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Minireview

# Structure, function and regulation of the vacuolar ($H^+$)-ATPases

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**Abstract The vacuolar ($H^+$)-ATPases (or V-ATPases) function to acidify intracellular compartments in eukaryotic cells, playing an important role in such processes as receptor-mediated endocytosis, intracellular membrane traffic, protein degradation and coupled transport. V-ATPases in the plasma membrane of specialized cells also function in renal acidification, bone resorption and cytosolic pH maintenance. The V-ATPases are composed of two domains. The $V_1$ domain is a 570-kDa peripheral complex composed of 8 subunits (subunits A–H) of molecular weight 70–13 kDa which is responsible for ATP hydrolysis. The $V_0$ domain is a 260-kDa integral complex composed of 5 subunits (subunits a–d) which is responsible for proton translocation. The V-ATPases are structurally related to the F-ATPases which function in ATP synthesis. Biochemical and mutational studies have begun to reveal the function of individual subunits and residues in V-ATPase activity. A central question in this field is the mechanism of regulation of vacuolar acidification in vivo. Evidence has been obtained suggesting a number of possible mechanisms of regulating V-ATPase activity, including reversible dissociation of $V_1$ and $V_0$ domains, disulfide bond formation at the catalytic site and differential targeting of V-ATPases. Control of anion conductance may also function to regulate vacuolar pH. Because of the diversity of functions of V-ATPases, cells most likely employ multiple mechanisms for controlling their activity.**

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*Key words:* V-ATPase; Vacuolar acidification; Proton transport; Endocytosis; Membrane traffic; Endosome; Lysosome; Clathrin-coated vesicle; Secretory vesicle; F-ATPase

## 1. Function of V-ATPases

The vacuolar ($H^+$)-ATPases (or V-ATPases) are a family of ATP-driven proton pumps responsible for acidification of a variety of intracellular compartments in eukaryotic cells, including clathrin-coated vesicles, endosomes, lysosomes, Golgi, secretory vesicles and the central vacuoles of plants and lower eukaryotes (for reviews, see [1–7]). We will begin with a brief review of the function of V-ATPases in eukaryotic cells.

V-ATPases within endosomes function to provide the acidic environment required for dissociation of internalized ligand-receptor complexes that is necessary for receptor recycling to the plasma membrane and delivery of dissociated ligands to lysosomes [1]. Thus, recycling of receptors for such ligands as low density lipoprotein (LDL), insulin and transferrin require exposure to an acidic endosomal pH. In addition, endosomal acidification appears to be required for budding of endosomal carrier vesicles that function to transfer ligands from early to late endosomes [8], a process that is sensitive to the specific V-ATPase inhibitor bafilomycin [9]. The V-ATPase dependent step appears to be binding of coat proteins involved in vesicle budding [10]. Finally, an acidic endosomal pH is utilized as a signal to activate entry of certain pathogens into the cytoplasm of infected cells. Thus, exposure of influenza virus to a low pH within endosomes induces formation of a fusion pore between the viral and endosomal membranes that permits entry of the viral RNA [11].

Vacuolar acidification plays an analogous role to that in the endocytic pathway in intracellular targeting of newly synthesized lysosomal enzymes from the Golgi to lysosomes [12]. Newly synthesized lysosomal enzymes are made with a mannose-6-phosphate recognition marker covalently attached that allows them to bind to the Man-6-P receptor in the trans-Golgi. These complexes are then delivered to an acidic, late endosomal compartment where the low pH activates release of the lysosomal enzymes and recycling of the Man-6-P receptors to the trans-Golgi. Although yeast does not possess a Man-6-P receptor system, an acidic pH appears to play a similar role in delivery of soluble proteins to the yeast vacuole [13,14].

In secretory vesicles, such as synaptic vesicles and chromaffin granules, the V-ATPases provide the driving force for coupled transport of small molecules, employing either the membrane potential or the pH gradient generated [15]. A similar function occurs in the yeast vacuole, where, for example, $Ca^{2+}$ uptake is coupled to proton efflux [5]. The acidic pH of digestive and secretory vesicles also plays an important role in facilitating protein processing and degradation by acid-dependent proteases.

V-ATPases in the plasma membrane of specialized cells have also been shown to function in a variety of important physiological processes. V-ATPases in the apical membrane of renal intercalated cells function in renal acidification [16] while V-ATPases in the plasma membrane of macrophages and neutrophils assist in maintaining a neutral cytoplasmic pH in the face of acid loads [17]. In osteoclasts, V-ATPases are targeted to a specialized domain of the plasma membrane that is involved in bone resorption, thus assisting in degradation of the bone matrix [18]. At least some tumor cells also appear to target V-ATPases to the plasma membrane [19], where they may function to provide an acidic extracellular environment necessary for the activity of secreted lysosomal enzymes, thus assisting in tumor metastasis. Finally, V-ATPases in the lumen of the midgut establish a positive exterior membrane potential which serves to drive potassium efflux via an electrogenic $H^+/K^+$ antiporter [20].

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## 2. Structure and subunit function of V-ATPases

Our current structural model of the V-ATPase is shown in Fig. 1. The V-ATPase is an 830-kDa multisubunit complex composed of two functional domains. The $V_1$ domain is a 570-kDa peripheral complex composed of eight subunits (subunits A–H) of molecular weight 70–13 kDa that functions in ATP hydrolysis. The subunits are present in a stoichiometry of $A_3B_3C_1D_1E_1F_xG_yH_z$ [21], where the relative number of copies of the F, G and H subunits has not yet been determined. The $V_0$ domain is a 260-kDa integral domain composed of five subunits (a, c, c′, c″, d) of molecular weight 100–17 kDa that functions in proton translocation. The subunits are present in a stoichiometry of $a_1c''_1d_1(c,c')_6$. The subunit names, molecular weights and genes encoding them in yeast are shown in Table 1.

### 2.1. Structure and function of $V_1$ subunits

The V-ATPases are known both from sequence homology [22,23] and overall structure [21,24] to be related to the F-ATPases (or ATP synthases), which are involved in ATP synthesis in mitochondria, chloroplasts and bacteria [25–30]. The recent X-ray crystal structure of the bovine mitochondrial $F_1$ domain [31] has revealed that it is composed of a hexamer of alternating alpha and beta subunits arranged around a central stalk consisting of the highly alpha helical gamma subunit. The six nucleotide binding sites are located at the interfaces of the alpha and beta subunits, with the three catalytic sites comprised mainly of beta subunit residues. Three additional sites, termed 'non-catalytic' nucleotide binding sites, are located primarily on the alpha subunits.

The V-ATPases also appear to contain both catalytic and non-catalytic nucleotide binding sites, with the catalytic sites located on the 70-kDa A subunits [32,33] and the non-catalytic sites located on the 60-kDa B subunits [34,35]. There is approximately 20–25% sequence identity between any pair of the A, B, alpha and beta subunits [22,23], indicating that they were derived from a common ancestral nucleotide binding protein. Mutational studies of the yeast V-ATPase A subunit [36–38] have identified a number of residues critical for activity, including E286, which corresponds to the glutamic acid residue in the F-ATPase beta subunit that is postulated to act in proton abstraction from the attacking water molecule, and K263, which is located in the 'glycine rich' loop consensus sequence and which is thought to interact with the negatively charged phosphate groups of bound ATP. Aromatic residues also appear to contribute to nucleotide binding at the catalytic sites of the V-ATPase [38], possibly through direct interaction with the adenine ring, as has been shown for the F-ATPase [31]. Interestingly, the A subunit contains a large insert, termed the 'non-homologous' region [22,23], which is not present in the F-ATPase beta subunit.

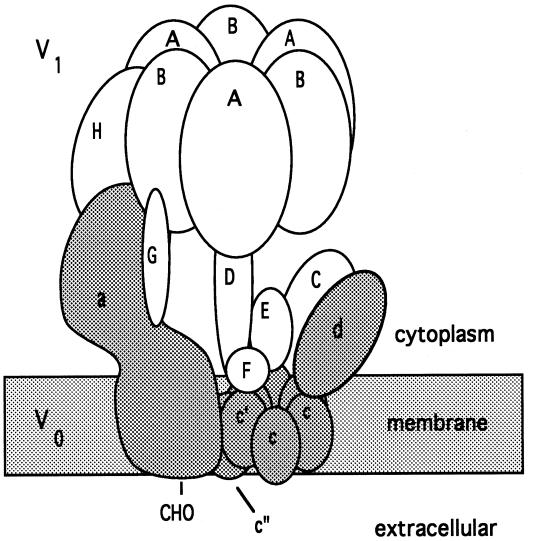



Fig. 1. Structural model of the V-ATPase. The V-ATPase complex is composed of a 560-kDa peripheral $V_1$ domain with the structure $A_3B_3C_1D_1E_1F_xG_yH_z$ (where the stoichiometry of the F, G and H subunits is unknown) which is responsible for ATP hydrolysis and a 260-kDa integral $V_0$ domain with the structure $a_1d_1c''_1(c,c')_6$ that is responsible for proton translocation (see text for details). The subunit composition of the second stalk (or stator) is uncertain, but candidates include subunit G, H and the amino terminal domain of the 100-kDa a subunit. CHO refers to carbohydrate covalently attached to the 100-kDa subunit.

Mutational analysis of the non-catalytic sites on the V-ATPase has revealed that changes generally lead to much less dramatic effects on activity [38,39], although chemical modification of these sites by BzATP does lead to inactivation [34,35]. Interestingly, mutation of R483 on the A subunit, which is postulated to be contributed to the non-catalytic sites, leads to a time-dependent increase in activity following addition of ATP [38], suggesting that occupancy of the non-catalytic nucleotide binding sites may be required for optimal

Table 1
Subunit composition of V-ATPases

| Domain | Subunit | Coated vesicles $M_r$ | Yeast vacuole $M_r$ | Yeast gene | Subunit function |
|---|---|---|---|---|---|
| $V_1$ | A | 73 | 69 | *VMA1* | Catalytic site, regulation (?) |
| | B | 58 | 57 | *VMA2* | Non-catalytic site, targeting (?) |
| | C | 40 | 42 | *VMA5* | Activity, assembly |
| | D | 34 | 32 | *VMA8* | Activity, assembly |
| | E | 33 | 27 | *VMA4* | Activity, assembly |
| | F | 14 | 14 | *VMA7* | Activity, assembly |
| | G | 15 | 13 | *VMA10* | Activity, assembly |
| | H | 50 | 54 | *VMA13* | Activity (not assembly) |
| $V_0$ | a | 100 | 95 | *VPH1/STV1* | $H^+$ transport, assembly, targeting |
| | d | 38 | 36 | *VMA6* | Activity, assembly |
| | c | 17 | 17 | *VMA3* | $H^+$ translocation, DCCD site |
| | c′ | 17 | 17 | *VMA11* | $H^+$ translocation, DCCD site (?) |
| | c″ | 19 | 23 | *VMA16* | $H^+$ translocation |

V-ATPase activity. It should be noted that the B subunit, unlike the A, alpha and beta subunits, does not possess a typical glycine rich loop consensus sequence [40], although the sequence that corresponds to this region has been highly conserved [41]. The function of the non-catalytic sites on the F-ATPases also remains uncertain, although they have been suggested to play a role in assembly of the F-ATPase complex [42,43].

The remaining $V_1$ subunits (C–H) have been shown from both genetic analysis [44–49] and biochemical studies [50–54] to play an important, although as yet undefined, role in V-ATPase activity. Two subunits (D and E) have been suggested from secondary structure analysis ([55]; B. Bowman, personal communication) as possible candidates to the alpha helical gamma subunit of $F_1$. The gamma subunit has been shown to rotate within the $alpha_3beta_3$ hexamer of $F_1$ during catalysis [56–58], and this rotation is central to the mechanism of energy coupling by $F_1F_0$. The 40-kDa C subunit of the V-ATPases appears to be located on the outside of the $V_1$ complex based upon the stability of $V_1$ formed in its absence [59], and is thus unlikely to correspond to the V-ATPase homolog of gamma. Interestingly, the C, D and E subunits can all be crosslinked to the c subunit of the $V_0$ domain [24], suggesting that they play a role in attachment and coupling of the $V_1$ and $V_0$ domains. Moreover, co-immunoprecipitation has demonstrated a direct interaction between the C and E subunits [59]. Unlike the corresponding $F_1$ domain, the $V_1$ domain does not carry out hydrolysis of MgATP [60], although a $(Ca^{2+})$-ATPase activity of $V_1$ has been reported [50,51].

Increasing evidence has suggested the existence of a second or peripheral stalk in both the V- and F-ATPases [61,62]. For the F-ATPases, this peripheral stalk is believed to function as a stator in holding the $alpha_3beta_3$ hexamer in place relative to the a and b subunits of the $F_0$ domain (see Section 3), and appears to be comprised of the delta and b subunits [61]. Electron micrographs of the V-ATPases ([61,63]; Wilkens and Forgac, unpublished results) suggest that the second stalk in the V-ATPases may be much more extensive than for the F-ATPases, more like a cuff covering three sides of the structure. While it is not yet certain what subunits constitute the second stalk in the V-ATPases, the F, G and H subunits are all possible candidates. In addition, the amino-terminal domain of the 100-kDa a subunit may serve to bind the $F_1$ subunits and anchor this second stalk to the membrane.

### *2.2. Structure and function of the $V_0$ subunits*

The $V_0$ domain is comprised of five different subunits (a, c,c′,c″ and d). The c, c′ and c″ subunits are termed proteolipid subunits because of their highly hydrophobic character and are homologous both to each other and to the 8-kDa c subunit of $F_0$ [64,26]. The F-ATPase c subunit, which contains two transmembrane helices, has been shown by NMR to form a hairpin in the membrane [65]. The V-ATPase c subunit, which contains four transmembrane helices, appears to have been derived by gene duplication and fusion from the F-ATPase c subunit gene [66]. Both the F- and V-ATPase c subunits have a single buried carboxyl group in the last transmembrane helix which is the site of reaction with DCCD and is essential for proton translocation through the $F_0$ and $V_0$ domains [26,67]. The c′ subunit has a similarly placed carboxyl group while the c″ subunit has five transmembrane helices with the critical carboxyl group present in the third [64]. Why the V-ATPase requires three proteolipid subunits whereas the F-ATPase functions with just one is unclear, but it has been definitively demonstrated by mutational analysis [64] that each V-ATPase complex in yeast must contain at least one copy of c, c′ and c″. The c and c′ subunits are together present in a stoichiometry of six copies per complex [21], thus contributing the same number of transmembrane helices (24) as the 12 copies of the $F_0$ subunit c [26]. Electron micrographs suggest that the $F_0$ c subunits are arranged in a ring with the one a and two b subunits to one side [68].

The 100-kDa a subunit is a transmembrane glycoprotein possessing an amino terminal soluble domain and a carboxy terminal hydrophobic domain containing 6–8 transmembrane helices [69]. In yeast, the a subunit is encoded by two homologous genes (*VPH1* and *STV1*), the products of which appear to be targeted to different intracellular membranes [70,71]. Vph1p is targeted to the central vacuole while Stv1p appears to be targeted to some other intracellular membrane, possibly the Golgi or endosomes [71]. While the topography of the 100-kDa subunit has not been established, preliminary results suggest that the amino terminal soluble domain may be exposed to the cytoplasmic side of the membrane (Leng and Forgac, unpublished results). Mutational analysis has identified a number of buried charged residues in the last two transmembrane segments which, while not absolutely essential for activity, may play a role in proton translocation [72,73]. These include E789 in the last transmembrane helix and H743 in the penultimate membrane span. Thus, as with the a subunit of $F_0$ [74,75,26], the 100-kDa subunit may function to allow protons to reach the buried carboxyl group of subunit c and to leave this site for the opposite side of the membrane. The 100-kDa a subunit also appears to serve an important role in assembly of the V-ATPase complex [73].

The 38-kDa d subunit is a cytoplasmically oriented polypeptide [76] that possesses no putative membrane spans but appears to remain tightly associated with $V_0$ through interactions with other $V_0$ subunits [77]. Unlike the $F_0$ domain, the free $V_0$ domain does not normally function as a passive proton channel [78], although it can be induced to passively conduct protons by dissociation and reassembly [79] or acid treatment [80].

## 3. Mechanism of V-ATPases

While the mechanism of neither the V- nor F-ATPases has been established, there has recently been made significant progress on the F-ATPase mechanism which is likely to be relevant to the mechanism of the V-ATPases. One current model for ATP coupled proton movement through $F_1F_0$ suggests that conformational changes in the beta subunit associated with ATP dissociation force the central gamma subunit to rotate within the $alpha_3beta_3$ hexamer [31]. Because gamma appears to be rigidly attached to the ring of c subunits [61], this also induces rotation of the ring of c subunits relative to the a subunit. The a subunit, which is held rigid relative to the $alpha_3beta_3$ hexamer by the b and delta subunits [61], is thought to form two hemichannels which are separated from each other in space [75,81]. For a proton to move from one hemichannel to the other, it must bind to one of the c subunit sites, rotate through the bilayer and dissociate at the other hemichannel. Because the c subunit carboxyl must be protonated to come in contact with the bilayer, this system

allows the rotational energy released on ATP hydrolysis to drive the formation of a linear proton gradient across the membrane. The reverse process happens during ATP synthesis. Interestingly, the V-ATPases possess only half the number of protonateable sites in the ring of c subunits as the F-ATPases [21], thus giving them a lower proton/ATP stoichiometry which is optimal for ATP-dependent proton transport as opposed to ATP synthesis [82].

## 4. Assembly and targeting of the V-ATPases

The most complete information on assembly of the V-ATPase complex has come from studies of the yeast enzyme. In general it has been found that the absence of any of the $V_1$ subunits results in the inability of the $V_1$ domain to attach to $V_0$, although $V_1$ subcomplexes have been observed under such conditions and the assembly and targeting of the $V_0$ domain is unimpaired [83,84]. One exception is the H subunit (product of the *VMA13* gene), whose absence results in an inactive complex in which $V_1V_0$ assembly still occurs, although the resulting complex is less stable [49]. A second exception is the F subunit (product of the *VMA7* gene). In the absence of a functional F subunit, even assembly of the $V_0$ domain appears to be defective, although some targeting of the 100-kDa subunit still appears to occur [47]. Analysis of $V_1$ subcomplexes observed in strains lacking specific $V_1$ subunits [85–87] has led to both the identification of a number of subunit contacts and to a putative assembly pathway in vivo. For the $V_0$ domain, the absence of any of the $V_0$ subunits results not only in the absence of attachment of $V_1$ to the membrane but also in the failure of the remaining $V_0$ subunits to correctly assemble and target to the vacuole [83].

It has also been demonstrated in yeast that there are several proteins (including the products of the *VMA12*, *VMA21* and *VMA22* genes) which function in assembly of the V-ATPase but do not form part of the final complex [88–90]. These proteins appear to be resident ER proteins which form transient associations with specific V-ATPase subunits and aid in the assembly process. A recent report has suggested, however, that the mammalian homolog to Vma21p may form part of the mature chromaffin granule V-ATPase complex, although the stoichiometry of this protein in the complex was not determined [91].

Several subunits have been implicated to play a role in intracellular targeting of the V-ATPases. As noted above, the two isoforms of the 100-kDa subunit in yeast (Vph1p and Stv1p) appear to be targeted to the central vacuole and Golgi or endosomal membranes, respectively [70,71]. Two isoforms of the B subunit have been identified in mammalian cells [41,92], one of which (the brain isoform) is expressed ubiquitously while the other (the kidney isoform) is expressed at high levels in cells (such as renal intercalated cells) which are able to target V-ATPases to the plasma membrane. Two isoforms of the A subunit have also been identified in plants, one of which appears to be localized to the central vacuole while the other localizes to the Golgi [93]. It is thus possible that targeting information exists at several sites within the V-ATPase complex.

## 5. Regulation of vacuolar acidification

One of the central questions concerning the V-ATPases is the mechanism by which cells regulate vacuolar acidification. A variety of studies indicate that cells are able to control the pH of different intracellular compartments independently [94] and evidence has been presented for a number of mechanisms of controlling vacuolar pH.

Studies in yeast [95] have indicated that reversible dissociation of the $V_1$ and $V_0$ domains represents an important mechanism for directly controlling V-ATPase activity. Thus, glucose deprivation causes a rapid dissociation of V-ATPases from approximately 80% assembled to approximately 20% assembled. This process is reversible and independent of new protein synthesis. Evidence for a similar dissociation of $V_1$ and $V_0$ domains during insect molting has been presented [96], although reutilization of $V_1$ domains has not been demonstrated. The existence of separate pools of $V_1$ and $V_0$ domains in mammalian cells [97] suggests that this may be a widely employed mechanism, although the signals that trigger dissociation and reassembly remain unclear.

A second mechanism that involves direct modification of V-ATPase activity is covalent modification of the active site through disulfide bond formation [98,99]. We have demonstrated that disulfide bond formation between $Cys^{254}$ of the bovine A subunit (located in the glycine rich loop consensus sequence) and $Cys^{539}$ (located in the C-terminal domain of the same A subunit) results in reversible inactivation of V-ATPase activity [95]. Moreover, at least half of the V-ATPase in native clathrin-coated vesicles exists in this inactive, disulfide bonded state [98]. We have suggested, therefore, that disulfide bond formation plays an important role in regulating V-ATPase activity in vivo. Mutagenesis studies [36] suggest that this mechanism may not be employed in controlling V-ATPase activity in the Golgi, at least in yeast.

Several other mechanisms have also been suggested to play a role in regulating V-ATPase activity in vivo. Both activator [100,101] and inhibitor [102] proteins have been isolated from tissue extracts, although their physiological role in controlling V-ATPase activity has not yet been established. Uncoupling of proton transport and ATPase activity has also been suggested as a regulatory mechanism [103,104], and several conditions have been shown to cause a change in the tightness of coupling these two activities, including mild proteolysis [76] and high concentrations of ATP [104], but the in vivo signals that control coupling efficiency remain uncertain. Control of pump density through intracellular targeting of V-ATPases has been shown to regulate proton transport across the apical membrane of renal intercalated cells [16], but this mechanism has not been shown to operate in regulation of pump density in intracellular compartments. Finally, because V-ATPases are electrogenic [104], vacuolar acidification can be controlled through regulation of counterion conductance, which in most cases in vivo appears to occur through the action of a parallel chloride channel. The chloride channel in both endosomes [105] and clathrin-coated vesicles [106] has been shown to be regulated by PKA-dependent phosphorylation, suggesting these channels may represent an important control point for vacuolar pH. Because of the diversity of functions that V-ATPases serve in eukaryotic cells, it is likely that the mechanisms for regulation of vacuolar acidification in vivo will reflect a comparable complexity.

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