Biochimica et Biophysica Acta 1837 (2014) 744–749

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

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

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ARTICLE INFO

Article history:
Received 22 November 2013
Received in revised form 10 February 2014
Accepted 12 February 2014
Available online 19 February 2014

Keywords:
Vacuolar-type ATPase
Osteoclast
Isoform
a3 knock out mouse

ABSTRACT

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*.

1. Introduction

Bone homeostasis is maintained through the equilibrium between resorption by osteoclasts and bonegenesis by osteoblasts. Reduced and increased bone resorptions cause osteopetrosis and osteoporosis, 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 V_1 and membrane V_0 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 V_0 (*a* and *d*) and V_1 (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.

Abbreviations: V-ATPase, vacuolar-type ATPase; RANKL, receptor activator of nuclear factor kappa B ligand; M-CSF, macrophage-colony stimulating factor; GST, glutathione S-transferase; DTT, dithiothreitol; EDTA, ethylenediamine-N,N,N′,N′-tetraacetic acid; Cath K, cathepsin K; TRAP, tartrate-resistant acid phosphatase

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http://dx.doi.org/10.1016/j.bbabio.2014.02.011
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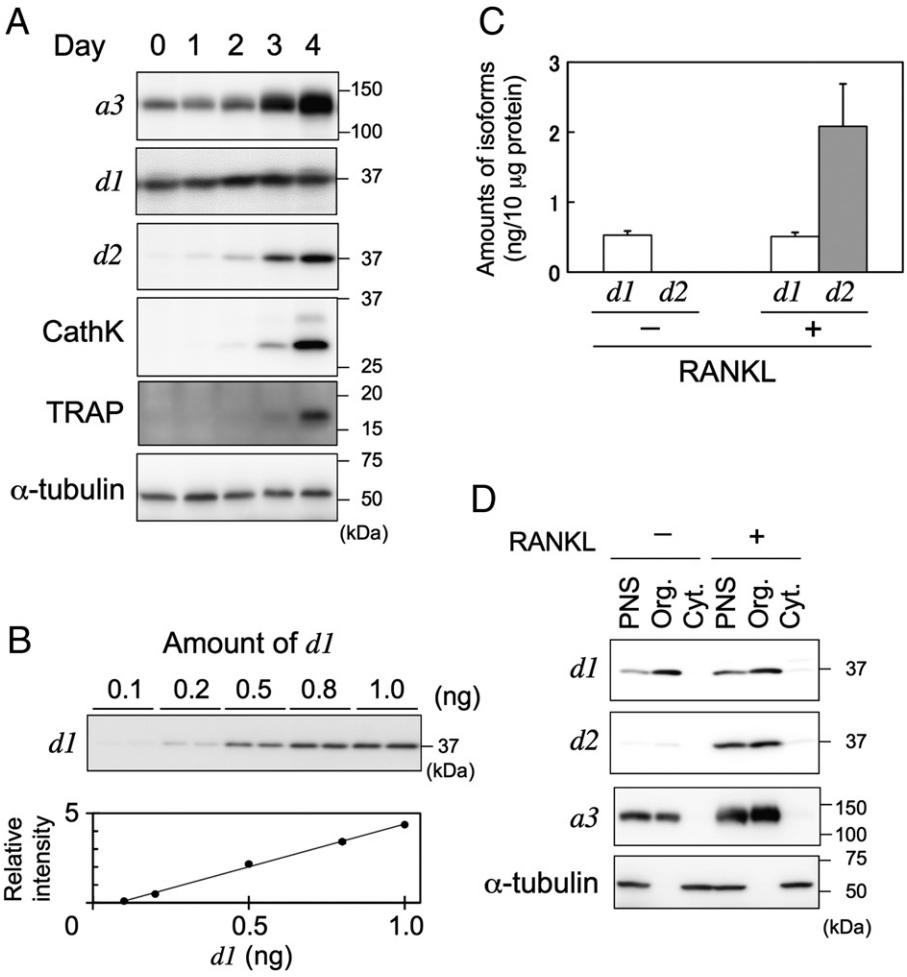

Fig. 1. Protein expression during differentiation into osteoclasts from RAW264.7 cells. (A) Time courses of protein expression during osteoclast differentiation. RAW264.7 cells were cultured for 0, 1, 2, 3, or 4 days with RANKL. After total cell protein (20 μg) had been separated by gel electrophoresis, *a3*, *d1*, *d2*, CathK, TRAP, and α-tubulin were detected with the corresponding antibodies. (B) Titration for estimating the amount of the *d1* isoform. The recombinant *d1* protein, 0.1–1 ng, was detected on Western blotting with antibodies against *d1* (*upper panel*), and the intensity of each signal was quantified with an LAS-3000. A typical titration for determining the amount of *d1* is shown (*lower panel*). Standard error bars are smaller than the symbols. Similar titration was carried out for the *d2* isoform (not shown). (C) Amounts of the *d1* and *d2* isoforms in progenitors and osteoclasts. After total protein had been obtained from the cells treated with (+) or without (−) RANKL, the amounts of *d1* (open bars) and *d2* (gray bar) isoforms were estimated by titration as shown in (B). The amount of *d1* or *d2* in 10 μg total cell protein is shown. (D) Presence of *d1*, *d2*, and *a3* isoforms in the organelle fraction. Cell lysates were prepared from RAW264.7 cells treated with (+) or without (−) RANKL. Post-nuclear supernatants (PNS), the organelle (Org.) and cytosol (Cyt.) fractions were subjected to Western blotting. The α-tubulin was used as a control cytosolic protein.

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′-GACAGGATCCTCGTTCTTCCCGGAGCTC-3′), anti-sense strand sequence for *d1* (5′-AATTGCGGCCGCCTAAAAGATG GGGATGTA-3′), sense strand sequence for *d2* (5′-ATGCGGATCCCTTG AGACTGCAGAGCTG-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

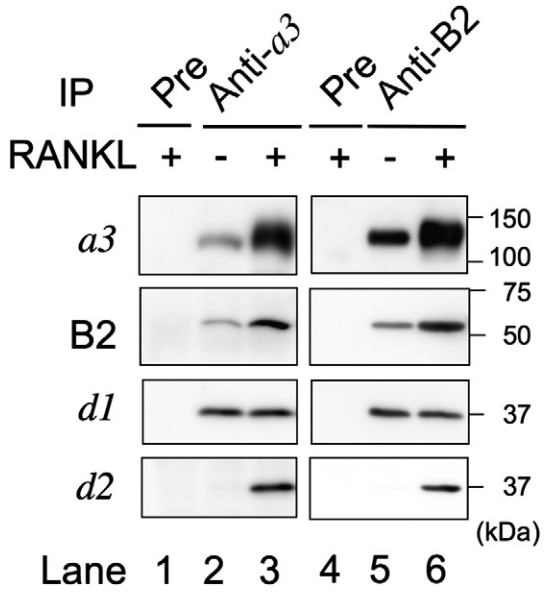

Fig. 2. V-ATPase with *d2* and *a3* isoforms in osteoclasts derived from RAW264.7 cells. RAW264.7 cells were treated with (+) or without (−) RANKL for 4 days. Cell lysates were subjected to immuno-precipitation using pre-immune serum (*lanes 1* and *4*), and antibodies against *a3* (*lanes 2* and *3*) and B2 (*lanes 5* and *6*). The precipitates were analyzed by blotting using antibodies against the indicated proteins.

[20]. Subunit isoforms in the precipitates were detected by Western blot analysis [18]. Immunodetection was carried out using horseradish peroxidase-conjugated antibodies and an ECL luminescence detection system (GE Healthcare). Antibodies against *a3*, *d2*, cathepsin K, and α-tubulin were obtained as described previously [18]. Antibodies against β-actin and B2 were purchased from Sigma-Aldrich (MO, USA). An antibody against TRAP (tartrate-resistant acid phosphatase) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A chicken monoclonal antibody against *a2* was generated as described previously [21]. Antibodies against the mouse *a1* and *d1* isoforms were prepared using synthetic peptides corresponding to Gly680–His693 of *a1* (Life Technologies) and to Val69–Tyr90 of mouse *d1* (Greiner Bio-one, Tokyo, Japan), respectively. Signals observed on Western blotting were quantified using an LAS-3000.

2.4. Subcellular fractionation

RAW264.7 and RAW264.7-derived osteoclasts were disrupted in 0.25 M sucrose containing 3 mM imidazole (pH–7.4), 0.5 mM EDTA, and protease inhibitors, and centrifuged at 750 ×*g* for 5 min. The resulting post-nuclear supernatant was centrifuged at 100,000 ×*g* for 30 min, organelle and cytosol fractions being obtained.

3. Results and discussion

3.1. Protein amounts of d1 and d2 isoforms in osteoclasts

We have reported that the V-ATPase *a3* isoform increased in RAW264.7 cells together with induced synthesis of osteoclast marker proteins upon stimulation with RANKL [6,18]. The *d2* isoform is also induced during osteoclast differentiation [15–17]. Consistent with previous results, the *d2* and *a3* isoforms increased to at least more than 100-fold and about 4-fold during differentiation, respectively (Fig. 1A). The amount of *d2* was slightly higher than the detection limit before stimulation, although it was difficult to quantify. The pattern of *d2* induction by RANKL was similar to that of osteoclast specific enzymes, TRAP and cathepsin K. On the other hand, ubiquitous *d1* was detectable before stimulation and did not increase during differentiation up to 4 days.

The amounts of *d1* and *d2* were estimated based on the signal intensities on Western blotting using purified *d1* and *d2* as standards (Fig. 1B). Titrations of signal intensities of standard *d* isoforms revealed that anti-*d2* antibodies were about 10-fold less sensitive than those of anti-*d1* antibodies (data not shown). Osteoclasts derived from RAW264.7 cells (4 days after stimulation) contained 2.1 ± 0.6 ng *d2* isoform per 10 μg cell lysate (Fig. 1C, gray bar), which is about 4 times higher than the level of *d1* (0.5 ± 0.05 ng/10 μg of lysate) (Fig. 1C, open bars). These results suggest that V-ATPase with *d2* comprises about 80 ± 23% of total enzyme, assuming that all *d2* are assembled. As expected, *d1* and *d2* were not detectable in the cytosol fraction, being only found in the organelle fraction (Fig. 1D). This finding suggests that most of *d1* or *d2* 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 d2 and a3 in osteoclast V-ATPase

To confirm the association of *d2* and *a3* 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-*a3* antibodies, and the presence of *d1* and *d2* was examined in the precipitate. As expected, *d2* was detected after differentiation but not before stimulation (Fig. 2, compare *lanes 2* and *3*). On the other hand, *d1* was immuno-precipitated with anti-*a3* antibodies, regardless whether differentiation had occurred or not (Fig. 2, *lanes 2* and *3*). These results clearly indicate that *d1* and *d2* are assembled, respectively, in V-ATPase together with *a3*.

Consistent results were obtained using anti-B2 antibodies (Fig. 2, *lanes 5* and *6*), indicating that both *a3* and *d2* 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 *a3* and *d2* ones of the V_0 sector.

3.3. Expression of V-ATPase with a3 and d2 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 *a3* and *d2*. 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*). *d2* and *a3* were induced during differentiation, similar to osteoclast markers (Fig. 3B, *left*). The amount of *d2*, estimated from the signal intensities on Western blotting using purified *d2* as a standard, comprised about 90% of total *d* subunit, confirming the result for osteoclasts induced from RAW264.7 cells. V-ATPase with the *a3* isoform was detected at the osteoclast periphery immunochemically using anti-*a3* antibodies, similar to those differentiated from RAW264.7 ones (data not shown) [6].

V-ATPase from wild-type osteoclasts was found to contain *d1* or *d2* when precipitated with anti-*a3* antibodies (Fig. 4A, *lane 3*), indicating that ubiquitous and osteoclast-specific V-ATPases having *a3*/*d1* and *a3*/*d2* 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 a3-knock-out mice macrophages

It became of interest whether or not osteoclasts can be formed when V-ATPase with *a3* is not expressed in the progenitors. *a3* may affect cell–cell fusion, since it is a trans-membrane protein. Furthermore, it was

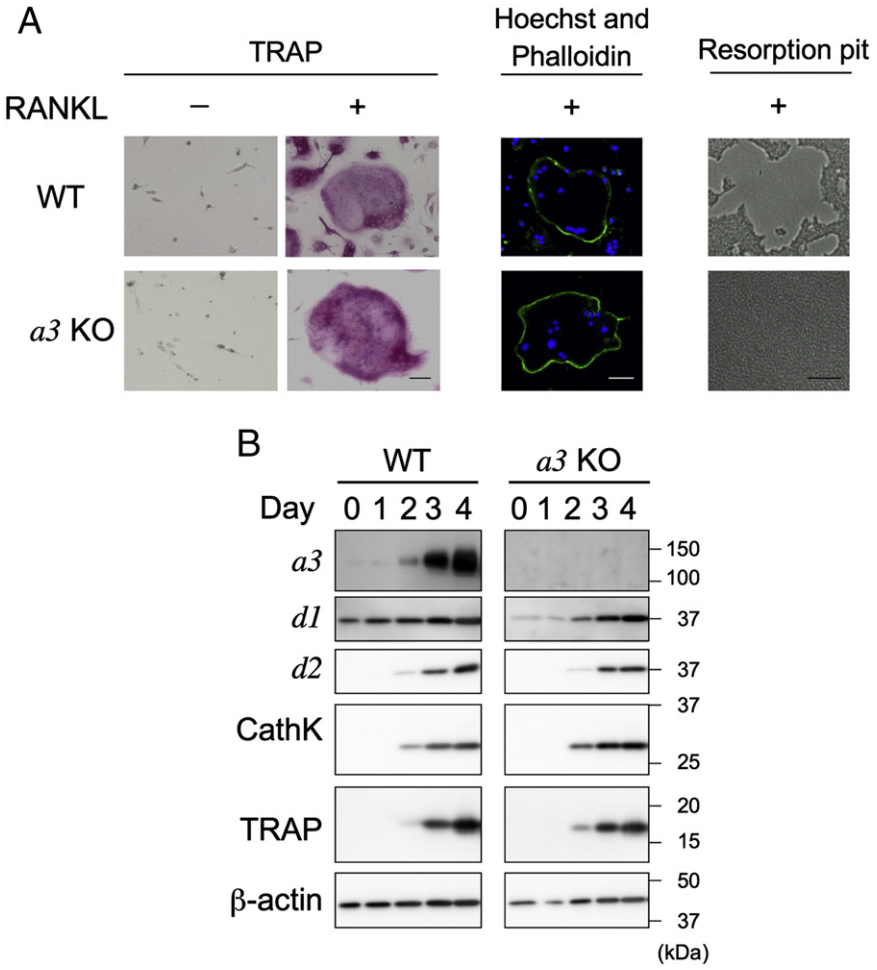

Fig. 3. Induction of *d2* in osteoclasts differentiated from wild-type and *a3*-deficient mice macrophages. (A) Formation of osteoclasts from mouse spleen macrophages. Macrophages obtained from the spleens of wild-type (WT) or *a3* gene-deficient mice (*a3* KO) were stimulated with RANKL for 4 days, fixed, and then subjected to TRAP-staining, or nuclei (blue) and actin (green) staining. For the pit formation assays, cells were cultured on calcium phosphate-coated dishes and allowed to differentiate into osteoclasts. Four days after stimulation, the formed pits were observed under a microscope. Cells shown in (A) are not identical. Scale bar, 50 μm. (B) Time courses of expression of *a3*, *d1*, *d2*, and osteoclast markers. Macrophages obtained from the spleens of wild-type (WT) or *a3* gene-deficient mice (*a3* KO) were allowed to differentiate into osteoclasts as in (A). After gel electrophoresis of cell lysates, V-ATPase isoforms and osteoclast marker proteins were detected by Western blotting with the corresponding antibodies. β-actin was used as a positive control.

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

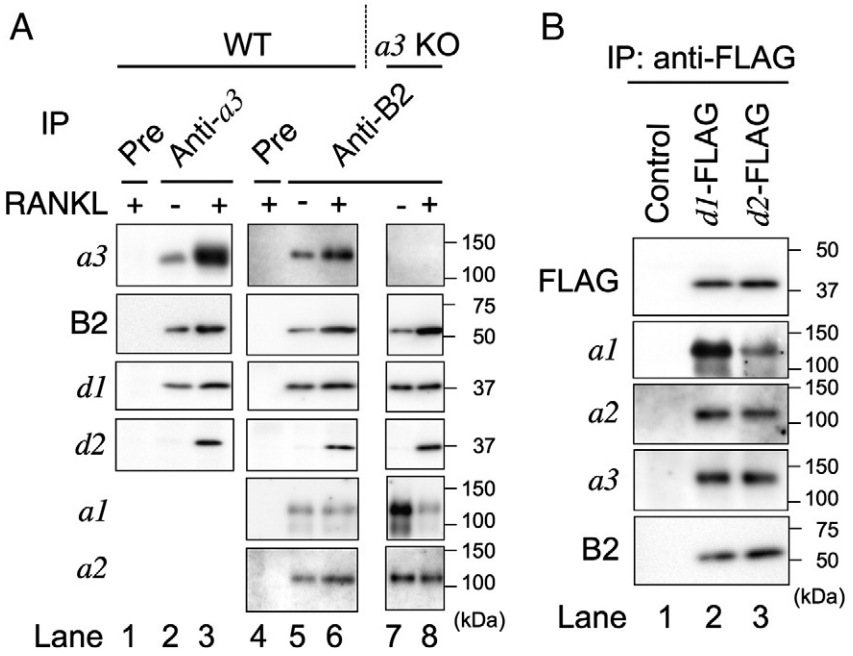

Fig. 4. Association of the *a* and *d* isoforms in osteoclast V-ATPase from mouse macrophages and RAW264.7 cells. (A) Presence of *d2* isoforms in V-ATPase in osteoclasts differentiated from wild-type and *a3*-deficient mice macrophages. Macrophages from wild type (WT) and *a3*-deficient (*a3*KO) mice were treated with (+) or without (−) RANKL, and cell lysates were prepared from the cells, and subjected to immuno-precipitation using pre-immune serum (*lanes 1* and *4*), and antibodies against *a3* (*lanes 2* and *3*) or B2 (*lanes 5–8*). The precipitates were analyzed using antibodies against the indicated proteins. The amount of *a1* was higher in *a3* knock-out mice macrophages than in other cells for an unknown reason (*lane 7*). (B) Co-precipitation of *a* isoforms with FLAG-tagged *d* isoforms. A FLAG-tag was introduced at the C-terminal of the *d1* or *d2* isoform, and expressed in RAW264.7 cells. Lysates of these cells were subjected to precipitation with anti-FLAG antibodies, and blotting was performed as in (A).

assembled in V-ATPase with *a3*. These results suggest that V-ATPase with *a3*/*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 *a3* [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 *a3* and *d2* localized in secretory lysosomes may be advantageous for trafficking and membrane fusion with the osteoclast plasma membrane.

Acknowledgements

This study was supported partly by the JSPS (Japan Society for the Promotion of Science), the Terumo Life Science Foundation, and the Japan Foundation for Applied Enzymology. This study was also supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan. The support of the Hayashi Memorial Foundation for Female Natural Scientists (to M. N.-M.) is also acknowledged.

We thank Shio Yano for the technical assistance. We also thank Yuko Warizaki and Namiko Segawa for the expression of the FLAG-tagged *d* subunits.

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