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Murine ameloblasts are immunonegative for Tcirg1, the v-H-ATPase subunit essential for the osteoclast plasma proton pump

Antonius L.J.J. Bronckers [a,*], Donacian M. Lyaruu [a], Theodore J. Bervoets [a], Juan F. Medina [b], Pamela DenBesten [c], Johan Richter [d], Vincent Everts [a]

[a] Dept of Oral Cell Biology ACTA, University of Amsterdam and VU University Amsterdam, Research Institute MOVE, Gustav Mahlerlaan 3004, Amsterdam, The Netherlands
[b] Division of Gene Therapy and Hepatology, University Hospital/School of Medicine/CIMA, University of Navarra, and Ciberehd, Pamplona, Spain
[c] Dept of Orofacial Sciences, School of Dentistry, University of California at San Francisco, CA, USA
[d] Molecular Medicine and Gene Therapy, Lund Stem Cell Centre, University of Lund, Lund, Sweden

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ABSTRACT

Maturation stage ameloblasts of rodents express vacuolar type-H-ATPase in the ruffled border of their plasma membrane in contact with forming dental enamel, similar to osteoclasts that resorb bone. It has been proposed that in ameloblasts this v-H-ATPase acts as proton pump to acidify the enamel space, required to complete enamel mineralization. To examine whether this v-H-ATPase in mouse ameloblasts is a proton pump, we determined whether these cells express the lysosomal, T-cell, immune regulator 1 (Tcirg1, v-H-Atp6v$_0$a$_3$), which is an essential part of the plasma membrane proton pump that is present in osteoclasts. Mutation of this subunit in *Tcirg1* null (or *oc/oc)* mice leads to severe osteopetrosis. No immunohistochemically detectable Tcirg1 was seen in mouse maturation stage ameloblasts. Strong positive staining in secretory and maturation stage ameloblasts however was found for another subunit of v-H-ATPase, subunit b, brain isoform (v-H-Atp6v$_1$b$_2$). Mouse osteoclasts and renal tubular epithelium stained strongly for both Tcirg1 and v-H-Atp6v$_1$b$_2$. In *Tcirg1* null mice osteoclasts and renal epithelium were negative for Tcirg1 but remained positive for v-H-Atp6v$_1$b$_2$. The bone in these mutant mice was osteopetrotic, tooth eruption was inhibited or delayed, and teeth were often morphologically disfigured. However, enamel formation in these mutant mice was normal, ameloblasts structurally unaffected and the mineral content of enamel similar to that of wild type mice.

We concluded that Tcirg1, which is essential for osteoclasts to pump protons into the bone, is not appreciably expressed in maturation stage mouse ameloblasts. Our data suggest that the reported v-H-ATPase in maturation stage ameloblasts is not the typical osteoclast-type plasma membrane associated proton pump which acidifies the extracellular space, but rather a v-H-ATPase potentially involved in intracellular acidification.

Introduction

Ameloblasts are epithelial cells that produce dental enamel principally in two stages. In the first (secretory) stage they deposit a protein-rich enamel matrix that provisionally mineralizes. In the second (maturation) stage the cells transform into cells with resorbing characteristics and periodically form a ruffled border in the apical plasma membranes facing the enamel space. The matrix in the enamel space is proteolytically degraded by enzymes such as MMP20 and kallikrein 4 (KLK4) secreted by the ameloblasts. The hydrolyzed matrix fragments are gradually removed from the enamel space, where simultaneously apatite crystals rapidly expand until enamel mineralization is completed and the tooth erupts [1,2].

Maturation ameloblasts have some structural similarity with osteoclasts, the bone resorbing cells. Both cell types have an actin-rich ruffled border [3,4] that can endocytose, resorb and digest extracellular matrix components, *i.e.* the forming enamel below ameloblasts [1,2], and the bone below osteoclasts [4,5]. However, with respect to the inorganic apatite mineral, the cells perform opposite functions. Ameloblasts are involved in *formation* of minerals in enamel at a pH that periodically alternates between 6.0 and 7.2 [6,7], whereas the osteoclasts *dissolve* the minerals of bone by acidifying the bone to which they are attached [4,5]. To dissolve bone mineral the osteoclast generates protons by the activity of intracellular carbonic anhydrase [4,5,8]. These protons are subsequently pumped into resorption pits that form beneath the osteoclast, a process mediated by a vacuolar type of H-ATPase located in the plasma membrane [9,10]. To control

* Corresponding author at: Dept of Oral Cell Biology, ACTA, Gustav Mahlerlaan 3004, 1081 LA Amsterdam, The Netherlands.
E-mail address: a.bronckers@acta.nl (A.L.J.J. Bronckers).

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intracellular pH the osteoclast uses a set of pH regulators in its membrane [8,11–13].

Recent data show that maturation ameloblasts also have a pH regulatory machinery, quite similar to osteoclasts ([14–17]. Immunohistochemical staining shows that also the ruffled border of rat incisor maturation stage ameloblasts is rich in v-H-ATPase [14,16]. In osteoclasts this v-H-ATPase is a proton pump to acidify the resorption pits, but its function in maturation ameloblasts has not been resolved. Several groups speculated that in analogy to osteoclasts the v-H-ATPase in maturation stage ameloblasts acts as a proton pump to acidify the enamel space [14–16]. This would prevent mineralization of the enamel surface and enables diffusion of mineral ions into deeper layers as long as these are not yet fully mature [16]. An alternate view of pH regulation proposes that maturation ameloblasts secrete bicarbonates into the forming enamel to buffer protons released by crystal growth [1,2,17–19]. In this model the v-H-ATPase in the ruffled border of ameloblasts would be associated with intracellular acidification, rather than serving as a plasma membrane proton pump.

In the present study we addressed two questions: first, does the ruffled border of mouse maturation stage ameloblasts contain typical osteoclast like-proton pump subunits? Secondly, are ameloblasts affected and is enamel mineralization incomplete when the typical osteoclast plasma proton pump is non-functional? To answer these questions we first tested if ameloblasts are immunopositive for the T-cell immune regulator 1 (Tcirg1; also called Atp6v$_0$a$_3$ subunit). In mice this subunit is a 116 kDa protein that is abundantly present in the ruffled border of acid-secreting osteoclasts and intercalating cells of acid-secreting renal tubular epithelium, where it forms an essential part of the transmembrane proton translation domain v_0 of the v-H-ATPase [20,21]. Its absence results in the dysfunction of osteoclasts leading to a severe osteopetrotic phenotype found in the *Tcirg1* null (*oc/oc*) mouse [4,9,20]. We furthermore localized the Atp6v$_1$b$_2$ subunit, also associated with murine osteoclasts [22]. Finally, we examined the effect of the disruption of the *Tcirg1* gene on enamel formation and on enamel mineral content in the *Tcirg1* null mouse. As positive control for enamel defects caused by disruption of pH regulation we used the anionic exchange-2 (*Ae2$_{a,b}$*) null mutant mouse model [15].

Materials and methods

Animals and tissues

The *Tcirg1* null mice (background: C57BL/6J_C3HheB/FeJ) were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Mutation in the *Tcirg1* null mouse is caused by a spontaneous 1.6 kp deletion starting in the middle of intron 1 and extending 62 bp into exon 3 in the genomic DNA. The deletion removes the translation start site of *Tcirg1* at the beginning of exon 2 [20]. Affected animals exhibit the characteristic radiologic and histologic features of osteopetrosis, including a generalized increase in skeletal density, absence of major marrow cavities and failure of teeth to erupt. Without treatment their life expectancy is about 3–5 weeks. In this study we used 6 homozygous *Tcirg1* null and 6 heterozygous/wild type littermates, 18–20 days old, and 2 *Tcirg1* null and 2 adult wild type littermates, 50 days old. These mice were used for gene cell therapy experiments of defective osteoclasts and received Green Fluorescent Protein (GFP) — labeled bone marrow cells at day of birth to rescue osteoclast function [23,24]. This therapy leads to a partial rescue of the phenotype, resulting in the animals living longer with some bone resorption by GFP-labeled active osteoclasts resulting in tooth eruption, with no effects on dental development (none of the dental cells was GFP-labeled, Bronckers, unpublished results). A strain of adult *anion exchanger-2*-deficient mice of moderate phenotype (*Ae2$^{-/-}_{a,b}$* mice) with a mixed 129/Ola and FVB/N background was used as an example of a severely disturbed enamel mineralization [15]. Details of the targeting strategy to generate *Ae2$^{-/-}_{a,b}$* mice have been reported elsewhere [25]. All animal handling at the University in Pamplona, Spain and at the Vrije Universiteit Amsterdam, The Netherlands complied with National and International regulations for Animal Care and permission was obtained from the Committee for Animal Care.

Histological procedures

Heads and kidneys were collected and fixed by immersion in 5% paraformaldehyde in 0.1 M phosphate buffer + 2% sucrose overnight. Lower jaws were split into two hemi-mandibles one of which was scanned by microcomputer tomography (microCT, see below). The other hemi-mandibles and maxillae were decalcified in 4.18% EDTA + 0.8% formalin at pH 7.2 for 2 to 3 weeks at 4 °C, rinsed with phosphate buffer, embedded in paraffin along with kidney samples and serially sectioned into 6 μm thick sections.

Immunostaining for v-H-ATPase subunits and enzyme histochemistry of TRAcP

Paraffin sections were stained with haematoxylin–eosin (HE) for a general survey. Selected sections with appropriate stages of enamel development were immunostained by the peroxidase procedure using an ABC Elite kit (Vector Labs, Burlingame, CA, USA) or an Envision kit (Dakopatt, Glostrup, Denmark). The human osteoclast specific TCIRG1 protein has a molecular weight of 110 kDa and contains 720 amino acids. Three different antibodies were used. The first antibody was an affinity purified goat IgG to the human ATP6V$_0$-A$_3$/TCIRG1 (Santa Cruz, catalog SC-162300, N13; referred to as "TC-S"). According to the manufacturer the synthetic peptide used to raise these antibodies was 15–25 amino acid long and maps within the N-terminal-end region, amino acid 75–125 of the human TCIRG1 (NCBI accession number Q13488). The second antibody was an affinity purified rabbit IgG against a 56 amino acid-long recombinant fragment with the sequence RPADRQEENKAGLLDLPDASVNGWSSDEEKAGGLDDEEEAELVP-SEVLMHQAIHTI of the human TCIRG1 (Atlas Antibodies, Sigma, St Louis, MO, USA, catalog HPA038742; referred to as "TC-A"). It locates at amino acid 664–720 of the long isoform of TCIRG1.The third antibody was a rabbit polyclonal antiserum against a 20 amino acid (1–20) long N-terminal peptide of the human ATP6V$_1$, subunit B$_2$, (referred to as anti-B2) kindly provided by Dr Shannon Holliday (University of Florida, USA). On western blots from the mouse osteoclast-like cell line (RAW264.7) extracts and isolated mouse v-H-ATPase this antiserum reacts as a single band of the expected size. Working concentrations of the primary antibodies were 1:600–1:800 (anti-B2), 2–4 μg/ml for anti-TC-S and 0.5–1 μg/ml for anti-TC-A. In some studies sections were subjected to antigen retrieval treatment before staining (10 mM citrate pH 6.0, 20 min at 95 °C; 1 mM EDTA pH 9.0, 20 min at 95 °C; or proteinase K 10 μg/ml in phosphate buffered saline, 20 min a 37 °C). Kidney samples contain endogenous biotin that also binds ABC-peroxidase complex [26] especially after antigen retrieval procedures. To suppress false positive staining for endogenous biotin using the ABC-peroxidase method, the kidney sections were first blocked using an avidin biotin blocking kit according to manufacturer's instructions (Vector Labs). To validate specificity of the anti-TCIRG1 antibodies kidney sections from *Tcirg1* null mice were immunostained (Fig. 3a–f). As negative controls the primary antibodies were replaced by matched non-immune antibodies from the same species and in the same concentrations. The peroxidase was visualized by DiaminoBenzidine (DAB) staining (brown) after which nuclei were counterstained with methyl green (green-blue) or haematoxylin (blue).To identify osteoclasts, sections were stained red for Tartrate Resistant Acid Phosphatase (TRAcP) using a kit from Sigma.