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Research Article

Cellular uptake of amelogenin, and its localization to CD63, and Lamp1-positive vesicles

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Abstract. Proteins of the developing enamel matrix include amelogenin, ameloblastin and enamelin. Of these three proteins amelogenin predominates. Protein-protein interactions are likely to occur at the ameloblast Tomes' processes between membrane-bound proteins and secreted enamel matrix proteins. Such protein-protein interactions could be associated with cell signaling or endocytosis. CD63 and Lamp1 are ubiquitously expressed, are lysosomal integral membrane proteins, and localize to the plasma membrane. CD63 and Lamp1 interact with amelogenin *in vitro.* In this study our objective was to study the molecular events of intercellular trafficking of an exogenous source of amelogenin, and related this movement to the spatiotemporal expression of CD63 and Lamp1 using various cell lineages. Exogenously added amelogenin moves rapidly into the cell into established Lamp1-positive vesicles that subsequently localize to the perinuclear region. These data indicate a possible mechanism by which amelogenin, or degraded amelogenin peptides, are removed from the extracellular matrix during enamel formation and maturation.

Keywords. Amelogenin, CD63, Emdogain®, enamel, endocytosis, Lamp1, late endosome, protein-protein interactions.

Introduction

The structural proteins relatively unique to the enamel matrix are amelogenin, ameloblastin and enamelin [1–7]. Structural proteins of the enamel matrix manifest specific protein-protein interactions required to produce a matrix capable of directing the highly ordered structure of the enamel crystallites [3, 6, 7]. Protein-protein interactions also occur between the secreted enamel proteins and proteins of the plasma membrane of the enamel producing cells (ameloblasts) [5, 8–10]. Implied protein-membrane interactions between ameloblasts and the enamel organic matrix have been discussed for ameloblastin [5], and also for amelotin [10]. Such protein-membrane interactions may be required to establish short-term order of the forming matrix, to mediate feedback signals to the transcriptional machinery of these cells, and to remove matrix protein debris during amelogenesis [5, 6, 9, 11]. Plasma membrane-bound proteins identified in ameloblasts, shown to directly interact with the struc-

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tural enamel proteins, include CD63 (cluster of differentiation 63 antigen; also known as lysosome-associated membrane glycoprotein 3 and melanoma 1 antigen) and Lamp1 (lysosomal-associated membrane glycoprotein 1) [9, 12–14]. CD63 and Lamp1 are also integral to the late endosome/lysosomal membrane [13]. Clathrin adaptor protein (AP) complexes are protein heterotetramers that cycle between the cytosol and membranes, and mediate the sorting of membrane-bound proteins in the secretory and endocytic pathways. Four distinct AP complexes have been identified (AP-1, AP-2, AP-3 and AP-4) and at least three of these AP complexes have isoforms that show tissue-specific expression [15, 16]. AP-3 has been directly associated with the intracellular trafficking of plasma membrane-bound CD63 and Lamp1 [13].

CD63 is a member of the transmembrane-4 glycoprotein superfamily, which is also known as the tetraspanin family [17, 18]. Family members are cell-surface proteins that are characterized by the presence of four transmembrane domains [17, 18]. Tetraspanins mediate signal transduction events that play a role in the regulation of cell development, activation, growth and motility [18, 19]. In particular, as a cell surface glycoprotein, CD63 and other tetraspanins are known to complex with integrins [18, 20]. Recent studies relating the tetraspanin-integrin protein interactions suggest that as a class of proteins, the tetraspanins act as organizers of membrane microdomains and signaling complexes [18]. CD63 resides not only in the plasma membrane of most cell types, but it also resides in late endosomes, lysosomes and secretory vesicles, and CD63 traffics among these different compartments [13]. This has led to the suggestion that CD63 may play a role in the recycling of membrane components, and the uptake of degraded proteins from the extracellular matrix [13].

At steady state, Lamp1 is a transmembrane protein highly expressed in late endosomes and lysosomes and is often used as a marker for these two organelles [21]. Lamp1 is involved in endocytosis, pinocytosis, or phagocytosis [21]. The movement of Lamp1 from the rough endoplasmic reticulum and Golgi to the lysosome membrane has been documented, and this pathway can be independent of movement through the plasma membrane [21]. However, Lamp1 immunoreactivity is also observed at the plasma membrane of most cell types [22–24], and it can also be observed in early endocytic compartments [22]. The presence of Lamp1 on the plasma membrane suggests that Lamp1 can act as a cell surface intermediary, and can traffic directly to the late endosome/lysosome during endocytotic events [13].

In eukaryotic cells, receptor-mediated endocytosis frequently occurs with the involvement of clathrin-coated pits and vesicles, and is distinguished from other types of endocytosis or pinocytosis that are independent of clathrin [25–27]. Over the past decade, the molecular mechanisms of non-clathrin associated endocytotic pathways have been investigated [15, 26–30]. The intracellular trafficking of plasma membrane-bound CD63 and Lamp1 involves the recruitment of the AP-3 complex [15, 27, 31], and the data suggest that AP-3-related intracellular trafficking may proceed independently of any association with clathrin. Although in mammals a clathrin consensus-binding motif is part of the AP-3 β3 subunit, there is little or no physiological evidence to support the association of clathrin and AP-3 *in vivo* [15, 27–29, 32, 33]. For example, while significant amounts of AP-1 and AP-2 are a feature of biochemically purified clathrin-coated vesicles, only trace amounts of AP-3 can be characterized in these same vesicles [15, 27, 28]. This lack of association between AP-3 and clathrin-coated vesicles is also supported by the fact that in yeast the AP-3 β3 subunit has no clathrin consensus-binding motif, and AP-3-related trafficking is totally independent of clathrin [15].

The data outlined above have directed the experimental strategy described in this current investigation. Amelogenin is the most abundant protein in the developing mammalian enamel extracellular matrix, accounting for greater than 90% of the total matrix protein content [7, 34–36]. Mature enamel is almost entirely inorganic, thus the process of amelogenesis must include an efficient mechanism for the removal of the organic matrix component and related organic debris. With the identification of two secreted, enamel-specific proteinases (matrix metalloproteinase-20 and kallikrein-4) [6, 37–39], it is conceivable that the enamel organic matrix is completely degraded extracellularly and then removed from this environment by macropinocytosis (fluid-phase endocytosis) [40]. However, if a mechanism for enamel matrix protein removal involves a significant uptake of partially degraded proteins (*i.e.* where the epitopes are recognizable), and their subsequent trafficking to late endosomes and lysosomes, then the presumption must be that ameloblasts absorb secreted proteins (and the debris of these secreted proteins) during amelogenesis [41–44]. This type of resorption would then be described more as a receptor-mediated endocytosis.

Polarized ameloblasts form a continuous monolayer over the forming enamel, with only their Tomes' processes (secretory surface) in direct contact with the extracellular matrix. If endocytosis or macropinocytosis is a functional and significant characteristic of secretory ameloblasts, then uptake is likely to occur at, or proximal to, the ameloblast apical membrane [42]. One of the challenges in studying the removal of the enamel matrix *in vivo* is to be able to distinguish between macropinocytosis and endocytosis and the relative contributions of each pathway to amelogenesis. Because of previously identified protein-interactions between Lamp1 and amelogenin [12], and CD63 and amelogenin [14], we sought to identify a possible pathway for the endocytosis of amelogenin in cell

lines of varying phenotypes, and relate our observations to the events of amelogenesis.

Materials and methods

Cell lines and culture conditions

The following cell lines have been used in this study: canine kidney MDCK cells (ATCC catalogue no. CCL-34); human osteoblast hFOB_1.19 (ATCC catalogue no. CRL-11372); mouse pre-osteoblast MC3T3-E1 (ATCC catalogue no. CRL-2595); and mouse ameloblast-like LS8 cells [45, 46]. Multiple cell lines have been included in this study to demonstrate that the cellular absorption of amelogenin epitopes is not limited to ameloblasts or ameloblast-like cells, but that this activity is a more general phenomenon seen in many cell types. In addition, it was necessary to exclude crinophagy [40, 47] as an explanation for the observations of amelogenin in the lysosomes. Crinophagy is the digestion of the contents of secretory granules following their fusion with lysosomes.

LS8 cells were originally derived from the first molar enamel organ epithelium cells of newborn mice [48] and have previously been used to study amelogenin [46, 49–51] and ameloblastin [45, 52] gene expression *in vitro*.

Canine kidney MDCK and mouse ameloblast-like LS8 cells were maintained in Dulbecco's modification of Eagles medium (DMEM) with high glucose (4.5 g/L) supplemented with 10% fetal calf serum (FCS). Human osteoblast hFOB_1.19 cells were maintained in a 1 : 1 mixture of Ham's F12 medium and DMEM with 2.5 mM L-glutamine, 0.3 mg/ml G418, and supplemented with 10% FCS. Mouse pre-osteoblast MC3T3-E1 were maintained in Alpha minimum essential medium with ribonucleosides and deoxyribonucleosides, 2 mM L-glutamine, 1 mM sodium pyruvate, and supplemented with 10% FCS.

Plasmid constructs

pAmel-DsRed. Mouse amelogenin M180 cDNA [53, 54] (NCBI reference sequence NM_009666), including the signal peptide region, was PCR amplified from a plasmid template using the following primers: forward 5′-GAATTCAAGAAATGGGGACCTGGATT, and reverse 5′-GGATCCACTTCTTCCCGCTTGGT. The forward primer included an *Eco*RI restriction site sequence (underlined), and the reverse primer a *Bam*HI restriction site (underlined) to allow for efficient, in-frame subclonings in subsequent steps. This PCR product was subsequently sub-cloned into a TA cloning vector (pCR®2.1; Invitrogen Corporation), released with restriction enzymes *Eco*RI and *Bam*HI, and subsequently subcloned into the vector pDsRed1-N1 (Clontech, Palo Alto, CA) at the *Eco*RI/*Bam*HI multicloning site. A two-step cloning strategy was used for this plasmid construct to ensure efficient subcloning of the PCR product, prior to its sequencing and movement into the red fluorescent vector that is not a TA cloning vector.

pLAMP1-GFP. Human LAMP1 cDNA (NCBI reference sequence NM_005561) was purchased from Origene (Rockville, MD), and amplified by standard PCR techniques using the following primers: forward 5′-GATATCCTCGGCATGGCGCCCCGC, and reverse 5′-GATAGTCTGGTAGCCTGCGTGACTCC. PCR amplified LAMP1 cDNA was subcloned into vector pcDNA3.1/CT-GFP-TOPO (Invitrogen Corporation, Carlsbad, CA). For both plasmid constructs, the fluorescent tag follows the entire coding regions for amelogenin and LAMP1. In both cases the entire coding regions were sequenced to identify correct orientation, to ensure that the correct open-reading frame was established, and that no PCR or cloning errors had occurred during their synthesis.

Porcine enamel matrix proteins

Emdogain® (previously owned by Biora AB, Malmö, Sweden but now distributed by Straumann, Basel, Switzerland; www.straumann.com) is a porcine-derived enamel matrix product and the principle component is the enamel matrix protein amelogenin [55]. Lyophilized porcine enamel protein (Emdogain®) was diluted to a final concentration of 50 μg/ml in sterile culture medium, and added freshly to cells.

Antibodies

LAMP1. A rat monoclonal antibody to mouse Lamp1 (Developmental Studies Hybridoma Bank or DSHB, University of Iowa; catalogue no. 1D4B) was used in this study.

CD63. A rabbit anti-peptide polyclonal mouse CD63 antibody was generated against a unique CD63 peptide region (amino acids 177–190; N-terminal CGND-FKESTIHTQG) by Zymed Laboratories Inc. (South San Francisco, CA, USA).

Mouse amelogenin. Chicken egg-derived polyclonal antibody prepared against recombinant mouse amelogenin rp(H)M180 [56] described previously [9, 46] was used in this study.

Stable transfection of pAmel-DsRed into MDCK cells and crude fluorescent-protein extracts

Plasmid pAmel-DsRed was successfully integrated into the genome of canine kidney MDCK cells using Lipofectamine2000 (Invitrogen) and standard laboratory meth-

odologies [57]. Cells were passaged multiple times and continued to express significant quantities of the red-fluorescent amelogenin protein as observed with fluorescence microscopy (Fig. 1a).

Red fluorescent MDCK cells were grown to confluence in a 60-mm cell culture dish (Corning Incorporated, Corning, New York), harvested, pelleted, resuspended in a minimal volume of fresh culture medium and then was subjected to repeated freeze-thawing cycles in 0.25 M Tris-HCl pH 7.8. The complete lysate was then filtered through a 0.45-µm membrane (Corning Inc.) to prepare a crude protein extract that included the red-fluorescent amelogenin. Crude protein extract, plus Emdogain® to bring the total concentration of exogenously added protein to 50 µg/ml, was then added to subconfluent human osteoblast hFOB_1.19 cells also in a 60-mm cell culture dish, and left for 3 h. Cells were subsequently washed three times in phosphate-buffered saline (PBS) at room temperature prior to their visualization using a fluorescence microscope (Fig. 1b).

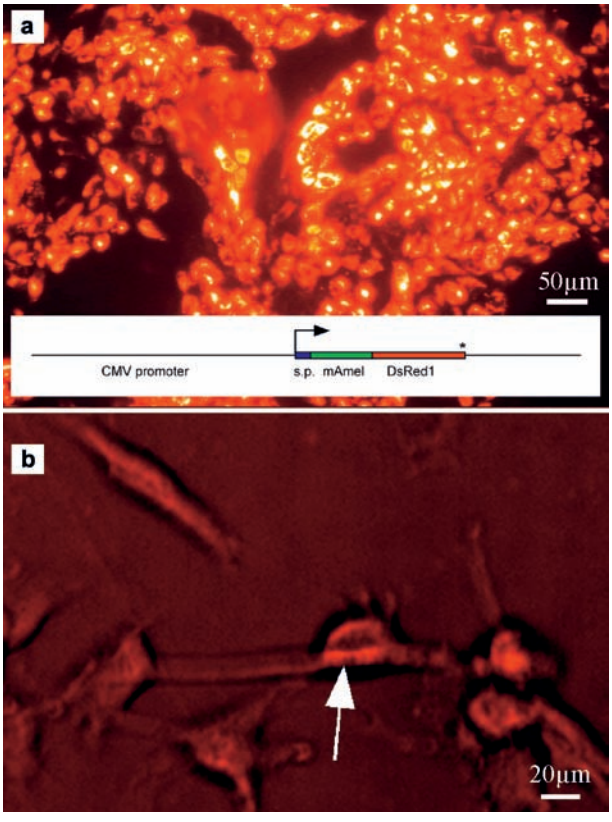

Figure 1. (*a*) MDCK cells stably transfected with amelogenin-red fluorescent protein construct as seen with fluorescent microscopy. Inset: schematic of hybrid fluorescent protein (pAmel-DsRed) involving the amelogenin signal peptide (s.p.), mouse amelogenin (mAmel) and red fluorescent protein (DsRed1). (*b*) Endocytosis of the amelogenin-red fluorescent protein in human osteoblast cells. Human osteoblast cells (hFOB_1.19) were exposed to a crude preparation of protein isolated from the MDCK cells expressing pAmel-DsRed as seen in (*a*). Emdogain® was also included in this crude protein extract so that the total concentration of added amelogenin protein was 50 µg/ml. The amelogenin red-fluorescent hybrid protein localized to the cell cytoplasm (arrows), and was excluded from the nucleus. Scale bars are included in the figures.

Immunohistochemistry

Antibodies against Lamp1 and CD63 were used to examine the spatiotemporal expression of these two proteins in mandibular incisor teeth in 3- and 4-day-old mice. Tissues were prepared as described previously [58–61]. Slides were counterstained with hematoxylin prior to making a digital photographic recording.

Immunofluorescence

For CD63 and amelogenin colocalization experiments mouse MC3T3-E1 cells were grown on glass coverslips to approximately 70% confluence. Emdogain® was added to the media to a final concentration of 50 µg/ml and incubated for 3 h. Cells were then washed with PBS, fixed with 2% paraformaldehyde/0.2% Triton X-100 for 10 min at room temperature, washed with PBS, and blocked 30 min with goat serum (Zymed Laboratories, San Francisco, CA). Cells were incubated overnight at 4 °C with chicken anti-mouse amelogenin antibody and rabbit anti-mouse CD63 (at dilutions of 1 : 2500 and 1 : 200 respectively). After PBS washes, goat anti-chicken Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 (Invitrogen Corporation; both at dilutions of 1 : 250) were used for fluorescence detection of amelogenin and CD63, respectively. Cells were left to incubate for 1 h at 37 °C. Finally, cells were washed with PBS for three times and then mounted with Pro-Long anti-fade reagent (Molecular Probes). Cells were visualized under confocal microscopy at 60× magnification using a Nikon PCM2000 confocal system.

For Lamp1 and amelogenin colocalization studies two experimental approaches were used; either unlabeled Emdogain® was added to cultured MC3T3-E1 cells, or Emdogain® that had been conjugated to Alexa Fluor 594 (Invitrogen Corporation, catalogue no. A30008) was added to cultured LS8 cells, and studied in an identical manner to that described immediately above. Visualization of Lamp1 was by the monoclonal anti-rat Lamp1 primary antibody and goat anti-rat Alexa Fluor 488 used at dilutions of 1 : 100 and 1 : 250, respectively. Visualization of intracellular Emdogain® in MC3T3-E1 cells followed primary and secondary antibody labeling. Visualization of Alexa Fluor 594-conjugated Emdogain® in LS8 cells required no additional antibodies.

Transient transfection and microscopy

For transient transfection assays, MC3T3-E1 or LS8 cells were grown on either glass cover slips in 3.5-cm

cell culture dishes or on 2-well Lab-Tek™chamber slides (Nalge Nunc International, Rochester, NY). Cells were transfected with pLAMP1-GFP using Lipofectamine Plus (Invitrogen Corporation) according to the manufacturer's instructions. The day after transfection, 50 µg/ml Emdogain® conjugated to Alexa Fluor 594 was freshly added to the culture medium for 3 h. Cells were then washed with PBS, fixed with 4% paraformaldehyde, washed again with PBS and mounted with Pro-Long anti-fade reagent (Molecular Probes). Images were taken from a 63x Carl Zeiss Plan Apo objective of an Olympus IMT-2 microscope and/or 60× lens of a Nikon PCM2000 confocal system, respectively.

Time-lapse confocal microscopy

MC3T3-E1 cells transfected with pLAMP1-GFP were grown to 70% confluency on 35-mm glass bottom culture dishes MatTek Corporation (Ashland, MA., catalogue no. P35G014C), and transfected with pLAMP1-GFP using Lipofectamine Plus (Invitrogen Corporation) according to the manufacture's instructions. After 24 h, Emdogain® conjugated to Alexa Fluor 594 (according to manufacturer's instructions; Invitrogen Corporation) was freshly added to the culture medium to reach a final concentration of 50 µg/ml. Images were taken from a 100× lens of a Nikon PCM2000 confocal system at 7-s intervals.

RT-PCR

Messenger RNA (mRNA) was isolated from LS8 cells, and used as template RNA for an RT-PCR using the Titanium™ One-step RT-PCR kit (Clontech Laboratories Inc, Mountain View, CA) and murine-specific CD63 (exon 3 forward 5′-CATTGGTGTAGCGGTTCAGGTTG and, exon 6 reverse 5′-CATTCCCACAGCCCACAGTTATG) and Lamp1 (exon 4 forward 5′-GTGACCGTTGTGCTCCGGGATGCC and, exon 8 reverse 5′-CCCTGCCAGGGCACCGCCCAC) primers. A primer set for murine β-actin (exon 3 forward 5′-CTGGCACCACACCTTCTACAATG and, exon 4 reverse 5′-GATGTCACGCACGATTTCCCTC) was also included as a control for RT-PCR. A DNA ladder (Promega, Madison, WI:

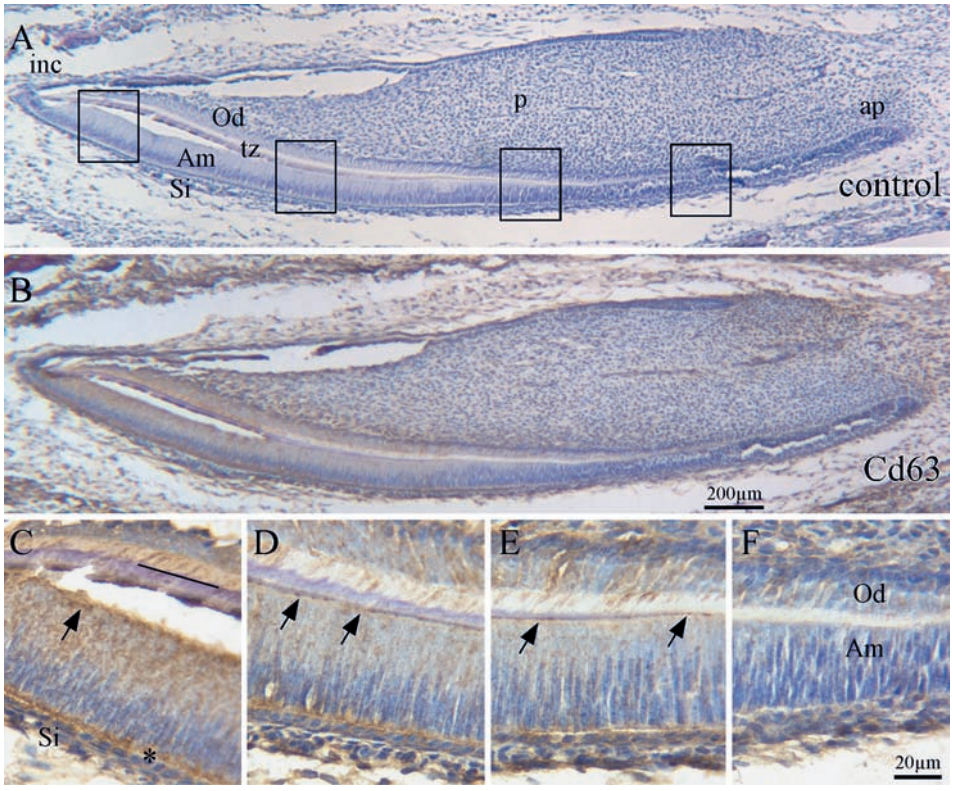

Figure 2. Immunolocalization of CD63 to ameloblast cells in a 3-day-old mouse mandibular incisor. (*a*) No primary antibody control section. No staining is seen in the control section. (*b–f*). Antibody to CD63 was used at a dilution of 1 : 30. (*c–f*) Magnification of regions approximating those boxed regions identified in *a* (from left to right). The dominant location of immuno-staining (brown chromophore) within ameloblasts is cytoplasmic, with immunostaining also apparent at Tomes' processes (arrows) and at their basal poles (asterisk; *c*). Pre-secretory ameloblasts (*f*) are located at the incisor's apical end, and the secretory ameloblasts (*e*, *d*) are prior to the transition zone. Post-secretory ameloblasts (*c*) are located at the incisal end of the tooth. All sections counterstained with hematoxylin prior to photograph. Ameloblasts (Am); odontoblasts (Od); stratum intermedium (Si); incisal end of incisor (inc); transition zone ameloblasts (tz); apical end of incisor (ap); and dental pulp (p). Scale bars are included in *b* (for *a* and *b*) and *f* (for *c–f*).

catalogue no. G210A) was used to confirm the amplified DNA size in base pairs (bp). Subsequently the entire PCR-generated products for CD63 and Lamp1 were sequenced to confirm their exact identities.

Results

Internalized red-fluorescent amelogenin localizes to the perinuclear region of osteoblasts

Exogenously added red-fluorescent amelogenin, produced *in vivo* from MDCK cells that had been stably transfected with pAmel-DsRed (Fig. 1a), was effectively endocytosed by the human osteoblast hFOB1_19 cells. This uptake was fairly rapid and could be visualized in hFOB1_19 cells after 3-h exposure to amelogenin. A 3-h time course for this, and the subsequent experiments, was chosen based on a similarly designed and previously published study [62]. The majority of the fluorescent-tagged amelogenin protein detected in the hFOB1_19 cells was localized to the perinuclear region, a region consistent with the passage of amelogenin from the culture medium to the late endosomes and lysosomes.

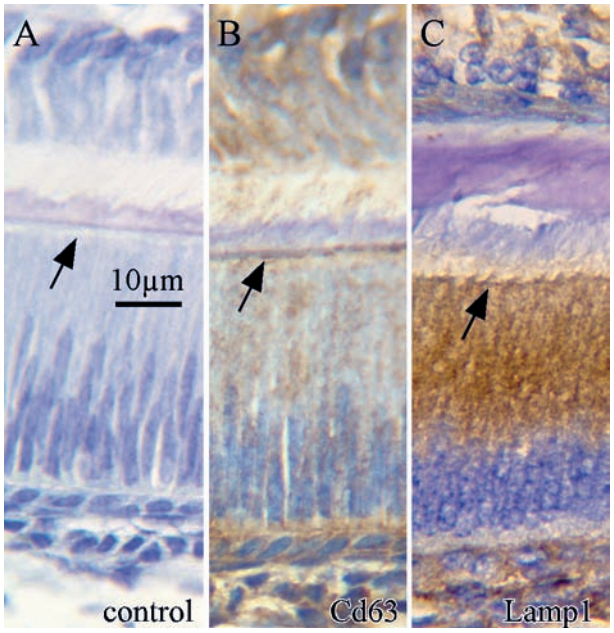

Figure 3. Immunolocalization of CD63 and Lamp1 to Tomes' processes of secretory ameloblasts. Arrows point to Tomes' processes of incisor secretory ameloblast cells. Immunostaining is apparent for CD63 (*b*) and Lamp1 (*c*). Control section (*a*) is prepared with secondary antibody only. Sections counterstained with hematoxylin. Scale bar is shown in *a* for all panels.

CD63 is expressed by ameloblasts at all stages of amelogenesis, but is more highly expressed in late-secretory and post-secretory ameloblasts

Immunohistochemistry was used to examine the distribution of CD63 in developing mouse mandibular incisor teeth. Spatial expression of CD63 to Tomes' processes of ameloblast cells has been previously demonstrated [9], and again demonstrated here (Fig. 2 and 3b). CD63 was unevenly distributed throughout the cytoplasm of ameloblast cells and all surrounding tissues (Fig. 2). Higher levels of CD63 expression were seen in late-stage amelogenesis (ameloblasts associated with maturing enamel) when compared to secretory or pre-secretory ameloblasts. This spatiotemporal expression pattern of CD63 in ameloblasts is suggestive of a functional role for CD63 in enamel formation.

CD63 and amelogenin colocalization

This experiment was performed to determine the intracellular spatial relationship of endogenous CD63 to exogenously added amelogenins that had been phagocytosed and/or endocytosed by MC3T3-E1 cells. Emdogain® was added to the culture medium of mouse MC3T3-E1 cells to a final concentration of 50 μg/ml, and left for 3-h prior to microscopic observation using antibodies specific to mouse CD63 and mouse amelogenin. Immunoreactive amelogenin epitopes could be recognized in the perinuclear region of MC3T3-E1 cell (Fig. 4). These data demonstrate that recognizable amelogenin epitopes survive the trafficking process from the culture medium to the cell perinucleus.

Lamp1 is expressed by ameloblasts at all stages of amelogenesis, but is more highly expressed in the early secretory stage

Immunohistochemistry was used to examine the distribution for Lamp1 in developing mouse mandibular incisor teeth. The data show that Lamp1 is immuno-localized to the apical region of pre-secretory ameloblast cells, and Lamp1 is evenly distributed throughout the cytoplasm of ameloblast cells and surrounding tissues (Fig. 5). Higher levels of Lamp1 expression were seen in the early stages of amelogenesis (non-polarized and pre-secretory ameloblasts) when compared to secretory or post-secretory ameloblasts from the more mature areas. The spatial expression of Lamp1 to Tomes' processes of secretory ameloblast cells is also evident (Fig. 3c and 5). The presence of Lamp1 in ameloblasts is suggestive of a functional role for Lamp1 in enamel formation, but this statement is made with the proviso that Lamp1 normally has a ubiquitous tissue expression profile [63–65] and that the presence of Lamp1 in ameloblasts was anticipated.

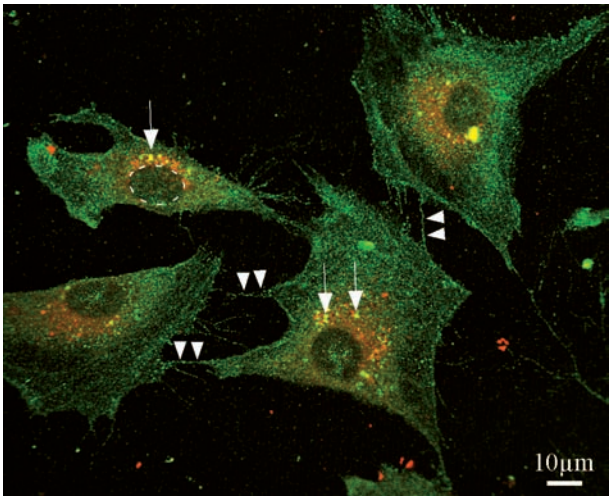

Figure 4. Confocal immunofluorescent images of CD63 and exogenously added porcine amelogenin colocalized in the perinuclear region of the mouse pre-osteoblast cell line MC3T3-E1. Endogenous CD63 (green) is distributed throughout the cytoplasm, but more highly concentrated in the perinuclear region. Amelogenin (red) is localized only to the perinuclear region. Colocalization of CD63 and amelogenin is seen as yellow (arrows). White, interrupted line defines the nuclear membrane of a single cell. A double arrow-head indicates expression of CD63 at the plasma membrane and extensions of the plasma membrane. Scale bar is shown in figure.

Lamp1 and amelogenin colocalization

This experiment allowed us to determine the intracellular spatial relationship of endogenous Lamp1 to exogenously added amelogenins that had been phagocytosed and/or endocytosed by MC3T3-E1 (Fig. 6a–c) and LS8 (Fig. 6d) cells. Unlabeled Emdogain® was added to the culture medium of mouse MC3T3-E1 cells to a final concentration of 50 µg/ml, and left for 3 h. The cells were then immediately processed for immunofluorescence microscopy using antibodies to Lamp1 and amelogenin (Fig. 6a–c). Emdogain® conjugated to Alexa Fluor 594 was added to LS8 cells previously transfected with pLAMP1-GFP. Data from MC3T3-E1 and LS8 cells were similar and demonstrated that Lamp1 expression was limited to the cell cytoplasm, and more specifically to the perinuclear regions (Fig. 6). This localization was the case for both endogenous Lamp1 (Fig. 6a–c) or transfected human LAMP1 (Fig. 6d). At higher magnification the bulk internalized amelogenin was seen completely contained within the vacuole space of Lamp1-positive vesicles (see inserts contained within Fig. 6b–d shown at higher magnification). These data demonstrate that exogenously derived amelogenin traffics to the cell perinucleus, and more specifically is localized to late endosomes and lysosomes.

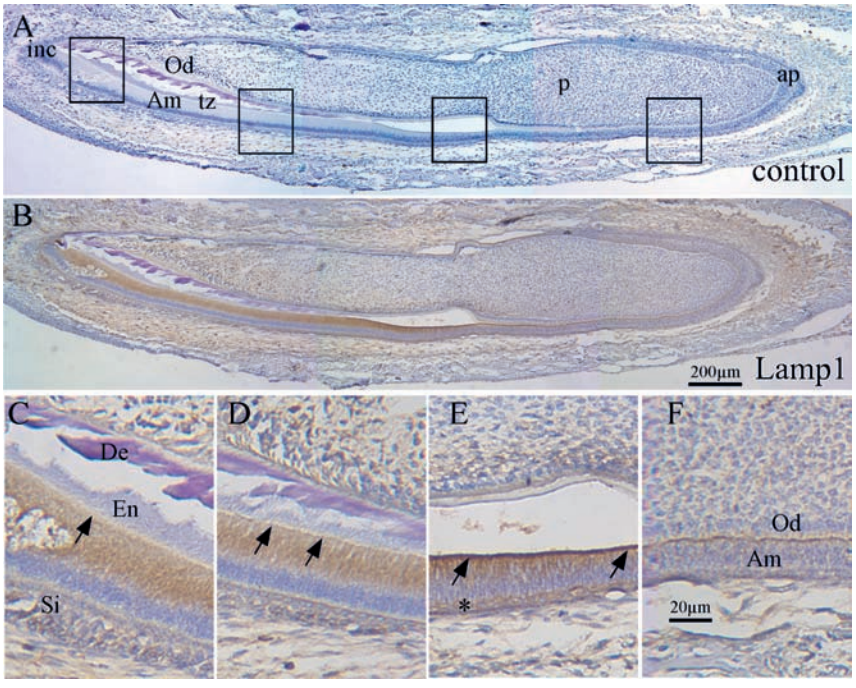

Figure 5. Immunolocalization of Lamp1 to ameloblast-like LS8 cells in a 4-day-old mouse mandibular incisor. (*a*) No primary antibody control section. No staining is seen in the control section. (*b–f*) Antibody to rat Lamp1 used at a dilution of 1 : 50. (*c–f*) Magnification of regions approximating those boxed regions identified in *a* (from left to right). Pre-secretory ameloblasts (*f*) are located at the incisor's apical end, and the secretory ameloblasts (*e*, *d*) are prior to the transition zone. Post-secretory ameloblasts (*c*) are located at the incisal end of the tooth. Lamp1 is clearly and evenly expressed in the cytoplasm of ameloblasts at all stages of amelogenesis (brown chromophore), and most highly expressed in the secretory ameloblasts within the transition zone of enamel formation (*e*). Immunostaining is apparent at Tomes' processes (arrows). High expression of Lamp1 is also noted at the basal poles of secretory ameloblasts (asterisk; *e*). Ameloblasts (Am); odontoblasts (Od); stratum intermedium (Si); incisal end of incisor (inc); transition zone ameloblasts (tz); apical end of incisor (ap); and dental pulp (p). All sections are counterstained with hematoxylin prior to photographing. Scale bars are included in *b* (*a* and *b*) and *f* (for *c–f*).

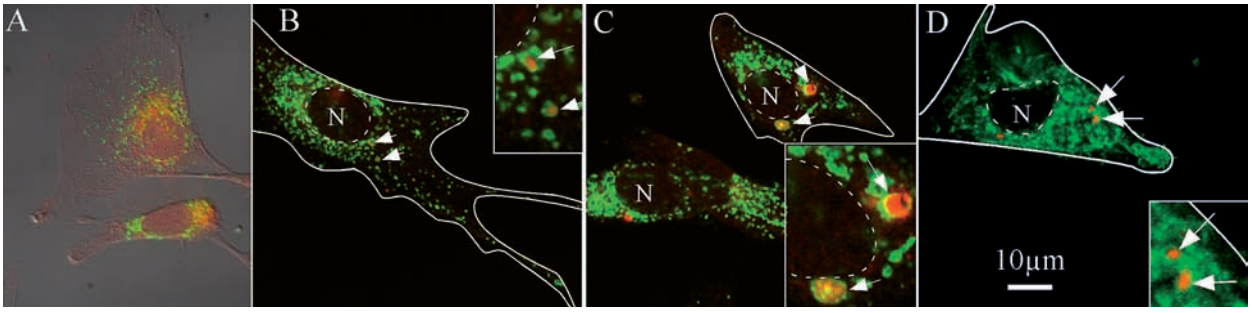

Figure 6. Colocalization of LAMP1 and exogenously added porcine enamel matrix proteins in the perinuclear region of mouse pre-osteoblasts MC3T3-E1 and ameloblast-like LS8 cells. (*a*) Combined differential interference contrast light microscopic and immunofluorescent image of MC3T3-E1 cells showing both the Lamp1 (green) and amelogenin (red) localization is exclusively to the perinuclear region. (*b* and *c*) Immunofluorescent confocal images of endogenous Lamp1 (green) and exogenously derived amelogenin (red) localized in MC3T3-E1 cells. Arrows point to amelogenin-containing Lamp1-coated vesicles. (*d*) Confocal images of a LS8 cells transfected with pLAMP1-GFP, and with the addition of Alexa Fluor 594-labeled Emdogain® (*b–d*) The arrows point to amelogenin-containing (*b*, *c*) and porcine Emdogain®-containing Lamp1-coated vesicles identified as late endosome/lysosome compartments. Colocalization of LAMP1 and amelogenin is seen as yellow. The white line is used to define the plasma membrane and the white interrupted line is used to define the nuclear membrane. Scale bar is included in *d* (for all panels). Inset images are enlarged 2.5×.

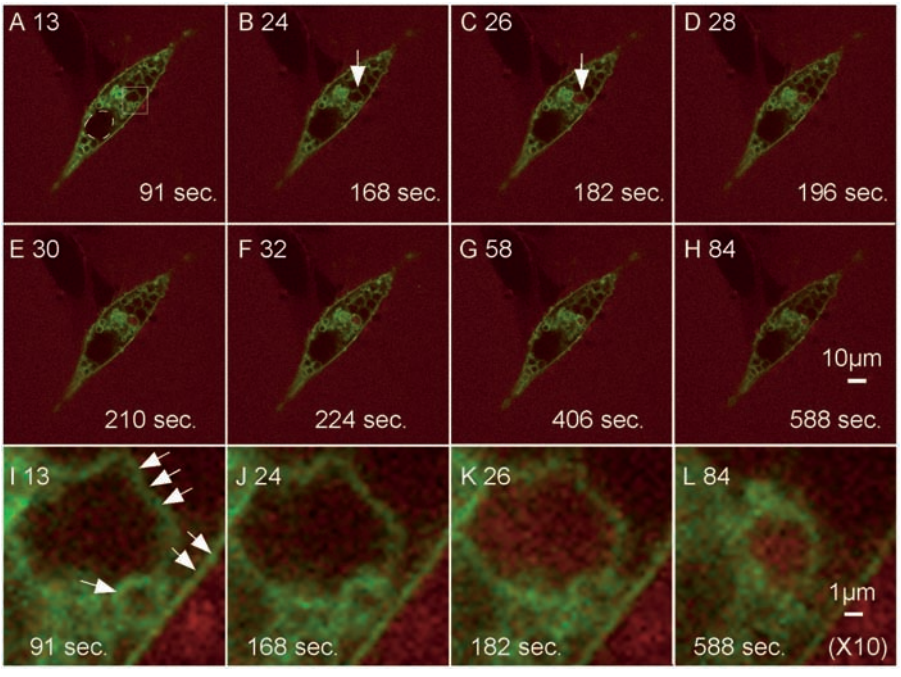

Figure 7. The movement of Alexa fluor 594-labeled Emdogain® into mouse pre-osteoblasts MC3T3-E1 involves the direct passage into pre-established LAMP1-coated vesicles. Time-lapse confocal microscopy was used to study the movement of exogenously added enamel matrix proteins into MC3T3-E1 cells expressing pLAMP1-GFP. Approximately 90 min after the addition of the Alexa Fluor 594-conjugated enamel matrix proteins into the culture medium, a single cell was observed at 7-s intervals for a total of 588 s (*a–h*, where the numbers seen in the top right indicate the frame number). In (*a*), the cell nucleus is highlighted with an interrupted white line, and a single boxed region containing a Lamp1-coated vesicle (also arrowed; *b* and *c*) subjacent to the plasma membrane is identified. This boxed region is enlarged 10× in (*i–l*), and shows the same vesicle (three parallel arrows; *i*) taken from frames 13, 24, 26 and 84, respectively. The two parallel arrows in (*i*) identify the plasma membrane positive for Lamp1. No red fluorescence is noted in the Lamp1-coated vesicle in frame 24, but is present in frame 26 which is taken 14 s after frame 24. An evenly timed, and significant shrinkage of this red-fluorescent Lamp1-coated vesicle occurred in an approximate 7-min interval (from frame 26 to 84). Scale bars are included in *h* (for *a–h*) and *l* (for *i–l*).

Transfer of extracellular amelogenin into the cell cytoplasm involves the direct passage of amelogenin into Lamp1-positive vesicles

Fluorescently labeled enamel matrix proteins (Emdogain® conjugated to Alexa Fluor 594) could be seen entering directly into Lamp1-positive vesicles that were located immediately subjacent to the plasma membrane. A significant aspect of this internalization process occurs in seconds as seen by rapid movement of the enamel matrix proteins into Lamp1-positive vesicles. For example,

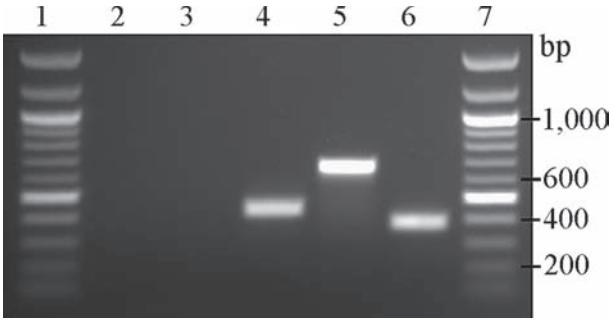

Figure 8. RT-PCR of CD63 and Lamp1 gene transcripts in ameloblast-like LS8 cells. Murine-specific CD63, Lamp1 and β-actin primers were used to amplify cDNA prepared from LS8 cells. Lane 4 is CD63 amplified cDNA at 449 bp. Lane 5 is Lamp1 amplified cDNA at 672 bp. Lane 6 is β-actin amplified cDNA at 382 bp. Lanes 2 and 3 are control lanes using CD63 and Lamp1 primer sets, respectively, with no added DNA template for the PCR. Lanes 1 and 7 show a 100-bp DNA ladder.

Figure 7 shows a Lamp1-positive vesicle approximately 90 min after the addition of Emdogain® (Fig. 7b and j, frame 24). Emdogain® is seen entering this Lamp1-positive vesicle during a 14-second time-lapse (Fig. 7c and k, frame 26). By observing this same vesicle for an additional 406 s (approximately 7 min), this vesicle contracted to 40% of its original diameter (Fig. 7h and l, frame 84). We can conclude from this data that the shrinking of the Emdogain®-containing Lamp1 vesicles (immediately following Emdogain® uptake) is likely to relate directly to the rate at which the enamel matrix proteins are degraded.

Ameloblast-like LS8 cells express CD63 and Lamp1 as determined by RT-PCR

RT-PCR was used to validate the use of LS8 cells to study amelogenin trafficking from the extracellular space to intracellular domains, and then relate this activity to the spatiotemporal profiles of CD63 and Lamp1 *in vitro.* CD63 and Lamp1 gene transcripts were identified in LS8 cells initially by gel electrophoresis (Fig. 8), and their identity confirmed by the sequencing of these PCR-generated products (data not shown).

Discussion

Amelogenin is the most abundant protein in the developing mammalian enamel extracellular matrix, accounting for greater than 90% of the total matrix protein content [7, 34–36]. Mature enamel is almost entirely inorganic, thus the process of amelogenesis must include an efficient mechanism for the removal of the organic matrix component and related organic debris. One potential pathway for their removal is through endocytosis and post-endocytotic degradation. In this study, evidence from tissue and cultured cells was presented that support a role for Lamp1 and CD63, membrane markers for late endosomes and lysosomes, in the endocytosis and post-endocytotic processing of amelogenin. Based upon immunohistochemical staining, Lamp1 and CD63 appear to be expressed at significant levels in ameloblasts *in vivo.* Moreover, both proteins localize to Tomes' processes of ameloblasts, placing these proteins in proximity with extracellular amelogenin, the putative cargo for endocytosis. In cultured ameloblast-like LS8 cells, PCR analysis confirmed the expression of Lamp1 and CD63. Based upon the results of the endocytosis studies performed here on these LS8 and MC3T3-E1 cells, they may represent a good model system to characterize the endocytosis and processing of amelogenin in a Lamp1-dependent and CD63-dependent manner. We have shown here that endocytosed amelogenin can be detected and appears to be concentrated in a subset of endogenous Lamp1-positive and CD63-positive membranes, as well as in Lamp1-positive and CD63-positive membranes in cells transfected with either of these proteins. In addition, the intracellular distribution of endocytosed amelogenin in select vesicles is consistent with specific uptake and post-endocytotic processing of amelogenin, rather than endocytosis by a fluid-phase mechanism, since uptake of fluid-phase markers typically results in a more uniform labeling of endosomal and lysosomal membranes after extended periods of endocytosis performed here.

A previous study has presented evidence supporting a receptor-like function of Lamp1 for amelogenin, by demonstrating binding of amelogenin to Lamp1 in biochemical assays and in cell surface binding assays [12]. The data here confirm and extend those findings by showing that endocytosed amelogenin accumulates in Lamp1-positive organelles, sometimes within a very rapid time frame. Taken together, these two studies suggest that Lamp1 may serve as a cell surface receptor for amelogenin, and Lamp1 may be involved with the trafficking of amelogenin to late endosomes or lysosomes. To our knowledge, these data are the first evidence that Lamp1 may function as a specific, endocytosing ligand-binding receptor at the cell surface.

We have shown previously by yeast two-hybrid analysis that CD63 interacts with amelogenin [14]. Here, we have shown that endocytosed amelogenin also accumulates in CD63-positive organelles. Together, these data suggest that CD63 may serve a role similar to that of Lamp 1 in the endocytosis or post-endocytotic processing of amelogenin. CD63 may function either cooperatively with Lamp1, for example, as essential proteins in the biogenesis of endocytotic organelles responsible for amelogenin uptake and processing. Alternatively, CD63 may function independently of Lamp1 in this process. Either scenario could account for the overlapping, yet slightly distinct dis-

tributions of Lamp1 and CD63 in ameloblasts observed by immunohistochemistry of tissue sections.

CD63 has been implicated in the regulation of trafficking of other transmembrane proteins [13, 66], although Lamp1 is not one of them, as mutations in CD63 that affect its localization do not appear to affect Lamp1 localization [31]. However, to our knowledge, the data presented here would be the first to suggest that CD63 itself may be a cell surface ligand-binding receptor, but further functional characterization of the endocytosis of amelogenin in a CD63-dependent fashion will be required before CD63 can actually be defined as a cell surface receptor for amelogenin.

Lamp1 and CD63 typically recycle between the Golgi and late endosomes/lysosomes [67]. However, a fraction of both of these proteins have been also shown to be present at the cell surface. It has been proposed that these two proteins may traffic through the plasma membrane as part of their normal itinerary, on their way to their ultimate steady-state distribution to late endosomes and lysosomes, although no function has been ascribed to their presence at the plasma membrane. It would be interesting to define whether Lamp1 and CD63 in Tomes' processes in ameloblasts are actually at the plasma membrane, perhaps by immunolocalization at the ultrastructural level. Some of the subcellular machinery involved in the trafficking of Lamp1 and CD63 has also been characterized, particularly with respect to trafficking dependent upon clathrin and associated proteins [67, 68]. Both appear to interact with AP-2 clathrin adaptors at the plasma membrane to regulate their endocytosis and with AP-3 adaptors at endosomes to regulate their trafficking to late endosomes/lysosomes [64, 67]. Thus, if Lamp1 and CD63 indeed serve a receptor-like function, the putative machinery for their endocytotic trafficking should also be present at significant levels in ameloblasts and LS8 cells. A corollary to this line of investigation is that there are mice strains deficient in AP-3, such as mocha [69] and pearl [70], and humans who lack AP-3 resulting in Hermansky-Pudlak syndrome [29, 71]; it would be interesting to determine whether amelogenesis is defective in these mice and humans. A more direct test of whether Lamp1 and CD63 are involved in amelogenesis would be to genetically eliminate either one or both of these proteins. Lamp1 has been deleted in mice [63], but there appears to be some functional redundancy between Lamp1 and the related protein Lamp2 [63, 72]. Lamp2-deficient mice show a more marked phenotype, and the double-knockout of Lamp1 and Lamp2 is embryonic lethal [67]. It is not known whether Lamp2 can bind to amelogenin or to compensate *in vivo* for Lamp1 in amelogenesis. There are no reports of organisms with CD63 deletions, but this knockout may also prove lethal.

There are other possibilities with respect to the role of Lamp1 and CD63 in amelogenesis. With the identification of two secreted, enamel-specific proteinases (matrix metalloproteinase-20 and kallikrein-4) [6, 39, 73, 74], it is conceivable that fragments of partially degraded amelogenin are endocytosed by Lamp1 and/or CD63; however, these endocytosed fragments would be restricted to those containing the binding site for Lamp1 or CD63. Another possibility is that the enamel organic matrix is completely degraded extracellularly and then removed from this environment by macropinocytosis or fluid-phase endocytosis [75, 76]. However, currently there is no evidence that CD63 or Lamp1 are involved in regulating macropinocytosis or fluid-phase uptake, and our data suggest that the uptake into cultured ameloblasts is not uniformly distributed among the various endocytotic organelles, as would be expected for fluid-phase uptake. Macropinocytosis is potentially a mechanism by which the internalization of amelogenin may occur directly into relatively large Lamp1-positive and CD63-positive vesicles. A precedent for this type of internalization is provided by the macropinocytosis of the epidermal growth factor receptor and the platelet-derived growth factor receptor [77–80].

If Lamp1 and CD63 are endocytotic receptors for amelogenin, they could also be operating in a novel fashion to initiate a signal transduction cascade downstream of amelogenin binding, ultimately to regulate gene expression. While the endocytosis of amelogenin has been shown to regulate amelogenin gene expression in ameloblasts [11, 81], there are no reports of Lamp1 or CD63 regulating signal transduction pathways. This system could provide an ideal opportunity to test this hypothesis. In summary, we have provided evidence for a novel association of Lamp1 and CD63 in the endocytosis and post-endocytotic processing of amelogenin. These proteins may be critical to the early formation of enamel by regulating the endocytosis of enamel matrix proteins.

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1 Hu, C. C., Hart, T. C., Dupont, B. R., Chen, J. J., Sun, X., Qian, Q., Zhang, C. H., Jiang, H., Mattern, V. L., Wright, J. T. and Simmer, J. P. (2000) Cloning human enamelin cDNA, chromosomal localization, and analysis of expression during tooth development. J. Dent. Res. 79, 912–919.

2 Hu, J. C., Zhang, C. H., Yang, Y., Karrman-Mardh, C., Forsman-Semb, K. and Simmer, J. P. (2001) Cloning and characterization of the mouse and human enamelin genes. J. Dent. Res. 80, 898–902.

3 Paine, M. L., White, S. N., Luo, W., Fong, H., Sarikaya, M. and Snead, M. L. (2001) Regulated gene expression dictates enamel structure and tooth function. Matrix Biol. 20, 273–292.

4 Lee, S. K., Kim, S. M., Lee, Y. J., Yamada, K. M., Yamada, Y. and Chi, J. G. (2003) The structure of the rat ameloblastin gene and its expression in amelogenesis. Mol. Cells. 15, 216–225.
5 Fukumoto, S., Kiba, T., Hall, B., Iehara, N., Nakamura, T., Longenecker, G., Krebsbach, P. H., Nanci, A., Kulkarni, A. B. and Yamada, Y. (2004) Ameloblastin is a cell adhesion molecule required for maintaining the differentiation state of ameloblasts. J. Cell Biol. 167, 973–983.
6 Bartlett, J. D., Ganss, B., Goldberg, M., Moradian-Oldak, J., Paine, M. L., Snead, M. L., Wen, X., White, S. N. and Zhou, Y. L. (2006) Protein-protein interactions of the developing enamel matrix. Curr. Top. Dev. Biol. 74, 57–115.
7 Margolis, H. C., Beniash, E. and Fowler, C. E. (2006) Role of macromolecular assembly of enamel matrix proteins in enamel formation. J. Dent. Res. 85, 775–793.
8 Iwasaki, K., Bajenova, E., Somogyi-Ganss, E., Miller, M., Nguyen, V., Nourkeyhani, H., Gao, Y., Wendel, M. and Ganss, B. (2005) Amelotin – a novel secreted, ameloblast-specific protein, J. Dent. Res. 84, 1127–1132.
9 Zhu, D., Paine, M. L., Luo, W., Bringas, P. Jr. and Snead, M. L. (2006) Altering biomineralization by protein design. J. Biol. Chem. 281, 21173–21182.
10 Moffatt, P., Smith, C. E., St-Arnaud, R., Simmons, D., Wright, J. T. and Nanci, A. (2006) Cloning of rat amelotin and localization of the protein to the basal lamina of maturation stage ameloblasts and junctional epithelium. Biochem. J. 399, 37–46.
11 Xu, L., Harada, H., Yokohama-Tamaki, T., Matsumoto, S., Tanaka, J. and Taniguchi, A. (2006) Reuptake of extracellular amelogenin by dental epithelial cells results in increased levels of amelogenin mRNA through enhanced mRNA stabilization. J. Biol. Chem. 281, 2257–2262.
12 Tompkins, K., George, A. and Veis, A. (2006) Characterization of a mouse amelogenin [A-4]/M59 cell surface receptor. Bone 38, 172–180.
13 Duffield, A., Kamsteeg, E. J., Brown, A. N., Pagel, P. and Caplan, M. J. (2003) The tetraspanin CD63 enhances the internalization of the H,K-ATPase beta-subunit. Proc. Natl. Acad. Sci. USA 100, 15560–15565.
14 Wang, H. J., Tannukit, S., Zhu, D. H., Snead, M. L. and Paine, M. L. (2005) Enamel matrix protein interactions. J. Bone Mineral Res. 20, 1032–1040.
15 Robinson, M. S. and Bonifacino, J. S. (2001) Adaptor-related proteins. Curr. Opin. Cell Biol. 13, 444–453.
16 Nakatsu, F. and Ohno, H. (2003) Adaptor protein complexes as the key regulators of protein sorting in the post-Golgi network. Cell Struct. Funct. 28, 419–429.
17 Stipp, C. S., Kolesnikova, T. V. and Hemler, M. E. (2003) Functional domains in tetraspanin proteins. Trends Biochem. Sci. 28, 106–112.
18 Yunta, M. and Lazo, P. A. (2003) Tetraspanin proteins as organisers of membrane microdomains and signaling complexes. Cell. Signal. 15, 559–564.
19 Mantegazza, A. R., Barrio, M. M., Moutel, S., Bover, L., Weck, M., Brossart, P., Teillaud, J. L. and Mordoh, J. (2004) CD63 tetraspanin slows down cell migration and translocates to the endosomal-lysosomal-MIICs route after extracellular stimuli in human immature dendritic cells. Blood 104, 1183–1190.
20 Berditchevski, F. (2001) Complexes of tetraspanins with integrins: more than meets the eye. J. Cell Sci. 114, 4143–4151.
21 Cook, N. R., Row, P. E. and Davidson, H. W. (2004) Lysosome associated membrane protein 1 (Lamp1) traffics directly from the TGN to early endosomes. Traffic 5, 685–699.
22 Kannan, K., Stewart, R. M., Bounds, W., Carlsson, S. R., Fukuda, M., Betzing, K. W. and Holcombe, R. F. (1996) h-LAMP2 (CD107b) are activation-dependent cell surface glycoproteins in human peripheral blood mononuclear cells which mediate cell adhesion to vascular endothelium. Cell. Immunol. 171, 10–19.
23 Holcombe, R. F., Baethge, B. A., Stewart, R. M., Betzing, K., Hall, V. C., Fukuda, M. and Wolf, R. E. (1993) Cell surface expression of lysosome-associated membrane proteins (LAMPs) in scleroderma: relationship of lamp2 to disease duration, anti-Sc170 antibodies, serum interleukin-8, and soluble interleukin-2 receptor levels. Clin. Immunol. Immunopathol. 67, 31–39.
24 Silverstein, R. L. and Febbraio, M. (1992) Identification of lysosome-associated membrane protein-2 as an activation-dependent platelet surface glycoprotein. Blood 80, 1470–1475.
25 Alberts, B., Bray, D. Lewis, J. Raff, M. Roberts, K. and Watson, J. D. (1994) Molecular Biology of the Cell, 4th edn, pp. 711–766, Garland Publishing, New York.
26 Robinson, M. S. (2004) Adaptable adaptors for coated vesicles. Trends Cell Biol. 14, 167–174.
27 Peden, A. A., Oorschot, V., Hesser, B. A., Austin, C. D., Scheller, R. H. and Klumperman, J. (2004) Localization of the AP-3 adaptor complex defines a novel endosomal exit site for lysosomal membrane proteins. J. Cell Biol. 164, 1065–1076.
28 Simpson, F., Bright, N. A., West, M. A., Newman, L. S., Darnell, R. B. and Robinson, M. S. (1996) A novel adaptor-related protein complex. J. Cell Biol. 133, 749–760.
29 Dell'Angelica, E. C., Klumperman, J., Stoorvogel, W. and Bonifacino, J. S. (1998) Association of the AP-3 adaptor complex with clathrin. Science 280, 431–434.
30 Johannes, L. and Lamaze, C. (2002) Clathrin-dependent or not: is it still the question? Traffic 3, 443–451.
31 Rous, B. A., Reaves, B. J., Ihrke, G., Briggs, J. A., Gray, S. R., Stephens, D. J., Banting, G. and Luzio, J. P. (2002) Role of adaptor complex AP-3 in targeting wild-type and mutated CD63 to lysosomes. Mol. Biol. Cell. 13, 1071–1082.
32 Shi, G., Faundez, V., Roos, J., Dell'Angelica, E. C. and Kelly, R. B. (1998) Neuroendocrine synaptic vesicles are formed *in vitro* by both clathrin-dependent and clathrin-independent pathways. J. Cell Biol. 143, 947–955.
33 Peden, A. A., Rudge, R. E., Lui, W. W. and Robinson, M. S. (2002) Assembly and function of AP-3 complexes in cells expressing mutant subunits. J. Cell Biol. 156, 327–336.
34 Termine, J. D., Belcourt, A. B., Christner, P. J., Conn, K. M. and Nylen, M. U. (1980) Properties of dissociatively extracted fetal tooth matrix proteins. J. Biol. Chem. 255, 9760–9768.
35 Deutsch, D., Palmon, A., Fisher, L. W., Kolodny, N., Termine, J. D. and Young, M. F. (1991) Sequencing of bovine enamelin ('tuftelin') a novel acidic enamel protein. J. Biol. Chem. 266, 16021–16028.
36 Robinson, C., Brookes, S. J., Shore, R. C. and Kirkham, J. (1998) The developing enamel matrix: nature and function. Eur. J. Oral Sci. 106 (Suppl. 1), 282–291.
37 Bartlett, J. D., Simmer, J. P., Xue, J., Margolis, H. C. and Moreno, E. C. (1996) Molecular cloning and mRNA tissue distribution of a novel matrix metalloproteinase isolated from porcine enamel organ. Gene 183, 123–128.
38 Simmer, J. P., Fukae, M., Tanabe, T., Yamakoshi, Y., Uchida, T., Xue, J., Margolis, H. C., Shimizu, M., DeHart, B. C., Hu, C.-C. and Bartlett, J. D. (1998) Purification, characterization and cloning of enamel matrix serine proteinase 1. J. Dent. Res. 77, 377–386.
39 Bartlett, J. D. and Simmer, J. P. (1999) Proteinases in developing dental enamel. Crit. Rev. Oral Biol. Med.10, 425–441.
40 Smith, C. E. (1998) Cellular and chemical events during enamel maturation. Crit. Rev. Oral Biol. Med. 9, 128–161.
41 Nanci, A., Slavkin, H. C. and Smith, C. (1987) Immunocytochemical and radioautographic evidence for secretion and intracellular degradation of enamel proteins by ameloblasts in rat incisors. Anat. Rec. 217, 107–123.
42 Franklin, D. L., Severs, N. J. and Katchburian, E. (1991) Development of the distal end and Tomes' processes of ameloblasts observed by freeze-fracture and ultrathin section electron microscopy. J. Anat. 174, 103–114.

43 Nanci, A., Fortin, M. and Ghitescu, L. (1996) Endocytotic functions of ameloblasts and odontoblasts: Immunohistochemical and tracer studies on the uptake of plasma proteins. Anat. Rec. 245, 219–234.

44 Reith, E. J. and Cotty, V. F. (1967) The absorptive activity of ameloblasts during the maturation of enamel. Anat. Rec. 157, 577–588.

45 Dhamija, S. and Krebsbach, P. H. (2001) Role of Cbfa1 in ameloblastin gene transcription. J. Biol. Chem. 276, 35159–35164.

46 Zhou, Y. L. and Snead, M. L. (2000) Identification of CCAAT/enhancer-binding protein alpha as a transactivator of the mouse amelogenin gene. J. Biol. Chem. 275, 12273–12280.

47 Nanci, A., Bendayan, M. and Slavkin, H. C. (1985) Enamel protein biosynthesis and secretion in mouse incisor ameloblasts as revealed by high-resolution immunocytochemistry. J. Histochem. Cytochem. 33, 1153–1160.

48 Chen, L. S., Couwenhoven, D., Hsu, D., Luo, W. and Snead, M. L. (1992) Maintainance of amelogenin gene expression by transformed epithelial cells of mouse enamel organ. Arch. Oral Biol. 37, 771–778.

49 Zhou, Y. L., Lei, Y. and Snead, M. L. (2000) Functional antagonism between Msx2 and CCAAT/enhancer-binding protein alpha in regulating the mouse amelogenin gene expression is mediated by protein-protein interaction. J. Biol. Chem. 275, 29066–29075.

50 Xu, Y., Zhou, Y. L., Ann, D. K., MacDougald, O. A., Shum, L. and Snead, M. L. (2006) Transcription factor sumoylation and factor YY1 serve to modulate mouse amelogenin gene expression. Eur. J. Oral Sci. 114 (Suppl. 1), 169–177.

51 Xu, Y., Zhou, Y. L., Luo, W., Zhu, Q. S., Levy, D., MacDougald, O. A. and Snead, M. L. (2006) NF-Y and CCAAT/enhancer-binding protein alpha synergistically activate the mouse amelogenin gene. J. Biol. Chem. 281, 16090–16098.

52 Dhamija, S., Liu, Y., Yamada, Y., Snead, M. L. and Krebsbach, P. H. (1999) Cloning and characterization of the murine ameloblastin promoter. J. Biol. Chem. 274, 20738–20743.

53 Snead, M. L., Zeichner-David, M., Chandra, T., Robson, K. J., Woo, S. L. and Slavkin, H. C. (1983) Construction and identification of mouse amelogenin cDNA clones. Proc. Natl. Acad. Sci. USA 80, 7254–7258.

54 Snead, M. L., Lau, E. C., Zeichner-David, M., Fincham, A. G., Woo, S. L. and Slavkin, H. C. (1985) DNA sequence for cloned cDNA for murine amelogenin reveal the amino acid sequence for enamel-specific protein. Biochem. Biophys. Res. Commun. 129, 812–818.

55 Maycock, J., Wood, S. R., Brookes, S. J., Shore, R. C., Robinson, C. and Kirkham, J. (2002) Characterization of a porcine amelogenin preparation, EMDOGAIN, a biological treatment for periodontal disease. Connect. Tissue Res. 43, 472–476.

56 Moradian-Oldak, J., Paine, M. L., Lei, Y. P., Fincham, A. G. and Snead, M. L. (2000) Self-assembly properties of recombinant engineered amelogenin proteins analyzed by dynamic light scattering and atomic force microscopy. J. Struct. Biol. 131, 27–37.

57 Sambrook, J. and Russell, D. W. (2001) Molecular Cloning: A Laboratory Manual, pp. 16.1–16.62, Cold Spring Harbor Laboratory Press, New York.

58 Luo, W., Wen, X., Wang, H. J., MacDougall, M., Snead, M. L. and Paine, M. L. (2004) *In vivo* overexpression of tuftelin in the enamel organic matrix. Cells Tissues Organs 177, 212–220.

59 Snead, M. L., Paine, M. L., Chen, L. S., Yoshida, B., Luo, W., Zhu, D.-H., Lei, Y.-P., Liu, Y.-H. and Maxson, R. E. Jr. (1996) The murine amelogenin promoter: Developmentally regulated expression in transgenic animals. Connect. Tissue Res. 35, 41–47.

60 Paine, C. T., Paine, M. L., Luo, W., Okamoto, C. T., Lyngstadaas, S. P. and Snead, M. L. (2000) A tuftelin-interacting protein (TIP39) localizes to the apical secretory pole of mouse ameloblasts. J. Biol. Chem. 275, 22284–22292.

61 Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (eds.), (1990) Current Protocols in Molecular Biology. pp. 14.0.1–14.14.8, Wiley, New York.

62 Reseland, J. E., Reppe, S., Larsen, A. M., Berner, H. S., Reinholt, F. P., Gautvik, K. M., Slaby, I. and Lyngstadaas, S. P. (2006) The effect of enamel matrix derivative (EMD) on gene expression in osteoblasts. Eur. J. Oral Sci. 114 (Suppl. 1), 205–211.

63 Andrejewski, N., Punnonen, E. L., Guhde, G., Tanaka, Y., Lullmann-Rauch, R., Hartmann, D., von Figura, K. and Saftig, P. (1999) Normal lysosomal morphology and function in LAMP-1-deficient mice. J. Biol. Chem. 274, 12692–12701.

64 Bonifacino, J. S. and Traub, L. M. (2003) Signals for sorting of transmembrane proteins to endosomes and lysosomes. Annu. Rev. Biochem. 72, 395–447.

65 Eskelinen, E. L., Illert, A. L., Tanaka, Y., Schwarzmann, G., Blanz, J., Von Figura, K. and Saftig, P. (2002) Role of LAMP-2 in lysosome biogenesis and autophagy. Mol. Biol. Cell 13, 3355–3368.

66 Jung, K. K., Liu, X. W., Chirco, R., Fridman, R. and Kim, H. R. (2006) Identification of CD63 as a tissue inhibitor of metalloproteinase-1 interacting cell surface protein. EMBO J. 25, 3934–3942.

67 Eskelinen, E. L., Tanaka, Y. and Saftig, P. (2003) At the acidic edge: emerging functions for lysosomal membrane proteins. Trends Cell Biol. 13, 137–145.

68 Di Pietro, S. M., Falcon-Perez, J. M., Tenza, D., Setty, S. R., Marks, M. S., Raposo, G. and Dell'Angelica, E. C. (2006) BLOC-1 interacts with BLOC-2 and the AP-3 complex to facilitate protein trafficking on endosomes. Mol. Biol. Cell 17, 4027–4038.

69 Kantheti, P., Qiao, X., Diaz, M. E., Peden, A. A., Meyer, G. E., Carskadon, S. L., Kapfhamer, D., Sufalko, D., Robinson, M. S., Noebels, J. L. and Burmeister, M. (1998) Mutation in AP-3 delta in the mocha mouse links endosomal transport to storage deficiency in platelets, melanosomes, and synaptic vesicles. Neuron 21, 111–122.

70 Feng, L., Seymour, A. B., Jiang, S., To, A., Peden, A. A., Novak, E. K., Zhen, L., Rusiniak, M. E., Eicher, E. M., Robinson, M. S., Gorin, M. B. and Swank, R. T. (1999) The beta3A subunit gene (Ap3b1) of the AP-3 adaptor complex is altered in the mouse hypopigmentation mutant pearl, a model for Hermansky-Pudlak syndrome and night blindness. Hum. Mol. Genet. 8, 323–330.

71 Di Pietro, S. M. and Dell'Angelica,E. C. (2005) The cell biology of Hermansky-Pudlak syndrome: recent advances. Traffic 6, 525–533.

72 Eskelinen, E. L., Schmidt, C. K., Neu, S., Willenborg, M., Fuertes, G., Salvador, N., Tanaka, Y., Lullmann-Rauch, R., Hartmann, D., Heeren, J., von Figura, K., Knecht, E. and Saftig, P. (2004) Disturbed cholesterol traffic but normal proteolytic function in LAMP-1/LAMP-2 double-deficient fibroblasts. Mol. Biol. Cell 15, 3132–3145.

73 Woessner, J. F. Jr (1998) Role of matrix proteases in processing enamel proteins. Connect. Tissue Res. 39, 69–73.

74 Hu, J. C., Sun, X., Zhang, C., Liu, S., Bartlett, J. D. and Simmer, J. P. (2002) Enamelysin and kallikrein-4 mRNA expression in developing mouse molars. Eur. J. Oral Sci. 110, 307–315.

75 Reith, E. J. and Cotty, V. F. (1967) The absorptive activity of ameloblasts during the maturation of enamel. Anat. Rec. 157, 577–587.

76 Smith, C. E. (1979) Ameloblasts: secretory and resorptive functions. J. Dent. Res. 58 (Spec. Issue B), 695–707.

77 King, A. C. and Cuatrecasas, P. (1981) Long-term epidermal growth factor-receptor internalization and processing in quiescent human fibroblasts. J. Supramol. Struct. Cell Biochem. 17, 377–387.

78 King, A. C., Willis, R. A. and Cuatrecasas, P. (1980) Accumulation of epidermal growth factor within cells does not depend on receptor recycling. Biochem. Biophys. Res. Commun. 97, 840–845.
79 Orth, J. D., Krueger, E. W., Weller, S. G. and McNiven, M. A. (2006) A novel endocytic mechanism of epidermal growth factor receptor sequestration and internalization. Cancer Res. 66, 3603–3610.
80 McNiven, M. A. (2006) Big gulps: specialized membrane domains for rapid receptor-mediated endocytosis. Trends Cell Biol. 16, 487–492.
81 Xu, L., Harada, H. and Taniguchi, A. (2006) The Exon 6ABC Region of amelogenin mRNA contribute to increased levels of amelogenin mRNA through amelogenin protein-enhanced mRNA stabilization. J. Biol. Chem. 281, 32439–32444.