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The V-type H$^+$-ATPase in vesicular trafficking: targeting, regulation and function

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Vacuolar-type H$^+$-ATPase (V-ATPase)-driven proton pumping and organellar acidification is essential for vesicular trafficking along both the exocytotic and endocytotic pathways of eukaryotic cells. Deficient function of V-ATPase and defects of vesicular acidification have been recently recognized as important mechanisms in a variety of human diseases and are emerging as potential therapeutic targets. In the past few years, significant progress has been made in our understanding of function, regulation, and the cell biological role of V-ATPase. Here, we will review these studies with emphasis on novel direct roles of V-ATPase in the regulation of vesicular trafficking events.

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Introduction

Vesicular trafficking is an essential cellular process in eukaryotic cells to deliver either membrane proteins or soluble cargos from one compartment to another. Defects of trafficking have been recently recognized as an important cell biological mechanism in various human diseases such as cancer, neurological disorders, and autoimmune and metabolic diseases including diabetes [1]. Both exocytotic and endocytotic traffic should be regulated by the different forms of 'protons' including transmembrane pH gradient, membrane potential and the acidic pH lumen of their intracellular organelles. The acidic environments outside the cells are also important for mammals, examples being osteoclast bone resorption lacuna, and the extracellular compartment of tumors as well as renal tubular and epididymal lumens. V-ATPase is a major proton pump in the proton homeostasis of eukaryotic cells. In accordance with their crucial roles in cellular function, V-ATPases have been also implicated in the pathophysiology of various human diseases [2,3••].

Most of the biochemistry, cell biology, and physiology related to V-ATPase have been recently reviewed [2,3••,4–6]. Generally accepted roles of V-ATPase include establishing acidic pH, which gives optimal conditions for enzymes in the lumens of compartments such as lysosomes and bone resorption lacuna. Acidic pH is also necessary for the entry of viruses, bacteria and dissociation of internalized ligand–receptor complexes in endosomes. The electrochemical proton gradient constitutes a driving force for the accumulation of neurotransmitters and hormones into secretory vesicles. However, recent studies revealed that, in addition to well-known functions of V-ATPase, its subunits may have direct roles in the regulation of vesicular trafficking.

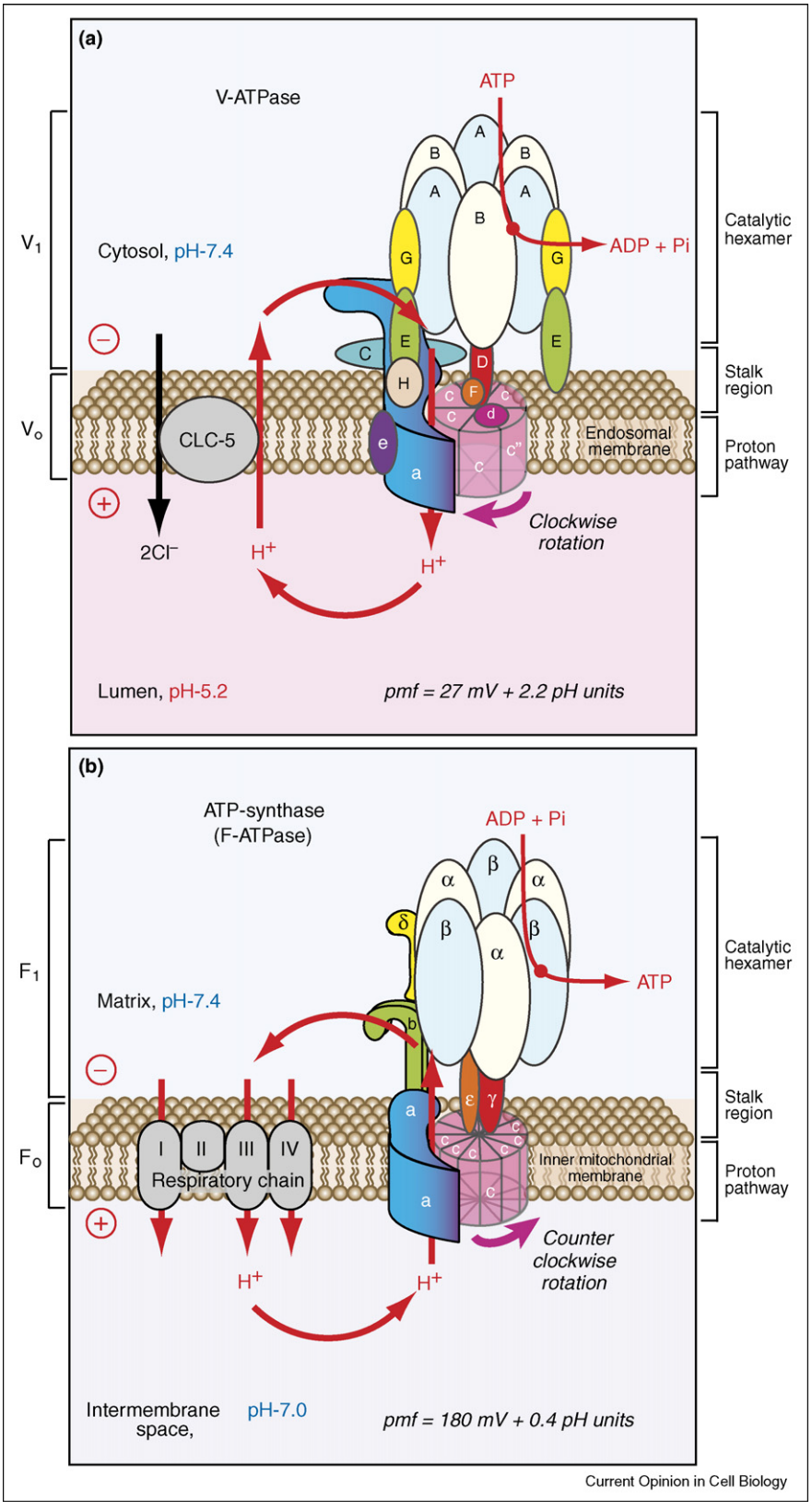
In this review, we will focus on targeting V-ATPase to the membranes of specific intracellular compartments. We will also discuss the roles of V-ATPase in vesicular trafficking between organelle and plasma membranes in the exocytotic pathway. Finally, we will discuss the emerging role of V-ATPase in interaction with small GTPases, well-known 'molecular switches', and the implication of this rendezvous for the regulation of the endocytotic pathway.

The V-ATPase: structure and function of the proton pumping rotary nano-motor

Structure and function of V-ATPase

The V-ATPase is a multimeric complex that functions as a proton pumping rotary nano-motor (Figure 1a). Both yeast and mammalian V-ATPases share a high degree of homology in their subunit compositions [7] and similarities in biochemical mechanism [3••,4,5]. The cytoplasmic V_1-sector is composed of eight different subunits with defined stoichiometry ($A_3B_3CDEFG_2H_{1\text{-}2}$) and is responsible for ATP hydrolysis. The transmembrane V_O-sector is composed of six different subunits ($ac_4c'c''de$) and is responsible for proton translocation. Functionally, the catalytic hexamer A_3B_3 is connected to the proton pathway by stalks. The central stalk (formed by subunits D and F) is attached to a ring of hydrophobic subunits (c, c,′ c″) and operates as a 'rotor' (Figure 1a, in red). The cytosolic N-terminal domain of the a-subunit together with the C, E, G, and H subunits form two peripheral stalks. They are attached to the A_3B_3 hexamer and form a 'stator'. Thus, ATP hydrolysis and proton pumping are coupled by a rotary mechanism, that is, rotation of a 'rotor' relative to a 'stator' in the nano-machine.

Figure 1


(a)
V-ATPase
ATP
ADP + Pi
Catalytic hexamer
Stalk region
Proton pathway
V1
Vo
Cytosol, pH-7.4
CLC-5
Endosomal membrane
2Cl−
H+
H+
Clockwise rotation
Lumen, pH-5.2
pmf = 27 mV + 2.2 pH units
(b)
ATP-synthase (F-ATPase)
ADP + Pi
ATP
Catalytic hexamer
Stalk region
Proton pathway
F1
Fo
Matrix, pH-7.4
I
II
III
IV
Respiratory chain
Inner mitochondrial membrane
H+
H+
Counter clockwise rotation
Intermembrane space, pH-7.0
pmf = 180 mV + 0.4 pH units
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Similarities and differences between V-ATPase and F-ATPase

V-ATPase shares similarities with ATP-synthase (F-ATPase) in subunit structure and rotational catalysis, as schematically shown in Figure 1b. Homologous subunits, such as the A-subunit of V-ATPase and the β-subunit of F-ATPase are shown in the same color. Both models (Figure 1a,b) are consistent with experiments showing subunit rotation in bacterial F-ATPase [5,8,9••,10•] and yeast V-ATPase [5,11•] (see Supplementary Information, Figures S1, S2 and Movies S1, S2). In spite of their similarities, there are important differences between F-ATPase and V-ATPase. F-ATPase is exclusively localized to the mitochondrial inner membrane, where it operates predominantly as an ATP-synthase, coupled with the *p*roton *m*otive *f*orce (*pmf*) generated by the respiratory chain (Figure 1b). By contrast, V-ATPases are found in diverse endomembrane organelles and plasma membranes (Figure 2), and function as proton pumps (Figure 1a). The targeting, function and regulation of V-ATPase is more complex than F-ATPase and depend on the specificity of cell biological function as well as membrane composition and cytosolic environment of these compartments.

Diverse V-ATPases targeting to unique acidic compartments

Multiple subunit isoforms of V-ATPase

Consistent with the presence of V-ATPases in diverse compartments [12,13], a large spectrum of subunit isoforms are found in mammals; two isoforms for the B, E, H and d subunits [14–18] and three isoforms for the C and G subunits [15,18–20]. The expression of these isoforms is tissue-specific and cell-specific. The B subunit isoform B1 is specific for kidney and inner ear, whereas B2 is ubiquitous. Thus, the mutations of B1 are not lethal, but cause human renal acidosis with hearing defects [14]. V-ATPase with C1 is found ubiquitously, whereas C2-a is found specifically in the lamellar bodies of lung alveolar epithelial cells responsible for surfactant secretion and C2-b is found in plasma membranes of renal α-intercalated and β-intercalated cells responsible for ion homeostasis [18,19]. V-ATPase with the E1 isoform is located specifically in developing acrosomes of spermatids and acrosomes in mature sperm, whereas E2 is expressed in all tissues that have been examined [17]. The G subunit isoform, G1 is expressed ubiquitously, whereas G2 and G3 are found in neuron synaptic vesicles and kidney, respectively [20]. Recent immunoprecipitation experiments have demonstrated that V-ATPases with unique combinations of subunit isoforms are localized in specific cellular membranes that could dictate their functions [18,21].

Role of a-subunit isoforms in intracellular targeting of V-ATPase

Mammalian transmembrane V_O of V-ATPase is more complicated than Fo of F-ATPase (Figure 1a,b). It is formed by a transmembrane ring and an adjacent a-subunit, containing a large N-terminal cytosolic tail and a C-terminal hydrophobic domain with six to nine putative membrane spanning helices [3••,4]. The interface between the a-subunit and the ring is a target for bafilomycin A_1 and concanamycin, macrolide antibiotics, which specifically inhibit V-ATPase catalysis and rotation. Their high specificities have contributed to the study of organelles with acidic luminal pH.

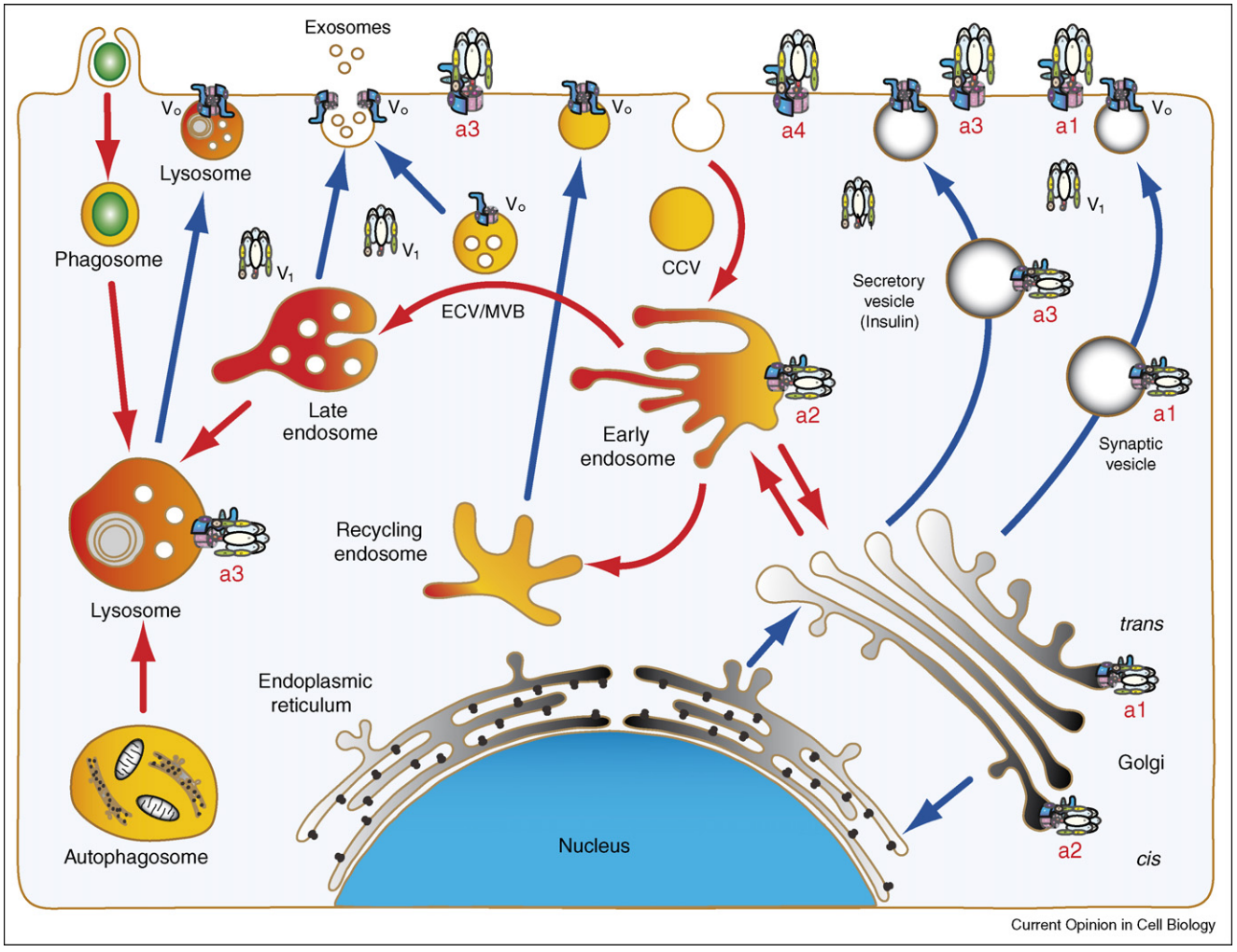
Multiple isoforms with unique intracellular localizations are also found for the a-subunit. In yeast the N-terminal cytosolic domains of two a-subunit isoforms (Vph1p and Stv1p) are responsible for the specific targeting of V-ATPase to vacuolar and Golgi compartments, respectively [22•]. By contrast, the C-terminal domain has been implicated in the specific targeting of 17 a-subunit isoforms recently identified in *Paramecium tetraurelia* [23•].

Four a-subunit isoforms (a1, a2, a3, and a4) were found in mice and humans [24–27]. They are localized in different endomembrane organelles and plasma membranes of specialized cells (Figure 2). The targeting of V-ATPases with different a-isoforms is a cell-specific dynamic process. During osteoclast differentiation, the a3-isoform relocates from lysosomes to the plasma membrane (Figure 3a,b) [28••]. It is noteworthy that this isoform is specifically (about 80%) targeted to insulin containing secretory granules in pancreatic β-cells (Figure 4a–d) [29•].

Targeting and localization of a-isoforms are also compartment-specific. All four isoforms are expressed in

(Figure 1 Legend) Comparative structural models and functional roles of V-ATPase and ATP-synthase (F-ATPase) expressed in endomembrane organelles and mitochondria, respectively. The comparative subunit composition of transmembrane (V_O and F_O) and peripheral (V_1 and F_1) sectors are indicated on the left, while the catalytic hexamers, stalks and proton pathways are indicated on the right. Homologous subunits, such as the A-subunit of V-ATPase and the β-subunit of F-ATPase are shown in the same colors. (**a**) V-ATPase is shown as a primary proton pumping nano-motor. ATP hydrolysis drives clockwise rotation of the central stalk and ring of proteolipid subunits indicated in red. This rotation leads to the translocation of protons from the cytosol to form an acidic lumen of endomembrane organelles, and generates an electrochemical proton gradient or *p*roton-*m*otive *f*orce (*pmf*) across the membrane. In endosomes the V-ATPase promotes the neutralizing current mediated by electrogenic CLC-5 (nCl^-/H^+-exchanger with unknown stoichiometry, which might be $n = 2$ as in its bacterial homologue [45]) and drives further acidification. The values of *pmf* components $\Delta\Psi$ and ΔpH were recently determined for the early phagosomal compartment [46••]. (**b**) Mitochondrial ATP-synthase (also called F-ATPase) is shown as a secondary pump. F-ATPase function (proton translocation, counter clockwise rotation, and coupled ATP synthesis) is driven by *pmf* that is primarily generated during the function of respiratory chain enzymes. The function and rotation of both V-ATPase and F-ATPase are reversible under certain experimental conditions. The rotation of yeast V-ATPase (see Supplementary Information, Figure S1 and Movie S1) [11•] and bacterial F-ATPase (see Supplementary Information, Figure S2 and Movie S2) [10•] nano-motors. Movies are adapted from Refs. [10•,11•].

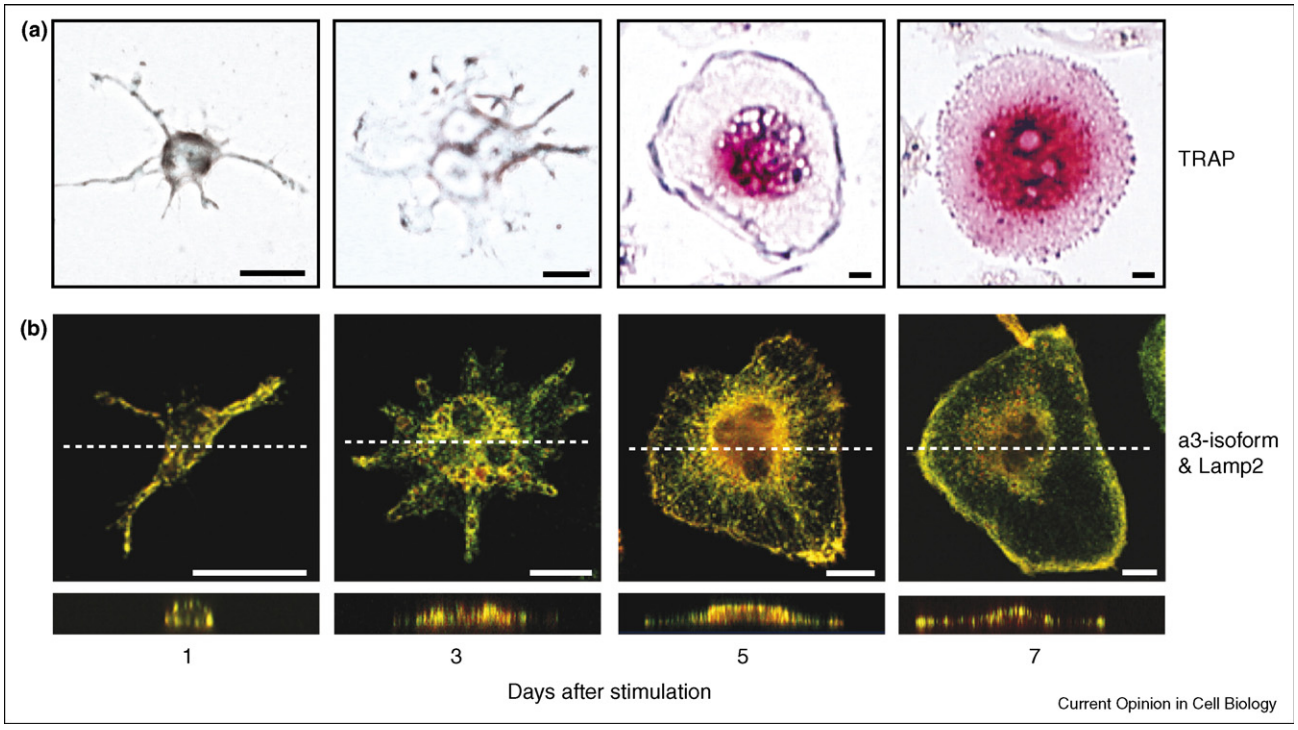
Figure 2



Differential targeting of the a-isoforms and vesicular trafficking of V-ATPase in eukaryotic cells. The scheme depicts the compartments of endocytotic (yellow/red) and exocytotic (gray) pathways. Vesicular trafficking steps are indicated for endocytosis in red arrows and for exocytosis in blue arrows. Differential targeting of V-ATPase is cell-specific and compartment-specific. Localization of V-ATPase a-isoforms is shown as demonstrated in Figure 4 and described in text. **(i)** In particular, V-ATPase with a1-isoform is targeted to Golgi and involved in synaptic vesicles fusion and secretion. It is also found on presynaptic plasma membrane. **(ii)** V-ATPase with a2-isoform is targeted either to early endosomes or to Golgi. In early endosomes a2-isoform functions as pH-sensor by recruiting small GTPases in acidification dependent manner and involved in the formation of endosomal carrier *v*esicles also known as *m*ultivesicular *b*odies (ECV/MVB). These vesicular intermediates are involved in the trafficking between early and late endosomes or in exosomes formation and secretion. **(iii)** V-ATPase with a3-isoform is targeted to lysosomes and in some cells is involved in lysosomal secretion and is also localized to plasma membrane. **(iv)** V-ATPase with a4-isoform is specifically targeted to plasma membrane of some cells.

mouse kidney proximal tubule cells, however, while a1-isoform, a3-isoform, and a4-isoform are targeted to the plasma membrane, the a2-isoform is targeted to early endosomes *in situ* (Figure 2) [30••]. Specific localization of a4 to the plasma membrane and a2 to early endosomes in proximal tubule cells is shown (Figure 4e,f) (see Supplementary Information, Figure S3 and Movie S3). It is noteworthy that in these cells a2-isoform does not target to Golgi (Figure 4g). By contrast, both a2-isoform and a1-isoform are targeted to the Golgi complex in cultured osteoclast cells (Figure 4h) [28••]. Interestingly, recent studies suggested that the a2-isoform is involved in Golgi function and development of congenital disorders of glycosylation in humans [31••]. The kidney specific a4-isoform is also specifically targeted to the apical plasma membrane of collecting duct intercalated and epididymal clear cells [32]. In nerve terminals, the a1-isoform is specifically delivered to synaptic vesicles from which it could also be relocated to the presynaptic plasma membrane [33]. Although it is generally accepted that a-subunit isoforms are crucial for trafficking V-ATPase, the mechanism of

Figure 3



Differential targeting of the a3-isoform of V-ATPase in osteoclasts. (**a**) Raw 264.7 cells were cultured for 7 days in medium containing sRANKL and M-CSF. Osteoclast-like cells were identified as multinuclear cells exhibiting positive staining for *t*artrate-*r*esistant *a*cid *p*hosphatase (TRAP). **(b)** Targeting and colocalization of a3 and Lamp2 in lysosomes of RAW 264.7 cells and their targeting to the plasma membrane during differentiation into osteoclast-like cells. Double immunochemical staining with anti-a3 and anti-Lamp2 antibodies was performed after different days of osteoclast differentiation followed by confocal microscopy analysis. Merged images are shown both in horizontal view (*x–y* sections, upper panels) and lateral view (*z–x* sections, lower panels). Adapted from Ref. [28••].

their specific targeting is currently unknown in mammalian cells.

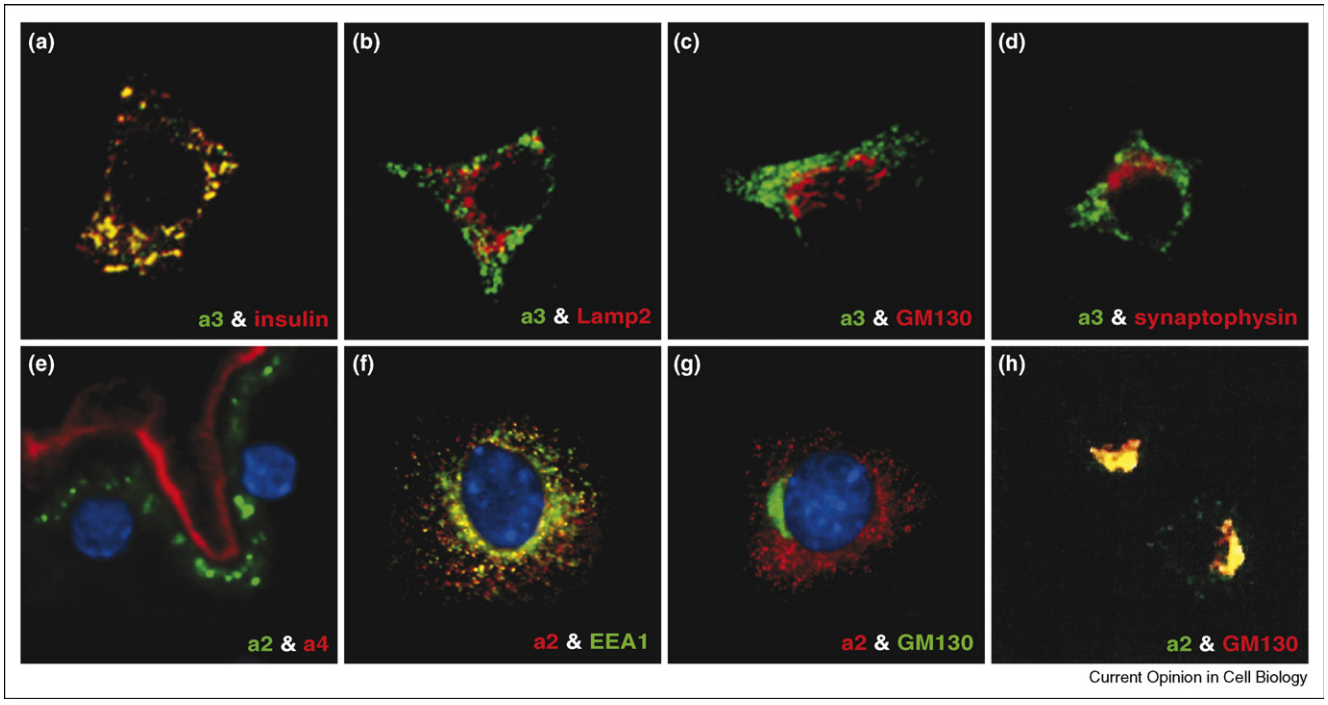
Regulation of V-ATPase and the luminal pH of organelles

Diverse acidic organelles are present in eukaryotic cells with different lumenal pH. The pH of compartments becomes more acidic as the exocytotic or endocytotic pathways approach their destination. The regulation of luminal acidity is selectively achieved by the combination of first, 'fine-tune' regulation of V-ATPase activity depending on isoform composition and cellular microenvironments (cytosolic and luminal environment, membrane composition, etc.) and second, specific targeting and trafficking of V-ATPase as well as other 'acidification machinery' proteins such as channels, exchangers to organelles.

Regulation of V-ATPase by reversible assembly/disassembly of V_1V_O sectors

Reversible assembly/disassembly of V_O and V_1 sectors is an important regulatory mechanism of V-ATPase, and observed in response to glucose depletion in *Saccharomyces cerevisiae* [34] and kidney proximal tubule epithelial cells [35••,36,37] as well as in response to ceased feeding in *Manduca sexta* [6]. In yeast, this mechanism is regulated by a-isoforms of V-ATPase and dependent on the cellular and membrane environment where the two isoforms are located [3••,38,39•,40]. V-ATPase (with Vph1p a-subunit) targeted to vacuoles is disassembled upon glucose depletion, whereas that (with Stv1p a-subunit) targeted to the Golgi does not [38]. However, V-ATPase with a chimeric a-subunit of Vph1p and Stv1p (N-terminal and C-terminal, respectively) and localized in vacuoles is disassembled. These results suggest that the N-terminal cytosolic tail of a-subunits function as a glucose sensor [3••] and its function also depends upon the cytosolic, lumenal and/or membrane environment where V-ATPase is localized [40]. The glucose-dependent assembly/disassembly of V-ATPase is partly controlled by vacuolar luminal pH [39•]; however, this is not the only cellular parameter involved [40]. The disassembly (but not assembly) involves the cytosolic microtubular network [3••], whereas the assembly (but not disassembly) requires the cytosolic RAVE (*r*egulator of H⁺-*A*TPase of *v*acuolar and *e*ndosomal membranes) complex [34].

Figure 4



Differential targeting of the a2-isoform, a3-isoform and a4-isoform of V-ATPase in eukaryotic cells. (**a–d**) Specific targeting and localization of the a3-isoform to insulin containing secretory granules in pancreatic β-cells. βTC6 cells were double stained with antibodies against the a3-isoform (green) and against either **(a)** insulin, **(b)** Lamp2, **(c)** GM130 or **(d)** synaptophysin (red) as indicated. Adapted from Ref. [29•]. **(e)** Differential targeting of a2-isoform to endosomes and a4-isoform to plasma membrane in kidney proximal tubule epithelial cells. Mouse kidney proximal tubules were double stained with antibodies against the a2-isoform (green) and against the a4-isoform (red). **(f, g)** Specific targeting of the a2-isoform to early endosomes in *m*ouse proximal *t*ubule *c*ells (MTC). Cells were double stained with antibodies against the a2-isoform (red) and against either **(f)** EEA1 or **(g)** GM130 (green) as indicated. Adapted from reference [30••]. **(h)** Localization of the a2-isoform to Golgi complex in osteoclasts. Raw 264.7 cells were double stained with antibodies against the a2-isoform (green) and against the GM130 (red). Adapted from Ref. [28••].

In mammalian renal epithelial cells, the effect of glucose on V-ATPase is mediated by phosphatidylinositol 3-kinase (PI3K)-dependent signaling [36]. The direct interaction of cytosolic aldolase (an enzyme in the glycolytic pathway) with a, B and E subunits has been also implicated in reversible assembly/disassembly of V-ATPase, and aldolase was suggested to be a glucose-sensor [35••]. Importantly, the crucial role of this regulatory mechanism for the endocytotic protein degradation pathway was recently demonstrated in mammalian dendritic cells [41••]. The regulation of V-ATPase activity by the assembly of V_OV_1 sectors onto the lysosomal membrane was observed during maturation of these cells. This mechanism is crucial for lysosomal acidification, activation of proteases, protein degradation and antigen presentation [41••].

Regulation of V-ATPase coupling efficiency by specific subunit isoforms

Modulation of coupling efficiency between ATP hydrolysis and proton pumping is also important regulatory mechanism of V-ATPase. The a-subunit isoforms and non-homologous 90 amino acid region unique to the A-subunit V-ATPase not found in F-ATPase have been implicated in regulating in yeast [38,39•]. However, the unique properties of mammalian V-ATPases with different isoforms are difficult to analyze because heterogeneous assemblies are present in the same cell. Thus 'mouse/yeast' hybrid V-ATPase, constructed by introducing mouse cDNA into mutant yeast lacking the corresponding gene, may shed light on the properties and function of subunit isoforms. This approach was successfully applied to study the roles of the mouse E-subunit and C-subunit isoforms in the regulation of V-ATPase coupling efficiency [17,19].

Modulation of organelle pH by vesicular trafficking and chemiosmotic mechanisms

Targeting and vesicular trafficking of V-ATPase to specific membranes is an important regulatory mechanism for acidification. In renal intercalated and epididymal clear cells, the density of V-ATPase in the plasma membrane is controlled by a balance of endocytosis and exocytosis of apical vesicles containing the same enzyme

[42,43]. The V-ATPase generates an electrochemical proton gradient or proton-motive force consisting of membrane potential ($\Delta\Psi$) and proton gradient (ΔpH). In endosomes, they promote electrophoretic chloride transport via the CLC5 exchanger ($2Cl^-/H^+$-antiporter) increasing the acidity of the compartment (Figure 1a) [44,45]. The values of $\Delta\Psi$ 27 mV and ΔpH 2.2 units were recently determined in direct FRET experiments in early phagosomes where the lumenal pH is 5.2 [46••]. Chloride accumulation during endosomal acidification (with a lumen pH of 5.3–5.6) was also shown *in vivo* [47]. The emerging roles of the CLC-family of chloride transporters in the endosomal/lysosomal pathway [44,45] as well as important roles of the NHE-family of cation/proton exchangers in organellar acidification and homeostasis were recently discussed [48].

V-ATPase and acidic organelles are essential from yeast to mammals: functional role of acidic pH

Yeast mutants lacking any gene encoding a V-ATPase subunit cannot grow at neutral pH (VMA phenotype). Silencing in *Caenorhabditis elegans* of three of the four a-subunit isoforms results in death during development [49]. Deletion of the mouse c-subunit, that is coded by a single gene, results in impaired acidification and causes defective intracellular trafficking essential for development [50]. These data indicate that V-ATPase is required for vesicular trafficking during early development from worms to mammals. The crucial role of V-ATPase in establishing acidic compartments is generally accepted [2–5,12,51,52•,53]; however, emerging evidence indicates that various subunits of V-ATPase may also have direct roles in the regulation of vesicular trafficking within both the exocytotic and endocytotic pathways.

Direct role of V-ATPase in vesicular trafficking of exocytotic pathway

Roles of V-ATPase in membrane fusion

Vesicular trafficking, essential for communication between organelles, involves a combination of two crucial steps: first, budding of vesicles from a donor and second, fusion with an acceptor compartment/membrane [54]. These two steps are tightly regulated to ensure efficient formation of vesicles during budding and to avoid mistargeting of their cargo during the fusion step. Recent emerging evidence indicates that providing an acidic environment is not the exclusive function of V-ATPase, and that it is directly involved in both budding and fusion events.

Direct participation of V_O sector c-subunits in membrane fusion has been proposed for yeast vacuole biogenesis. According to this model, the c-subunits of V_O are directly involved in the fusion of two vacuoles (V_O *trans*-complex formation) which also depends on Rab-GTPase Ypt7 and calmodulin [55••]. Inactivation of the Vph1p a-subunit also blocks fusion between V_O sectors [56,57]. It is noteworthy that in contrast to fusion, vacuole fission and fragmentation *in vivo* depends on proton pumping by V-ATPase [57]. Although the exact molecular mechanism of V_O *trans*-complex formation remains controversial, recent studies suggest a direct role of V-ATPase in fusion in other organisms (Figure 2). In *Drosophila melanogaster*, the V_O a-subunit has been implicated in synaptic vesicle fusion [58] and the a1-isoform directly interacts with calmodulin at fly synapses [59]. In *Caenorhabditis elegans*, the a-subunit mediates secretion of Hedgehog-related proteins from exosomes to the apical membrane [60]. Finally, crystallographic studies of the c-ring of *Nephrops norvegicus* V-ATPase also suggest the direct role of the V_O-sector in membrane fusion [61].

Role of V-ATPase in mammalian hormone exocytosis

In mouse pancreatic β-cells, the V-ATPase containing the a3-isoform is specifically targeted to insulin-containing secretory vesicles (Figure 4a–d) [29•]. *Oc/oc*-mice, containing a null mutant of the *a3* gene, are unable to secrete insulin in response to glucose or depolarization, suggesting that they are defective in insulin exocytosis. Increased levels of the a2-isoform in *oc/oc*-mice did not replace the function of the a3-isoform. However, while the inhibition of V-ATPase by bafilomycin results in disappearance of endomembrane acidic lumens it does not prevent secretion of insulin in βTC9 cell line. These results suggest that insulin secretion does not require proton pumping, but intact V-ATPase with a3-isoform is necessary. The a3-isoform is highly expressed in endocrine tissues including adrenal, parathyroid, thyroid and pituitary glands, suggesting that function of the a3-isoform could be commonly involved in the regulation of the exocytotic pathway and secretion (Figure 2) [62].

Trafficking of V-ATPase to the plasma membrane: secretory lysosome of the osteoclast

Bone homeostasis in vertebrates depends on bone formation by osteoblasts and resorption by osteoclasts. During the resorption process, V-ATPase secrets protons into 'bone resorption lacuna', a compartment formed between the plasma membrane and the bone surface. This compartment is rich with proteases and an acidic lumen is essential for mineral dissolution and matrix protein degradation. The a3-isoform of V-ATPase is specifically localized to the plasma membrane of osteoclasts [24]. Consistent with these findings, mutations in the a3-isoform result in deficient bone resorption and osteopetrosis in humans [63,64] and mice [65].

The RAW264.7 cell is an established macrophage line that can form osteoclast-like cells upon differentiation. The a3-isoform is localized in lysosomes before stimulation, whereas after stimulation the same isoform is targeted to the plasma membrane together with lysosomal enzymes, suggesting that lysosome exocytosis forms osteoclast plasma membranes (Figure 3) [28••]. Thus,

localization of the V-ATPase a3-isoform is a dynamic membrane process. In human osteoclasts the a3-subunit is colocalized with the V_O d2-isoform [66]. Knockout of d2-isoform in mice results in markedly increased bone mass because of defective osteoclasts and enhanced bone formation [67••]. However, the disruption of the d2-gene did not affect osteoclast differentiation or V-ATPase activity, suggesting the direct role of the d2-isoform in the fusion of osteoclast progenitors. Exocytosis of lysosomes is an important function in phagocyte-derived, neutrophil-derived, and monocyte-derived cells including macrophages and osteoclasts. Thus, the fusogenic role of V-ATPase could play an important biological role in general.

Direct role of V-ATPase in vesicular trafficking of endocytotic pathway

Role of V-ATPase in endocytosis of viruses, microorganisms, and toxic molecules

The endocytotic pathway is used by viruses and bacteria to enter into eukaryotic cells. They have developed a variety of strategies in order to reach their site of replication (cytosol or intracellular organelles) and/or to avoid degradation in lysosomes [68,69]. One strategy of the infection process requires a V-ATPase-driven acidic environment in early or late endosomes. For example, membrane fusion of vesicular stomatitis virus and its escape to the cytosol depends upon the endosomal acidic lumen. The function of the M2-protein from influenza A-virus and SARS coronavirus proteinase also need acidification. The translocation of various toxins from endosomes to the cytosol also depends upon acidification and includes among others: anthrax, diphtheria, and clostridial toxins [68,70,71]. An alternative survival strategy is applied by *Mycobacterium tuberculosis*. These microorganisms diminish the acidity of phagosomes and impair their fusion with lysosomes [69].

An acidification-independent strategy of internalization is employed by HIV (*h*uman *i*mmunodeficiency *v*irus) and requires the direct interaction with V-ATPase. *N*egative *f*actor (Nef) of HIV plays a crucial role in viral pathogenesis and promotes the progression to AIDS. Nef directly interacts with the H-subunit of V-ATPase and promotes CD4 internalization at the plasma membrane [72]. The H-subunit is homologous to β-adaptins [73], interacts with the μ2-chain of AP2 adaptor and promotes *c*lathrin-*c*oated *v*esicle (CCV) formation [74], suggesting that V-ATPase is acting as a scaffolding complex. Thus this strategy of viral infection involves direct interaction with V-ATPase in the early stages of budding and coat formation at the plasma membrane.

When V-ATPase meets with small GTPases: functional significance of this rendezvous

Organelles along the endocytotic pathway have acidic lumenal pH that is crucial for vesicular trafficking. Acidification in early endosomes of BHK cells is required for the formation of *e*ndosomal *c*arrier *v*esicles (ECV) or *m*ulti*v*esicular *b*odies (MVB) (Figure 2), that mediate either trafficking between early and late endosomes [75] or involved in exosomes formation and secretion [76••]. It has been proposed that biogenesis of ECV/MVB vesicles is a tightly regulated budding process coupled to the acidification of endosomes. Indeed, it has been shown the acidification-dependent recruitment of cytosolic coatomer proteins (β-COP, ε-COP) and Arf1 small GTPase onto early endosomes. Furthermore, the involvement of a hypothetical *p*H-*s*ensing *p*rotein (PSP) in direct interaction with β-COP during the formation of endosomal carrier vesicles has been suggested [77,78].

In kidney physiology, receptor-mediated endocytosis by proximal tubule epithelial cells plays an important role in protein homeostasis via reabsorption of albumin, hormones, chemokines, vitamin-binding proteins, etc. [53]. This protein degradation pathway also depends on endosomal acidification, with defects in this process leading to proximal tubulopathies in humans and mice [53]. Early studies on small GTPases demonstrated the colocalization of V-ATPase with Arf6 and *A*DP-*r*ibosylation factor *n*ucleotide site *o*pener (ARNO) in early endosomes of the degradation pathway [79]. The recruitment of these small GTPases was driven by intra-endosomal acidic pH, and the presence of PSP in the endosomes was proposed [80]. Recently, V-ATPase has been identified as pH-sensor that directly interacts with small GTPases in an acidification-dependent manner [30••,81,82]. In particular, the a2-isoform is targeted to early endosomes of the proximal tubule (Figure 2) (Figure 4e,f) (see Supplementary Information, Figure S3 and Movie S3) and directly interacts with ARNO in acidification-dependent manner, while Arf6 specifically interacts with the c-subunit of the V_O-sector of V-ATPase. Importantly, these studies also demonstrated that the acidification-dependent interaction between V-ATPase and small GTPases is crucial for protein trafficking between early and late endosomes [30••] (see Supplementary Information, Figure S4 and Movies S4,S5). Although these studies showed that V-ATPase could modulate vesicular trafficking by scaffolding and recruiting small GTPases, the downstream targets of this V-ATPase/ARNO/Arf6 complex as well as functional significance of this rendezvous remains to be elucidated.

The Ras-superfamily of small GTPases function as 'molecular switches' and regulate an extraordinary variety of cell functions [83]. The transition between the 'on' and 'off' states of this molecular device is mediated by the GDP/GTP cycle. The *A*DP-*r*ibosylation *f*actor (Arf) family belongs to the Ras-superfamily and also functions as a molecular switch to regulate vesicular traffic and organelle structure [84]. The acti-

vation of Arfs (GTP-bound conformation, 'on'-state) is mediated by *g*uanine-nucleotide *e*xchange *f*actors (GEFs), while deactivation (GDP-bound conformation, 'off'-state) is catalyzed by *G*TPase-*a*ctivating *p*roteins (GAPs). Both small GTPase Arf6 and its cognate GEFs and GAPs have been implicated in the regulation of the endocytotic pathway and organelle biogenesis by: first, recruiting coat components; second, modifying phospholipids; and third, remodeling the cytoskeleton near vesicular membranes [30••,85••]. Thus, the acidification-dependent recruitment of ARNO/Arf6 and their binding to endosomal V-ATPase may trigger these downstream pathways giving rise to the endosome carrier vesicles (ECV/MVB) formation.

Finding the interaction between V-ATPase and small GTPases pointed out the intriguing possibility that small GTPases might function as 'molecular on/off switches' for V-ATPase function. The following results suggest possible 'cross-talk' between V-ATPase/small GTPase interaction and disassembly/assembly of V-ATPase. First, the glucose-dependent disassembly of V-ATPase is also controlled by the acidification of vacuoles suggesting the presence of a vacuolar pH-sensor [3••,39•,40]. Second, V-ATPase is known to directly interact with the actin-microfilament cytoskeleton [3••,6,86] and requires an intact microtubular network for the dissociation of V_OV_1 complex [3••]. Thus, when recruited to V-ATPase, Arf6 could be involved in cytoskeleton remodeling [3••,6,85••,87]. Third, binding of V-ATPase to F-actin [86] and glucose-dependent assembly of the V_OV_1 complex [36] depend on PI3-kinase activity, that is also necessary for specific recruitment of ARNO [88] and Arf6-mediated actin dynamics [89]. Finally, aldolase modulates assembly/disassembly of V-ATPase by the interaction with the a, B, and E subunits [35••,36,37]. Recently, the direct and specific interaction of aldolase with ARNO has been demonstrated (Marshansky Laboratory, unpublished data) suggesting that ARNO can modulate the assembly/disassembly of the V-ATPase/aldolase complex. On the basis of these data it is tempting to propose that the pH-sensing function of V-ATPase and the acidification-dependent recruitment of small GTPases are integral parts of the glucose/aldolase-dependent regulation of V-ATPase. Thus, the generally accepted function of small GTPases as 'molecular switches' may directly be applied to the assembly/disassembly of V-ATPase and turning 'on/off' this remarkable nano-machine.

Conclusions and perspectives

The function of V-ATPases in different compartments is tightly coordinated with the unique roles of different organelles and their cytosolic microenvironments. Identification of the mechanisms of isoform-specific targeting and assembly of V-ATPase at specific organelles in mammalian cells is an important challenge for the years to come. The mechanism of glucose-dependent reversible dissociation of V-ATPase is starting to be resolved. Its significance in vesicular trafficking is of interest because the levels of glucose are tightly controlled in multicellular organisms. It becomes increasingly clear that reversible dissociation of V-ATPase is controlled by a variety of other cellular pathways and the potential regulatory role of small GTPases in the assembly/disassembly of V_OV_1 awaits further studies.

Our understanding of vesicle trafficking will be reinforced by further studies of V-ATPase as a pH-sensor and identification of effectors downstream of V-ATPase/GTPase complex. In particular the direct role of V-ATPase as pH-sensor and recruitment of small GTPases is of interest for the formation of endosomal carrier vesicles (ECV/MVB) because emerging evidence suggest that these vesicles are involved in biogenesis and function of exosomes. Thus the potential regulatory role of V-ATPase in the formation and/or secretion of exosomes also awaits further studies.

Emerging evidence also suggests possible interplay between assembly/disassembly of V_OV_1 and the direct role of V_O in membrane fusion during vesicular trafficking. The relevant important questions that remain to be addressed are: first, does disassembly of V-ATPase take place during the budding process and, thus, is associated with the formation of carrier vesicles from the donor membrane? or second, does disassembly of V-ATPase take place nearby the acceptor-membrane before fusion and, is associated with uncoating of vesicles?

Increasing evidence has shown that V-ATPase can interact with numerous regulatory proteins. Cascades of different protein–protein interactions modulate targeting, assembly and activity of V-ATPase, followed by the regulation of intravesicular acidification and trafficking. Defective V-ATPase function can impair vesicular trafficking and give rise to human diseases. Thus, future studies will further shed light on the direct roles of this magnificent nano-motor in the regulation of vesicular trafficking and will undoubtedly contribute to the development of new drugs specifically targeting V-ATPase function.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceb.2008.03.015.

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