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Function, structure and regulation of the vacuolar (H⁺)-ATPases

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Abstract

The vacuolar ATPases (or V-ATPases) are ATP-driven proton pumps that function to both acidify intracellular compartments and to transport protons across the plasma membrane. Intracellular V-ATPases function in such normal cellular processes as receptor-mediated endocytosis, intracellular membrane traffic, prohormone processing, protein degradation and neurotransmitter uptake, as well as in disease processes, including infection by influenza and other viruses and killing of cells by anthrax and diphtheria toxin. Plasma membrane V-ATPases are important in such physiological processes as urinary acidification, bone resorption and sperm maturation as well as in human diseases, including osteopetrosis, renal tubular acidosis and tumor metastasis. V-ATPases are large multi-subunit complexes composed of a peripheral domain (V₁) responsible for hydrolysis of ATP and an integral domain (V₀) that carries out proton transport. Proton transport is coupled to ATP hydrolysis by a rotary mechanism. V-ATPase activity is regulated *in vivo* using a number of mechanisms, including reversible dissociation of the V₁ and V₀ domains, changes in coupling efficiency of proton transport and ATP hydrolysis and changes in pump density through reversible fusion of V-ATPase containing vesicles. V-ATPases are emerging as potential drug targets in treating a number of human diseases including osteoporosis and cancer.

Function of V-ATPases

Functions of intracellular V-ATPases

V-ATPases are ATP-dependent proton pumps that couple the energy released upon hydrolysis of ATP to the active transport of protons from the cytoplasm to the lumen of intracellular compartments or, if localized to the plasma membrane, the extracellular environment (for reviews of V-ATPase function in mammalian and yeast systems, see references [1–3]). V-ATPases were early identified in lysosomes and the central vacuoles of fungi and plants where they provide the acidic environment required for degradation of macromolecules by digestive enzymes having a low pH optimum [4]. V-ATPases were also identified in secretory vesicles, such as synaptic vesicles and chromaffin granules, where they provide the driving force for the uptake of secreted small molecules, such as neurotransmitters and ATP (for review of V-ATPase function in neurotransmitter uptake, see reference [5]). These small molecules are taken up by secretory vesicles either via proton-coupled antiporters that employ the acid-interior proton gradient generated by the V-ATPases or, for negatively charged species such as glutamate and ATP, by transporters driven by the interior positive membrane potential generated by the V-ATPases (Figure 1a). Secretory vesicles involved in secretion of peptide

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hormones and growth factors, such as insulin and EGF, also require a low pH to activate processing enzymes involved in conversion of the precursor forms of these molecules to their mature forms [6].

Intracellular V-ATPases are particularly important for various membrane traffic processes ([1,3]). Acidification of early endosomes promotes the release of internalized ligands (such as low density lipoprotein and insulin) from their receptors, thus facilitating the recycling of the unoccupied receptors to the cell surface and the targeting of the released ligands to the lysosome for degradation [7]. A similar acid-activated release is required for delivery of newly synthesized lysosomal enzymes from the Golgi to the lysosome (Figure 1a) (for reviews of endocytosis and lysosomal targeting see references [8] and [9]). This process involves the binding of these enzymes to the mannose-6-phosphate receptor in the TGN and delivery of the complexes to late endosomes where the low pH triggers dissociation. The low luminal pH within endosomes is required not only for dissociation of ligand receptor complexes but also for the budding of endosomal carrier vesicles that carry released ligands from early to late endosomes [10]. This budding process requires the binding of beta-COP coat proteins to the early endosomal membrane, and this budding is in turn dependent upon the acidic luminal pH generated by the V-ATPase. The V-ATPase itself has been suggested to serve as a scaffold for the binding of proteins involved in vesicle budding, including ARF-6 (a low molecular weight G protein) and ARNO (a guanine-nucleotide exchange factor that activates ARF6) [11]. These proteins are involved in budding of endosomal carrier vesicles in renal proximal tubule cells and their binding to the V-ATPase is also dependent upon endosomal acidification.

A novel role of the integral V_0 domain of the V-ATPase (see “Structure of V-ATPases” below) has been proposed in membrane fusion. Based upon studies of homotypic vacuole fusion in yeast [12], the V_0 domain was suggested to directly participate in the fusion process independent of its role in acidification. Following tethering of donor and acceptor membranes via SNARE complex formation, V_0 is suggested to directly catalyze the mixing of the two lipid bilayers by virtue of its highly hydrophobic subunit composition. Evidence supporting a direct role of V_0 in membrane fusion has emerged from studies of synaptic vesicle release in *Drosophila* [13], exosome release of Hedgehog-related proteins in *C.elegans* [14] and secretion of insulin by pancreatic islet cells [6]. Evidence for association of specific V-ATPase subunits with other components of the fusion machinery, including VAMP-2, syntaxin-1 and a Ca^{2+} -release channel has also recently appeared [13–15].

V-ATPase-dependent acidification of endosomal compartments is also crucial for the entry of the cytotoxic portions of various envelope viruses and bacterial toxins (Figure 1b) [16]. The hemagglutinin (HA) coat protein of influenza, for example, acts as a pH dependent fusagen, mediating fusion between the viral membrane and the endosomal membrane following endocytic uptake of the virus particle. This facilitates the release of the viral nucleic acid into the cytoplasm of the host cell, which is required for viral replication. Similarly, toxins, such as diphtheria toxin and anthrax toxin, contain pore-forming subunits which, in response to the low endosomal pH following their internalization, facilitate the insertion into the cytoplasm of the cytotoxic portion of the molecule.

Functions of plasma membrane V-ATPases

Plasma membrane V-ATPases are present in a variety of specialized cells, particularly various epithelial cells, where they play important functions in acid secretion. V-ATPases are present in the plasma membrane of proximal tubular cells as well as both alpha and beta-intercalated cells of the distal tubule and collecting duct in the mammalian kidney (Figure 2a) [17]. Acid secretion across the apical membrane of proximal tubular cells aids in the re-absorption of bicarbonate by these cells by acidifying the tubular lumen and promoting conversion of filtered bicarbonate into carbon dioxide, a species which can readily diffuse across the apical plasma

membrane. In alpha-intercalated cells of the distal tubule and collecting duct, V-ATPases present in the apical membrane are involved in secretion of acid into the urine. Genetic defects in the V-ATPase isoforms expressed in these cells that are responsible for this acid secretion lead to the inherited human disorder distal renal tubular acidosis, in which patients are unable to secrete sufficient acid into the urine [18]. Interestingly, many of these patients also suffer from deafness due to the role of V-ATPases in control of the ion composition of the fluid surrounding hair cells of the inner ear [18]. Alpha-intercalated cells also contain chloride-bicarbonate exchangers in the basolateral membrane that secrete bicarbonate back into the plasma to avoid alkalization of the intercalated cell cytoplasm during acid secretion. The distal tubule and collecting duct also contain beta-intercalated cells which have an opposite polarity to alpha-intercalated cells [17]. Thus, these cells express V-ATPases at the basolateral membrane and chloride-bicarbonate exchangers at the apical membrane. These cells are thus able to reabsorb acid equivalents under conditions where the plasma pH is too alkaline. The balance in activity between alpha and beta intercalated cells is thus important in maintaining the correct pH of the plasma.

Plasma membrane V-ATPases play an especially important role in osteoclasts, where they are directly involved in bone resorption [19]. Osteoclasts have the ability to attach to bone and seal off a portion of the extracellular space, into which they secrete acid and digestive enzymes (Figure 2b). The acidic environment both directly dissolves the bone matrix and activates secreted proteases that participate in bone resorption. Patients with genetic defects in the V-ATPase isoform expressed in osteoclast plasma membranes develop an inherited disorder known as osteopetrosis that is characterized by an increase in bone mineral density and developmental defects due to the inability to remodel bone [20]. The importance of V-ATPase function in bone resorption by osteoclasts has also led to interest in the V-ATPase as a potential target in treating osteoporosis, which is characterized by excessive bone loss.

V-ATPases present in the apical membrane of clear cells present in the vas deferens and epididymus have been shown to be important in the maturation and storage of sperm in the male reproductive tract (Figure 2c) [21]. As a result, plasma membrane V-ATPases in these cells are a potential target in the development of male contraceptives. A variety of cells also employ plasma membrane V-ATPases in pH homeostasis, including macrophages and neutrophils (Figure 2d) [22], which often encounter acidic environments in their normal physiological function.

V-ATPases have been shown to play a novel and unique role in cells of the insect midgut [23]. In the tobacco hornworm *Manduca sexta*, the V-ATPase is present in the apical membrane of cells lining the gut known as goblet cells (Figure 2e). These cells are charged with secretion of K^+ into the midgut, necessitated by the high K^+ diet of these insects. Potassium secretion is accomplished by goblet cells using two transport proteins. The first is the apical V-ATPase, which secretes protons into the luminal space and, as a result, creates a luminal positive membrane potential. The second is an electrogenic $K^+/2H^+$ antiporter which transports two protons into the cell for every one K^+ transported into the lumen, thus utilizing the membrane potential established by the V-ATPase to drive K^+ secretion. Because of the $K^+/2H^+$ stoichiometry, the luminal space actually becomes quite alkaline (pH~11), unlike the digestive tract of most animal species. Thus in insect goblet cells, the primary function of the V-ATPase is in creation of a transmembrane electrical potential rather than in creation of a pH gradient.

Plasma membrane V-ATPases have also been identified in cells possessing an invasive phenotype, including vascular endothelial cells and certain tumor cells [24,25]. In vascular endothelial cells, plasma membrane V-ATPases have been identified at the leading edge, where they appear to participate in both migration and invasion (Figure 2f). In the highly invasive human breast cancer cell line MB-231, plasma membrane V-ATPases were shown to be

functional and to be involved in the invasive phenotype of these cells, as demonstrated by inhibition of both invasion and migration using the specific V-ATPase inhibitor bafilomycin. These results suggest that plasma membrane V-ATPases are a potential target in treating both tumor metastasis and the excessive vascularization associated with diabetes.

Structure and Mechanism of a Rotary Motor

The V-ATPases are large multi-subunit complexes that hydrolyze ATP to pump protons across biological membranes [1,3]. The subunit model of the yeast V-ATPase is shown in Figure 3a. The enzyme is composed of two functional domains, termed V_1 and V_0 . The 640-kDa membrane peripheral V_1 domain is composed of eight different subunits in a stoichiometry of $A_3B_3CDE_2FG_2H$ [26–28], and houses the three catalytic ATP binding sites. The membrane integral V_0 domain is a 260-kDa complex that is composed of six subunits with a stoichiometry of $ac4c'c''de$ in the yeast enzyme [26,29] and is responsible for proton translocation. The mammalian V_0 lacks the c' subunit and contains an additional glycoprotein named Ac45 [30]. The V-ATPases are structurally and mechanistically related to the well known F-ATPase and operate via a rotary mechanism [31,32]. Even more closely related are the archaebacterial ATPases (or A-ATPases)[33] including the Na^+ -ATPase from *Enterococcus hirae* and the H^+ -ATPase from *Thermus thermophilus*. ATP hydrolysis in V_1 drives the rotation of the central stalk (or rotor) that contains the D, F, d, and proteolipid subunits (c , c' , and c''). Rotation of the proteolipids past the a subunit (see below) drives the translocation of protons from the cytoplasmic to the luminal side of the membrane.

Structure and function of V_1 subunits

Much of what is known about the structure and mechanism of the V-ATPase has been inferred from its homology with the F-ATPase. The A and B subunits show a high degree of sequence homology with the β and α subunits, respectively, and are arranged in an alternating hexameric ring [34]. The six ATP binding sites are located at the interfaces between subunits, and have been probed using both site-directed mutagenesis and chemical modification [35–40]. There are 3 catalytic binding sites, the majority of which are formed by residues from subunit A, with minor contacts taking place from subunit B. Interspersed between the catalytic sites are three non-catalytic ATP binding sites, the function which is unclear, but which have been suggested to play a regulatory role [35,36,38,40].

Hydrolysis of ATP causes conformational changes in the catalytic A subunits that drive rotation of the central stalk or rotor that is composed of subunits D and F of V_1 , and subunit d and the proteolipid subunits of V_0 . Subunit D is highly helical and predicted to extend up into the A_3B_3 hexamer of V_1 , in a similar fashion to the homologous F-ATPase γ subunit. As with the F_1 -ATPase γ [41] and ϵ [42] subunits, rotation of subunits D and F has been directly observed using fluorescence microscopy [32]. Moreover, subunit F can be cross-linked to D [27] and forms a tight heterodimeric complex with subunit D [43], showing that the rotor assembly of V_1 is composed of the D and F subunits. A high resolution structure of the isolated *Thermus thermophilus* F subunit has been reported [44]. The remainder of the V_1 subunits (C, E, G, and H) will be discussed in the section “The Peripheral stator of V-ATPase”.

Structure and function of V_0 subunits

As with the F-ATPase, proton translocation through the V_0 domain is driven by rotation of the proteolipid subunits past the a subunit and direct visualization of this rotation has been observed [31]. The proteolipid subunits of V_0 are highly hydrophobic proteins that form a ring-like structure in the lipid bi-layer [45], and are essential for function [46]. Subunit d is shaped like a cork that sits on top of the proteolipid ring [47] and provides the interface that contacts the D and F subunits. This interaction drives rotation of the proteolipids (Figure 3a). Quantitative

amino acid analysis with the bovine enzyme [26], and genetic studies in yeast [29] suggest the presence of four or five copies of c and single copies of c' and c'', however, a crystal structure of the *Enterococcus hirae* Na⁺-translocating A-ATPase contains a ring of ten identical c subunits [48]. For this discussion we will assume a stoichiometry of four copies of subunit c and single copies of c' and c'', although the exact number of proteolipid subunits in the ring remains uncertain. Subunits c and c' are 16-kDa proteins containing 4 transmembrane helices with N and C-termini on the luminal side of the membrane [49]. Subunit c'' is 23 kDa and contains five transmembrane helices with a cytoplasmic N-terminus and a luminal C-terminus [49], although the N-terminal helix can be removed with no effect on the function of the enzyme [50]. The subunits are arranged in a specific orientation, with subunit c' adjacent to and clockwise from subunit c'' when viewed from the cytoplasmic side of the membrane [51]. The remainder of the ring is therefore made of up 4 consecutive c subunits (Figure 3b). Each proteolipid subunit contains an essential glutamic acid residue, located in TM4 on c and c' and TM3 on c'' [46], that interacts with the a subunit and undergoes reversible protonation/deprotonation during proton transport.

The 100-kDa a subunit of V₀ is a critical component of the proton transport mechanism. Topological studies suggest a two-domain structure with an N-terminal hydrophilic domain of approximately 50 kDa that is located on the cytoplasmic side of the membrane, and a membrane integral C-terminal domain that contains nine putative transmembrane helices [52]. In yeast, the two isoforms show differences in targeting, assembly, and coupling efficiency [53,54]. The later function has been mapped to the C-terminal domain while the former two functions have been mapped to the N-terminus [54]. The C-terminal domain is analogous to the a subunit of the F-ATPase, providing two hemi-channels that allow access of protons to the membrane embedded glutamic acid residues that reside on the proteolipids. Figure 3c outlines the mechanism of rotation dependent proton transport via V₀, which is again based upon the mechanism proposed for the F-ATPases [55]. Protons from the cytoplasmic side of the membrane gain access to and protonate the proteolipid glutamate residues that are exposed by the incoming hemi-channel. The proteolipid ring rotates in a clockwise fashion (as viewed from the cytoplasmic side of the membrane) allowing the glutamic acid residues to travel through the hydrophobic lipid milieu in an uncharged state, eventually returning back to subunit a and making contact with the second hemi-channel. An essential Arg residue on subunit a (Arg 735 in the yeast a subunit Vph1p)[56] promotes the deprotonation of the glutamate and the release of the proton through the hemi-channel to the luminal side of the membrane. Thus, in the V₁V₀ complex the energy that is released from ATP hydrolysis drives rotation of the rotor, and the rotating proteolipid ring results in the net movement of protons from the cytoplasmic to the luminal side of the membrane.

The peripheral stator of V-ATPase

An essential requirement for the rotary mechanism is that the A₃B₃ complex must be tethered to the a subunit of V₀, a task that is accomplished by the peripheral stalk. Crosslinking and electron microscopy studies have provided evidence that C, E, G, H, and the N-terminal domain of the a subunit all form part of the peripheral stalk, while D and F form the central stalk [57–61]. Subunits E and G form a tight helical interaction and form a long extended complex of ~220 Å [62] that is able to form crosslinks along the entire length of the B subunit [57,58]. Subunits C and H are both located at the interface between the V₁ and V₀ domains [59,60], consistent with their proposed function in regulation. High resolution structures are available for both C [63] and H [64], and have provided the basis for the cysteine mediated crosslinking studies [59,65] that have allowed us to orient these subunits in our model (Figure 3a). Subunit C completely dissociates from the V₁ and V₀ complex during reversible dissociation (see below) indicating a potential role in regulation. It has a two domain structure connected by a flexible linker that has been proposed to facilitate elastic energy coupling [63,66]. Subunit H

is unique as in its absence the V-ATPase can still assemble, however the pump remains inactive [67]. Differences in expression levels of H affect enzyme assembly [68]. H also has two structural domains and it has been shown that the N-terminal domain is required for activation of ATP hydrolysis while the C-terminal domain is required for proper energy coupling [69].

Unlike the single peripheral stalk of the F-ATPase, a picture is emerging of the V-ATPase containing multiple peripheral stalks. Recently both E and G have been shown to be present in more than one copy per enzyme [28]. The EG complex has been shown to interact with both C [59,70] and H [60,70,71], and in recent EM images identifying the location of H and C by difference mapping there appear to be two stalks, one containing H and the other containing C [60,72,73]. The N-terminal domain of a has been shown to interact with C [59], H [61], and A [61]. This data supports the hypothesis that there are two peripheral stalks in the V-ATPase, one containing an EGC complex and another containing and EGH, and each complex interacts with the N-terminal domain of a. The reason for this complexity is not certain but may be related to the role of the V-ATPase stalks in both stabilizing the complex during catalysis and yet remaining sufficiently labile to separate during *in vivo* dissociation of the complex (see below).

Assembly and Targeting of V-ATPases

V-ATPases are large, multi-subunit complexes that begin assembly in the ER prior to completion in the Golgi [74,75]. In yeast, the dedicated chaperones Vma12p, Vma21p, Pkr1p, and Vma22p are required for proper assembly of the V_O domain in the ER, although the function of higher eukaryote homologs of these subunits has not yet been established. In the ER of yeast, proteolipid ring formation and subunit d assembly is facilitated by Vma21p. Vma12p and Vma22p are responsible for subunit a association with this nascent ring, while Pkr1p appears to increase the efficiency of V_O domain assembly and export [74,76]. Significantly, a partial complex composed subunit A of the V₁ domain and subunit a of V_O has been observed before leaving the ER [2], suggesting a coordinated parallel assembly pathway. The V_O domain can assemble and reach the vacuole in the absence of V₁ subunits, and a nearly complete V₁ complex can also assemble in the absence of the V_O domain [2]. In the latter case, a V₁ complex lacking subunit C is formed. All subunits are required for complete V₁V_O complex assembly of the V-ATPase in yeast except subunit H, as complexes that lack subunit H assemble on the vacuolar membrane but are unstable and inactive [67].

Although multiple isoforms of several subunits are found in higher organisms, in both yeast and mammals, intracellular targeting appears to be directed by the a subunit [54]. In yeast, there are two isoforms of the a subunit – Vph1p and Stv1p [77]. Enzyme complexes containing Vph1p are targeted to the vacuole, whereas complexes containing Stv1p are localized to the late Golgi. Differences in the sequence of the cytosolic N-terminal domain of Vph1p and Stv1p have been shown to direct intracellular trafficking [54].

In mammalian cells, V-ATPases are targeted to both the plasma membrane of certain cell types in addition to specific intracellular compartments [78,79]. The four isoforms of the a subunit – a1, a2, a3, and a4 – are generally tissue-specific [3]. Complexes containing the a1 isoform have been shown to localize to pre-synaptic nerve terminals in the brain where they are present in both vesicles and the pre-synaptic plasma membrane [80]. The a2 isoform localizes to apical endosomes in renal proximal tubule cells [11], while complexes containing the a3 isoform are targeted to the plasma membrane of osteoclasts [19]. It should be noted, however, that a3-containing complexes have also been identified in lysosomes of pre-osteoclasts [19] and insulin-containing secretory vesicles in pancreatic islet cells [6]. The a4 isoform targets the V-ATPase to the apical membrane of renal intercalated cells and epididymal clear cells [17,21].

In yeast, with the exception of subunit a, each subunit has only one isoform. In mammalian cells however, several subunits are expressed as multiple isoforms. Subunits B, E, H, and d, are all expressed as two isoforms [17,81–84], while subunits C and G exist in three isoforms [82,84]. Like the isoforms of subunit a, these tend to be expressed in a tissue-specific manner. For example, a4, B1, C2b, d2, G3 are found in the kidney and epididymis [21,81,82,84,85], a1 and G2 in the brain [83], and a3 and d2 in osteoclasts [86]. Isoform B1 has been shown to interact with the PDZ protein Na/H exchange regulatory factor (NHE-RF) [87], which may help to retain it in the appropriate membrane. Specific mutations in isoforms that are known to be highly expressed in different cell types have been linked to disease phenotypes. Mutations in the a3 isoform, which is highly-expressed in osteoclasts, causes autosomal recessive osteopetrosis [20]. In renal intercalated cells, mutations in both the a4 and B1 isoforms cause renal tubule acidosis [18,88]. Patients with mutations in the B1 isoform also suffer from deafness, as noted earlier [18].

These unique subunit profiles may provide for independent regulation of V-ATPases present in the same cell and open the possibility of the development of drugs that target particular subunit combinations for inhibition. Such drugs may be useful in the treatment of such diseases as osteoporosis and cancer.

Mechanisms of Regulation of the V-ATPase

A unique mechanism of regulation of the V-ATPase involves the reversible dissociation of the V_1 domain from the V_O domain and the subsequent silencing of ATP hydrolysis and blocking of proton passage (Figure 4a) [2]. In yeast [2], and in the midgut of some insect cells [23], this mechanism is thought to be an energy-conserving response, as dissociation occurs upon a decrease in glucose levels. Subunit H of the peripheral stalk of the V_1 domain has been shown to play a critical role in the silencing of V_1 ATP hydrolysis, as complexes depleted of subunit H retain ATPase activity [89]. It has also been proposed that subunit H imparts this catalytic inhibition by bridging rotor and stator in the free V_1 domain, as a direct interaction between subunit H and the rotary subunit F has been observed only in the free V_1 complex [65].

A similar glucose-induced effect has been reported in the mammalian kidney cell line LLC-PK1 [90], although the purpose of this event in the normal physiology of renal cells is unknown. Increased assembly of the V-ATPase complex occurs during dendritic cell maturation, which facilitates higher levels of acidification of lysosomes thus activating antigen processing [91].

Glucose dependent dissociation of the V-ATPase complex occurs rapidly - less than two minutes after glucose removal - and does not require direct interaction with glucose [2]. It is likely that a signaling pathway mediates disassembly and reassembly of the V-ATPase. Among the glucose-dependent responses that have been tested and shown not to be involved in yeast are the Ras-cAMP pathway, the main Snf1p kinase-mediated glucose repression/derepression pathway, and a protein kinase C-dependent pathway believed to be involved in glucose-dependent phosphorylation events [92]. Nevertheless, recent studies have implicated the PI-3 kinase pathway in renal tubule cells [90] and the protein kinase A pathway in insect cells [93].

Although our understanding of reversible dissociation is incomplete, it is known that an intact microtubular network is required for dissociation [94], and that a protein complex called RAVE (regulator of the ATPase of vacuolar and endosomal membranes) is required for reassembly (Figure 4a) [95]. RAVE is composed of the proteins Rav1p, Rav2p, and Skp1p, which belongs to the SCF ubiquitin ligase family of proteins. RAVE has also been shown to be involved in biosynthetic assembly of the V-ATPase and to bind to subunits E or G of free V_1 [96]. Interaction between RAVE and subunit C is also essential for RAVE function [97]. Since an intact microtubular network is unnecessary for reassembly and the RAVE complex is not

required for dissociation, it appears that disassembly and reassembly are independently controlled processes.

The glycolytic enzyme aldolase also plays a critical role in glucose dependent assembly as it has been found to interact with the α , β , and ϵ subunits of the V-ATPase and this interaction increases dramatically in the presence of glucose (Figure 4a) [98]. Aldolase facilitates the formation of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate from fructose 1,6 biphosphate during glycolysis. Disruption of the interaction between aldolase and the β subunit has also been shown to cause disassembly and a loss of V-ATPase function [99]. This evidence suggests that aldolase may be the “glucosensor” that signals the V-ATPase to dissociate, although aldolase activity is not required for this effect.

Recent studies point to a unique region of the catalytic α subunit as playing an important role in glucose-dependent dissociation. This region, known as the “non-homologous region” (so named due to its absence from the otherwise homologous F-ATPase catalytic subunit), is a 90 residue insert found in the V-ATPases of many species [100]. Specific point mutations in certain conserved residues of this region, including those maintaining nearly full wild-type activity, result in complete loss of V-ATPase dissociation in the absence of glucose [100]. It was found that when expressed by itself, the non-homologous region can independently bind the V_O domain, and this interaction is significantly reduced upon removal of glucose *in vivo* [101]. These results suggest that either the non-homologous region or the N-terminus of the subunit α , or both, may function as glucose sensors.

The membrane environment in which the V-ATPase resides also appears to contribute to glucose-dependent dissociation. In yeast, the subunit α isoform Stv1p is found in V-ATPases that target to the Golgi and do not dissociate upon a drop in glucose concentration. The other subunit α isoform, Vph1p, is found in V-ATPases present in the vacuole that do dissociate upon glucose depletion [53]. It has been shown that if Stv1p-containing V-ATPases are re-directed to the vacuole, they are able to dissociate and reassociate in response to changing glucose levels [53]. Moreover, the glucose-dependent dissociation of both isoforms has been shown to depend on cellular environment [53,102]. The nature of the environmental signal controlling dissociation is not certain, but the luminal pH of the compartment appears to play some role [101].

A different means of regulation involves the reversible formation of disulfide bonds between conserved cysteine residues at the nucleotide-binding site of the catalytic subunit α . Disulfide bond formation between Cys254 and Cys532 of the bovine V-ATPase results in inactivation of the V-ATPase, whereas disulfide interchange within the catalytic subunit restores activity (Figure 4b) [103–105]. It has furthermore been shown that the redox state of the environment can modulate V-ATPase activity by reversible disulfide bond formation *in vivo* [106].

Some cell types that express V-ATPases on the plasma membrane have evolved a means to regulate the V-ATPase density on the plasma membrane and thus control local acid levels. This is accomplished by reversible exocytosis of intracellular vesicles that contain a large amount of V-ATPase which fuse to the plasma membrane, thus increasing the density of V-ATPases on the cell surface and increasing proton efflux [3,17]. V-ATPases in the apical membrane of type-A renal intercalated cells use this regulatory mechanism to control acid/base balance in the kidney (Figure 2a) [17]. Indeed, mutations in the human isoforms of both the β and α subunits have been shown to cause defects in renal acidification which result in the condition metabolic acidosis [88]. In the epididymis and proximal vas deferens, plasma membrane V-ATPases in narrow and clear cells help acidify the luminal pH which is necessary for maintaining spermatozoa in a quiescent state during maturation (Figure 2c) [21]. As the pH in epididymal clear cells increases, sAC (soluble Adenylyl Cyclase) is activated, which

increases cyclic (c)AMP concentration and, through regulation of exocytosis or endocytosis or a combination of both, increases the density of V-ATPases at the plasma membrane (Figure 4c) [107].

It has also been reported that modulation of the actin cytoskeleton plays an important role in regulating V-ATPase density at the plasma membrane. Both isoforms of subunit B, B1 and B2, have been found to bind directly to actin through sites near their N termini [108]. In clear cells, inhibition of gelsolin, one of many factors that are involved in remodeling of the actin cytoskeleton, blocks the pH induced translocation of V-ATPases to the plasma membrane [109]. Gelsolin functions by capping the growing ends of actin filaments which blocks elongation and promotes filament degradation [110,111]. Additionally, activation of bone resorption in osteoclasts disrupts the subunit B-actin interaction, yet inhibition of PI3K preserves this interaction [90,112]. A potential role in actin bundling has been proposed for subunit C, which has also been shown to directly interact with actin [113,114], although additional studies will be required to fully define the role of the cytoskeleton in regulation of plasma membrane V-ATPase activity.

Differences in the efficiency with which protons are pumped during ATP hydrolysis, or the “coupling efficiency”, has emerged as yet another V-ATPase regulatory mechanism (Figure 4d) [3, 115]. In yeast, V-ATPase complexes containing the Vph1p subunit a isoform localize to the vacuoles and have a much higher coupling efficiency than Stv1-containing V-ATPases, which are found in the Golgi [53]. This difference in efficiency can be attributed to unique sequences within the hydrophobic carboxy-terminal half of subunit a [54]. This difference in coupling efficiency may also help to explain the observation that vacuoles are more acidic than the Golgi.

Mutations in other subunits have been shown to radically change the coupling efficiency of the complex. Specific point mutations in the d subunit of the V_O domain [116], and in the non-homologous region of the A subunit [100] have been shown to actually increase coupling efficiency to levels greater than wild-type. This suggests that the wild-type enzyme is not optimally coupled, although further work is necessary to determine the precise mechanisms by which coupling efficiency is regulated *in vivo*.

Though not directly affecting V-ATPase activity, control of the pH of intracellular compartments can also be attributed to other passive ion transport proteins, such as chloride and proton channels, that are present in these compartments. In endosomes and clathrin coated vesicles, the pH depends on the activity of a chloride channel that dissipates the membrane potential generated by the V-ATPase (Figure 4e). Defects in chloride channel protein 5 (CLC-5) have been linked to defective endosomal acidification in the kidney [117], while defects in chloride channel protein 7 (CLC-7) are associated with defects in bone resorption in osteoclasts [118]. Passive proton conductance also plays an important role in balancing pH levels, as it has been shown that a Zn²⁺ sensitive proton channel contributes to control of luminal pH of the Golgi [119].

Conclusions

V-ATPases are an important class of ATP-driven proton pumps present in intracellular and plasma membranes of eukaryotic cells that serve a wide array of functions in both normal and disease processes. They resemble in structure and mechanism the ATP synthases, but the greater structural complexity of V-ATPases likely reflects their diverse biological roles. This diversity of function has also necessitated a complex array of regulatory mechanisms, which include reversible dissociation, selective targeting and changes in coupling efficiency. V-

ATPases are emerging as potential drug targets in the treatment of a number of human diseases, including cancer and osteoporosis.

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Figure 1. Role of intracellular V-ATPases in normal and disease processes

Panel A, role of intracellular V-ATPase in membrane trafficking, endocytosis, and secretion. Extracellular ligands are internalized by receptor mediated endocytosis and trafficked to the sorting endosome. Acidification of the endosome by the V-ATPase allows for release of the ligand and the recycling of the receptor back to the membrane. Budding of endosomal carrier vesicles and multivesicular bodies are also dependent on the acidic environment. Lysosomal proteins are synthesized in the trans Golgi network and are trafficked to the lysosome via the mannose-6-phosphate receptor. Acidification of the late endosome allows for the release of the lysosomal proteins and recycling of the Man-6-P receptor back to the Golgi. In the lysosome acidification activates cathepsins and other degradative enzymes. The pH gradient created by

the V-ATPase in secretory vesicles drives the uptake of neurotransmitters and other molecules to be secreted. The V_0 domain, has also been proposed to play an important role in membrane fusion. Panel B, role of intracellular V-ATPase in the entry of envelope viruses and toxins. Envelope viruses and bacterial toxins such as diphtheria toxin enter the cell via endocytosis where they are trafficked to the sorting endosome. The low pH generated by the V-ATPase causes the viral coat to fuse with the endosomal membrane releasing the viral m-RNA into the cytoplasm. The acidic environment also induces the diphtheria toxin B chain (shown in green) to form a pore in the membrane that facilitates the entry of the A chain (shown in red) into the cytoplasm.

Figure 2. Function of plasma membrane V-ATPases

Panel A, function of V-ATPase in renal alpha-intercalated cells. To increase secretion of acid into the late distal tubule and collecting duct of the kidney, vesicles containing a high density of V-ATPases fuse with the apical membrane. These cells also absorb CO_2 , and release HCO_3^- into plasma through a $\text{Cl}^-/\text{HCO}_3^-$ exchanger, preventing the cytoplasm from becoming too alkaline. Panel B, during bone resorption V-ATPase are targeted to the membranes of osteoclasts that are in contact with bone, acidifying the extracellular matrix and activating degradative enzymes. Panel C, in epididymal clear cells, V-ATPases are targeted to the luminal membrane, providing the decreased pH of the lumen required for sperm maturation. Panel D, in neutrophils and in macrophages, plasma membrane V-ATPases are involved in pH homeostasis. Panel E, in insect midgut cells, V-ATPases are used to drive a H^+/K^+ antiporter resulting in the net secretion of K^+ into the gut. Panel F, some invasive tumor cells target V-ATPase to the plasma membrane where they create an acidic extracellular environment that aids invasion, likely through activation of cathepsins that breakdown matrix proteins.

Figure 3. Structural and mechanistic features of the V-ATPase

Panel A, Schematic representation of the *Saccharomyces cerevisiae* V-ATPase. Subunit placement and orientation are based on a combination of homology to the F-ATPase, electron microscopy studies and cysteine-mediated cross-linking studies. ATP hydrolysis by the V_1 domain drives the rotation of a rotor composed of the D, F, d, and proteolipid subunits (c, c', and c''). Rotation of the proteolipid ring past the a subunit drives the movement of protons from the cytoplasmic to the luminal side of the membrane. Two peripheral stalks, the EGC and EGH subcomplexes, hold the A_3B_3 hexamer stationary with respect to a. *Panel B*, Schematic representation of the helical arrangement in the proteolipid ring showing the unique orientation of the proteolipid subunits embedded in the membrane. The view is from the cytoplasmic side of the membrane. *Panel C*, Mechanism of proton translocation in the V_0 domain. Only the membrane integral C-terminal domain of a (yellow) and the proteolipid ring (blue) are shown. The unprotonated form of the proteolipid's essential glutamate residue enters the incoming hemi-channel on a. A proton from the cytoplasmic side of the membrane (the top in this diagram) enters the channel and protonates the exposed glutamic acid residue. As the proteolipid ring rotates, the now neutral glutamic acid is exposed to the hydrophobic lipid bilayer. As the ring rotates, the glutamic acid approaches the a subunit again, and interacts with a second hemi channel on the luminal side of the membrane. Residue Arg-735 on a (shown in green) interacts with the glutamic acid, changing its pKa and promoting the deprotonation and release of the proton into the luminal side of the membrane through the hemichannel. The net result is the rotation driven pumping of protons from the cytoplasmic to the luminal side of the membrane.

Figure 4. Regulation of V-ATPase activity

Panel A, Glucose-dependent reversible dissociation. Upon glucose depletion the V-ATPase dissociates into a soluble V_1 minus subunit C, a V_0 , and a soluble subunit C. In their free form, neither the V_1 , nor the V_0 has any ATPase or proton permeability, respectively. Assembly of V_1 back onto V_0 is mediated by the RAVE complex and aldolase, and dissociation requires intact microtubules, suggesting that dissociation and reassembly may be regulated independently. Panel B, reversible disulfide bond formation between subunit A residues Cys-254 in the catalytic site and Cys-532 locks the catalytic site into a conformation that is unable to undergo ATP hydrolysis. Panel C, changes in pump density in epididymal clear cells is regulated by HCO_3^- . When the luminal pH is raised, there is an increase in HCO_3^- , which

is transported into the clear cell and activates an HCO_3^- sensitive adenylyl cyclase. The resulting increase in cAMP results in an increased rate of exocytosis (or decreased rate of endocytosis) of vesicles containing a high density of V-ATPases, thus increasing the density of V-ATPases at the apical membrane. While the mechanism of regulation is different, the process of vesicular fusion to deliver V-ATPase to the plasma membrane occurs in many cell types, including those shown in Figure 2. Panel D, changes in the coupling efficiency of the pump. Panel E, changes in vesicle pH though changes in Cl^- or H^+ conductance through distinct channels.