Rotational Catalysis and Diverse Functions of Proton-Pumping ATPases

Review

V-ATPase a3 Subunit in Secretory Lysosome Trafficking in Osteoclasts

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Vacuolar-type ATPase (V-ATPase) shares its structure and rotational catalysis with F-type ATPase (F-ATPase, ATP synthase). However, unlike subunits of F-ATPase, those of V-ATPase have tissue- and/or organelle-specific isoforms. Structural diversity of V-ATPase generated by different combinations of subunit isoforms enables it to play diverse physiological roles in mammalian cells. Among these various roles, this review focuses on the functions of lysosome-specific V-ATPase in bone resorption by osteoclasts. Lysosomes remain in the cytoplasm in most cell types, but in osteoclasts, secretory lysosomes move toward and fuse with the plasma membrane to secrete lysosomal enzymes, which is essential for bone resorption. Through this process, lysosomal V-ATPase harboring the a3 isoform of the a subunit is relocated to the plasma membrane, where it transports protons from the cytosol to the cell exterior to generate the acidic extracellular conditions required for secreted lysosomal enzymes. In addition to this role as a proton pump, we recently found that the lysosomal a3 subunit of V-ATPase is essential for anterograde trafficking of secretory lysosomes. Specifically, a3 interacts with Rab7, a member of the Rab guanosine 5’-triphosphatase (GTPase) family that regulates organelle trafficking, and recruits it to the lysosomal membrane. These findings revealed the multifunctionality of lysosomal V-ATPase in osteoclasts; V-ATPase is responsible not only for the formation of the acidic environment by transporting protons, but also for intracellular trafficking of secretory lysosomes by recruiting organelle trafficking factors. Herein, we summarize the molecular mechanism underlying secretory lysosome trafficking in osteoclasts, and discuss the possible regulatory role of V-ATPase in organelle trafficking.

Key words vacuolar-type ATPase; osteoclast; secretory lysosome; proton pump; organelle trafficking

1. INTRODUCTION

To maintain bone homeostasis, it is essential to balance bone formation by osteoblasts and bone resorption by osteoclasts; excessive bone resorption reduces bone density and causes osteoporosis, while inadequate bone resorption increases bone density and causes osteopetrosis.1–3 In both cases, bone fracture often occurs, and QOL is severely decreased. It is estimated that there are more than 10 million people suffering from osteoporosis in Japan.4,5 Therefore, simple and minimally invasive clinical treatments for the disease are urgently needed.

Osteoclasts are multinuclear cells differentiated from monocytic/macrophage hematopoietic lineages that are stimulated by the receptor activator of nuclear factor κB ligand (RANKL) released by osteoblasts.6–8 During differentiation into osteoclasts, the expression level of the marker enzyme tartrate-resistant acid phosphatase (TRAP) is strongly induced. Osteoclasts attach tightly to the surface of bone through actin rings, and form bone resorption lacunae between cell and bone (Fig.1). The basal membrane facing bone has a complicated folded finger-like structure known as a ruffled border. Lysosomal digestive enzymes are secreted into the bone resorption lacunae to digest collagen, a major protein component of bone matrix.5–6 The lysosomes involved in secretion are called secretory lysosomes.7,8 Vacuolar-type ATPase (V-ATPase) in the lysosomal membrane is transported to the plasma membrane through secretory lysosomes, and its proton-pumping activity acidifies the resorption lacunae to provide optimal acidic conditions for secreted lysosomal enzymes.4–6,9–12 Bone resorption lacunae appear to be large lysosomes able to digest objects larger than cells. Secretory lysosomes are essential for lysosomal enzyme secretion and resorption lacunae acidification by osteoclasts.7,8 V-ATPase transports protons across the membrane using energy obtained from ATP hydrolysis. As shown in Fig.2, V-ATPase is composed of V$_1$ and V$_o$ sectors. The V$_1$ sector is a soluble complex with three catalytic A subunits, and the V$_o$ sector is a membrane-embedded complex with a proton pathway formed by the a subunit and the c-ring.13–18 Similar to F-ATPase (ATP synthase), V-ATPase couples catalysis and proton transport through subunit rotation, which is called rotational catalysis.16–18 The mechanism has been analyzed in detail using bacterial enzymes, and these studies have been covered elsewhere.19–21 Among the subunits composing mammalian V-ATPase, six subunits have cell- and/or organelle-specific isoforms, resulting in diversity in isoform composition.13–18 For example, the a subunit in the V$_1$ sector has four isoforms (a1, a2, a3, and a4). The a4 isoform is specifically expressed in kidney, while the other three isoforms are ubiquitously expressed, but localized in specific organelles. The a1, a2, and a3 isoforms localize in coated vesicles, early endosomes/Golgi apparatus, and late endosomes/lysosomes, respectively. The d subunit also has ubiquitous d1 and kidney.

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epididymis, and osteoclast-specific d2 isoforms.\textsuperscript{15–18} The a3 and d2 isoforms are strongly induced during differentiation into osteoclasts, and V-ATPase with a3 and d2 is the predominant form in osteoclasts.\textsuperscript{22–24} Interestingly, d2-knockout (d2KO) mice possess smaller osteoclasts than wild-type (WT) mice, presumably due to inefficient cell–cell fusion, and they can resorb bone, but not efficiently.\textsuperscript{25} Osteoclasts of a3-knockout (a3KO) mice cannot resorb bone, and mice develop osteopetrosis. Thus, a3 is indispensable for bone resorption.\textsuperscript{26,27}

Human infantile malignant osteopetrosis is an autosomal recessive disease caused by impaired bone resorption.\textsuperscript{28–31} Genomic analysis of osteopetrosis patients revealed that, in more than 50% of cases, the locus responsible for the disease was mapped to 11q13, where the a3 gene (T cell immune regulator 1 (TCIRG1)) is located.\textsuperscript{30} To date, more than 90 mutations in the human a3 gene have been identified in patients suffering from the disease.\textsuperscript{33} Analysis of the expression and functions of four a3 mutants using a3KO osteoclasts revealed that short deletions in the cytoplasmic amino-terminal domain of a3 impair its expression, and thereby disrupt V-ATPase subunit assembly essential for bone resorption.\textsuperscript{32} These observations clearly indicate that a3 is critical for bone resorption in human, and mutations lead to osteopetrosis, consistent with the observation of osteoclasts in a3KO mice.\textsuperscript{26,27}

It was recently demonstrated that the lysosomal a3 subunit of V-ATPase is involved in trafficking of secretory lysosomes in osteoclasts; a3 binds to and recruits Rab7, a Rab small guanosine 5’-triphosphatase (GTPase) regulating late endosome trafficking, to secretory lysosomes, which is essential for triggering anterograde trafficking of secretory lysosomes.\textsuperscript{33} In this review, we cover recent findings on the functions of a3 in osteoclasts, and discuss a possible molecular mechanism underlying anterograde trafficking of osteoclast secretory lysosomes.

2. V-ATPASE IN SECRETORY LYSOSOME TRAFFICKING

Osteoclasts are multinuclear cells equipped with well-developed ruffled borders and actin rings (Fig. 1). Electron microscopy has revealed that the morphology of osteoclasts in a3KO mice is similar to that in WT mice.\textsuperscript{33} However, the electron density of bone in a3KO mice is clearly increased compared with WT mice, indicating that bone density is increased in a3KO mice.\textsuperscript{33} This observation is consistent with the previously reported phenotype of osteopetrosis caused by a defective a3 gene.\textsuperscript{26,27,30} It was also reported that osteoclasts do not acidify the bone resorption lacunae in a3KO mice.\textsuperscript{26} Based on these observations, it has been suggested that in osteoclasts, the main role of V-ATPase with a3 is acidification of bone resorption lacunae, but whether there are other functions of lysosomal V-ATPase with a3 remains unknown.
As described above, secretory lysosomes move toward ruffled borders in an anterograde manner during osteoclast differentiation.\(^{10,11}\) Thus, it was considered interesting to explore whether the V-ATPase α3 subunit is involved in secretory lysosome trafficking. To this end, secretory lysosomes were observed in osteoclasts by immunoelectron microscopy using antibodies against CD68, a marker protein of lysosomes.\(^{33}\) In WT osteoclasts, CD68 was detected both in ruffled borders and the perinuclear region, whereas in α3KO cells it was barely present in ruffled borders\(^{33}\) (Fig. 3A). These results indicate that α3 is required for secretory lysosome trafficking toward ruffled borders.

For further analysis, osteoclasts were differentiated in vitro from mouse spleen macrophages through stimulation with the soluble part of RANKL, and lysosomes were observed by immunostaining using antibodies against CD68, a marker protein of lysosomes.\(^{33}\) In WT osteoclasts, CD68 was detected both in ruffled borders and the perinuclear region, whereas in α3KO cells it was barely present in ruffled borders\(^{33}\) (Fig. 3A). These results indicate that α3 is required for secretory lysosome trafficking toward ruffled borders.

For further analysis, osteoclasts were differentiated in vitro from mouse spleen macrophages through stimulation with the soluble part of RANKL, and lysosomes were observed by immunostaining with anti-CD68 antibody.\(^{33}\) In WT cells, CD68 was well colocalized with α3 and accumulated at the cell periphery (Fig. 3B, arrowhead), whereas in α3KO cells, peripheral localization of CD68 was rarely observed. When α3 was exogenously expressed in α3KO cells, peripheral localization of lysosomes was recovered.\(^{33}\) Organelles other than lysosomes, such as early endosomes and Golgi apparatus, were normally localized in α3KO cells, and abnormal accumulation of cholesterol in the lysosomal membrane, which affects lysosome trafficking in lysosomal storage disease cells, was not observed.\(^{33}\)

Therefore, the in vivo and in vitro studies demonstrated that α3 is essential for secretory lysosome trafficking in osteoclasts. Interestingly, lysosomal V-ATPase with the α3 subunit has a dual function in osteoclasts: α3 is essential not only for acidification of bone resorption lacunae, but also for outward trafficking of secretory lysosomes.\(^{33}\)

3. RAB7 RECRUITMENT TO SECRETORY LYOSOMES BY α3

It has been shown that Rab7 is involved in secretory lysosome trafficking in osteoclasts.\(^{34,35}\) The function of Rab7 is regulated by exchanging guanine nucleotides. A guanine nucleotide exchange factor (GEF) activates Rab7 by exchanging guanine nucleotide bound to Rab7 from guanosine 5’-diphosphate (GDP) to GTP. Activated GTP-bound Rab7 stably localizes to the target organelle membrane, where it forms the trafficking machinery to combine with microtubules.\(^{36–39}\)

Therefore, Rab7 recruitment is the crucial first step in organelle trafficking. Localization of endogenous Rab7 was investigated in osteoclasts by immunostaining.\(^{33}\) In WT cells, Rab7 partly colocalized with lysosome marker CD68, especially at the cell periphery (Fig. 3C, arrowhead); in α3KO cells, it was dispersed throughout the cytoplasm, and colocalization with CD68 was significantly reduced, indicating that Rab7 requires α3 for its lysosomal localization. To explore whether α3 is also important for lysosomal localization of activated Rab7, a constitutively active GTP-fixed form of Rab7 in which Gln67 was mutated to Leu was expressed in osteoclasts.\(^{33}\) In WT cells, constitutively active Rab7 clearly colocalized with CD68, but in α3 KO cells it was diffusely distributed throughout the cytoplasm, and colocalization with CD68 was significantly decreased. This result suggests that α3 is essential for lysosomal localization of Rab7 even after its activation.
The interaction between a3 and Rab7 was subsequently examined. The three a subunit isoforms (a1, a2, a3) and Rab7 derivatives (WT, GDP-fixed form, and GTP-fixed form) were expressed in HEK293T human embryonic kidney cells, and immunoprecipitation was performed. The a3 subunit specifically interacted with the dominant-negative GDP-fixed form of Rab7 harboring the Thr22 to Asn mutation, but not with the WT or constitutively active GTP-fixed forms. Similar to a3, a1 and a2 isoforms interacted with the GDP-fixed form of Rab7, but less effectively than a3. It is likely that a3, whose expression is strongly induced during osteoclast differentiation, recruits GDP-bound Rab7 to secretory lysosomes to trigger their trafficking.

Furthermore, a3 was also shown to bind to the GDP-fixed form of Rab27A, and to recruit it to secretory lysosomes in osteoclasts. Rab27A, another member of the Rab small GTPase family, is involved in membrane fusion between secretory lysosomes and the plasma membrane to release lysosomal enzymes. Osteoclasts derived from Rab27AKO mice exhibited abnormal subcellular localization of lysosomes. Thus, lysosomal V-ATPase with a3 appears to play a role in membrane fusion by recruiting Rab27A to secretory lysosomes.

4. PROTON PUMP ACTIVITY OF V-ATPASE IN SECRETORY LYSOSOME TRAFFICKING

Voronov et al. showed that the lysosomal acidic pH generated by V-ATPase is essential for osteoclast differentiation, but the role of acidic pH in secretory lysosome trafficking remained unknown. To investigate whether the proton pump activity of V-ATPase is required in the process of Rab7 recruitment to lysosomes, osteoclasts were treated with bafilomycin A1, a V-ATPase inhibitor, and lysosomes were stained with anti-CD68 antibody. In cells treated with bafilomycin A1, lysosomes were dispersed throughout the cytoplasm, and lysosomal localization of Rab7 was rarely observed, suggesting that acidic conditions generated by V-ATPase are essential for recruitment of Rab7 to lysosomes. On the other hand, bafilomycin A1 did not significantly affect either the interaction between a3 and the GDP-fixed form of Rab7, or activation of Rab7.

In general, the GTP-fixed form of Rab proteins localize to corresponding organelle membranes when expressed exogenously. Consistently, the GTP-fixed form of Rab7 localizes to lysosomes when expressed in WT osteoclasts, whereas its lysosomal localization was significantly reduced in bafilomycin A1-treated cells. These results suggest that acidic conditions generated by V-ATPase are important for stable lysosomal localization of activated Rab7.

How the lysosomal luminal acidic conditions affect Rab7 recruitment outside the organelle is an interesting question. Given the fact that constitutively active Rab7 does not interact with a3, an unknown lysosomal membrane factor other than a3 appears to be required. If conformation of the hypothesized factor is affected by luminal acidic conditions, and if its interaction with activated Rab7 depends on the protein conformation, lysosomal luminal acidic conditions may affect the stable lysosomal localization of activated Rab7. It is of interest to identify such a factor in future work.

5. FUNCTIONAL COMPLEMENTATION BY A SUBUNIT ISOFORMS

As mentioned above, in addition to a3, a1 and a2 subunit isoforms interact with the GDP-fixed form of Rab7 to some extent. The expression levels of these a isoforms in a3KO osteoclasts are similar to those in WT cells. However, these a isoforms do not compensate for a3 function in organelle trafficking in a3KO osteoclasts. This is probably because a1 and a2 mainly localize to coated vesicles and early endosomes/Golgi apparatus, respectively. The a1 and a2 subunits were overexpressed in a3KO osteoclasts, and their localization was observed. Interestingly, a small portion of overexpressed a1 and a2 localized to lysosomes, and outward trafficking of secretory lysosomes was partially recovered. These results indicate that a1, a2, and a3 isoforms all possess the ability to induce trafficking of secretory lysosomes if they are sorted to lysosomes. Given the fact that a1 and a2 do not compensate for a3 function at their endogenous expression levels, the amount of a subunit isoforms in the lysosome membrane appears to be important for inducing trafficking. It is likely that triggering trafficking depends on both the amount of a subunit isoforms in lysosomes and their affinity for Rab7.

As expression of a3 is strongly induced during differentiation of osteoclasts, the local concentration of a3 is increased on the lysosomal membrane, which may make Rab7 recruitment more efficient. To fully understand the mechanism triggering secretory lysosome trafficking, it is essential to compare secretory and general lysosomes in detail.
6. CONCLUSION AND PERSPECTIVES

Based on recent findings, we propose the following working hypothesis for the molecular mechanism underlying secretory lysosome trafficking in osteoclasts (Fig. 4). (1) Lysosomal α3, whose expression is highly induced during osteoclast differentiation, recruits GDP-bound Rab7 to secretory lysosomes; (2) Rab7 is activated by a GEF, probably on the lysosomal membrane; (3) activated Rab7 dissociates from α3, but the acidic conditions generated by V-ATPase are required for stable lysosomal localization of activated Rab7; and (4) α3 also recruits GDP-bound Rab27A involved in fusion between the plasma membrane and the secretory lysosome membrane during later stages of secretion from lysosomes.

Recent studies partially illuminated the molecular mechanism of secretory lysosome trafficking in osteoclasts, and the findings revealed an unexpected function of the V-ATPase α3 subunit in organelle trafficking. In addition to osteoclasts, secretory lysosomes are indispensable for several types of cells to perform cell-specific functions. Melanocytes employ secretory lysosomes to secrete melanin, a black pigment, and cytotoxic T lymphocytes employ them to secrete perforin, a glycoprotein forming a pore in the plasma membrane of target cells. It is interesting that different types of cells involved in bone homeostasis, pigment secretion, and immunity share secretory lysosomes for secretion of cell-specific materials. Insulin secretory granules in pancreatic β cells are not lysosomes, but they have V-ATPase with α3 on their membrane. Interestingly, oc/oc mice with a null mutation in the α3 gene exhibit low levels of insulin in the blood, and insulin secretion stimulated by high glucose was significantly decreased in islets isolated from oc/oc mice, indicating that the α3 isoform also plays a role in insulin secretion.

Furthermore, isoforms of V-ATPase possessing the α3 subunit have attracted attention in the field of cancer malignancy. Increasing evidence demonstrates that some types of cancer cells target V-ATPase with α3 and/or α4 to the plasma membrane, which is related to high metastasis. V-ATPase on the plasma membrane forms an abnormally acidic extracellular microenvironment that facilitates extracellular matrix digestion by cathepsin and metalloproteases, which provides cancer cells the opportunity to move through the extracellular matrix. It is unclear whether the mechanism by which V-ATPase with α3 or α4 is targeted to the plasma membrane, but it is possible that α3 may be transported to the plasma membrane via secretory lysosomes. Consistent with this, Funato et al. showed that cancer cells that use secretory lysosomes and secrete protons proliferate in the acidic tumor microenvironment. Taken together, secretory lysosomes and V-ATPase targeted to the plasma membrane, presumably via secretory lysosomes, have pivotal roles in various important physiological events. It is of interest to examine whether the molecular mechanism of secretory lysosomes in these cells is similar to that in osteoclasts.

In the context of V-ATPase function in organelle trafficking, one group reported that V-ATPase with the α2 subunit isoform in early endosomes is involved in inward trafficking of the organelle in renal proximal tubule epithelial cells during protein resorption from primitive urine. The group also revealed that c subunit and α2 subunit isoforms of early endosomal V-ATPase bind to the small GTPase Arf6 (ADP-ribosylation factor 6), and its GEF ARNO (ADP-ribosylation factor nucleotide-binding site opener), respectively, and that V-ATPase drives recruitment of these trafficking factors to early endosomes. These findings, together with our own on secretory lysosomes, led to the idea that the α subunit isoforms of V-ATPase in different organelles determine the direction of organelle trafficking by recruiting specific trafficking regulators to the organelle membrane. Further studies are required to elucidate the entire molecular mechanism by which the α subunit isoforms of V-ATPase spatiotemporally regulate the trafficking of organelles where they localize.

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