Review Article

Vacuolar H⁺-ATPase: An Essential Multitasking Enzyme in Physiology and Pathophysiology

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Vacuolar H⁺-ATPases (V-ATPases) are large multisubunit proton pumps that are required for housekeeping acidification of membrane-bound compartments in eukaryotic cells. Mammalian V-ATPases are composed of 13 different subunits. Their housekeeping functions include acidifying endosomes, lysosomes, phagosomes, compartments for uncoupling receptors and ligands, autophagosomes, and elements of the Golgi apparatus. Specialized cells, including osteoclasts, intercalated cells in the kidney and pancreatic beta cells, contain both the housekeeping V-ATPases and an additional subset of V-ATPases, which plays a cell type specific role. The specialized V-ATPases are typically marked by the inclusion of cell type specific isoforms of one or more of the subunits. Three human diseases caused by mutations of isoforms of subunits have been identified. Cancer cells utilize V-ATPases in unusual ways; characterization of V-ATPases may lead to new therapeutic modalities for the treatment of cancer. Two accessory proteins to the V-ATPase have been identified that regulate the proton pump. One is the (pro)renin receptor and data is emerging that indicates that V-ATPase may be intimately linked to renin/angiotensin signaling both systemically and locally. In summary, V-ATPases play vital housekeeping roles in eukaryotic cells. Specialized versions of the pump are required by specific organ systems and are involved in diseases.

1. The Importance of “Housekeeping” Acidification in Eukaryotic Cells

Eukaryotic cells use the localized concentration of protons in vesicles, powered by ATP hydrolysis by the vacuolar H⁺-ATPase (V-ATPase), for essential purposes [1]. Acidic proteases in the lysosome are converted by changes in pH to active forms that can degrade other proteins as in the case of acid cysteine proteinases of the cathepsin family [2]. The low pH also triggers changes in the conformation of proteins that make them more susceptible to proteolytic degradation. In a more selective sense, many proteins are processed by proteolytic enzymes from a pro form, and this is often linked to cycling of the proteins through acidic compartments on their way to their final destination (Figure 1) [3]. In compartments for uncoupling receptors and ligands (CURL compartments) receptors make use of V-ATPase-dependent

acidification to release their ligand in order to allow recycling of the receptor to the plasma membrane and reuse [1].

Equally important, V-ATPases energize membranes and this can be used to power the movement of molecules across membranes by coupled transporters [4]. For example, the uptake of glutamate, a neurotransmitter, into presynaptic vesicles is coupled to proton secretion by V-ATPases.

Accumulating evidence shows that the pH of the lumen of vesicles can be sensed by transmembrane proteins or complexes and that information can lead to changes in the destiny of the vesicle. The V-ATPase has been reported to have such acid sensing ability [5–7]. As will be described in greater detail below, the V-ATPase senses the pH of the lumen of vesicles into which it is embedded and based on that information recruits factors that regulate vesicular trafficking and cytoskeletal reorganizations. In a recently identified variation of this theme, it was reported that sensing of aminoacids

FIGURE 1: Roles of V-ATPases in cell physiology. V-ATPases have vital roles in the Golgi apparatus. Mutations in the $\alpha 2$ -subunit, which is found in the Golgi, result in a form of cutis laxa due to impaired glycosylation. In secretory pathways, energy from a proton gradient generated by V-ATPases may be used to load molecules like glutamate. V-ATPases in endosomal compartments are involved in disassociating complexes, like the mannose-6 phosphate receptor from cathepsins. Acid-dependent enzymes may also become active due to V-ATPase acidification to modify proteins in the lumen of the vesicles. There is also increasing evidence that the V-ATPase may be able to recruit regulators of vesicular trafficking like ARF6. V-ATPases in the lysosome provide an acidic environment for the degradation of proteins by acidic proteases.

by mTORC1 requires ATPase activity by the V-ATPase [8–11]. This central nutrient sensing apparatus is a vital node in physiological signaling, but also represents a potential “Achilles heel” of cancer cells which may be made vulnerable as a more sophisticated understanding of the role of the V-ATPase in the process emerges [9].

Given the importance of acidification, it is not surprising that most eukaryotic cells cannot survive if V-ATPase activity is lost either due to the administration of a selective inhibitor or molecular genetic loss of a ubiquitous subunit [12–14]. An important exception is the yeast *Saccharomyces cerevisiae* which is able to take up protons from acidic media to compensate for lack of V-ATPase activity. V-ATPase-deficient yeast thrive in media at pH 5, but fail to grow in alkaline media. This allows knockouts and replacement knockouts to be performed to analyze functions of specific subdomains of V-ATPase subunits [15]. Yeast has become one of the most powerful model systems for studying the V-ATPase [16]. The fact that the V-ATPase is highly conserved also makes the yeast V-ATPase a semiuniversal model for understanding the basic mechanisms of the pump.

2. Structure and Enzymatic Function of V-ATPases

The mammalian V-ATPase is composed of 13 subunits that can be divided into 8 peripheral proteins which is also called

the V1 and 5 membrane intrinsic proteins are called V0 [17, 18] (Figure 2). The V-ATPase is referred to formally as ATP6 [19]; V1 subunits are ATP6V1A (A-subunit), ATP6V1B (B-subunit), ATP6V1C (C-subunit), ATP6V1D (D-subunit), ATP6V1E (E-subunit), ATP6V1F (F-subunit), ATP6V1G (G-subunit), and ATP6V1H (H-subunit). The V0 domains are ATP6V0a (a-subunit), ATP6V0b (c⁺-subunit), ATP6V0c (c-subunit), ATP6V0d (d-subunit), and ATP6V0e (e-subunit). As indicated in Figure 2, many of the subunits have isoforms. In higher organisms, there are ubiquitous isoforms and isoforms that are selectively expressed in specific cell types. These are associated with subsets of V-ATPases that perform specialized functions. However, specialized V-ATPases represent a mixture of cell type selective isoforms and ubiquitous isoforms. Indeed, some specialized functions may be carried out by V-ATPases that are indistinguishable from the ubiquitous enzyme.

As an example, in mammals there are 4 isoforms of the a-subunit; ATP6V0a1 (a1) and ATP6V0a2 (a2) are ubiquitously expressed and are associated with subsets of housekeeping V-ATPases. ATP6V0a3 (also referred to as TCIRG1, Atp6i, TIRC7, and a3) is found at high levels in osteoclasts [20], microglia [21], gastric parietal cells [22], pancreatic beta cells [23], and perhaps a few other cell types and associates with a specialized subset of V-ATPases that perform nonhousekeeping functions. Osteoclasts, for instance, express a1, a2, and a3 [24]. The first two are thought to perform housekeeping

FIGURE 2: Structure and subunit composition of V-ATPases. The model represents recent proposals regarding the subunit composition, stoichiometry, and organization of V-ATPases. A list of subunits with isoforms is provided. ATP hydrolysis occurs on the A-subunit and it is thought that the three subunits have at any specific time ATP, ADP-Pi, or ADP bound. The conformational changes in the AB heterohexagon promote turning of the central rotor and the ring of c and c' (b) subunits. The pathway of protons through the membranes includes both the rotating cb-ring and the a-subunit.

functions. The last is a component of V-ATPases targeted to a plasma membrane domain and is crucial to osteoclasts bone resorptive function. Knockout of a3 in transgenic mice or mutations that reduce or eliminate its expression or ability to form functional pumps in humans leads to autosomal malignant osteopetrosis [20, 25–27]. Thus, even though a3-knockout osteoclasts contain functional V-ATPases, the housekeeping V-ATPases are unable to compensate for the loss of a3. This appears to be a general theme. The a4-subunit is restricted to kidney intercalated cells, proximal tubules and a few other types of epithelium [28, 29]. There is evidence that a4 may be upregulated and may be able to compensate for lack of a3 in some situations [30]. As with a3, V-ATPases with a1 or a2 do not compensate for the lack of a4 [31].

The V-ATPase can be divided into functional subdomains [1, 18, 32] (Figure 2). The ATP catalytic domain is composed of a heterohexamer of alternating A- and B-subunits. Hydrolysis

of ATP on the A-subunit drives a conformational change in the heterohexamer, which powers rotation of a central rotor. The rotor contains an evolutionarily conserved D-subunit, which serves as the main axle of the rotor, the F-subunit which is associated with the axle and may serve a regulatory function, and d-subunit which couples the axle to the ion pump components of the rotor. The ion pump component is composed of a rotating ring of b- and c-subunits which along with the a-subunit create the channel through which protons move across the membrane as the b/c-subunit ring rotates. A collar domain containing C-subunit and H-subunit is thought to wrap around the stator and link to the transmembrane a-subunit. Three stator arms composed of dimers of E- and G-subunits emanate from three nonequivalent attachment sites [33]. Two attach to the aminoterminal domain of the a-subunit. This large domain (500 aminoacids or so) varies amongst a-subunit isoforms and almost certainly contains information that is involved in differently regulating isoforms of a-subunit. The three stators are known to interact with H-, C-, and a-subunits although the exact binding sites have not yet been determined [34–38]. The stators terminate at and interact with the aminoterminal domain of the B-subunit and the a-subunit interacts directly with the B-subunit [39–41].

V-ATPases contain two coupled rotary motors; one driven by ATP hydrolysis, the other by a proton gradient [42]. Typically, V-ATPases pump protons powered by ATP hydrolysis with the ion motor resisting the pumping activity based on the size of the electrochemical gradient, which consists of both the proton gradient and the membrane potential, against which the V-ATPase is working. The pH of the lumen of vesicles is thought to be “fine tuned” through the actions of voltage-gated chloride channels which have the ability to dissipate membrane potential by allowing entrance of chloride ions, negatively charged counter ions, to protons [43–45].

3. Isoforms and Splice Variants of V-ATPase Subunits

Numerous V-ATPase subunits are present as isoforms. In addition, splice variants have been identified for a number of subunits, although little is known about the functional significance of these variations. Here is a brief summation of the current knowledge of the subunits and their isoforms.

3.1. A-Subunit (70 kDa). The A-subunit contains a Walker consensus sequence which binds ATP. ATP hydrolysis occurs at the interface of the A- and B-subunits [46, 47]. Most of the catalytic site residues are found in the A-subunit [48]. In mammals, only one isoform of A-subunit is present. A splice variant of A-subunit was reported in chickens that lacked the Walker consensus ATP binding sequence, and when expressed it prevented assembly of the V-ATPase [49, 50]. The physiologic significance of this splice variant is not known and no mammalian version has yet been identified.

A number of studies suggest that phosphorylation of the A-subunit by cyclic-AMP-dependent kinase (PKA) may

play a vital role in regulating V-ATPase [51–54]. The key phosphorylation site was identified as serine-175. While data show that A-subunit is phosphorylated and that PKA phosphorylation regulates V-ATPase activity, the underlying molecular mechanism remains unresolved.

3.2. B-Subunit (B2 56 kDa/B1 58 kDa). Two isoforms of B-subunit are expressed in mammals [55]. B2 is expressed ubiquitously and is an element of the “housekeeping” V-ATPase subsets. The B2 mRNA was originally isolated from human brain and it was initially called the brain isoform [56]. It is also present in V-ATPases performing specialized functions including those osteoclasts and in presynaptic neurons [57]. The mRNA for the B1 isoform was first isolated and sequenced from human kidney [58]. It is expressed in type A intercalated cells in the kidney as well as cochlea and the endolymphatic sac of the inner ear, and in epithelial plasma membranes of the epididymis [59, 60].

The B1-isoform has a PDZ-binding domain present at the extreme C-terminus that is missing in B2 [61]. Both B1 and B2 have a high affinity (100–200 nM) microfilament binding domain in the aminotermus [62]. Binding to microfilaments can be achieved by 44 aminoacids, 23–67 in B1 and 29–73 in B2 [63]. Within the overall microfilament binding domain a subdomain called the “profilin-like” domain is present that has sequence similarity to a portion of the actin binding domain of mammalian profilin 1 [63]. Thirteen aminoacid peptides derived from B1 or B2, that included the profilin-like domain, bound monomeric actin with an affinity of 20 μ M and microfilaments with an affinity of about 200 μ M [63]. Other features in the overall binding domain conferred specificity for microfilaments over monomeric actin and the high affinity. A key residue in the overall sequence was phenylalanine 59 in B1 or 65 in B2 [63, 64]. Change of that residue to an alanine or glycine reduced the affinity of the binding interaction by an order of magnitude. Although the sequence of the actin binding domain of B1 and B2 varies substantially, both bound microfilaments with similar affinities [62]. This implies that specialized epithelial cells expressing B1 may make use of microfilament binding. The current understanding of the function of actin binding will be described in a subsequent section.

Yeast only express one isoform of B which displays microfilament-binding activity [64]. However, the actin binding domain starts at aminoacid 10 rather than 23 or 29. The functions of the aminoterminal additions to the B-subunit in B1 and B2 are not known. Figure 3 shows an overall comparison of B2, B1, yeast B, and a B-subunit from an Archaean.

3.3. C-Subunit (C1 42 kDa, C2 48 kDa). Two isoforms of C-subunit are present in mammals [65]. C1 is ubiquitous and C2 is found in the kidney and lung. The C-subunit is thought to wrap the rotor structure and supply an attachment site for the EG stator arm [35–37]. The C-subunit is elongated and dumbbell shaped [66]. It is present in the V1-V0 interface and is thought to contact two of the three EG stator arms [33]. An EG contact site in the head domain (aminoacids 158–277)

FIGURE 3: Location of the actin-binding site on mammalian B2 and B1-subunits and in yeast B compared with an Archaean B-subunit that does not bind microfilaments. Identical colors indicate high conservation. The position of the minimal actin binding domain is indicated by the blue transparent square. As depicted, the C-terminus of the actin binding domain is highly conserved between B2, B1, and yeast, but the N-terminal portion is less well conserved. Part of the sequence that makes up the actin binding domain is missing in the Archaean, and the rest is only modestly conserved. The sequence of the “profilin-like domain,” which is in the red conserved domain, is provided below. Mutation of the phenylalanine to a glycine is sufficient to reduce the binding affinity for actin about 10-fold.

holds a high affinity binding site for an EG stator [36, 37]. Interaction with that binding site stabilizes the EG dimer. The second site is thought to be of lower affinity and may coordinate with one of the a-subunit binding sites.

One mode of regulation of the V-ATPase is the reversible disassembly of the V1 from the V0 leaving a membrane bound V0 and cytosolic V1 [67, 68]. The C-subunit has been suggested to be involved in this reversible disassembly. It was proposed that an unknown environmental stimuli leads to a conformational change in the C-subunit (two different conformations of C-subunit have been observed in different crystals). This change breaks the low affinity EG stator interaction with C-subunit. This is followed by rotation, driven by ATP hydrolysis, that breaks the high affinity interaction. It was noted that the bond energy of the high affinity C-subunit interaction with EG is roughly equivalent to the amount of energy released by the hydrolysis of 1 ATP molecule. This idea may be oversimplified in that there is certainly more bonds than those between EG and C-subunit that must be broken in order to release V1.

Interestingly, like the B-subunit, the C-subunit has been shown to bind actin [69, 70]. Unlike B-subunit, C-subunit binds both monomeric and filamentous actin and C-subunit holds two actin binding domains, one in either end of the barbell. Because of this, evidence was presented showing that C-subunit could crosslink microfilaments into higher order structures [70].

FIGURE 4: Models of C1 and C2a produced by Swiss-Model are shown. Note the insertion in C2a that is derived from the differential splicing (arrow). C2b, like C1, lacks that interruption of the helix found in the splice variant.

It is not clear that the C-subunit, while wrapped around the rotor near the membrane surface, can interact with the relatively massive microfilament. It has been proposed that interactions with actin occur after V-ATPase disassembly [71]. C-Subunit interaction with microfilaments could maintain the location of C-subunit to the area of the disassembled V0, or could be involved in organizing the microfilament network in the area in which the V-ATPase has disassembled. Further studies that precisely identify the actin binding sites and mutate the sites so that actin binding activity is lost will be required to answer these questions.

The C2-subunit has two splice variants, one containing a 48 aminoacid insert not present in C1 [72]. Modeling C1 and C2 using Swiss-Model suggests that the insert breaks a long helical domain and certainly is positioned to have an important regulatory role (Figure 4). The C2 splice variant with the insert (C2a) is located in the lamellar bodies of the lung, whilst the other splice variant (C2b) is found in the plasma membrane of intercalated cells in the kidney. One possibility is that the insert may extend toward the membrane raising the possibility it may interact with membrane lipids or proteins that are tightly associated with the membrane. There are relatively low levels of sequence homology between yeast C-subunit and the human C-subunits (30% identity). Caution must be exercised in interpreting results from studies of yeast or *Manduca* to humans. Given the potential regulatory role suggested for C-subunit, examinations of differences in isoforms and splice variants may prove crucial to a general understanding of how the V-ATPase is regulated.

3.4. D-Subunit (34 kDa). Only one isoform of D-subunit exists in mammals, and the D-subunit has been highly conserved (99% identity between mouse and human and 52% identity between human and yeast). Like the A- and B-subunits, the pressure to maintain the structure for its key role as the rotor likely explains the conservation. In addition, its position and function would suggest that it is unlikely to be directly interacting with elements outside of the pump.

3.5. E-Subunit (31 kDa). There are two isoforms of E-subunit expressed in mammals; E1 is ubiquitous and E2 is found in the testis. The two isoforms are the same length and share over 80% identity.

In recent years it has become well established that E subunit forms a dimer with G-subunit and the two intertwine to make a long extended helical structure that projects from the collar of the V-ATPase to the top of the AB heterohexagon [36]. Although for many years it was accepted that each V-ATPase only contained 1 E-subunit and 1 G-subunit, it is now thought that fully assembled and functional V-ATPases have 3 of each [73].

The EG-dimers represent possible regulatory targets; total disassembly of the V-ATPases into V1 and V0 sections would seem to require disruption of the connections mediated by all three stator arms. This could be accomplished either by regulation at the sites from which the stators emanate from the collar, for example, at subunit C as described above. This would seem to require three different regulatory signals to disrupt the three at the collar. Alternatively regulation of EG dimerization, overall conformation, or at the interaction site at the top of the B-subunit could provide the opportunity to regulate all three stators at once.

The E-subunit has been identified as the binding site of the glycolytic enzyme aldolase both in mammals and in yeast and a variety of evidence suggests that this interaction is a crucial element of a more extensive set of interactions between V-ATPase subunits and glycolytic enzymes [7, 74–82]. These have been proposed to represent a metabolon in which physical interactions between V-ATPase subunits and glycolytic enzymes create functional relationships allowing rapid access for the V-ATPase to ATP and protons, both byproducts of glycolysis [75, 80].

3.6. F-Subunit (14 kDa). Only one isoform of the F-subunit exists in mammals. The F-subunit is required for assembly of the V-ATPase [83–86]. It is not required for rotation of the rotor but is involved in stimulating ATPase activity, perhaps simply by being required for assembly of the pump.

3.7. G-Subunit (13 kDa). Three isoforms of G-subunit are present in mammals. G1 is ubiquitous, G2 is found in the brain, and G3 is expressed in the kidney [65]. As described previously, the G-subunit forms a heterodimer with the E-subunit and the dimers form the three stators. The important role of these stators, the fact that they are exposed on the outside of the V-ATPase, and the fact that disassembly of the stators from the pump must occur for disassembly of the V1 from the V0 and the reverse must occur for

assembly make G-subunit an attractive potential regulatory subunit.

3.8. H-Subunit (50/57 kDa). The H-subunit is known to activate ATP-powered proton pumping in intact V-ATPases and block ATPase activity in free V1 sectors after disassembly [87]. The H-subunit (then called SFD) was originally identified as a component of the bovine V-ATPase that had the capacity to activate isolated pumps in an in vitro system [88]. Work in yeast then confirmed the original observation and also showed the ability of the H-subunit to inhibit the activity of disassembled V1. This prevents hydrolysis of ATP when proton pumping is not possible [87].

H-subunit is located, like C-subunit, at the base of the V1; it interacts with the E-subunit of at least one stator arm. Thus, like the C-subunit, the H-subunit is positioned to be involved in regulatory functions. The H-subunit has armadillo-like repeat domains that bind the medium chain ($\mu 2$) of adapter protein complex-2 [89]. This was shown to interact with the Nef protein from HIV, providing a mechanistic basis for earlier studies that documented an association between V-ATPase and Nef [90, 91]. It was proposed that this interaction might play a vital role in HIV infections. However, after an initial series of articles, nothing further has emerged regarding this intriguing interaction.

3.9. α -Subunit (115 kDa). The α -subunit is of critical importance to V-ATPases. As described previously mammals have four isoforms [31, 92]. Indeed multicellular organisms as simple as *C. elegans* have four isoforms [93] and yeast has two [94]. The $\alpha 1$ -subunit is ubiquitously expressed and likely plays the role of α -subunit for many of the housekeeping V-ATPases. In addition to its role in housekeeping V-ATPases, evidence has emerged that it may be involved in the fusion of synaptic vesicles in presynaptic neurons [95]. It is expressed at high levels in the brain which may reflect a specialized role in neural tissues beyond its normal housekeeping functions.

The $\alpha 2$ -subunit was recently shown to be involved in the pathology of a form of autosomal recessive cutis laxa [96–98]. This $\alpha 2$ mutation results in severe skin wrinkling, it induces generalized connective tissue weakness leading to hernias and hip dislocations and triggers osteopenia or osteoporosis with increased fracture risk, cardiovascular and pulmonary dysfunction, and in some cases, mental retardation that may be associated with brain malformations [96, 99–101]. This syndrome is associated with alterations in patterns of glycosylation and defects in vesicular trafficking in the Golgi apparatus [97, 98, 102]. These results are consistent with previous studies that indicated that $\alpha 2$ is localized to the Golgi [24, 103].

Subunit $\alpha 3$ was first identified as required for osteoclast function in studies of a transgenic knockout mouse [20]. A spontaneous mutation that triggered osteopetrosis in an often studied mouse model proved also to be a mutation in $\alpha 3$ [25]. Soon thereafter, it was shown that about half of the human patients that suffer from autosomal malignant osteopetrosis have mutations in the $\alpha 3$ -subunit [27, 104]. The $\alpha 3$ -subunit is expressed at high levels in osteoclasts, in pancreatic beta

FIGURE 5: Location of the $\alpha 3$ -containing V-ATPases in resorbing osteoclasts. Osteoclasts require expression of functional $\alpha 3$ -subunit even though they also express $\alpha 1$ and $\alpha 2$. The top panels show confocal images of a resorbing osteoclast on a bone slice stained with phalloidin to detect microfilaments and with an anti-E-subunit antibody to detect V-ATPases. Notice that the microfilaments are concentrated in a structure called the actin ring. The actin ring surrounds the V-ATPases in the ruffled plasma membrane where most V-ATPases are concentrated. Below is a schematic showing a side view of a resorbing osteoclasts with actin filaments green and V-ATPases red. The resorption compartment is the site of bone degradation.

cells, in microglia in the brain, in gastric parietal cells, and a few other cell types [21–23, 105–107]. It was found in a variety of tissues in early screens, but it is not clear which cell types [106] it was derived from. There is evidence that it might be linked to protection from pathogen infection [108]. Recently evidence was presented that $\alpha 3$ is expressed in the stomach and that its lack of expression in the stomach might be linked to osteomalacia [22, 109]. It has also been shown to be expressed in cancer cells [110] and to play an important role in cancer growth and metastasis [111].

The $\alpha 3$ -subunit has been studied most extensively in osteoclasts (Figure 5). It is required for the transport of V-ATPases to the ruffled plasma membrane of osteoclasts [20]. Until recently little was known regarding the mechanism by which $\alpha 3$ conferred its ability to target V-ATPases to the plasma membrane. A recent study showed that in osteoclasts the V-ATPases containing the $\alpha 3$ -subunit bound microfilaments whereas the housekeeping V-ATPases, in the same osteoclasts, did not [112]. Evidence has been presented suggesting that the interaction between the B-subunit and microfilaments is also necessary for plasma membrane targeting [113]. It is possible that exposure of the actin binding

sites in the B-subunit to allow microfilament binding may be regulated through the a3-subunit by displacing one or more EG-stators that normally block access [114].

The gene for the a4-subunit was identified as the cause for a form of distal renal tubular acidosis [115]. It is found at high levels in intercalated cells and proximal tubules of kidneys [115]. Like the a3-subunit, the a4-subunit is present in V-ATPases that are targeted to the plasma membrane. Because the B1-subunit, the specialized isoform of B found in these cells, binds microfilaments (at least *in vitro*), it is possible that a4 also modulates microfilament binding. The mechanism by which V-ATPase binding to microfilaments may modulate trafficking of V-ATPases and other proteins will be discussed below.

3.10. b-Subunit (21 kDa). Mammals contain two varieties of the proteolipid subunits (b and c) that make up the spinning transmembrane ring that is directly involved in proton transport [116]. The b-subunit is the less abundant of the two and probably only 1 is present in each V-ATPase [117, 118]. Of the two, the b-subunit is larger (21 kD compared with 16 kD) and has 5 membrane spanning domains compared with 4 in the c-subunit. In yeast there are 3 proteolipid subunits, and all three are required [1].

3.11. c-Subunit (16 kDa). Multiple c-subunits together with a b-subunit compose a rotating membrane-embedded ring. This ring, along with the a-subunit, forms half channels that allow protons to move across the membrane in conjunction with the rotation of the ring [1]. In addition to its vital role in the machinery of the pump, the c-subunit has been shown to interact with Arf6, a small GTPase that directs membrane trafficking and cytoskeletal dynamics [6].

3.12. d-Subunit (38 kDa). There are two isoforms of the d-subunit. The d1-subunit is ubiquitously expressed while the expression of d2 is restricted. In mice, mutation of d2 leads to a mild form of osteopetrosis [119]. To date, no human pathology resulting from d2 mutation has been identified. Mutation of d2 in mice reduces the fusion of osteoclast precursors to form the characteristic giant cells. This is associated with reductions in resorption capacity. It has been proposed that d2 may function independently from its role in V-ATPases as a fusogenic factor [119]. Alternatively, d2 may play a role in osteoclasts that allows the sorting of fusogenic factors to the plasma membrane [114].

Knockout of d2 in mice led to both decreased osteoclast bone resorption and increased rates of bone formation [119]. This suggested that agents might be identified that were directed against d2 and were bone anabolic. Initial enthusiasm has been somewhat tempered by the finding that these mice lose bone at the same rate as wild type rats in a model of post menopausal osteoporosis [120].

3.13. e-Subunit (9 kDa). The e-subunit is probably associated with the a-subunit and is highly hydrophobic. Its position has not yet been definitively reported. It is essential in yeast [121] and is present in *Manduca* [122] and mammals [123].

4. V-ATPase Accessory Proteins

Two accessory proteins to the V-ATPase have been identified: ATP6AP1 also known as Ac45 and ATP6AP2, also known as the (pro)renin receptor. The precise relationship between these accessory proteins and V-ATPase is at best tenuous, and numerous questions remain unanswered. Are these proteins associated only with certain specialized V-ATPases? How do they interact with V-ATPases? What subunits, if any, bind the accessory proteins? How do they affect and regulate V-ATPase activity?

4.1. ATP6AP1. ATP6AP1 (known initially as Ac45) was first found in V-ATPase preparations from bovine chromaffin granules [124, 125]. It can be separated with the V0 complex. It contains an internalization sequence in a 26 amino acid sequence in the C-terminus [126]. Knockout of ATP6AP1 in transgenic mice is lethal in embryos [127]. ATP6AP1 is upregulated during osteoclastogenesis [128]. When knocked down by RNA interference (RNAi) in osteoclast-precursors less fusion was detected, the osteoclast cytoskeleton was altered, and less bone resorption occurred in *in vitro* assays [128, 129]. Overexpression of a form of ATP6AP1 that lacked the internalization domain also impaired bone resorption [130].

In osteoclasts, ATP6AP1 interacts with the small GTPase Rab7 [128], which regulates vesicular trafficking. However, it remains to be demonstrated whether the effects of ATP6AP1 require its association with V-ATPase and its hypothesized ability to regulate V-ATPase activity.

4.2. ATP6AP2. It was a surprise that the V-ATPase accessory protein ATP6AP2, originally referred to as M8/9, proved to be the long sought (pro)renin receptor [131]. This protein has the capacity to stimulate angiotensin signaling by activating (pro)renin, and to directly stimulate ERK1/2, MAPK, and PI 3-kinase signaling pathways [132–135]. ATP6AP2 was also shown to serve as a scaffolding protein linking V-ATPase to the WNT-signaling pathway [136].

ATP6AP2 was originally detected as a 10 kD peptide in V-ATPase isolates prior to the identification of the (pro)renin receptor [123]. Full length ATP6AP2 is 38 kD and the 10 kD portion proved to be the membrane spanning domain left after the (pro)renin receptor portion was cleaved. ATP6AP2 is cleaved by furin, perhaps in a regulated manner, in some cell types to produce 28 kD and 10 kD (M8/9) fragments [137]. Cleavage of furin may be a cell type specific process. Recent genetic data supports ATP6AP2 as a protein intimately linked to V-ATPase [138, 139].

The renin-angiotensin system (RAS) is a systemic hormone system that regulates blood pressure and fluid balance [140]. Hypertension is commonly treated using drugs that block the RAS [141]. Emerging data suggest that local RAS regulation occurs in various tissues including bone [142–145]. In rodents, stimulation of the RAS leads to decreases in bone mass and inhibition of RAS increases bone mass [143, 146–148]. It is possible that both systemic and local RAS regulation may affect bone remodeling and bone quality.

Bone cells express elements of the RAS signaling making local RAS regulation of bone remodeling plausible [147]. RAS regulation of bone remodeling may play a role in integrating bone remodeling with systemic calcium and phosphate regulation.

Binding of either renin or (pro)renin to ATP6AP2 stimulates the enzymatic activity of renin (4-fold) or (pro)renin [131]. ATP6AP2 forms a necessary, renin-independent link between LRP5/6 and Frizzled (Fz) [136] and acidification of endosomes by V-ATPase is required for LRP5/6 phosphorylation and activation of the WNT/ β -catenin pathway.

Full length ATP6AP2 might serve both as the (pro)renin receptor and as a V-ATPase-associated scaffolding protein, but this has not yet been demonstrated [135]. Furin-cleavage of ATP6AP2 yields a soluble 28 KD and an integral 10 kD (M8/9) fragment [137]. It was reported that full length ATP6AP2 represses WNT signaling, perhaps by blocking proton pumping activity. Cleavage of ATP6AP2 by furin released this inhibition [149].

5. Coordinate Expression and Assembly of V-ATPases

V-ATPases are large multisubunit complexes. For them to function the constituent proteins must be expressed in correct quantities and the subunits must be assembled into the active enzymes. This is particularly challenging in that the V-ATPases contains both peripheral and integral components. In addition, there is coordinate regulation required that includes both ubiquitous and cell-specific isoforms. Evidence has accumulated to suggest that both transcriptional and post transcriptional mechanisms are in play to produce the correct amount of V-ATPase proteins in a specific cell at a particular time.

5.1. Transcriptional Regulation. Transcriptional regulation of the ubiquitous V-ATPase isoforms involves TATA-less, G + C-rich regulatory regions containing multiple Sp1, and/or AP-2-like binding sites [150]. This type of promoter is found in other housekeeping enzymes and contains promoters with CpG islands, areas rich in C next to G [151, 152]. The p in CpG refers to the phosphodiester bond joining the C and G residues. CpG islands are usually defined as a region with at least 200 base pairs that is greater than 50% C + G and which has an observed to expected CpG ratio ((Num of CpG)/(Num of C $\times$ Num of G)) greater than 60%. CpG islands are relatively rare in vertebrate genomes. Methylation of cytosines in CpG islands is established as a method for regulation of transcriptional activity; it seems likely that expression of housekeeping V-ATPases may be subject to this form of epigenetic regulation [152–156]. Moreover, because the specialized V-ATPases in cells like osteoclasts require both specialized isoforms and over expression of housekeeping isoforms, methylation of CpG islands may be of particular importance.

mRNAs of genes expressing V-ATPase isoforms that are selectively expressed in the kidney (B1 and a4) are regulated by the forkhead transcription factor Foxl1 [157].

This transcription factor is expressed in relevant tissues in the kidney and is also found in cochlea and the endolymphatic sac of the inner ear and in epithelial plasma membranes of the epididymis, where V-ATPases are required for maintaining spermatozoa in a quiescent state [158].

The expression of a3 is induced by a factor termed the receptor activator of NF- κ B ligand, or RANKL, which is essential for osteoclast differentiation [159]. RANKL also upregulates a3 in microglia [160]. The sequence responsible for RANKL sensitivity is located about 1.6 kb upstream of the a3 coding region. Basal transcriptional activity of a3 is suppressed by the binding of poly (ADP-ribose) polymerase-1 (PARP-1) to this sequence, and RANKL treatment causes degradation of PARP-1, thereby resulting in increased expression of a3 [161, 162]. PARP-1 is a highly conserved nuclear protein that modulates chromatin structure, in some genes causing transcriptional inhibition [163]. A second PARP-1 binding site may similarly regulate binding of a JunD/Fra2 heterodimer which is the most abundant form of AP-1 in mature osteoclasts. It is reasonable to hypothesize that ubiquitously expressed PARP-1 may continually keep the expression of a3 at low levels in cells that are not responding to RANKL signaling, but this remains to be confirmed experimentally [161, 162].

Mechanisms must be in place to coordinate expression of ubiquitous isoforms and cell type specific isoforms. As will be described below, posttranscriptional regulation of the stability and translation of mRNAs likely has a role. It is, however, intriguing that PARP-1 regulation is known to be integrated with DNA methylation [164, 165]. Indeed, PARP-1 has been shown to regulate expression and activity of Dnmt DNA methyltransferase activity. PARP-1 interacts with newly synthesized polymers of ADP-ribose, which inhibits Dnmt methyltransferase activity [166, 167]. Satisfyingly, degradation of PARP-1 downstream of RANKL activity could both allow a3 transcription and block methylation, which might trigger increased expression of housekeeping subunits.

5.2. Posttranscriptional Regulatory Mechanisms. Recent studies point to a key role for V-ATPase 3' untranslated regions in regulating posttranscriptional processing. For example, promoter activity of the c-subunit in RAW 264.7 cells and NIH3T3 fibroblasts was similar despite RAW 264.7 cells expressing 6–8-fold more mRNA [168]. Likewise, mRNA stability of the ubiquitous B2-, E1-, F-, c-, and a1-subunits between the same two cell lines showed that all but B2 were more stable in RAW 264.7 cells. These studies suggest that expression of housekeeping V-ATPases is regulated at both transcriptional and posttranscriptional levels. Molecular genetic analysis of the ubiquitously expressed E1-subunit mRNA showed that an AU-rich element (ARE) near the polyadenylation site of the 3' untranslated region was crucial for regulation of the mRNA's stability. AREs associate with RNA binding proteins and regulate the stability of mRNA transcripts. Pull-down assays showed interaction of the E1 and c-subunit mRNAs with regulatory proteins called HuR, and hnRNP D [169, 170]. A similar result was obtained in studies of colorectal carcinoma cells [171]. These studies

suggest that regulators of mRNA stability play a vital role in modulating V-ATPase expression levels.

HuR has antiapoptotic functions in mammalian cells. It is usually found in the nucleus but it shifts to the cytoplasm in response to various types of stress. In the cytosol HuR is capable of binding its mRNA targets and thereby stabilizing the mRNA. HuR can be activated by hypoxia [172], UV irradiation [173], heat shock [174], and nutrient and energy depletion [169, 170].

Recent studies have identified a second mechanism by which the 3' untranslated regions are involved in regulating V-ATPase subunit expression. Large numbers of microRNAs (miRNAs) and small (22 nucleotides) noncoding RNAs are expressed in higher vertebrates [175]. These miRNAs bind by base pairing with complementary sequences in mRNAs, usually resulting in gene repression via translational suppression or mRNA degradation. A genome-wide linkage analysis of twins in the US and Australia uncovered a role for the $\alpha 1$ -subunit in influencing hypertension and this was linked to miRNA regulation. A single nucleotide polymorphism identified in approximately 5% of the population substitutes a cytosine (C) for a thymidine (T) at position 3246, resulting in lowered systolic blood pressure [176]. Detailed analysis of the $\alpha 1$ mRNA demonstrated that lowered expression was found to be due to the creation of a higher efficiency binding site for miRNA, miR-637 in the C-allele variant. This miRNA, then, is predicted to reduce $\alpha 1$ translation, resulting in alteration of chromogranin A processing and impairment of its progress into the regulated secretory pathway, which has a prohypertension effect. This identifies a functional miRNA binding site within a V-ATPase subunit with human health.

6. V-ATPase Inhibitors

The V-ATPase is important in diseases and disease states and for that reason considerable efforts have been made to identify V-ATPase inhibitors and to develop them for therapeutic purposes. However, to date, V-ATPase inhibitors have not been employed in the clinic. Although specialized V-ATPases (like those in the ruffled plasma membrane of osteoclasts) differ in the inclusion of specific isoforms (i.e., the $\alpha 3$ -subunit) for most other V-ATPases, this does not seem to alter them so that specific or sufficiently selective inhibitors of those V-ATPases are easily identified. In addition, the molecular mechanisms underlying the selective activities associated with the isoforms (e.g., altered vesicular trafficking associated with $\alpha 3$) are not understood. Finally, the inhibitors identified have been from natural sources and are produced as toxins; it may be unlikely that a natural product would develop to inhibit osteoclasts. It is possible that as the structure and mechanisms of the V-ATPase become better known, reverse chemical genetic approaches can be utilized to identify novel selective inhibitors of the specialized subset of pumps. Cancer in particular has shown selective susceptibility to V-ATPase inhibitors and is sufficiently grave to warrant the risk of off target effects. V-ATPases inhibitors have been recently reviewed by several groups [177–179]. A brief summary will be provided below.

6.1. Plecomacrolides. Bafilomycins A_1 , B_1 , C_1 , and D_1 were isolated from *Streptomyces* [180]. These are macrolide antibiotics with a 16-membered lactone ring. Bafilomycin A_1 was identified as the first selective inhibitor of V-ATPases [181]. Concanamycin was isolated in the mid-1980s from *Streptomyces*. [182, 183]. Studies from various groups have identified the c-subunit as being the primary binding site for plecomacrolides [184–186]. There is some evidence also for a-subunit contributing to binding of plecomacrolides. Currently it is thought that these inhibitors function by mechanically perturbing rotation of the b/c-ring with relation to a-subunit.

6.2. Archazolid. Archazolid is produced by the myxobacteria *Archangium gephyra* and *Cystobacter violaceus* and blocks growth of mammalian cells [187] and inhibits mammalian V-ATPases in the nanomolar range [188]. Archazolid competes with concanamycin for a binding site on the c-subunit. Efforts to improve the pharmaceutical properties of archazolids by structure function analysis and characterization of the binding site and mechanism of action are underway [189, 190].

6.3. Benzolactone Enamides. These inhibitors have been extracted from a variety of sources. These molecules have IC_{50} s in the nanomolar range against mammalian V-ATPases [188, 191], but surprisingly do not affect fungal V-ATPases [191].

6.4. Pea Inhibitor. Recently a new V-ATPase inhibitor was identified from peas (pea albumin 1, subunit b) that is active against the V-ATPases from several types of insects that feed on peas, but does not interact with mammalian V-ATPases. This inhibitor is unique for two reasons. It is the first peptide inhibitor of V-ATPases. It is also selective for certain types of insects and does not affect mammalian V-ATPases [192].

7. Rational Approaches to the Identification of New V-ATPase-Directed Therapeutic Agents

To date, inhibitors of the enzymatic or proton pumping activity of V-ATPases that are selective for specialized V-ATPases like those found in the plasma membrane of osteoclasts or intercalated cells of the kidney have not been identified. It is not clear that a natural product that has such specificity would be selected for by evolution. However, it seems likely that such cell type specific inhibitors exist or can be synthesized. Such inhibitors that are useful for application like blocking the plasma membrane V-ATPases of osteoclasts will likely require either isolation of osteoclasts V-ATPases intact and large scale assay of small molecules or rational, structural based approaches. Recently two groups have pioneered such rational approaches.

Manolson and colleagues used yeast two-hybrid screening to identify an interaction between the $\alpha 3$ -subunit and B2-subunit of V-ATPase, and this interaction was confirmed using fusion proteins [40]. Small molecules that disrupt binding between $\alpha 3$ and B2, but not B2 with other

FIGURE 6: Identification of new antiresorptives by disrupting subunit-subunit interactions unique for the a3-containing V-ATPases in osteoclasts. (a) A binding interaction between a3 and B2 was identified, and a high throughput screen was developed to identify small molecules that blocked the interaction. Such molecules would be expected to block V-ATPase assembly. (b) A binding interaction between a3 and d2 was detected and small molecules were sought to block this interaction. In this case, exclusion of d2 from an otherwise assembled pump might be expected. Mice in which d2 was knocked out suggested that this might both disrupt osteoclast resorption and also lead to stimulation of bone formation by osteoblasts.

a-subunit isoforms, and thus might represent a means to selectively target the a3-containing ruffled plasma membrane V-ATPases, were sought (Figure 6(a)). An ELISA system was utilized to determine that in fact B2 binds all isoforms of a-subunit, but with different affinities. The basic system was adapted to use to screen of 10,000 small molecules seeking selective inhibitors of the a3-B2 interaction. This screen resulted in the identification of the molecule 3,4-dihydroxy-N'-(2-hydroxybenzylidene) benzohydrazide. This molecule inhibited the a3-B2 interaction and the ability of osteoclasts to resorb bone in vitro with an IC_{50} of 1.2 μM [40].

The osteoclasts ruffled membrane V-ATPases contain both the a3 and d2 isoforms and interaction was identified between these two subunits as well. A similar strategy was employed to identify luteolin as an inhibitor of a3-d2 interaction [193] (Figure 6(b)). Luteolin inhibited bone resorption, but did not perturb fusion of osteoclast precursors to form giant cells, or formation of V-ATPases. The IC_{50} in vitro for inhibiting bone resorption was 2.5 μM and it did not affect viability of osteoclasts or other cells at concentrations as high as 40 μM . Other biological activities of luteolin include inhibitor of phosphodiesterase [194], tumor necrosis factor α , and interleukin 6 activities [195], as well as a stimulator of heme oxygenase expression [196]. Luteolin was recently shown to reduce wear particle osteolysis in a mouse model [195].

The fact that these strategies have identified fairly potent and selective osteoclast inhibitors from a screen of only 10,000 small molecules is encouraging. It is reasonable to propose

that large scale screens of 100,000's of small molecules may yield very potent and selective osteoclast inhibitors.

Data from our group suggested that direct binding between the B2-subunit of V-ATPase and microfilaments is vital for V-ATPase function in osteoclasts [114]. These data led to the hypothesis that a small molecule that bound the microfilament-binding site on the B2-subunit and sterically-inhibited the interaction between B2 and microfilaments would be a new type of osteoclast inhibitor (Figure 7). A reverse chemical genetic approach making use of supercomputer-based virtual screen initially identified 100 candidates predicted to bind that microfilament binding domain of B2-subunit. A number of the candidates also blocked binding between B2-subunit and microfilaments in test tube assays [197]. The lead molecule chosen from that screen, enoxacin, inhibited osteoclastogenesis and bone resorption by osteoclasts without affecting osteoblasts [197]. Detailed characterization of enoxacin showed that it likely functions by disrupting vesicular trafficking [112]. More recent data showed that a bone-targeted version of enoxacin inhibits orthodontic tooth movement in a rat model [198].

8. Regulation of V-ATPase

8.1. Reversible Assembly into V1 and V0 Components (Figure 8). This process has been described and characterized in detail in yeast [32, 199] and in *Manduca sexta* [71, 200]. The same mechanism has been reported in mammalian dendritic cells [201] and kidney cells [202], but characterization in

FIGURE 7: Identification of new antiresorptives by disrupting binding between microfilaments and the B2-subunit. (a) V-ATPases bind microfilaments through an actin binding site in the B-subunit. Here we have depicted a conformational change in an EG stator arm to allow access to the actin binding site. Alternatively, the EG may disassemble. (b) Experimental evidence suggested that a molecule that bound the B-subunit and prevented interaction with microfilaments might prevent trafficking of V-ATPases to the ruffled plasma membrane and thereby block bone resorption.

FIGURE 8: Regulation of V-ATPase by reversible disassembly of V1 and V0 components. Reversible disassembly might be triggered by phosphorylation by kinases, like protein kinase A, or this may be regulated by signaling lipids. The H-subunit blocks ATP hydrolysis by the free V1 and the V0 does not serve as a proton channel without the presence of V1.

mammalian systems has not been as detailed. Disassembly in yeast and *Manduca* were both shown to be fully reversible [67, 199, 203].

Initial studies showed that reversible disassembly of V-ATPase could be triggered by glucose deprivation. A number of regulatory pathways have now been described that

modulate this process. These include RAVE/rabconnectin [204], aldolase [75, 76], and other glycolytic enzymes, phosphatidylinositol 3-kinase [202] and protein kinase A [205, 206].

When V1 and V0 are disassembled both the ATPase activity of V1 and the ion transport capacity of V0 are inactive.

In the case of V1, inhibition of ATPase activity has been shown to be an activity of the H-subunit [87].

Although the reversible assembly hypothesis is well supported by studies performed by a number of groups in different systems, some vital questions remain unresolved. Perhaps the most perplexing is how, once the V-ATPase is disassembled, the V1 component finds its way back to the V0 for reassembly. It is known that initial V-ATPase assembly in yeasts requires assembly factors that dwell in the endoplasmic reticulum [207] and similar factors are present in mammals.

9. V-ATPase-Binding Proteins

9.1. Neuronal Proteins. Neurotransmitter release and membrane fusion has been tied to activities of the V0 domain. The protein components of the *Torpedo marmorata* electric organ synaptosomes “mediatophore”, a pore-forming protein complex capable of Ca^{2+} -dependent secretion of acetylcholine [42], was shown to include the c-subunit [208]. Evidence from studies on yeast vacuole [209, 210], fly neurons [211], and mouse pancreatic β -cells [23] supports the hypothesis that the V0 domain plays a role in Ca^{2+} -dependent exocytosis. Evidence from developing zebrafish suggest that V-ATPase is involved in brain lysosome-phagosome fusion during phagocytosis [95].

The V-ATPase V0 domain has been shown to interact with the SNARE system (synaptobrevin and synaptophysin) [212], involving Ca^{2+} /calmodulin binding to synaptobrevin [213]. This finding, together with the fact that in *Torpedo marmorata* nerve terminals the a1-subunit interacts with synaptobrevin [214], provides a possible molecular link between V0 and Ca^{2+} -dependent membrane fusion. Disruption of the a1-subunit gene in *Drosophila* inhibited vesicle fusion with the presynaptic membrane, further implicating a post-SNARE role for a1 and V0 in synaptic membrane fusion [211].

9.2. Microfilaments. It was first suggested that V-ATPase might associate with the cytoskeleton in 1997 by Suda's lab [215]. Study of osteoclasts in osteosclerotic mice, which proved to harbor a mutation in the a3-subunit of V-ATPase [25], showed that V-ATPases did not interact with the detergent-insoluble cytoskeleton of osteoclasts. This suggested that a3 might be crucial for a linkage of the V-ATPase to the cytoskeleton. Because the osteoclast from osteosclerotic mice was not able to transport V-ATPases to the ruffled membrane, that suggested that an interaction with the cytoskeleton might be vital for that specialized targeting. In 1999, a direct interaction between microfilaments was detected in osteoclasts and reconstituted using isolated kidney V-ATPases [216]. In addition to showing that this binding interaction was present, evidence was presented that the percentage of V-ATPases bound to microfilaments changed with the activation state of the cells. Resorbing osteoclasts displayed little binding between V-ATPase and microfilaments and little colocalization between the two. Unactivated osteoclasts in contrast displayed a high percentage of V-ATPase bound to microfilaments and high levels of

colocalization. It was then shown that the B-subunit of V-ATPases contained a high affinity microfilament binding site [62]. Both the B2 isoform, which is found in ubiquitously and at high levels in osteoclasts, and B1, which is restricted to certain kidney epithelial cells and a few other epithelial cells, bound microfilaments with similar affinities and intact V-ATPases could be competed from filaments by competition with recombinant B2- or B1-subunits [62].

The precise aminoacids required for binding to microfilaments were identified using fusion proteins, mutagenesis, and peptide analysis and conservative substitutions that eliminated microfilament binding were identified [63]. B-subunits that lacked microfilament-binding activity were tested in osteoclasts and in yeast. In osteoclasts the mutant B-subunit assembled with other V-ATPase components but were not targeted to the ruffled plasma membrane [217]. This suggested that microfilament-binding activity was required for the specialized transport of V-ATPases required for osteoclast activity. In yeast, actin binding activity was not required for growth on standard laboratory media, but was required for growth in the presence of sublethal concentrations of certain pharmacological toxins [217].

Studies from *Dictyostelium* provide a potential underlying mechanism for the V-ATPase-microfilament binding interaction. V-ATPase recruited the Wiscott-Aldrich and Scar Homolog (WASH) complex to acidic vesicles. The WASH complex then triggered local actin polymerization into microfilaments and V-ATPase bound the newly formed dynamic microfilaments and this was required for the sorting of V-ATPases into a daughter vesicle [218] (Figure 9). The WASH complex is evolutionarily conserved and it will be of interest to determine whether the WASH complex is involved in sorting of V-ATPases required for osteoclast formation and ruffled membrane formation.

9.3. Aldolase and Glycolytic Enzymes. In 2001 Lu and colleagues reported that V-ATPase binds directly to the glycolytic enzyme aldolase and presented evidence that that interaction occurred in osteoclasts and proximal tubules of the kidney [219]. This suggested the possibility that the glycolytic metabolon, consisting of binding interactions between various glycolytic enzymes that increases the efficiency of glycolysis [220–222], may at least in some cells include V-ATPases [80]. This idea is attractive because it would provide a ready source of both ATP and protons for the V-ATPase as both are produced by glycolysis. Evidence has been provided that this interaction between V-ATPase and glycolytic enzymes occurs in diverse species ranging from humans to yeast [7, 75, 76].

9.4. Arf6 and ARNO. Both ARF6, a small regulatory GTPase which is a member of the Ras superfamily, and its activator ARNO (cytohesin2) bind V-ATPases [6]. Arf6 bind the transmembrane c-subunit and ARNO binds the a2-subunit [6]. When this occurs ARNO can then binds ARF6 which enhances the rate of nucleotide of change, a process that activates [223, 224] ARF6. ARF6 regulates membrane trafficking and cytoskeletal organization in various cells [223, 224].

FIGURE 9: Proposed mechanism by which binding of microfilaments by V-ATPase can be linked to actin polymerization stimulated by the WASH complex [218]. (A) V-ATPases in a vesicle (or any membrane) may recruit the (B) WASH complex which then stimulates actin polymerization. (C) Actin filaments elongate by addition of monomers at the boundary of the membrane. (C) and (D) V-ATPases bind to an actin in the polymerizing filament and are drawn away from the original membrane as the filament elongates. (D) At some point the V-ATPase-rich membrane separates from the mother vesicle to form a new vesicle. (E) Actin filaments depolymerize and the WASH complex is released.

Importantly, the ability of the $\alpha 2$ -subunit to bind ARNO was dependent on the luminal pH of the associated vesicle allowing V-ATPase to recruit ARF6 and activate it in response to pH [6].

9.5. Mammalian Target of Rapamycin Complex 1 (mTORC1). The mTORC1 complex protein kinase has a vital role as a nutrient/energy/redox sensor and controller of protein synthesis [10, 225]. It is composed of mTor, regulatory-associated protein of mTor (raptor), mammalian lethal with SEC13 protein (MLST8), proline-rich AKT1 substrate 40 (PRAS40), and DEP domain-containing mTOR-interacting protein (DEPTOR). Controllers of mTORC1 include insulin, growth factors, aminoacids, and oxidative stress [9, 225, 226].

V-ATPase is required for aminoacids present in the lumen of the lysosome to activate mTORC1 on the outside of the lysosome [8–11, 227]. This is achieved through aminoacid sensitive interaction between the V-ATPase and Ragulator, a scaffolding complex that links the Rag GTPase to the lysosome. The Rag GTPase then recruits mTorc1 to the lysosomal surface where it is activated and phosphorylates downstream targets like the translation inhibitor 4E-BP1 and the kinase S6 K to promote protein synthesis [225].

9.6. V-ATPase and Exosomes. Exosomes are a class of secreted vesicles [228–230]. The lumen of these vesicles often contains cytosolic proteins like the cytoskeletal proteins actin and myosin, glycolytic enzymes, and mRNAs and microRNAs. The membranes are oriented so that the cytosolic domain of plasma membrane proteins (when they are present in exosomes) faces the lumen and the extracellular domain is exposed to the external milieu. Communication mediated by exosomes includes interaction with cell surfaces and

the delivery of proteins, mRNA, and microRNAs to target cells. V-ATPase subunits have been identified in exosomes but most importantly molecular genetic analysis in *C. elegans* identified an α -subunit of V-ATPase as being vital for the release of certain types of exosomes [231].

9.7. V-ATPases and Cancer. Specialized V-ATPases have been shown to be involved in cancer growth and metastasis. Cancer cells were shown to express high levels of V-ATPase in their plasma membranes [232, 233]. The $\alpha 3$ - and $\alpha 4$ -isoforms were shown to be expressed at high levels in breast cancer cells [110] and knockdown of the $\alpha 3$ -subunit by RNA interference was shown to disrupt the capacity of cancer cells to metastasize in a mouse model [111]. V-ATPase inhibitors have been the subject of interest as potential tools for treating cancer both for direct effects and to prevent multidrug resistance [9, 81, 177, 178, 234–241]. Despite the clear involvement of V-ATPases in cancer, to date therapeutic use of V-ATPase-targeted agents has not reached the clinic. The key may be in identifying regulatory processes (either targeting or regulation of enzymatic activity) that are vital for cancer growth and metastasis, but not for most cells. Because of similarities in the mechanisms involved in osteoclast bone resorption and cancer invasion, efforts to take rational approaches to blocking V-ATPase activity in osteoclasts may prove useful in identifying new agents for treating cancer [41, 114, 242].

10. Summary

V-ATPases play a central role in the physiology of eukaryotic cells. It is increasingly apparent that their ability to pump protons is integrated with many cell processes, often in surprising ways potentially regulating vesicular trafficking and cytoskeletal rearrangements via interaction with Arf6 and ARNO, regulating vesicular trafficking through associations with microfilaments, coupling glycolysis to acidifying vesicles and extracellular spaces, sensing and responding to nutrient levels, and directly linking to the RAS and WNT signaling. In hindsight, it is perhaps not surprising that these roles and others would be taken up by V-ATPases; they are among the oldest, most complex, and powerful of enzymes. It is logical that evolution would find ways to fit the cell's needs to the structure and functions of this enzyme. We expect that as more information emerges, we will learn that V-ATPases are tied to more surprising processes and that these ties once understood will open doors to new understanding of physiology that will result in new approaches to the treatment and prevention of pathologies. It can be expected that soon atomic level structures of V-ATPases will become available that will provide great insight and that these will be indispensable tools for the next generation of V-ATPase researchers. At the same time, in order to make use of detailed structural knowledge, greater understanding of the cell biology of V-ATPases and their place in the networks of regulation within cells will be vital. Because of the immense complexities, this will likely be the work not of decades but of generations.

Conflict of Interests

The author declares that he has no conflict of interests regarding the publication of this paper.

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