e $\pm$ 0.030	0.112 ^e $\pm$ 0.018	10.122 ^f $\pm$ 1.064	0.441 ^f $\pm$ 0.455
Dense bodies ^g /vacuoles	0.596 $\pm$ 0.023	0.885 $\pm$ 0.025	3.779 $\pm$ 0.331	2.105 $\pm$ 0.144	2.514 $\pm$ 0.154	4.764 $\pm$ 0.856
Filaments ^h	0.093 $\pm$ 0.013	0.355 $\pm$ 0.025	0.452 $\pm$ 0.032	1.003 $\pm$ 0.057	5.619 $\pm$ 0.467	13.188 $\pm$ 1.276
Pts. counted/ameloblast	108,110	213,647	243,513	133,109	62,060	30,851

^aSEM, standard error of the mean.

^bSEC, secretory stage of enamel development.

^cMAT, maturation stage of enamel development.

^dRegress, regression stage of enamel development.

^eThe classic small secretory granules ($\sim$ 200 nm) are associated with active secretion of enamel proteins.

^fThe large irregular and dense ferritin-containing granules accumulate intracellularly as maturation proceeds, and, when secreted, they give rodent incisor enamel its characteristic yellow color.

^gThese are classic lysosomes and endosomes.

^hThe filaments represent bundles of tonofilaments associated with desmosomes that become more prominent as ameloblasts age.