Diversity of proton pumps in osteoclasts: V-ATPase with a3 and d2 isoforms is a major form in osteoclasts

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Osteoclasts acidify bone resorption lacunae through proton translocation by plasma membrane V-ATPase (vacuolar-type ATPase) which has an a3 isoform, one of the four isoforms of the trans-membrane a subunit (Toyomura et al., J. Biol. Chem., 278, 22023–22030, 2003). d2, a kidney- and epididymis-specific isoform of the d subunit, was also induced in osteoclast-like cells derived from the RAW264.7 line, and formed V-ATPase with a3. The amount of d2 in osteoclasts was 4-fold higher than that of d1, a ubiquitous isoform. These results indicate that V-ATPase with d2/a3 is a major osteoclast proton pump. Essentially the same results were obtained with osteoclasts derived from mouse spleen macrophages. Macrophages from a3-knock out mice could differentiate into multi-nuclear cells with osteoclast-specific enzymes. In these cells, the d2 isoform was also induced and assembled in V-ATPase with the a1 or a2 isoform. However, they did not absorb calcium phosphate, indicating that V-ATPase with d2/a1 or d2/a2 could not perform the function of that with d2/a3.

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1. Introduction

Bone homeostasis is maintained through the equilibrium between resorption by osteoclasts and bonegenesis by osteoblasts. Reduced and increased bone resorpions cause osteoporosis and osteopetrosis, respectively [1,2]. Osteoclasts tightly attached to the bone surface secrete protons and lysosomal enzymes into bone resorption lacuna, a compartment between osteoclasts and bone [3]. Proton pumping vacuolar-type ATPase (V-ATPase) in the osteoclast plasma membrane is responsible for this acidification [4,5]. Murine macrophage line RAW264.7 can form osteoclast-like multinuclear cells upon incubation with the extracellular domain of RANKL (receptor activator of nuclear factor κB ligand) [6]. The differentiated cells express osteoclast-specific enzymes in addition to V-ATPase.

V-ATPase is a multi-subunit enzyme formed from peripheral V0 and membrane V1 sectors, which function as a catalytic sector and a proton channel, respectively [7,8]. Consistent with its diverse physiological roles, V-ATPase has a number of subunit isoforms of V0 (a and d) and V1 (B, C, E, and G) [8–10]. One of the four isoforms (a1, a2, a3, and a4) of the a subunit, a3, is a component of lysosomal and osteoclast enzymes [6,11]. Detailed studies on RAW264.7-derived osteoclasts suggested that lysosomes having V-ATPase with a3 are targeted to the cell periphery and then become localized in the plasma membrane [6]. Consistently, disruption of the gene for a3 causes osteopetrosis with impaired bone resorption [12–14].

Similar to a3, d2 is induced during osteoclast differentiation [15–17]. Analysis of osteoclasts from d2 gene-deficient mice or from knock-down cells revealed that d2 plays important roles in cell fusion during differentiation into osteoclasts and in acidification of bone resorption lacunae [16,17]. However, association of a3 and d2 in the same V-ATPase has not been shown in osteoclasts, although d2 is immuno-precipitated with a3 when the tagged proteins are expressed in HEK-293T cells derived from the human embryonic kidney [17]. It is also of interest to determine whether or not a3 and d2 are expressed similarly during differentiation.

In this study, we found in mature osteoclasts that d2 is expressed at a 4-fold higher level than d1, a ubiquitous isoform, and assembled in V-ATPase together with a3. Spleen macrophages from a3 gene-deficient mice could differentiate into osteoclasts, however, they did not absorb calcium phosphate. Interestingly, the d2 isoform was induced similar in wild-type macrophages, and formed a V-ATPase with a1 or a2 in these cells. These results suggest that V-ATPase with a3/d2, not a1/d2 and a2/d2, plays a pivotal role in bone resorption.
2. Materials and methods

2.1. Cell culture and expression of FLAG-tagged d isoforms

RAW264.7 cells obtained from the European Collection of Cell Culture (ECACC Cat. 91062702) were grown in Dulbecco’s Modified Eagle Medium as described previously [18]. A stable RAW264.7 line expressing FLAG-tagged d1 or d2 was isolated after retrovirus infection using a Platinum Retrovirus Expression System (Cell Biolabs, San Diego, USA) and selection with 5 μg/mL puromycin. All reagents for cell culture were from Life Technologies (NY, USA). The a3-deficient mice were generated by crossing C57BL/6-a3+/- mice (BRC No. 04421) obtained from the RIKEN BioResource Center (Tsukuba, Japan) [19]. Macrophages were isolated from spleen cells of C57BL/6 or C57BL/6-a3−/− mice (2 weeks old), and cultured in MEMα containing 10% fetal bovine serum, antibiotics, and 25 ng/mL M-CSF (macrophage-colony stimulating factor) (R&D Systems, Minneapolis, USA). For osteoclast differentiation, RAW264.7 cells and spleen macrophages were cultured in the presence of 100 and 200 ng/mL RANKL (Peprotech, Rocky Hill, NJ), respectively. Actin and nuclei were stained with phalloidin and Hoechst33342, respectively [18]. The pit formation assay was performed using calcium phosphate coated dish (Corning, NY, USA).

2.2. Purification of the d1 and d2 isoforms

The cDNAs encoding d1 and d2 were synthesized by RT-PCR using mRNA from RAW264.7-derived osteoclasts and specific primers, sense strand sequence for d1 (5’-GACAGGATCCCTTGAGACTGCAGAGCTG-3’), anti-sense strand sequence for d1 (5’-AATTGCGGCCGCCTAAAAGATGGGGATGTA-3’), sense strand sequence for d2 (5’-ATGCGGATCCCTTGAGACTGCAGAGCTG-3’), and anti-sense strand sequence for d2 (5’-GTACGCGGCCGCTTATAAAATTGGAATGTA-3’), and cloned into the pGEX-6p vector (GE Healthcare, Buckinghamshire, UK). The GST (glutathione S-transferase)-fused d1 and d2 isoforms were expressed in Escherichia coli and purified with glutathione-Sepharose. After removal of the GST tag, d1 and d2 were used as standards for estimation of their amounts in osteoclasts.

2.3. Immuno-precipitation and Western blot analysis

Cells cultured with or without RANKL were lysed with IP buffer (1% Triton X-100, 10% glycerol, 50 mM Tris–HCl (pH7.4), 150 mM NaCl, 1 mM DTT, 1 mM EDTA, protease inhibitors). The lysates were subjected to immuno-precipitation as described previously.
The amounts of $d_1$ and $d_2$ were estimated based on the signal intensities on Western blotting using purified $d_1$ and $d_2$ as standards (Fig. 1B). Titrations of signal intensities of standard d isoforms revealed that anti-$d_1$ antibodies were about 10-fold less sensitive than those of anti-$d_1$ antibodies (data not shown). Osteoclasts derived from RAW264.7 cells (4 days after stimulation) contained $2.1 \pm 0.6$ ng$d_2$ isoform per 10 μg cell lysate (Fig. 1C, gray bar), which is about 4 times higher than the level of $d_1$ (0.5 ± 0.05 ng/10 μg of lysate) (Fig. 1C, open bars). These results suggest that V-ATPase with $d_2$ comprises about 80 ± 23% of total enzyme, assuming that all $d_2$ are assembled. As expected, $d_1$ and $d_2$ were not detectable in the cytosol fraction, being only found in the organelle fraction (Fig. 1D). This finding suggests that most of $d_1$ or $d_2$ is assembled in V-ATPase localized in the membranes. It is noteworthy that both proteins expressed in E. coli were recovered in a soluble fraction (data not shown), suggesting that they are soluble proteins when not assembled in V-ATPase.

### 3.2. Assembly of $d_2$ and $a_3$ in osteoclast V-ATPase

To confirm the association of $d_2$ and $a_3$ in osteoclasts, V-ATPase was precipitated with antibodies. The total cell lysate derived from osteoclasts or undifferentiated cells were subjected to immuno-precipitation using anti-$a_3$ antibodies, and the presence of $d_1$ and $d_2$ was examined in the precipitate. As expected, $d_2$ was detected after differentiation but not before stimulation (Fig. 2, compare lanes 2 and 3). On the other hand, $d_1$ was immuno-precipitated with anti-$a_3$ antibodies, regardless whether differentiation had occurred or not (Fig. 2, lanes 2 and 3). These results clearly indicate that $d_1$ and $d_2$ are assembled, respectively, in V-ATPase together with $a_3$.

Consistent results were obtained using anti-B2 antibodies (Fig. 2, lanes 5 and 6), indicating that both $a_3$ and $d_2$ are associated with B2. These results indicate that $V_1$ and $V_0$ sectors are assembled together in osteoclasts, since B2 is a component of the $V_1$ sector, and $a_3$ and $d_2$ ones of the $V_0$ sector.

### 3.3. Expression of V-ATPase with $a_3$ and $d_2$ in osteoclasts derived from spleen macrophages

For confirmation, we followed osteoclast differentiation from mouse spleen macrophages upon addition of RANKL, and examined the presence of V-ATPase with $a_3$ and $d_2$. Basically the same results as for RAW264.7 cells were obtained: TRAP-positive multinuclear cells forming calcium phosphate resorption pits were observed upon stimulation of RANKL (Fig. 3A, upper panels). $d_2$ and $a_3$ were induced during differentiation, similar to osteoclast markers (Fig. 3B, left). The amount of $d_2$, estimated from the signal intensities on Western blotting using purified $d_2$ as a standard, comprised about 90% of total $d$ subunit, confirming the result for osteoclasts induced from RAW264.7 cells. V-ATPase with the $a_3$ isoform was detected at the osteoclast periphery immunocytochemically using anti-$a_3$ antibodies, similar to those differentiated from RAW264.7 ones (data not shown) [6].

V-ATPase from wild-type osteoclasts was found to contain $d_1$ or $d_2$ when precipitated with anti-$a_3$ antibodies (Fig. 4A, lane 3), indicating that ubiquitous and osteoclast-specific V-ATPases having $a_3/d_1$ and $a_3/d_2$ isoforms, respectively, are both present in osteoclasts. These isoforms were also detectable on precipitation with anti-B2 antibodies (Fig. 4A, lane 6), confirming that $V_0$ and $V_1$ are associated in the V-ATPase studied, since the B subunit is a component of the $V_1$ sector.

### 3.4. V-ATPase in osteoclasts formed from $a_3$-knock-out mice macrophages

It became of interest whether or not osteoclasts can be formed when V-ATPase with $a_3$ is not expressed in the progenitors. $a_3$ may affect cell-cell fusion, since it is a trans-membrane protein. Furthermore, it was
unknown whether or not d2 assembles only with the a3 isoform. To address these issues, we studied osteoclast differentiation from macrophages lacking the a3 gene. Spleen macrophages from a3-knock-out mice formed TRAP-positive multinuclear cells on stimulation (Fig. 3A, lower panels), and osteoclast marker enzymes were induced following essentially the same time courses as in wild-type macrophages (Fig. 3B, right). The number of osteoclasts formed from a3-knock-out macrophages was similar to that from wild-type ones. However, those from the knockout mice did not form calcium phosphate resorption pits (Fig. 3A, right). These results suggest that the a3 isoform is not required for osteoclast differentiation itself, but plays a role in bone resorption, consistent with previous findings [12].

As shown in Fig. 3B, right, d2 was induced and expressed in a3 knock-out cells following essentially the same time course as in wild type cells, suggesting that d2 is expressed independently from the a3 isoform, and possibly assembled in V-ATPase with a1 or a2. It is noteworthy that the amount of d1 was increased during osteoclast differentiation from a3-knockout macrophages (Fig. 3B, right). The increased amount of d1 possibly corresponds to that of a subunit: a2 isoform is induced during differentiation (data not shown).

a1 and a2 were detected in the a3-knock-out mice V-ATPase precipitated with anti-B2 antibodies (Fig. 4A, lane 8), suggesting that they could form V-ATPase with d1 or d2. As expected, no a3 was detectable in a3-knock-out mice (Fig. 4A, lane 8). These results suggest that the d2 isoform could form V-ATPase with a1 or a2.

Furthermore, we expressed FLAG-tagged d isoforms in RAW264.7 cells, and immuno-precipitated V-ATPase with these isoforms using anti-FLAG antibodies. a1, a2, and a3 were co-precipitated with the d1 or d2 isoform (Fig. 4B), indicating that both d1 and d2 can form a complex with one of these three a subunit isoforms. These results confirm the presence of V-ATPase with diverse isoform combinations in osteoclasts.

In this study, we observed that V-ATPase with a3 and d2 was induced and became a major proton pump for bone resorption in osteoclasts. However, this V-ATPase may not play roles in cell fusion leading to multinuclear cells, since a3-deficient macrophages could form multinuclear osteoclast-like cells. These cells did not show calcium phosphate resorption activity due to the absence of V-ATPase with a3/d2. Interestingly, V-ATPases with a1/d2 and/or a2/d2 were expressed in a3-knockout cells. However, they could not function as V-ATPase with a3/d2.

We suggested previously that late endosomes/lysosomes having a unique V-ATPase with a3 move to the cell periphery and fuse with the plasma membrane facing bone resorption lacunae [6]. At the same time, cathepsin K and TRAP are secreted possibly from lysosomes at the site of bone resorption. As found in this study, d2 was induced following similar kinetics as those for lysosomal enzymes, and
assembled in V-ATPase with α3. These results suggest that V-ATPase with α3/d2 is localized in the membrane of secretory lysosomes containing cathepsin K and TRAP, and plays a role in bone resorption.

Similar to ARNO, a regulator at vesicle trafficking, the d2 isoform is indicated to interact with the amino terminus of α3 [17,22,23], suggesting their roles in organelle trafficking. Moreover, d2 is involved in membrane fusion between osteoclast progenitors [16,17]. Taken together, V-ATPase with α3 and d2 localized in secretory lysosomes may be advantageous for trafficking and membrane fusion with the osteoclast plasma membrane.

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References


