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Vacuolar-type proton ATPase as regulator of membrane dynamics in multicellular organisms

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Abstract Acidification inside membrane compartments is a common feature of all eukaryotic cells. The acidic milieu is involved in many physiological processes including secretion, protein processing, and others. However, its cellular relevance has not been well established beyond the results of in vitro studies involving cultured cell systems. In the last decade, human and mouse genetics have revealed that the acidification machinery is implicated in multiple pathophysiological disorders, and thus our understanding of physiological consequences of the defective acidification in multicellular organisms has improved. In invertebrates including *Drosophila* and nematodes, mutations of V-ATPase were found to lead the development of rather unexpected phenotypes. Studies have suggested that V-ATPase may be involved in membrane fusion and vesicle formation, important processes for membrane trafficking, and have further implied its involvement in cell–cell fusion. This rather novel idea arose from the phenotypes associated with genetic disorders involving V-ATPase genes in various genetic model systems. In this article, we focus and overview the non-classical, beyond proton-pumping function of the vacuolar-type ATPase in exo/endocytic systems.

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Intracellular membrane transport

All eukaryotic cells develop an elaborate array of membrane compartments, which are distinguishable morphologically and functionally from one another. Various biochemical and physicochemical reactions, which frequently require different environments to proceed, occur in the distinctive compartments separated by limiting membranes. These subcellular compartments are highly dynamic structures, and exchange material and information with one another via membrane-vesicle mediated transport or direct interactions. The acidification inside transport vesicles and the lumina of the destinations are thought to play regulatory roles in this process. It has been long known that the acidification is required for membrane trafficking along endocytic pathways. The pH of the lumina of membrane compartments progressively decreases, however, the underlying mechanism by which this is achieved has not been fully elucidated yet. The luminal pH plays an important role in proper membrane trafficking, as dissipation of the pH gradient across organelle membranes by V-ATPase inhibitors results in malfunctions of the sorting and transport of materials. However, again, the underlying mechanism was not elucidated until recently.

Vesicle formation

ARNO, an ARF exchange factor, is recruited to the endosome membrane in a V-ATPase-mediated acidification dependent manner (Maranda et al. 2001). Considering that ARNO and ARFs regulate the formation and budding of

transport vesicles by recruiting coat proteins to the budding sites of endosomes, detachment of the ARF/ARNO complex from the donor compartment causes severe perturbation of vesicle-mediated trafficking. This hypothesis explains why the endosomal acidification is prerequisite for delivery of internalized materials from one compartment to the next one. In this scenario, the key molecule is the sensor of the pH difference across the endosomal membrane. As the cytosolic pH is maintained at a rather constant value, a certain mechanism is required to convey information of the lumenal environment to the cytosolic phase to recruit the cytosolic components like ARFs/ARNO and coat proteins.

This important issue has been solved recently, at least regarding the membrane transport from early endosomes to late endosomes in renal epithelial cells (Hurtado-Lorenzo et al. 2006). Biochemical studies have demonstrated that ARNO/ARF6 specifically interacts with the Vo sector of V-ATPase. Importantly, this protein–protein interaction observed in vitro is dependent on the acidification inside organelles, reflecting that the recruitment of ARFs/ARNO depends upon the luminal acidification in vivo. These observations provide enough rationale for the mechanistic link between the luminal acidification and transport vesicle formation, and further the V-ATPase plays dual roles as a proton pump that acidifies the lumen and as the membrane anchor for the cytosolic machinery for vesicle formation.

The above observations were made for the early and late endosome transport in the renal epithelium, where the V-ATPase *a*2 subunit isoform is a component of the membrane intrinsic Vo sector. Four distinct isoforms of the *a* subunit are expressed in various tissues, and these isoforms are localized to specific subcellular locations along the endocytic and exocytic pathways (Toyomura et al. 2000; Oka et al. 2001; Toyomura et al. 2003). Thus, the specific interaction between the V-ATPase Vo sector and distinct ARFs may regulate the vesicle formation and budding in each subcellular compartment with a distinct threshold of luminal acidification. This attractive hypothesis is supported in part by the fact that the V-ATPase with a different *a* subunit interacts with ARNO/ARFs (Hurtado-Lorenzo et al. 2006).

V-ATPase as the fusion machinery—intracellular membrane trafficking

Transport vesicles detached from the original compartment (donor) travel through the cytosol, reach their destination (acceptor), and fuse with the membrane of the acceptor compartment to deliver their cargoes. The importance of SNAREs in vesicle docking and fusion has been well established. In addition to the SNARE complex, several lines of evidence have suggested the involvement of the Vo sector in the membrane fusion. The *c* subunit, a membrane intrinsic subunit of the Vo sector, was shown to have the ability to induce calcium-dependent neurotransmitter release from vesicles (Brochier and Morel 1993). This observation was followed by the finding that a component of vacuolar-ATPase functions as an accessory molecule for the neuronal SNARE complex, which places two separate membranes in close proximity enough to evoke the fusion (Galli et al. 1996). These studies raised the novel idea that V-ATPase may be involved in membrane fusion. This has been further substantiated by a series of studies on homotypic fusion of yeast vacuoles, organelles equivalent to lysosomes in animal cells. The yeast vacuole is a prominent organelle occupying more than a quarter of the cell volume, and is highly dynamic in its shape and function (Raymond et al. 1992; Wada et al. 1992). In the assay system for monitoring vacuole–vacuole fusion in vitro, Mayers and colleagues found that the Vo sector provides a membrane pore that is required for the formation of a continuous membrane from two separate membranes. In addition to the SNARE-mediated process, the final step of membrane fusion, the Vo–Vo trans-complex on the paired membranes provides the initial point of membrane continuity between the two separate membranes (Muller et al. 2002; Bayer et al. 2003; Muller et al. 2003; Fig. 1).

Although these in vitro studies established that V-ATPase plays a role in membrane fusion, in vivo evidence was lacking (Kane 2006). In yeast, the most amenable system for genetic studies, a lack of V-ATPase or *c*-subunits does not lead impaired vacuolar morphology. Indeed, yeast cells lacking V-ATPase subunits have a single, large central vacuole as a common Vma^{-} (for *v*acuolar *m*embrane *A*TPase) phenotype (Anraku et al. 1992), in contrast to the clear and severe fusion defects of the vacuoles lacking the Vph1 protein (*a* subunit) in the in vitro assay.

Recently, the first in vivo evidence emerged from a genetic study on a synaptic transmission mutant of the fruit fly. *Vha-100*, encoding the *a* subunit of the Vo sector, is expressed in neural tissues. Mutations in this gene result in severe defects in neural transmission. The V-ATPase activity acidifies the lumina of synaptic vesicles, and drives neurotransmitter accumulation inside the vesicles (Moriyama and Futai 1990). The above observation would reflect the reduction of neurotransmitters in the vesicles. However, electrophysiological recordings have indicated that the amount of transmitters in each vesicle is not affected but that the exocytic frequency is significantly reduced by the mutations. The expression of this *Drosophila a* subunit in a yeast mutant lacking the *a* subunits (Vph1p and Stv1p) rescues the defective vesicle trafficking, but does not the impaired acidification, showing that these two phenotypes can be separated. These observations demonstrate a role of the Vo sector in the neurotransmitter release, most likely at

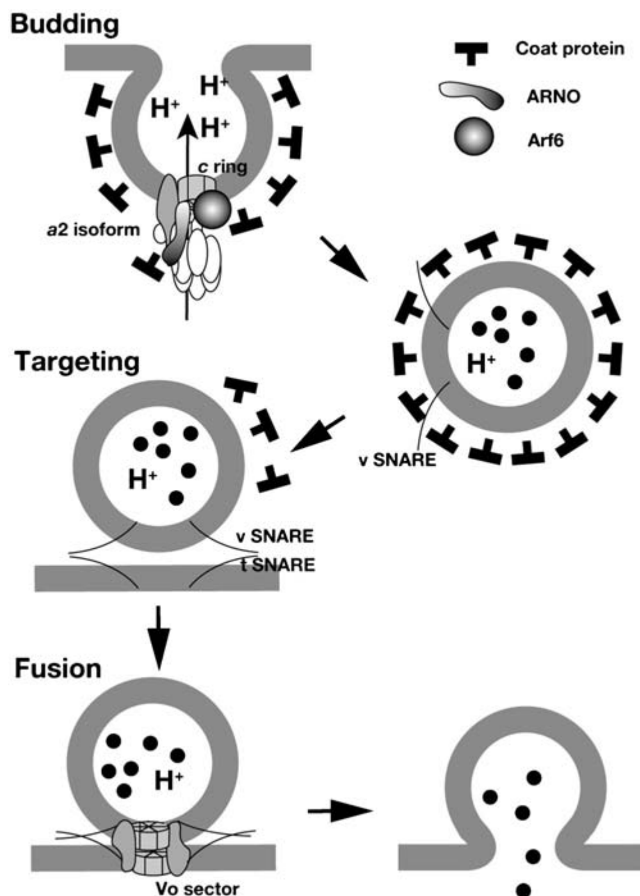

Fig. 1 Principle of membrane trafficking and V-ATPase. The *a* subunit isoforms of the V-ATPase Vo sector are commonly involved in the elementary processes of exocytosis and endocytosis. Vesicle assembly on the donor membrane requires the recruitment of budding machinery. This process is regulated by an acidification-dependent interaction between ARNO/ARFs and the Vo sector of the V-ATPase. At the final step of vesicle fusion, the Vo sectors in the donor and acceptor membranes interact to form a *trans*-Vo complex, which provides an initiating pore where the fusion starts

the final step of membrane fusion, and interestingly, this function seems to be independent of the proton translocating activity.

The second compelling evidence is the finding that mice lacking the V-ATPase *a*3 subunit exhibit a defect in insulin secretion, another physiologically important exocytic event. During studies on the expression patterns of different isoforms of the *a* subunit in various tissues (Toyomura et al. 2000; Oka et al. 2001; Toyomura et al. 2003), we noted that the *a*3 subunit isoform is highly accumulated in endocrine tissues including the pancreas and others. This isoform is mainly localized to late endosomes and lysosomes in tissue culture cells (Toyomura et al. 2003), however, they turned out to be residing in dense-core vesicles containing hormones in endocrine cells (Sun-Wada et al. 2007).

Osteosclerosis (*oc*) is an autosomal recessive mutation in mice that is initially identified as defective bone morphogenesis (Schlager and Dickie 1967; Scimeca et al. 2000). The *oc* mutation comprises a 1.6-kb deletion in the *a*3 subunit locus, which is involved in translation initiation (Scimeca et al. 2000). Because V-ATPase with the *a*3 subunit constitutes a major proton pump on the plasma membrane of osteoclasts, loss of the *a*3 subunit results in a lack of V-ATPase, which allows the extracellular acidification for bone resorption, and thereby the mutation causes development of a severe bony phenotype (Li et al. 1999; Toyomura et al. 2003). With a mutant with loss of the major V-ATPase on insulin granules, we examined the cellular and subcellular phenotypes of finding no obvious disorganization of insulin granules or other membrane compartments, and further that the molecular size of insulin is normal in the mutants (Sun-Wada et al. 2006). These results indicate that the dense-core formation and the processing of proinsulin are not dependent on the V-ATPase *a*3 subunit function. Although the mutants do not exhibit acute and chronic hyperglycemia, the blood insulin levels are lower than in the wild-type, especially after glucose intake. Islets isolated from *oc*/*oc* mutants are impaired in insulin secretion in response to glucose or depolarizing stimulation. These observations show that the *oc*/*oc* mutants are defective in insulin secretion in vivo and in vitro despite that they produced and contained substantial amounts of insulin.

From these results regarding insulin secretion, we propose that the regulated secretion is dependent on the V-ATPase subunit *a*3 isoform. This function is not directly related to the acidification inside the secretory vesicles, since pharmacological inhibition of V-ATPase does not impair insulin secretion in vitro. We have shown that the *a*3 isoform is localized to secretory granules in a variety of endocrine tissues (Sun-Wada et al. 2007). These observations, collectively, further imply that that V-ATPase Vo sector is commonly involved in the process of exocytosis in regulated secretion including neurotransmitter release and hormone secretion.

V-ATPase regulates cell–cell fusion

Cell–cell fusion, another highly dynamic membrane fusion event

Highly regulated intercellular fusion has great importance in the physiology of multicellular organisms. The life of multicellular organisms begins with transcellular fusion known as fertilization. The fusion of the plasma membranes of two (or more) distinct cells generates a multinuclear cell. This process is involved in the differentiation of highly specialized cell types, and the generation of specific tissues including muscle, bone, and so on (Chen et al. 2007).

The osteoclast, of bone marrow lineage, is a large multinuclear cell that plays indispensable roles in bone

formation and remodeling (Vaananen et al. 2000). This multinucleated cell arises through sequential fusion of precursor cells, rather than uncoupled cytokinesis and DNA replication, as suggested by the fact that the number of nuclei is not restricted to multiples of 2 in mature osteoclasts. This morphologically and functionally highly specialized cell is one of the best examples demonstrating the physiological significance of V-ATPase: it presents the proton pump at specialized domains on the plasma membrane attached to a bone surface, and secretes protons toward the space between the cell and bone, to facilitate bone resorption. Loss of the plasma membrane ATPase due to a genetic lesion (the *oc* mutant, for example) results in a severe defect in bone resorption, a pathological status known as osteopetrosis.

Recently, a genetic study showed that V-ATPase is involved in this osteoclast development by regulating cell–cell fusion (Lee et al. 2006). Genetic deletion of the *d*2 subunit (encoding a component of the Vo sector) in mice causes mild osteopetrosis. Two structural genes for the *d* subunit of V-ATPase have been identified in the mouse, as well as in the human genome (Sun-Wada et al. 2003). The expression profiles of these *d* subunit isoforms suggest that most V-ATPase is composed of the *d*1 subunit, whereas the expression of this *d*2 subunit is restricted to only a few tissues. This seems to be also true for osteoclasts: *d*1 is major, whereas *d*2 is indeed expressed but at a low level, if at all, therefore the osteopetrotic phenotype is not responsible for the loss of proton pumping activity, as V-ATPase with *d*1 remains functional. Bone marrow cells obtained from mutant mice can differentiate and acquire the osteoclast characteristics as shown by the gene expression profile, however, most of them remain as small mononucleated cells. This genetic and cell biological study showed that V-ATPase with the *d*2 subunit isoform participates in cell–cell fusion (Lee et al. 2006; Fig. 2).

This study implies a pro-fusion function of V-ATPase in cell–cell fusion. However, another result suggesting that V-ATPase acts in an anti-fusion manner was also obtained. In nematode development, prominent cell fusion occurs in the epidermis during body elongation. Genetic screening for defective patterning in the epidermis morphology revealed several mutations causing hyperfusion of epidermal cells (Kontani et al. 2005). One of these mutations was identified as a loss of function allele for the *fus-1* gene, which encodes a small membrane protein (80–90 amino acids) known as the *e* subunit of the V-ATPase. As loss of the *e* subunit of yeast (encoded by the *VMA9* gene) leads to loss of the V-ATPase in yeast vacuoles, this subunit is essential for functional assembly of the proton pump (Sambade and Kane 2004; Compton et al. 2006), therefore, the *fus-1* phenotype reflects the consequence of the loss of V-ATPase function. Further, the loss of other V-ATPase subunits due to RNAi-mediated perturbation in nematodes results in

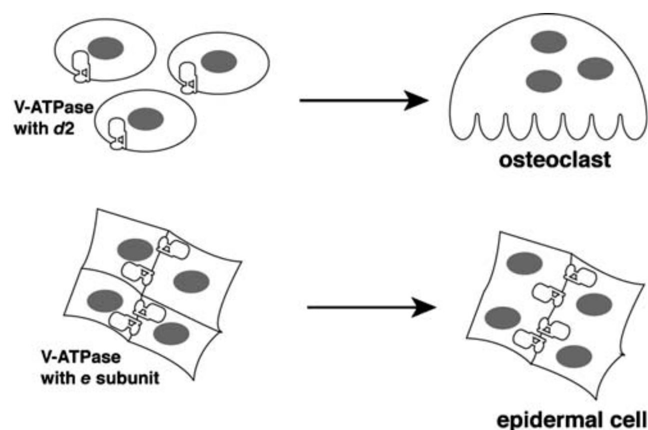

Fig. 2 The functions of V-ATPase required for cell–cell fusion. Multinucleated osteoclasts are formed through intercellular fusion of mononucleated precursor cells. Loss of the V-ATPase *d*2 subunit hinders the maturation of osteoclasts by preventing cell–cell fusion. In contrast, *Caenorhabditis* epidermal cells ectopically fuse with each other upon loss of V-ATPase from their apical surface, suggesting that V-ATPase restricts transcellular fusion

similar hyperfusion of epidermal cells. Collectively, these observations suggest that the V-ATPase function is required for normal development of epidermal tissue because it ‘restricts’ the cell–cell fusion (Kontani et al. 2005).

What has been found in mice and nematodes, as consequences of genetic loss of the V-ATPase function, is apparently contradictory, leaving the important question of whether V-ATPase (or its component) acts in a pro-fusion or anti-fusion manner. Further, these studies did not address whether the relevant intracellular events depend on the proton pumping and acidification or the presence of the Vo sector. Therefore, these questions should be addressed in the future. Nevertheless, it has become very clear that V-ATPase is involved in various biological activities.

Concluding remarks

Biochemical and molecular biological studies have revealed the structure, bioenergetics and mechanochemistry of V-ATPase in detail, while knowledge on the physiological aspects of this unique proton pump is largely limited to unicellular organisms. In recent years, it has become increasingly clear that this proton pump is essential for regulation of cellular activities through creating specific ionic environments along the endocytic and exocytic pathways. Simultaneously, it has also become evident that this ‘proton pump’ plays certain roles other than proton translocation, opening an entirely unexpected paradigm. Not plentiful, but steadily increasing lines of evidence suggest that these ‘non-classical’ functions of V-ATPase are involved in the occurrence of highly differentiated biological functions. Most recently, Zhang and colleagues showed that the V-ATPase *a*1 subunit interacts directly with

calmodulin, an important regulator of the neurotransmitter release in Drosophila neurons (Zhang et al. 2008). We anticipate seeing new views of V-ATPase as more than a simple proton pump.

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