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Regulation of V-ATPase activity

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TABLE OF CONTENTS

1. ABSTRACT

V-ATPases are ATP-driven proton pumps present in both intracellular and cell surface membranes of eukaryotes that function in many normal and disease processes. V-ATPases are large, multi-subunit complexes composed of a peripheral domain (V_1) that hydrolyzes ATP and a membrane integral domain (V_0) that translocates protons. Because of the diversity of their functions, V-ATPase activity is controlled by a number of mechanisms. Regulated assembly of the V_1 and V_0 domains rapidly modulates V-ATPase activity in response to a variety of cues, including nutrient availability, growth factor stimulation and cellular differentiation. Considerable information has recently emerged concerning the cellular signaling pathways controlling regulated assembly. Acid secretion by epithelial cells in the kidney and epididymus is controlled by regulated trafficking of V-ATPases to the cell surface. Isoforms of subunit a of the V_0 domain both control trafficking of V-ATPases to distinct cellular membranes and confer properties to the resultant complexes that help account for differences in pH between cellular compartments. Finally, differential expression of genes encoding V-ATPases subunits occurs in a number of contexts, including cancer.

2. INTRODUCTION

The vacuolar (H^+)-ATPases are a family of ATP-dependent proton pumps that function to acidify a variety of intracellular compartments as well as to transport protons from the cytoplasm to the extracellular space (1–6). Intracellular V-ATPases function in endocytosis and intracellular membrane traffic to activate dissociation of ligand-receptor complexes necessary for receptor recycling (2). This is important for the continued uptake of ligands such as low density lipoprotein (LDL), a major carrier of plasma cholesterol, as well termination of signaling from receptors such as epidermal growth factor receptor (EGFR). Acidification of endocytic compartments is required for the entry of many envelope viruses, including influenza and Ebola virus, and toxins, such as diphtheria and anthrax toxin (7). These pathogens employ V-ATPase-mediated acidification as a trigger to infect or kill cells. V-ATPases provide the acidic environment within lysosomes essential for protein degradation and for the recovery of the resultant amino acids via proton-coupled amino acid transporters. Within secretory vesicles, they both activate processing of precursor molecules and drive uptake of small molecules, such as neurotransmitters (2). Plasma membrane V-ATPases function in a number of cellular processes, including acid secretion by renal epithelial cells, epididimal clear cells and osteoclasts, which are necessary for plasma pH homeostasis, sperm maturation and bone resorption, respectively (3 6). Plasma membrane V-ATPases are also important in the survival and invasiveness of tumor cells (8-11). Tumor cells are particularly susceptible to V-ATPase inhibition, likely due to the high rate of acid production derived from their dependence on glycolytic metabolism. Moreover, many tumor cells secrete acid-dependent proteases (cathepsins) which depend upon an acidic extracellular environment in order to participate in tumor cell invasion and metastasis (12).

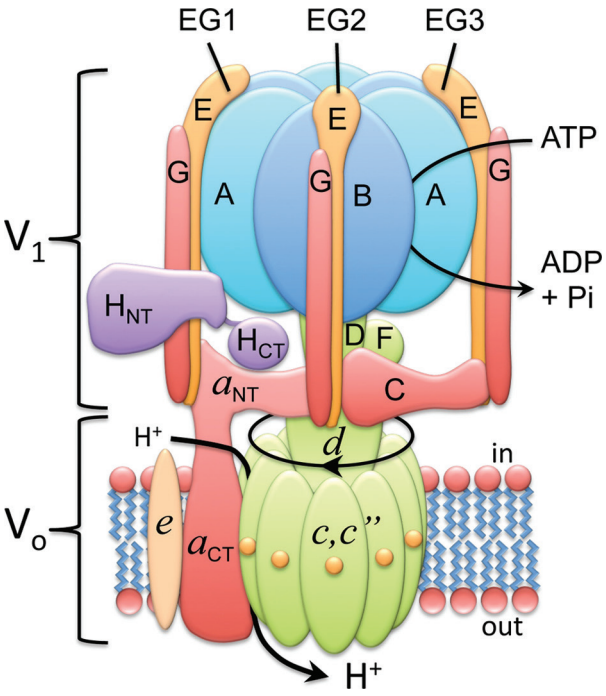

Figure 1. Structure and mechanism of the V-ATPase. The V-ATPase is composed of a peripheral V_1 domain that hydrolyzes ATP and is composed of subunits A-H, and an integral V_0 domain that translocates protons and, in animals, is composed of subunits a, c, c", d and e (1-6). V-ATPases operate by a rotary mechanism in which ATP hydrolysis in V_1 drives proton transport through V_0 (see text for details). The model shown is adapted from reference (99).

V-ATPases are composed of a peripheral V_1 domain that contains eight subunits (A-H) and is responsible for ATP hydrolysis as well as a membrane-integral V_0 domain that (in mammals) contains five subunits (a,c,c",d and e) and carries out proton translocation (Figure 1) (1 2 13). Yeast V_0 contains an additional subunit (c'). V-ATPases operate by a rotary mechanism in which ATP hydrolysis at catalytic sites located at the interface of the A and B subunits drives rotation of a central stalk composed of subunits D,F,d and a ring of proteolipid subunits (c,c") (14 15). The A_3B_3 catalytic domain is prevented from rotating relative to subunit a by peripheral stalks composed of EG heterodimers linked to subunits C, H and the N-terminal cytoplasmic domain of subunit a (16). Each proteolipid subunit contains a buried glutamate residue which undergoes reversible protonation during transport. Protons enter via a cytoplasmic hemichannel located in subunit a and protonate each of the c subunit glutamate residues as they rotate past (17). Once rotation of the ring brings the protonated glutamate residues into contact with the luminal hemichannel of subunit a, protons are released into this channel through stabilization of the deprotonated glutamate by interaction with a critical arginine residue in subunit a (18).

Regulation of V-ATPase activity occurs in both a spatial and temporal fashion. Spatial regulation is observed in the distinct pH values at which various organelles are maintained. Thus, lysosomes are more acidic than late endosomes, which in turn are more acidic than early endosomes (19). This gradient in luminal pH among compartments of the endocytic pathway is important to ensure ligand-receptor dissociation and recycling of cell surface receptors from early endocytic compartments in which lysosomal proteases have not yet been fully activated. Similarly, in intracellular trafficking, late endosomes are more acidic than the trans-Golgi network (TGN), which is more acidic than the cis and medial Golgi compartments (19). This allows binding of lysosomal enzymes to Mannose-6-phosphate receptors in the TGN and dissociation in late endosomes, facilitating recycling of the receptors to the TGN. Examples of temporal regulation of V-ATPase activity include changes in assembly in response to various environmental cues and changes in localization in polarized epithelial cells in response to changes in cytoplasmic pH (see below). This review will focus first on current knowledge of the function and mechanisms of regulating V-ATPase assembly in yeast, insects and mammalian cells. Next, we will consider regulated trafficking of V-ATPases in epithelial cells followed by control of expression of V-ATPase subunits. Finally, we will consider other mechanisms of regulation for which data has emerged.

3. REGULATED ASSEMBLY OF THE V-ATPASE IN YEAST

Reversible dissociation/reassembly of the V-ATPase into its component V_1 and V_0 domains (Figure 2) was first demonstrated in yeast in response to glucose depletion (20) and in insect midgut cells during molting (21; see below). In both cases, dissociation is observed under conditions of limiting nutrient availability and, because the dissociated complexes are inactive with respect to both ATP hydrolysis and proton translocation (22 23), likely reflects an attempt by cells to conserve cellular stores of ATP. In yeast, dissociation occurs rapidly in response to glucose starvation (or replacement with a poorly fermentable carbon source), is reversible and does not require new protein synthesis (20). Interestingly, dissociation requires catalytic activity (24 25), suggesting that the complex must be in the appropriate conformational state to undergo dissociation. Dissociation and reassembly are to some degree independently controlled processes, as dissociation (but not reassembly) requires an intact microtubular network (26) while reassembly (but not dissociation) requires the heterotrimeric RAVE complex (discussed below). Reassembly also requires physical interaction of the V-ATPase complex with the glycolytic enzyme aldolase, which binds to subunits B and E of the V_1 domain and subunit a of the V_0 domain (27). Interaction of the V-ATPase with aldolase is glucose-dependent and mutants of aldolase which fail to bind the V-ATPase but retain catalytic activity are unable to support V-ATPase

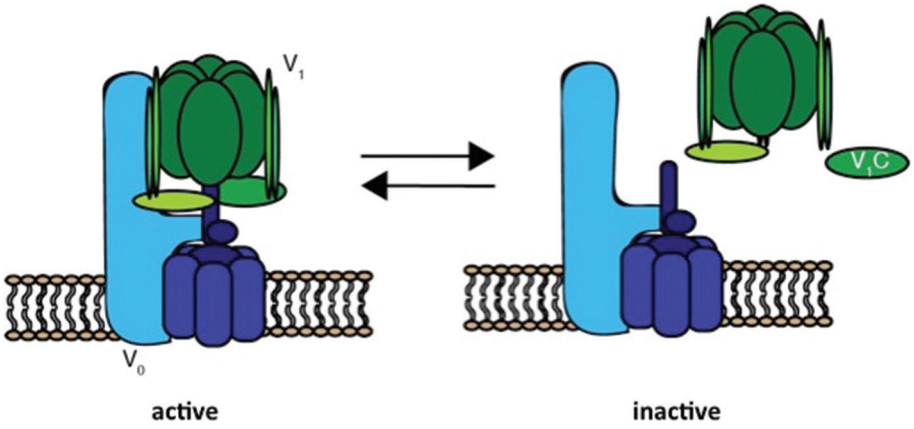

Figure 2. Regulated assembly of the V-ATPase. V-ATPase activity is controlled *in vivo* by reversible dissociation of the active holoenzyme into its component V_1 and V_0 domains, which are inactive. Dissociation is rapid, reversible and occurs in both animal cells and yeast in response to a variety of environmental and temporal cues (see Table 1). In addition, a number of signaling pathways have been shown to participate in regulation of V-ATPase assembly, including PKA, PI3K and mTORC1 (see Table 1 and text for details).

assembly (28). Interestingly, the V-ATPase also interacts with the glycolytic enzyme phosphofructokinase (29), and disruption of this enzyme reduces V-ATPase dissociation in response to glucose withdrawal (30). It is possible that the proximity of the V-ATPase to glycolytic enzymes expedites the export from the cell of metabolically generated acid. While it is true that glycolytic enzymes are likely more abundant than V-ATPases and can exist throughout the cytoplasm, for glycolytic complexes which associate with V-ATPases at the cell surface, glucose that enters the cell via plasma membrane glucose transporters could be rapidly metabolized and the protons generated exported via the associated V-ATPases. Glucose metabolism by glycolytic/V-ATPase complexes associated with intracellular organelles, such as lysosomes, would similarly optimize the export from the cytoplasm of acid generated by glycolysis.

Our laboratory has demonstrated that protein kinase A (PKA) plays a key role in glucose-regulated assembly of the V-ATPase in yeast (31). Using a novel genetic screen, IRA2 was identified as a gene whose disruption results in the V-ATPase remaining assembled even in the absence of glucose. Ira2p is a Ras GAP which, through activation of Ras GTP hydrolysis, converts Ras to the inactive, GDP-bound form (32). In the presence of glucose, Ira2p activity is suppressed, leading to activation of Ras. We further showed that expression of the constitutively active Ras(Val19) mutant also maintains the pump in an assembled state independent of glucose concentrations (31). In yeast, Ras activates adenylate cyclase, leading to increased concentrations of cAMP and activation of PKA (33). PKA is a heterotetramer containing two catalytic subunits and two regulatory subunits (the latter encoded by the BCY1 gene; 33). Disruption of bcy1 leads to constitutive activation of PKA (34). We demonstrated that in bcy1D mutants, the V-ATPase again showed assembly even in the absence of glucose (31). Thus activation of Ras/cAMP/PKA pathway leads to assembly of the V-ATPase in a glucose-independent manner. Interestingly, it has been reported that in yeast elevated glucose leads to increased cytosolic pH, which may in turn promote V-ATPase assembly (35), although interpretation of these results is complicated by the use of a proton ionophore to equilibrate intracellular and extracellular pH. Moreover, the V-ATPase is reported to be required for glucose activation of PKA. Thus, there may exist a positive feedback loop between PKA and V-ATPase assembly in response to elevated glucose.

Several other observations suggest that pH plays a role in controlling V-ATPase assembly in yeast. First, extracellular pH has been shown to be an important determinant in controlling V-ATPase assembly, with dissociation in response to glucose depletion reduced at elevated extracellular pH (36). Importantly, this was shown not to be due to changes in cytoplasmic pH, which was constant under the conditions employed. It has also been shown that neutralization of the yeast vacuole with chloroquine prevents disassembly of the V-ATPase upon glucose starvation (37). Thus the V-ATPase itself or some other pH-sensing mechanism may prevent dissociation of the complex under conditions where some minimal level of vacuolar acidification must be maintained.

An important regulator of V-ATPase assembly is the RAVE complex (38). RAVE is a heterotrimeric complex composed of two novel proteins (Rav1p and Rav2p) and Skp1p, a component of the SCF ubiquitin ligase, and is required for both biosynthetic and glucose-regulated assembly of the V-ATPase (39). RAVE binds to subunits E,G and C of the V_1 domain and subunit a of the V_0 domain (40). Interestingly, although RAVE binds to the V_1 domain in a glucose-independent manner (39), its association with vacuolar membranes (presumably

Table 1. Known regulators of V-ATPase assembly

System	Stimulus	Known signaling pathway	Physiological significance	Reference
Yeast	Glucose	Ras/cAMP/PKA	Energy conservation	(20, 31)
Yeast	Alkaline extracellular/ Vacuolar pH	Unknown	To prevent dissociation of the complex under conditions where some minimal level of vacuolar acidification must be maintained.	(36, 37)
Yeast	Salt stress	PI (3,5) P_2	To increase sodium export from the cytosol.	(47)
Insect midgut cells	Molting	PKA	Energy conservation.	(21, 51-53)
Dendritic cells	Maturation	PI3K/Akt/mTOR	Lysosomal acidification for antigen processing.	(55, 58)
Hek293T cells	Amino acid starvation	Unknown	To increase lysosomal protein degradation and generate amino acid levels sufficient for mTORC activation.	(61)
Primary Rat Hepatocytes	EGF	PI3K	To increase lysosomal protein degradation and generate amino acid levels sufficient for mTORC activation.	(64)
Renal epithelial, MDCK, A549, and HeLa cells	Glucose	PI3K	Thought to reduce acid load due to increased glycolysis. Relevant for cases in which cells are exposed to high glucose levels.	(59, 60, 63)
MDCK and A549 cells	Influenza infection	PI3K Erk	To facilitate cytoplasmic entry of the virus via low-pH mediated fusion of the viral coat with the endosomal membrane	(60, 63)

through interaction with subunit a) is glucose-dependent (40). RAVE has thus been proposed to bind to both V_1 and V_0 as well as subunit C (41), which dissociates from both domains in the absence of glucose, positioning them to promote assembly. Mutagenesis studies of the non-homologous region of the catalytic subunit A suggest that this novel domain, unique to and highly conserved among V-ATPases, also plays a role in glucose-regulated assembly of the V-ATPase (42). Interestingly, the non-homologous region, when expressed in the absence of the remainder of the A subunit, also binds to vacuolar membranes in a glucose-dependent manner (37).

Several other determinants of V-ATPase assembly in yeast have been identified. In particular, the membrane environment has been shown to be important in controlling V-ATPase dissociation. Targeting of V-ATPase complexes to different cellular membranes is controlled by isoforms of subunit a (1 2). Vph1p targets V-ATPases to the vacuole while Stv1p targets complexes to the Golgi (43), with the targeting information localized to the N-terminal cytoplasmic domain of subunit a (44). Vph1p-containing complexes localized to the vacuole undergo dissociation upon glucose depletion whereas Stv1p-containing complexes localized to the Golgi do not (43). Interestingly, if Stv1p is overexpressed, sending Stv1p-containing complexes to the vacuole, dissociation upon glucose depletion is observed (43), suggesting that the vacuolar environment rather than the a subunit isoform is crucial in determining whether the V-ATPase dissociates in the absence of glucose. The importance of membrane environment in controlling V-ATPase assembly has been supported by further studies of dissociation behavior in compartments intermediate between the Golgi and the vacuole (45). Interestingly, RAVE promotes assembly of Vph1p-containing complexes but not those containing Stv1p (46), suggesting that a subunit isoforms play additional roles in controlling assembly beyond their role in targeting of complexes to specific cellular membranes.

Glucose is not the only regulator of V-ATPase assembly in yeast (Table 1). Thus, salt stress also promotes assembly as a way to increase sodium export from the cytosol, and the inositol lipid $PI(3,5)P_2$ is important in this response (47). The function of $PI(3,5)P_2$ in assembly is thought to be through its ability to bind to the N-terminal cytoplasmic domain of subunit a (47).

In vivo dissociation of the V-ATPase complex has typically been measured by Western blot following cell disruption using either membrane fractionation or co-immunoprecipitation of V_1 and V_0 subunits. Under these conditions, subunit C as well as the other V_1 subunits (which remain together as a soluble complex), dissociate from the V_0 domain. Recently, using GFP-tagged subunits, it has been suggested that, under *in vivo* conditions, only subunit C dissociates upon glucose starvation, with the remainder of the V_1 subunits loosely associated with the membrane (48). Given the unstable properties of V-ATPase complexes formed in the absence of subunit C (49), it is likely that dissociation of subunit C upon glucose removal weakens the remaining complex such that, upon cell disruption, the resultant complexes dissociate.

4. REGULATED ASSEMBLY OF THE V-ATPASE IN INSECTS

In goblet cells of the insect midgut, the V-ATPase creates a luminal positive membrane potential across the apical membrane which drives potassium efflux via an electrogenic $K^+/2H^+$ antiporter (50). This facilitates excretion of the large amount of potassium ingested by the insect surviving on a plant diet. During molting, when ingestion of plant material ceases and the need to excrete large amounts of potassium into the gut is reduced, the V_1 domain dissociates into the cytosol (21). While this was not directly shown to be a reversible process, it is likely that at least some of the same dissociated V_1 domains reassemble following molting. Subunit C of the V-ATPase from Manduca sexta was shown to be phosphorylated by PKA as either the free subunit or when part of V_1, but not as part of the V_1V_0 holoenzyme (51). Moreover, following earlier observations that agents that increase cAMP concentrations in blowfly salivary glands increase V-ATPase-dependent luminal acidification (52), it was shown that the same treatments stimulate phosphorylation of subunit C (51). While not directly demonstrating that PKA-dependent phosphorylation of subunit C directly triggers assembly of the V-ATPase, these results are consistent with this hypothesis. In particular, it has not been demonstrated that blocking PKA-dependent phosphorylation of subunit C by site-directed mutagenesis prevents PKA-dependent assembly of the V-ATPase. A PKA-dependent increase in V-ATPase-dependent proton transport has also been demonstrated in Malphigian tubules (53).

5. REGULATED ASSEMBLY OF THE V-ATPASE IN MAMMALIAN CELLS

The first demonstration of regulated assembly of the V-ATPase in mammalian cells came from work on dendritic cells. Dendritic cells internalize and proteolytically process foreign antigens in lysosomes before presentation of the antigen fragments in complex with MHC class II molecules on the cell surface to stimulate T cells to mount an immune response (54). Because antigen processing occurs in lysosomes, it requires a low luminal pH generated by the V-ATPase. It was shown that when dendritic cells are induced to mature by exposure to LPS (lipopolysaccharide, a bacterial antigen), the lysosomes become more acidic and antigen processing is increased (55). Importantly, this increase in V-ATPase-dependent acidification of lysosomes is achieved through increased assembly of the V1 and V0 domains. When dendritic cells in the periphery break cell-cell contacts, they are induced to achieve a semi-mature state in which self-antigens which are internalized and processed are presented on the cell surface and suppress an immune response (56). This is important in immune tolerance. Cluster disruption of dendritic cells *in vitro* mimics the breakage of cell-cell contacts and induces this semi-mature state (57). Our laboratory recently demonstrated that cluster disruption also increases lysosomal acidification and increased assembly of the V-ATPase (58). Importantly, increased assembly of the V-ATPase in dendritic cells is dependent upon both PI-3 kinase and mTORC1 (58).

Exposure of various renal cell lines to elevated glucose concentrations increases both V-ATPase-dependent acidification of intracellular compartments and increased assembly of the V-ATPase (59 60). Cell lines investigated include MDCK, LLCPK and HK2 cells. Acidification of intracellular compartments was measured by staining with the weak base DAMP (59) or the pH sensitive fluorescent probe SNARF-1 (60), while assembly was measured by co-immunoprecipitation of V_1 and V_0 subunits or a change in distribution of V_1 from a diffuse to a punctate pattern as measured by immunofluorescence. Glucose-stimulated assembly could be blocked by inhibition of PI-3 kinase and assembly stimulated at low glucose by increased PI-3 kinase activity (59). It is important to note that increased assembly is reported to occur at a glucose concentration of 25 mM. which is much higher than normal physiological glucose concentrations (4-5 mM), and likely to occur only in patients with uncontrolled diabetes mellitus.

Our laboratory recently observed that amino acid starvation of HEK293T cells increases both V-ATPase-dependent lysosomal acidification and V-ATPase assembly (61). Unlike increased assembly during dendritic cell maturation, upon viral infection (see below) and in response to elevated glucose, increased assembly in response to amino acid withdrawal does not depend upon PI-3 kinase activity, nor does it require mTORC1 activity (61). Thus, a novel signaling pathway is involved in amino acid regulation of V-ATPase activity and assembly. Although the V-ATPase is required for amino acid activation of mTORC1 (62), several lines of evidence indicate that amino acid-dependent changes in V-ATPase assembly are not required for amino acid-dependent changes in mTORC1 activity. First, activation of mTORC1 requires an active V-ATPase whereas elevated amino acid concentrations actually decrease V-ATPase activity. Thus, there is likely some minimal level of V-ATPase activity preserved when levels of amino acids are elevated which is necessary for activation of mTORC1. Second, there is no correlation between the effects of withdrawal of individual amino acids on V-ATPase assembly and mTORC1 activity. If the amino acid-dependent changes in V-ATPase assembly and activity are not involved in regulation of mTORC1, what is their physiological function? We hypothesize that amino acid starvation increases V-ATPase assembly and lysosomal acidification as a means to increase lysosomal protein degradation, thereby increasing the supply of amino acids.

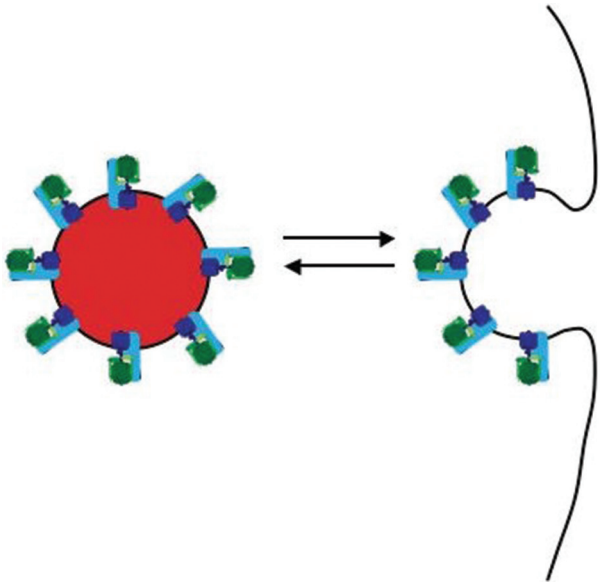

Figure 3. Regulated trafficking of V-ATPases. V-ATPase activity is controlled in various animal systems by regulated trafficking of V-ATPases between intracellular vesicles and the plasma membrane. This mechanism has been shown to occur in a number of epithelial cell types in the kidney, epididymus and insect midgut and to be regulated by PKA (see text for details).

A number of other conditions have also been shown to increase V-ATPase assembly and activity in mammalian cells (Table 1). Infection of cells with influenza virus increases acidification of intracellular compartments, presumably as a means to facilitate cytoplasmic entry of the viral nucleic acid via low-pH mediated fusion of the viral coat with the endosomal membrane following endocytic uptake of the virus (63). This increased acidification was dependent upon PI-3 kinase and ERK (extracellular signal-regulated kinase), which are both activated upon viral infection. Treatment of cells with epidermal growth factor (EGF) stimulates V-ATPase assembly and lysosomal acidification (64), and this is postulated to increase lysosomal protein degradation and generate amino acid levels sufficient for activation of mTORC1. Finally, comparison of V-ATPase assembly in early and late endosomes suggests that assembly and activity increase along the endocytic pathway, consistent with the lower pH of later endocytic compartments (65). This suggests that changes in assembly can result in spatial as well as temporal differences in V-ATPase-dependent acidification.

6. REGULATED TRAFFICKING OF THE V-ATPASE

Several mammalian cell types display regulation of V-ATPase activity via modulation of V-ATPase trafficking (Figure 3). In renal alpha intercalated cells, which are involved in acid secretion into the collecting duct, the density of V-ATPases in the apical membrane is controlled by reversible fusion and budding of a population of apical membrane vesicles containing a high density of V-ATPases with the apical surface (3). Apical insertion of pumps is induced by cytoplasmic acidification and is reversed by endocytic uptake. Microtubules are important in this process as disruption of the microtubular network causes a shift of pumps into intracellular vesicles (66). Interaction with SNARE proteins important for membrane fusion is also involved in V-ATPase redistribution in intercalated cells (67). Increased cAMP levels have been shown to induce translocation of V-ATPases to the apical surface (68), and this signal is thought to be generated by a bicarbonate-sensitive, soluble adenylate cyclase expressed in intercalated cells. Increased cAMP levels in turn activate PKA, leading to direct phosphorylation of the catalytic A subunit of the V-ATPase and translocation to the apical surface (69). AMP kinase also regulates trafficking of V-ATPases in renal intercalated cells via phopshorylation of the A subunit at a distinct site, with increased AMP kinase activity resulting in decreased proton secretion (70). Acid secretion by alpha intercalated cells is also under the control of angiotensin II and aldosterone, both of which stimulate V-ATPase dependent luminal acidification in a Ca/PKC-dependent manner (71 72).

In epididymal clear cells, V-ATPases also cycle between the apical membrane and intracellular vesicles (3). Increased luminal pH leads to increased bicarbonate levels which activate the soluble adenylate cyclase described above (73). This in turn leads to increased PKA activity (74). Involvement of the actin cytoskeleton is supported by the observation that actin depolymerization induced by treatment with inhibitors of RhoA (Ras homolog gene family, member A) or ROCK II (Rho-associated protein kinase II) leads to accumulation of V-ATPases at the apical surface (75). Angiotensin II also stimulates acid secretion from clear cells, but this involves adjacent basal cells and stimulation of the cGMP pathway in clear cells (76).

An additional example of regulation of V-ATPase activity through trafficking comes from osteoclasts. Osteoclasts utilize V-ATPases at the plasma membrane to acidify the extracellular space, a process that is crucial for bone resorption. To accomplish this, osteoclasts express high levels of the a3 isoform of the a subunit, which targets pumps to the ruffled border adjacent to bone (77). Mutations in the gene encoding a3 lead to the disease osteopetrosis, characterized by a lack of osteoclast-dependent bone resorption and a resultant thickening and brittleness of bone (78). In osteoclast precursor cells, a3 is localized to late endosomes and lysosomes (77). Upon induction of osteoclast differentiation, a3-containing V-ATPases translocate to the plasma membrane, likely as a result of fusion of lysosomes with the cell surface. This highlights the fact that cellular localization of particular a subunit isoforms may be cell context dependent. This is supported by

the observation that the a3 isoform localizes to insulin-containing secretory vesicles in pancreatic islet cells (79).

7. REGULATED EXPRESSION OF V-ATPASE SUBUNITS

Another potential means of regulating V-ATPase activity is by regulating expression of V-ATPase subunits. Our laboratory has demonstrated that the a3 isoform of the a subunit is overexpressed in highly invasive breast cancer cell lines (MDA-MB231 and MCF10CA1a cells) relative to poorly invasive lines (MCF7 and MCF10a cells), and that knockdown of a3 reduces invasiveness of the highly invasive but not the poorly lines (8 11). Importantly, knockdown of a3 also reduces plasma membrane localization of V-ATPases in MCF10CA1a cells and overexpression of a3 in the parental MCF10a cells increases both invasiveness and targeting of V-ATPases to the cell surface (8). The importance of plasma membrane V-ATPases for breast cancer cell invasion has been demonstrated by the inhibitory effect of selectively ablating surface pumps on cell invasion and migration (10). The a4 isoform is also overexpressed in MB231 cells and its knockdown reduces both invasiveness and plasma membrane localization of the V-ATPase (11). These results suggest that breast cancer cells are able to up-regulate expression of a subunit isoforms (a3 and/or a4) capable of targeting of V-ATPases to the cell surface, where they promote cell migration and invasion (8 10 11).

Although the mechanism by which plasma membrane V-ATPases promote tumor cell invasiveness is not known, we hypothesize they may promote the activity of secreted, acid-dependent proteases capable of directly promoting invasion. The a3 isoform is also upregulated in highly invasive melanoma cells and its knockdown inhibits both *in vitro* invasion and *in vivo* metastasis (80). The a4 isoform is upregulated in human glioma samples and its expression is correlated with particular subtypes (81). The a2 isoform is upregulated in ovarian cancer tissue and cell lines, although higher levels of a3 expression relative to normal tissue were also reported (82). Cell surface V-ATPases are likely to be important for more than invasion of tumor cells. In particular, cancer cells are more sensitive to induction of apoptosis by V-ATPase inhibitors than normal cells (9). This is likely due to their dependence upon plasma membrane V-ATPases for the excretion of the large amount of metabolic acid generated by their reliance on glycolysis for ATP production. Upregulation of V-ATPase subunits is not restricted to isoforms of subunit a. Thus, subunit C is overexpressed in oral squamous cell carcinoma and its knockdown reduces both tumor growth and metastasis (83), and subunit c is overexpressed in both human pancreatic cancer (84) and human hepatocellular carcinoma and its knockdown in the latter reduces tumor growth and metastasis (85).

How is regulation of expression of V-ATPase subunit genes achieved? The transcription factor TFEB (and the Drosophila homolog Mitf) controls expression of V-ATPase subunit genes as well as other autophagy related genes and is in turn under the control of mTORC1 (86-88). Thus, for example, amino acid starvation decreases mTORC1 activity, which then activates entry of TFEB into the nucleus where it turns on transcription of genes required for autophagy. This makes sense because of the importance of V-ATPase-mediated acidification in lysosomal proteolysis and autophagic flux. The specific V-ATPase inhibitor concanamycin is a commonly used inhibitor of autophagy, blocking both protein degradation and autophagosome-lysosome fusion (89). Whether there are transcription factors responsible for upregulation of particular V-ATPase subunits or isoforms and how these are controlled are important but unanswered questions.

8. OTHER REGULATORY MECHANISMS

Results suggesting additional modes of regulating V-ATPase activity have emerged. Given the function of isoforms of subunit a in targeting of V-ATPase complexes to distinct cellular compartments and the observation that these compartments are often maintained at different pH values (see above), it is logical to ask whether the a subunit isoforms themselves help to determine the pH of the compartment to which they are localized. In fact, data supporting this hypothesis have been published. Thus, comparison of V-ATPase complexes containing the two a subunit isoforms in yeast (Vph1p and Stv1p) has revealed that Vph1p-containing complexes (which are localized to the vacuole) are both better assembled with V1 and are more tightly coupled than Stv1p-containing complexes (which are localized to the Golgi) (43). Coupling refers to the extent to which ATP hydrolysis is coupled to proton translocation, with more tightly coupled complexes displaying a higher level of proton transport per unit of ATP hydrolysis. This higher degree of V_1V_0 assembly and tighter degree of coupling of Vph1p-containing complexes relative to Stv1p containing complexes thus likely contributes to the vacuole being a more acidic compartment than the Golgi (43). Whether similar differences in coupling and assembly of mammalian a subunit isoforms occurs and helps account for differences in acidification of cellular compartments of higher eukaryotes remains to be determined. It is interesting to note, however, that mutations in the non-homologous region of subunit A have been identified that actually increase the coupling efficiency of V-ATPase complexes in yeast (37), suggesting that the wild type complex is not optimally coupled but is instead poised at an intermediate level of coupling which could be either increased or decreased depending upon the needs of the cell. Cellular factors which control the tightness of coupling remain to be elucidated.

Another mechanism proposed to regulate V-ATPase activity involves the reversible formation of a disulfide bond between conserved cysteine residues located at the catalytic site of subunit A (90–92). Disulfide bond formation occurs between a cysteine residue located in the Walker A sequence (GCGKTV) and a cysteine in the C-terminal domain of subunit A involved in binding the adenine ring. Disulfide bond formation likely inhibits ATP hydrolysis by preventing separation of the N and C-terminal domains required for achieving an "open" state associated with nucleotide binding and release (93). A significant fraction of the V-ATPase in clathrin-coated vesicles exists in this disulfide bonded state, suggesting that it represents an important means of regulating activity *in vivo* (90). Interestingly, cleavage of this inhibitory disulfide bond does not occur by simple reduction but rather by internal disulfide exchange with a second cysteine residue in the same A subunit (92). Further evidence for a role of disulfide bond formation in *in vivo* regulation of V-ATPase activity comes from studies in yeast in which alterations in red-ox regulation in cells alters vacuolar acidification (94). How the balance between the active and inactive forms of the A subunit is controlled is an unanswered question.

A variety of regulatory factors have been identified that may also modulate V-ATPase activity. For example, the protein HRG-1 has been shown to associate with the V-ATPase in mammalian cells and to increase V-ATPase activity (95). HRG-1 is an endosomal protein that is upregulated upon treatment of cells with IGF-1 and which is required for endocytic traffic, cell migration and survival. Some of the factors recently identified from a proteomic screen of V-ATPase-associated proteins in the kidney (96) may also play a role in regulation of V-ATPase activity. Regulation of acidification of intracellular compartments is dependent not just upon the rate of proton transport but also upon the rate of proton leakage, making study of the regulation of passive proton channels of prime importance (97). In addition, because acidification of intracellular compartments is an electrogenic process, it is dependent upon the activity of ion channels that can dissipate the luminal positive membrane potential generated during proton transport by the V-ATPase, in particular intracellular chloride channels (98). Regulation of such channels thus represents yet another mechanism of controlling the pH of intracellular compartments.

9. CONCLUSIONS

The diversity of functions performed by the V-ATPases has led to the complexity of mechanisms used to regulate its activity. These include regulated assembly of the peripheral and integral domains, regulated trafficking of the assembled complex, regulated expression of V-ATPase genes, differential properties of complexes containing different isoforms (including differences in assembly and coupling) as well as a number of other mechanisms. Greater understanding of the mechanisms employed to control V-ATPase activity is important to both our basic understanding of cell physiology and to the efforts to therapeutically target the V-ATPase in the development of treatments for diseases in which it participates, including viral infection, osteoporosis and cancer.

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