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REVIEW

Mechanisms controlling vacuolar H$^+$-adenosine triphosphatase activity: targets for the development of new therapeutic agents for the management of osteoporosis

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Abstract: Recent genetic studies show that mutations in vacuolar H$^+$-adenosine triphosphatase (V-ATPase) subunit isoforms that are selectively expressed in osteoclasts (a3 and d2) lead to both reduced bone resorption and increased bone formation. This implies that pharmaceuticals targeting these subunits or activities that are linked to these subunits might prove to be bone anabolic. The fact that V-ATPase is a ubiquitous "housekeeping" enzyme has made it challenging to directly target the enzymatic activity of the subset of V-ATPases involved in bone resorption; however, the unique mechanisms that control the V-ATPases involved in bone resorption have begun to emerge. These include binding interactions with the cytoskeleton and with proteins involved in regulating the cytoskeleton and membrane trafficking, links to glycolysis, and surprising ties to the renin–angiotensin signaling network. Links between controlling mechanisms and subunit a3 have been identified, suggesting that it may be possible to develop agents that disrupt bone resorptive V-ATPase activity while leaving the housekeeping activities unhindered. The first steps toward using this new information to rationally design novel classes of therapeutic agents have been taken. Such agents might selectively act against osteoclasts both to prevent the initiation of osteoporosis and to restore already-compromised bone.

Keywords: vacuolar H$^+$-ATPase, enoxacin, luteolin, computational chemistry, microfilaments, ARF6

Introduction

The vacuolar H$^+$-adenosine triphosphatases (V-ATPases) are complex, elegant, and multifunctional enzymes.[1–11] V-ATPases utilize energy derived from adenosine triphosphate (ATP) hydrolysis to pump protons against an electrochemical gradient.[2,12] In eukaryotic cells, V-ATPases are vital for acidifying various vesicular populations, including late endosomes, lysosomes, phagosomes and elements of the Golgi.[9] V-ATPases are therefore "housekeeping" enzymes. However, in addition to their housekeeping roles, in a few cell types V-ATPases perform specialized, nonhousekeeping functions.[13–15] These cells are thought to express both housekeeping V-ATPases and an extra subset of V-ATPases that perform specialized function(s). An example is the osteoclast, a cell that has the ability to degrade mineralized extracellular matrix.[16]

Osteoclasts have housekeeping V-ATPases that are located in lysosomes, endosomes, and the Golgi.[17] In addition, they contain a subset of V-ATPases that enter the plasma membrane and acidify an extracellular resorption compartment, an activity essential for bone degradation (Figure 1).[17,18]

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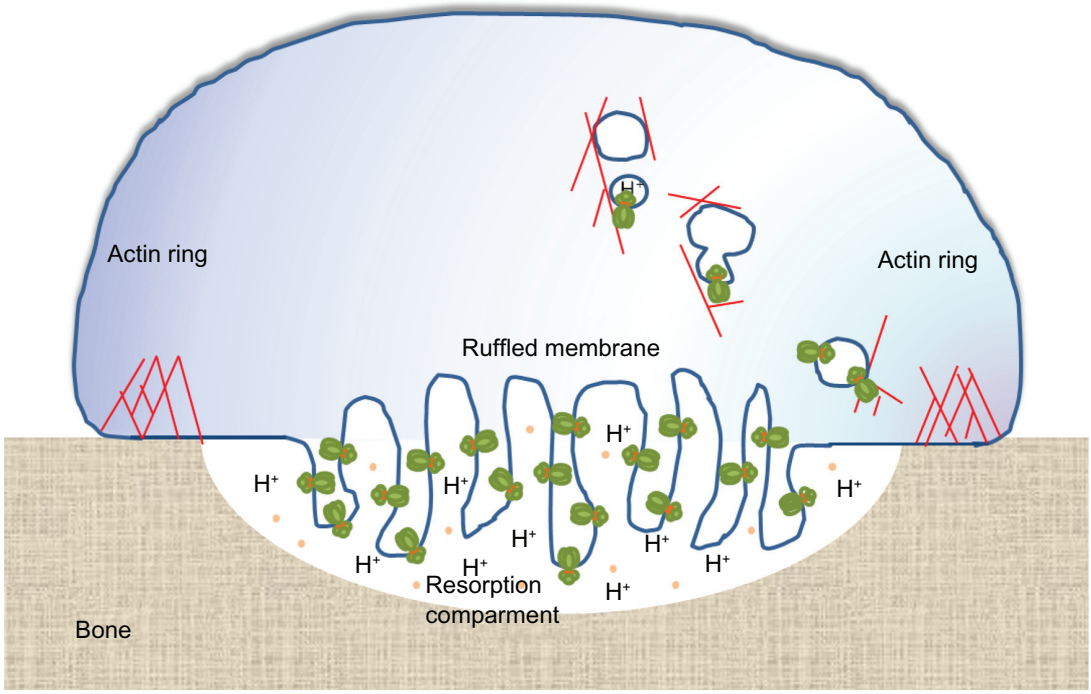

Figure 1 Schematic depiction of a resorbing osteoclast.
Notes: The osteoclast contacts the bone to form a sealing zone that is associated, on the cytoplasmic side, with the actin ring (red lines). Vesicular sorting in the cytoplasm generates vacuolar H+-adenosine triphosphatase (V-ATPase)-rich vesicles that fuse with the plasma membrane to form the ruffled plasma membrane. V-ATPases (green) in the ruffled membrane extrude protons that lower the pH in the resorption compartment to 5.0 or less. This solubilizes the bone mineral and provides an environment for the degradation of the organic matrix by cathepsin K, which is also secreted by osteoclasts.

Just as V-ATPases are complex and elegant nanomachines that utilize ATP hydrolysis with extraordinary efficiency to drive protons across a membrane, osteoclasts gracefully utilize V-ATPases for bone resorption, making use of an unusual, highly dynamic, and coordinated web of interactions involving the cytoskeleton and various membranes.[16] The regulation and molecular machinery involved in this unique use of V-ATPases to degrade bone are candidates to target for antiresorptives directed against the pump.[8,19–24]

A number of specific and potent inhibitors of V-ATPase activity have been identified, including bafilomycin,[25,26] concanamycin,[27,28] SB242784,[29] lejimalides, FR167356,[30] FR202126, FR177995, and diphyllin.[8,22,31] Despite much effort, none of the inhibitors of V-ATPase enzymatic activity have been advanced to the clinic for the treatment of bone disease. Because these agents inhibit both housekeeping and specialized V-ATPases, they are toxic to all cells.

If directly targeting the enzymatic activity of the pumps involved in resorption is impractical, another plausible approach would be to identify the unique elements found in osteoclasts that allow the insertion of V-ATPases into the plasma membrane.[18,32] Because V-ATPases are not normally inserted into the plasma membrane of cells, there may be regulatory elements that are specific for osteoclasts and therefore potential targets for osteoclast-specific therapeutic agents. Understanding of the regulation of V-ATPases in osteoclasts remains surprisingly incomplete. However, as the mechanistic details begin to emerge, the pathways that control V-ATPases in osteoclasts may provide new targets for antiresorptive agents. The ultimate prize could prove to be new therapeutic agents that may be superior to those currently available for the treatment of osteoporosis.[15,31,33–35]

Structure of V-ATPases

Recent and excellent reviews provide current views of V-ATPase structure and function.[6,9,11] Briefly, V-ATPases can be divided into a peripheral domain, referred to as the V_1, and an integral domain called the V_0. The V_1 is a complex of eight subunits and 16 distinct peripheral proteins with the stoichiometry 3A:3B:1C:1D:3E:1F:3G:1H. The subunits are indicated by the nomenclature ATP6V1(A–H)(1–3) where A–H indicates the specific subunit and the number (if present) indicates the isoform.[36] For example, the common designation B2 indicates the number 2 isoform of subunit B with the formal designation ATP6V1B2.[37] Likewise, the V_0 is composed of six subunits that are integral membrane

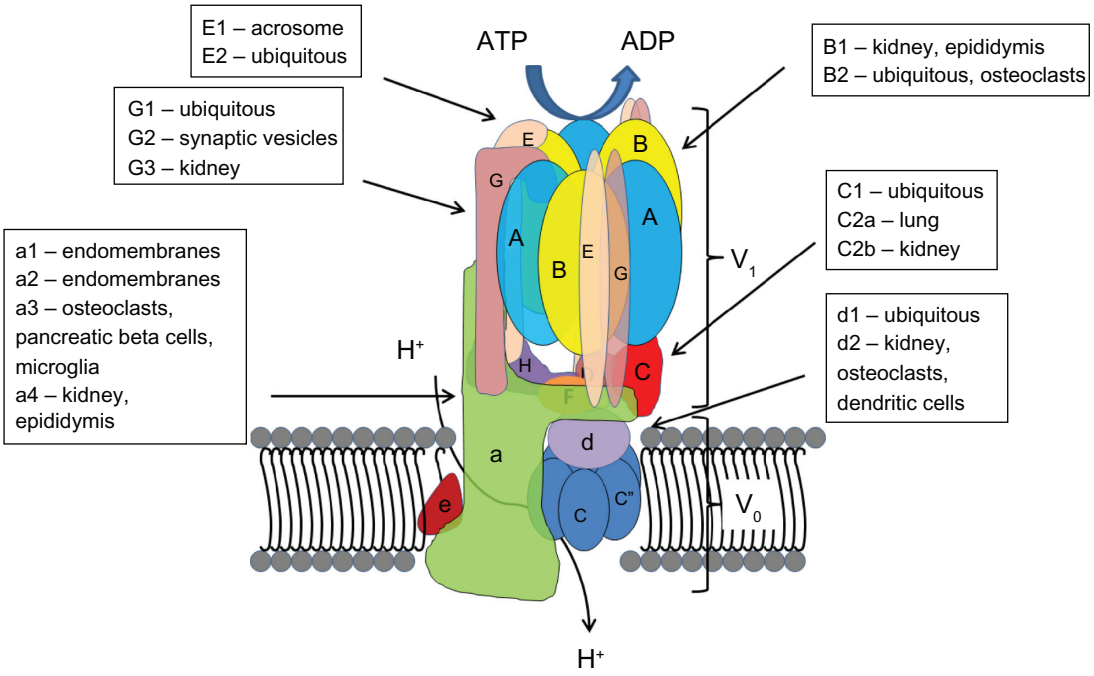

Figure 2 Schematic of generic vacuolar H⁺-adenosine triphosphatase.
Notes: Postulated positions of various subunits are shown and the isoforms of specific subunits are indicated. Schematic is based primarily on Muench et al.[6] The V_1 and V_0 components are indicated by brackets. The subunits of the V_1 are denoted by capital letters and those of the V_0 by small letters, which are the conventional designations.
Abbreviations: ADP, adenosine diphosphate; ATP, adenosine triphosphate.

proteins with the stoichiometry of 1a:1b(c″):?c:1d:1e:1f.[6] These are designated ATP6V0(A–E)(1–4) (Figure 2). For example, a3 is formally designated ATP6V0a3, although it has various names in the literature, including a3, Atp6i, TIRC7, and TCIRG7. Hereafter, subunits will be referred to by their common designations. Additionally, there are two accessory proteins, ATP6AP1 (commonly known as Ac45)[38,39] and ATP6AP2 (commonly known as M8/9 or the [pro]renin receptor).[39,40]

Functionally, the V-ATPase can be reduced to four vital structural elements: the ATPase domain, a rotor, a stator, and the proton channel (Figure 2). The stator includes subunits A, B, C, E, G, H, a, and perhaps e.[6] The rotor consists of subunits D, F, c, c″, and d.[6] ATP hydrolysis occurs in the A subunit, and this powers conformation changes in the heterohexagon, composed of A and B subunits, which turns a central rotor with the force being transduced into a ring of c subunits,[a] which spin in the membrane. These subunits spin in relation to the large integral a subunit and the closely approximating c/b and a subunits provide elements of the pathway for protons to pass through the membrane.[41,42] This pathway is composed of dynamic half-channels that are created as the c subunits rotate past the a subunit.[43]

The a subunit is fixed to the AB heterohexagon through three stator arms. Each arm is composed of a dimer of E-subunit and G-subunit (EG). Which probably contacts the same sites on the B-subunit. Each EG dimer emerges from a different location in the base of the stator apparatus (two distinct sites in the amino terminal domain of the a subunit and from the C subunit).[44] Very recently, a crystal structure of the EG heterodimer in complex with the C subunit was presented.[44]

It is well established that the a-subunit isoforms are tied to the control of different subpopulations of V-ATPase. To illustrate this concept, there are two isoforms of the a subunit present in baker's yeast. One (called Vph1) is normally targeted to the vacuole, while the other (Stv1) is linked to the endosomal pathway.[45] When one is deleted, there is some ability of the other subunit to compensate.[46] The mechanisms by which the differential localization of the a-subunit isoforms are regulated have not been identified, although it is known that the amino terminal domain controls targeting.[47]

Osteoclasts present a more complex picture. Three of the four mammalian isoforms of the a subunit are expressed in osteoclasts.[48] Subunits a1 and a2 are housekeeping isoforms, and are found mainly in endosomes and Golgi. Subunit a3 is upregulated upon stimulation with receptor activator of nuclear factor kappa B-ligand (RANKL, which

[a]In addition to c subunit, mammals also express the b, also called the c′ subunit, and other organisms have a related subunit called c′. It appears that either/or c″ or c′ is included in the ring of c subunits. The number of c subunits per ring has not been established and may vary among organisms.

is required for osteoclast differentiation from hematopoietic precursors). The presence of functional a3-subunit is required for bone resorption. The housekeeping isoforms are unable to compensate for the absence of a3, which results in severe osteopetrosis (autosomal malignant osteopetrosis), leading to death in mice and humans before adulthood.[49,50] Although the bones of patients suffering from autosomal malignant osteopetrosis are much more dense than normal, they are also more brittle, as normal formation of mature bone requires cycles of resorption and formation.

In addition to the a3 subunit in osteoclasts, the d2 subunit is also upregulated and selectively expressed.[15,51] Evidence suggests that d2 may preferentially associate with V-ATPases that also contain a3.[51] However, unlike a3, loss of d2 in mice leads to only a mild osteopetrotic phenotype and a phenotype from mutations of the d2 gene in humans has not been reported.

The fact that a3 and d2 are selectively expressed and trafficked in osteoclasts indicates that specific activities associated with these subunits exist that might be targeted for inhibition of osteoclasts. Below, we will provide a more detailed description of osteoclasts for background and then discuss four topics that relate to the development of therapeutic agents that are directed against V-ATPase for treatment of bone disease. First, evidence suggesting that therapeutic targeting of the controlling mechanisms of V-ATPase in osteoclasts may be particularly useful for the treatment of osteoporosis will be briefly discussed. Second, interactions between V-ATPase and other proteins that may play a role in controlling V-ATPase activity in osteoclasts will be described. Third, evidence that the accessory proteins ATP6AP1 and ATP6AP2 might play a role in regulating bone remodeling will be detailed. Finally, strategies for selectively targeting the unique controlling mechanisms of V-ATPase in osteoclasts have been reported in the literature. The results for these studies will be examined.

Osteoclasts

Osteoclasts arise from multipotential hematopoietic precursors.[52] At early stages, these cells have the potential to differentiate into a variety of cells, ranging from macrophages and T and B cells to osteoclasts, but as they pass through several checkpoints, they become increasingly limited in their potential. Among the last branch points is the decision between dendritic cells and osteoclasts. Indeed, it has been reported that transdifferentiation from dendritic cells to osteoclasts is possible.[53]

An unusual feature of osteoclast differentiation is the tendency of precursors to fuse to form giant cells. Although resorbing osteoclasts both in vitro and in vivo can range from mononuclear cells to giant cells with many nuclei, the formation of multinuclear cells by fusion of mononuclear precursors is usually a characteristic of osteoclasts.[16]

Differentiation of osteoclasts involves up- or downregulation of numerous genes, and the expression of a few genes that are very selective (if not specific) for osteoclasts.[54] The case of V-ATPase subunits in osteoclasts provides illustrative examples. The A and B2 subunits are found in every nucleated eukaryotic cell. However, along with most other components of housekeeping V-ATPases, they are upregulated as osteoclasts differentiate, and are found both in the subset of housekeeping enzymes and also in the specialized enzymes that make their way to the ruffled membrane.[55] The concept that there are multiple subsets of V-ATPases with different roles in the osteoclast is important. Osteoclasts live for many days after they begin resorbing, and like any other cell-type they require V-ATPases for housekeeping functions. An important and still controversial question is whether the housekeeping and specialized V-ATPases have any overlap in function. It has been suggested that the specialized V-ATPases of osteoclasts represent overexpression of a housekeeping lysosomal subset of V-ATPases.[29,56] Alternatively, the ruffled membrane V-ATPases may be a specialized type of V-ATPase whose expression, marked by the presence of the a3 subunit, is normally confined to very few types of cells, including osteoclasts and osteoclast precursors,[49] a subset of microglia,[57] pancreatic beta cells,[58] and perhaps a few other cell types. In targeting a3-containing V-ATPases for pharmaceutical development, it is vital to clarify this issue. The a1 and a2 isoforms of a subunits are components of housekeeping enzymes in osteoclasts, and are not upregulated during osteoclastogenesis.[17,48] The a3 subunit is expressed at low levels in osteoclast precursors, and its expression increases as osteoclasts differentiate.[48]

Even when a cell biochemically expresses the markers of the mature osteoclast, it does not express the morphological features of the resorbing osteoclast until it encounters activation signals associated with the bone surface.[59] Activation typically involves transport of V-ATPases to the ruffled plasma membrane,[18] coordinated formation of the actin ring,[60] and the initiation of uptake of degraded bone mineral and organic components and its transport, by the process of transcytosis to the opposite membrane of the osteoclasts, where these waste components are exocytosed.[61,62]

V-ATPases are on the plasma membrane of osteoclasts only during resorption events; insertion into the membrane is coupled to the formation of the actin ring.[63,64] Initially, both a patch of podosome-like actin filaments and V-ATPases

appear at the site of the nascent ruffled membrane.[64] Over the next few minutes, the ring expands around the forming ruffled membrane. When osteoclasts inactivate, which can be experimentally triggered using phosphatidylinositol 3-kinase inhibitors, the reverse process occurs.[63,65] As the actin ring tightens, the V-ATPases of the ruffled membrane are internalized in endocytosed vesicles.[66] This process is reversible, and under normal conditions osteoclasts undergo numerous cycles of resorption, inactivation, migration, and reactivation.[67]

Targeting V-ATPases in osteoclasts: a route to bone anabolic therapeutic agents?

Osteoclast-directed antiresorptives that are currently used in the clinic are useful for stopping bone loss and have been reported to have modest bone anabolic effects.[68,69] However, these agents (nitrogen-containing bisphosphonates and denosumab) reduce fracture risk only by 50% or less per year.[69] Nitrogen-containing bisphosphonates are targeted to bone by their phosphate backbone and have various side chains that inhibit an enzyme in the mevalonate pathway that disrupts osteoclasts.[70] Denosumab is a humanized monoclonal antibody that blocks binding of RANKL with its receptor RANK, an interaction that is required for osteoclast formation.[69] Given the entry of the post-Second World War baby-boomer generation cohort into their years of greatest risk for osteoporotic fractures, the United States and the rest of the world will be burdened by increasing health-care costs and human suffering due to osteoporotic fractures, unless better methods for treating osteoporosis become available. Estimates suggest that by 2025, treatment of osteoporotic fractures will cost the United States health-care system over $25 billion (in 2005 constant dollars).[71]

In 2006, it was reported that knockout of the d2 isoform of the d subunit of V-ATPase in mice resulted in a phenotype in which bone resorption was reduced and bone formation was stimulated.[15] Because the d2 isoform is expressed in osteoclasts, but not osteoblasts, the implication was that it might be possible to produce a bone anabolic agent by targeting the osteoclast V-ATPase. Similar supporting data were soon produced working with mutants of the a3 subunit.[34] Furthermore, there is evidence that d2 and a3 subunits in osteoclasts are normally found in the specialized, ruffled membrane-targeted V-ATPase subset.[51] It has been formally proposed that agents that target the a3/d2-containing V-ATPases in osteoclasts may yield osteoclasts with reduced resorptive activity, but which produce bone anabolic factors that stimulate bone formation.[31,35]

Despite these exciting results, there are some questions regarding these conclusions. Surprisingly, there was a difference between the osteoclast phenotype comparing the d2 and a3 mutants. Osteoclasts with mutations in a3 fused and appeared normal, but did not make normal ruffled membranes when they encountered bone and could not resorb bone.[49] In contrast, osteoclasts from the d2 knockout did not fuse efficiently, but were able to resorb.[15] It was suggested that d2 might have a non-V-ATPase related "moonlighting" role in cell fusion.[15] In that scenario, the phenotype observed in d2-knockout mice might have nothing to do with the V-ATPase enzyme, but might be linked to disruption of cell fusion. An alternative explanation is that the d1 isoform (unlike the a1 and a2 isoforms of a) may be able to participate in ruffled membrane V-ATPases and allow for some resorption to occur. The failure of the cells to fuse could be the result of disruptions in the trafficking of proteins that are required for fusion to the plasma membrane that are the result of V-ATPase-dependent events. A recently published study shows that a small molecule that blocks interaction between d2 and a3 inhibits bone resorption but not osteoclast differentiation.[72]

Although mice that lack d2 from birth have much higher bone density, they lose bone just as rapidly after ovariectomy as wild-type mice.[73] This may indicate that elimination of the activity that d2 contributes to will not block bone loss associated with menopause. However, the interpretation of this result is greatly complicated by the large difference in bone mass between the d2-mutant mice and wild type at the time of the ovariectomy. Even if knocking out the d2-associated activity does not block the initial large reduction in bone mineral density linked to ovariectomy (or menopause), blocking the d2 activity may alter bone remodeling to help rebuild damaged bone. Further studies will be required to establish whether the role of d2 in osteoclasts is as a component of the V-ATPase complex and/or as a moonlighting fusogen. Also, it will be important to determine whether elimination of the d2-related activity contributes to rebuilding of bone after ovariectomy (in rodents) or after menopause. Without knowing the underlying mechanism for the effects of the mutation, it is difficult to rationally design therapeutic agents that target an activity associated with d2.

Mice lacking the a3 subunit lack osteoclast bone resorption and display enhanced bone formation. Osteoclasts are abundant but inactive with respect to resorption.[49] It is thought that despite their inability to resorb bone, the osteoclasts may still produce factors that stimulate bone formation.[35] A recent study sought to confirm that knocking out a3-associated

activity would improve bone quality.[31,34] They showed that by replacing the marrow of normal a3-containing mice with fetal liver cells from a3-deficient mice, they were able to populate mice with a3-deficient osteoclasts. Measures of bone mineral density and bone strength increased after 12 and 28 weeks.[34] The conclusive test of this hypothesis will ultimately depend on identifying the mechanism by which a3 enables transport of proton pumps to the plasma membrane and then disrupting that activity.

Recently, our group reported that one activity linked to a3-containing V-ATPases is their ability to bind microfilaments directly through the B2 subunit.[74] This will be described below, as well as initial tests of the hypothesis that disruption of a3-linked activities might be a route to novel antiosteoclastic agents.

V-ATPase-binding proteins linked to ruffled membrane V-ATPases

A number of V-ATPase-binding proteins have been identified. These include actin/microfilaments,[75] aldolase and other glycolytic enzymes,[76–79] ADP-ribosylation factor 6/ARF6 nucleotide-binding site opener (ARF6/ARNO),[80,81] HIV Nef protein,[82,83] synaptobrevin and synaptophysin,[84,85] and the Wiskott–Aldrich and scar homolog (WASH) complex.[86] From these, only microfilaments,[63,64,87] aldolase,[76] and ARF6/ARNO[88] have been directly linked to the transport of V-ATPase to ruffled membranes in osteoclasts. Interactions between microfilaments with V-ATPase and aldolase with V-ATPase in osteoclasts have been shown directly.[64,76] Interaction between V-ATPase and ARF6/ARNO in osteoclasts is a plausible and attractive idea, and ARF6 has been shown to be an important regulator of formation of the resorptive apparatus of osteoclasts.[88] Direct evidence for interaction between V-ATPase and ARF6 in osteoclasts has yet to be reported. The binding interaction between V-ATPase and microfilaments is the best characterized. It is mediated by an actin-binding domain in the B2 subunit and is linked to the a3 subunit.[74]

V-ATPase binding to microfilaments in osteoclasts

In osteoclasts, unlike most other cell types and tissues, V-ATPases were isolated bound to large amounts of actin and nonmuscle myosin II.[64] The amount of actin and myosin bound to V-ATPase varied with the activation state of the osteoclasts.[64] It was demonstrated that V-ATPase bound to microfilaments directly and to myosin II indirectly through the microfilaments. In inactive osteoclasts, V-ATPase colocalized with microfilaments as dispersed punctate (probably vesicular) arrays throughout the cell, which were associated with loosely organized microfilaments. During osteoclast activation to resorb bone, microfilaments and V-ATPase concentrated and colocalized in patches at the site of the nascent ruffled membrane.[64] Gradually, microfilaments were organized into a dynamic network of discrete microfilament-based structures called podosomes. Linked together, the podosomes form the actin ring, a specialized structure that delineates the boundaries of the ruffled membrane and which is associated with the sealing zone.[89] An extracellular resorption compartment is "sealed off" by an area of very tight contact between the osteoclast membrane and the bone. These data provided a basis for the hypothesis that regulated binding between the V-ATPase and microfilaments might be required for the trafficking of V-ATPases to the ruffled membrane. It also raised numerous questions. Which subunit(s) mediate interaction with microfilaments? Is the interaction between V-ATPase and microfilaments required for osteoclast function? What is the mechanistic purpose of the binding interaction? How is the interaction regulated? These questions are addressed below.

B2 subunit is required for interaction between V-ATPase and microfilaments in osteoclasts

We identified the B2 subunit as a likely microfilament-binding protein based on several criteria, including blot overlays (also known as far-Western blots) of V-ATPases using microfilaments as a probe, and controlled disassembly of immunoprecipitates of V-ATPase with bound microfilaments.[90] Next, we used recombinant B-subunit fusion proteins to confirm binding between the B subunit and microfilaments and to identify the region in the B subunit that contained the microfilament-binding site. Finally, we were able to narrow the actin-binding site to a 44-amino acid stretch in the amino terminal domain of the B subunit.[63] The domain contained a smaller region that had clear homology to part of the actin-binding domain of the well-characterized actin-binding protein profilin. We were able to eliminate the actin-binding activity of the B subunit by replacing the profilin-like domain with a spacer derived from an Archaea.[63] We then showed in a yeast model that disrupting the actin-binding activity of the yeast B subunit (which also binds microfilaments) does not affect the ATPase or proton-pumping activities of the pump.[91] It did increase the sensitivity of yeast to specific drugs. We added B subunits that lacked microfilament-binding activity to osteoclasts using adeno-associated virus vectors, and found that although they assembled with endogenous subunits to

make pumps, they were not targeted to ruffled membranes.[87] Taken together, these data suggested that the interaction between the B2 subunit and microfilaments in osteoclasts may be required for proper V-ATPase transport in osteoclasts and therefore may be required for osteoclast bone resorption. Along with the data described previously that suggest that V-ATPase-directed pharmaceuticals might be bone anabolic, these data supported the hypothesis that agents that disrupt interaction between the B2 subunit and microfilaments might prove to be a new class of antiresorptives, which may also be bone anabolic.

The B subunit for *Manduca sexta* was shown to bind microfilaments.[92] In addition, the C subunit of *Manduca* is also an actin-binding protein.[92,93] Unlike the B subunit, the C subunit interacts with both microfilaments and monomeric actin. The C subunit contains two actin-binding domains, giving it the ability to cross-link microfilaments into orthogonal arrays. The actin-binding site in the B subunit is physically located in the region farthest away from the associated membrane (if the pump is assembled), suggesting the possibility that the B subunit might mediate the interaction between intact or nearly intact pumps with the microfilaments. Therefore, the B subunit could be involved in transporting or sorting intact V-ATPases. The location of the C subunit within the intact pump would seem to make it difficult for interactions between the C subunit and microfilaments or actin to occur while the pump is intact. It was proposed that the C subunit might interact with microfilaments when pumps are disassembled, perhaps as part of the regulatory mechanism to modulate reversible disassembly.[10] Alternatively, the C subunit may have a housekeeping role outside of its role in the pump that involves interaction with microfilaments. To date, only the *Manduca* C subunit has been shown to bind microfilaments, and evidence that mammalian C subunits have similar activity is lacking. Evidence for the reversible assembly of the pump into discrete V_1 and V_0 subcomponents in osteoclasts has not been reported either.

The B2–microfilament interaction in osteoclasts is associated with the a3 subunit

Data indicating that interaction between V-ATPase and microfilaments is required for V-ATPase transport to the ruffled membranes strongly suggested an association between a3 and the V-ATPase–microfilament interaction. We tested whether a3 was bound to the microfilament-based detergent-insoluble cytoskeleton in osteoclasts. We found that a3, but not a1, isolated by immunoaffinity chromatography from osteoclasts, was bound to microfilaments.[74] To our knowledge, this represents the first evidence for an activity that might explain isoform-dependent localization of V-ATPases.

Why might a3 or a3-containing V-ATPases bind microfilaments, in contrast with a1 or a1-containing V-ATPases? One possibility is that a3 might bind directly to microfilaments, but no evidence that a3 is an actin-binding protein has emerged. A second possibility is that a3 modulates the interaction with microfilaments through the B or C subunits. The recent development of a three-stator arm model of the V-ATPase suggests a possible explanation for how a3 could regulate interaction with B2 in osteoclasts.[6] Until recently, V-ATPases were thought to have one stator arm. During the past decade, advances in methods of isolating and preserving V-ATPases and in the analysis of electron microscope images have led to the transformation of models of the V-ATPase from a one-stator structure to the now widely accepted three-stator model. Each stator terminates in an EG heterodimer that binds the B subunit in a region very near the profilin-like domain. In yeast, the G subunit can be cross-linked to the B subunit with a chemical cross-linker to lysine 45 in the middle of the profilin-like domain.[94] It is very unlikely that microfilaments could interact with B subunits in an intact pump with all three stators in the positions indicated in solved structures. This could explain why V-ATPases in most cells are not bound to microfilaments, despite having three high-affinity microfilament-binding sites in B subunits per pump. However, this implies that for V-ATPase to bind microfilaments, at least one of the stators must be positioned to allow microfilaments access to the actin-binding domain in the B subunit. Since in osteoclasts, V-ATPases containing a3 are isolated bound to microfilaments through the B2-subunit, this subset of pumps must have at least one EG in a position that allows microfilaments access (Figure 3). One possibility would be for the EG to change conformation to allow access of microfilaments to the actin-binding site on the B2 subunit (Figure 3A). Alternatively, disassembly of one or more stators from the enzyme could achieve this regulation (Figure 3B). As a first test of the latter model, we reanalyzed published and unpublished immunoprecipitation data by densitometry.[55,63,64,95] These experiments involved immunoprecipitation with anti-E-subunit antibodies from osteoclasts and other cell types. We found that when actin was not pulled down, the ratio between the A subunit and E subunit was $1.0{:}1.1 \pm 0.2$ ($n = 12$), consistent with three E subunits per V-ATPase. In immunoprecipations where more than four actin molecules were pulled down per A-subunit, the

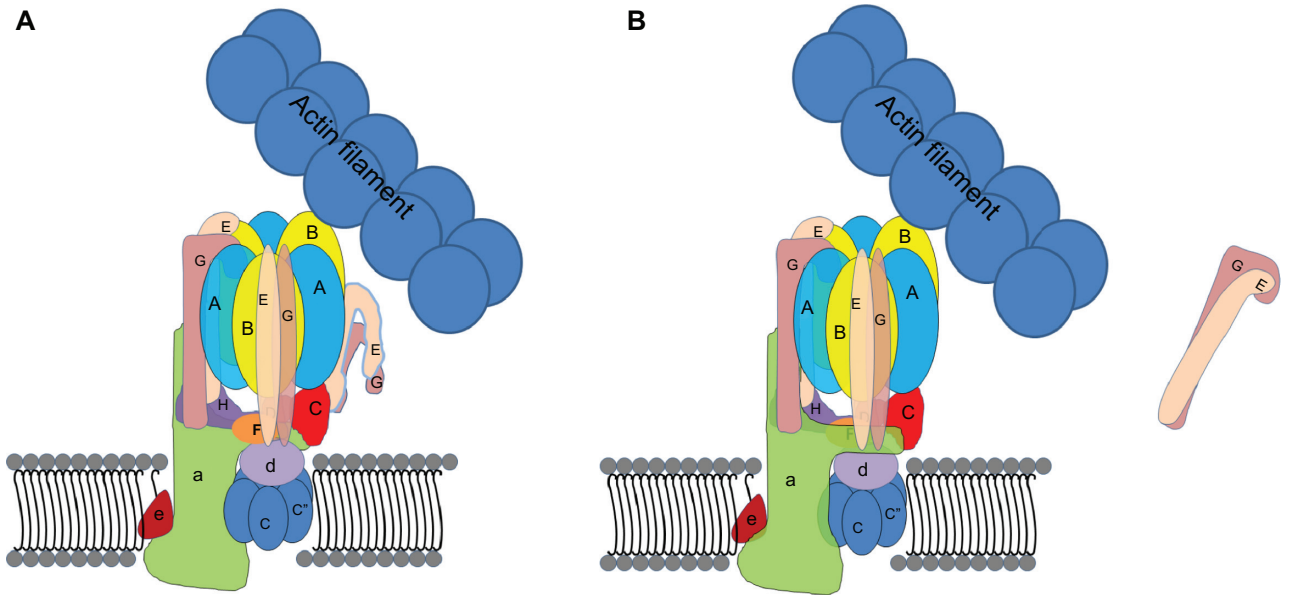

Figure 3 (**A** and **B**) Speculative models depicting vacuolar H^+-adenosine triphosphatase (V-ATPase) bound to microfilaments. (**A**) The actin-binding sites in the three B subunits are predicted to be blocked by the three E-subunit and G-subunit (EG) dimer stator arms. A conformational change has been induced in one of the EGs to allow the microfilament access to the actin-binding site. (**B**) An alternative model is that one of the EGs may be released from the V-ATPase.
Note: Stochiometric data derived from densitometry of V-ATPase immunoprecipitations is consistent with this idea (see text).

ratio between the A subunit and E subunit was $1.0:0.59 \pm 0.3$ ($n = 4$), most consistent with two E subunits per pump.

Whatever the mechanism, regulation of one or more EG stators to V-ATPase could be tied to the reduction of microfilaments bound to V-ATPases in resorbing osteoclasts and the increase in microfilaments linked to V-ATPase during reinternalization from the ruffled plasma membrane as resorbing osteoclasts inactivate. It was shown that V-ATPase–microfilament interaction could be reversibly regulated in osteoclasts by blocking phosphatidylinositol 3-kinase activity.[63] Whether this regulation was achieved by the direct effects of the phosphoinositide or by a downstream effector has not been determined.

To summarize, it has been demonstrated that binding between V-ATPase and microfilaments in osteoclasts is regulated. Binding between V-ATPase and microfilaments appears structurally implausible if three EG stators are positioned as described in solved structures.[6,44] For now, we can only speculate how exposure of the actin-binding domain is regulated, but we believe this is a vital unanswered question.

V-ATPase binding to aldolase in osteoclasts

Yeast two-hybrid analysis was performed seeking binding partners for the E subunit of V-ATPase.[76] The position of E subunits on the outside of the pump in the stator makes it an attractive candidate for interacting with non-V-ATPase proteins.[96] From two hybrid screens, the glycolytic enzymes aldolase and the H subunit of V-ATPase were identified. The identification of the H subunit in the screen was not only of interest for understanding the structural basis of the pump[97] but also increased the confidence that aldolase might also be a true E-subunit binding protein. It was also demonstrated that aldolase could be isolated from osteoclasts in V-ATPase immunoprecipitates and that aldolase concentrated near the ruffled membrane region of resorbing osteoclasts.[76] These data supported the concept that V-ATPases might directly bind aldolase in cells. Because aldolase in turn binds other glycolytic enzymes to form a metabolon,[98] these data suggested that in osteoclasts, V-ATPases might be included in a larger proton-pumping metabolon. Later reports further confirmed a functional binding interaction between aldolase and V-ATPase and provided evidence that glycolysis is coupled to proton pumping in organisms ranging from yeast to mammals.[77,99] Subsequently, phosphofructokinase-1 was shown to bind the a-subunit.[78,79] Coupling between V-ATPase and glycolysis was postulated to directly link both ATP and protons generated by glycolysis to their transport across membranes by way of V-ATPase (Figure 4).

ARF6/ARNO and V-ATPase in osteoclasts

Hurtado-Lorenzo and colleagues reported in 2006 that ARF6, a small regulatory GTPase that is a member of the

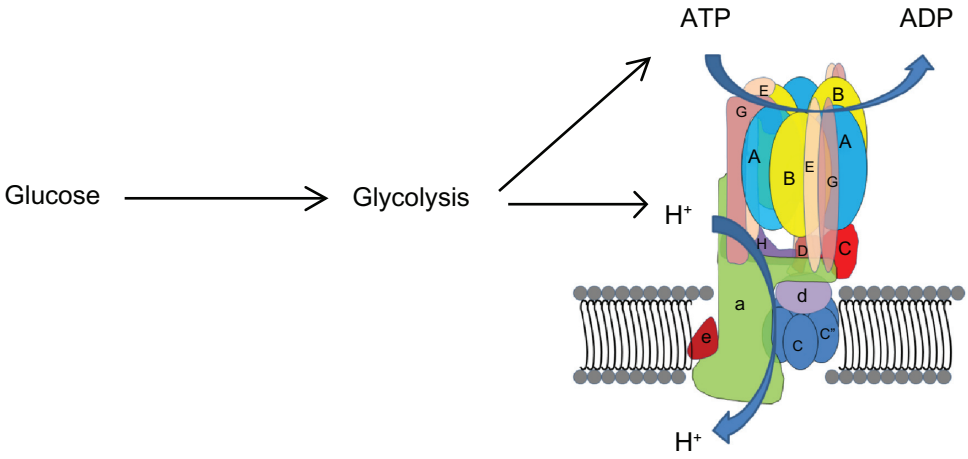

Figure 4 Coupling of glycolysis to vacuolar H+-adenosine triphosphatase (V-ATPase).
Notes: Glycolysis has the capacity to provide both protons and ATP for proton pumping against an electrochemical gradient. In the osteoclast, where large numbers of V-ATPases in the plasma membrane pump copious numbers of protons out of the cell, physical coupling may be required to prevent local pH increases.
Abbreviation: ADP, adenosine diphosphate; ATP, adenosine triphosphate.

Ras superfamily, binds the transmembrane c subunit.[100] They also showed that the ARF6 activator ARF nucleotide-binding site opener (ARNO) binds the a2 subunit.[100] ARNO in turn binds ARF6 and enhances the rate of nucleotide change, a process that activates ARF6.[101,102] ARF6 has been shown to be involved in the regulation of membrane trafficking and cytoskeletal organization in a variety of cells.[101,102] It was also demonstrated that the ability of the a2 subunit to bind ARNO was dependent on the luminal pH of the associated vesicle. This scheme would allow V-ATPase to recruit ARF6 and activate it in response to pH.[100]

In osteoclasts, it was shown that ARF6 played a key role in the regulation of the actin ring in osteoclasts.[88] The actin ring is a higher-level organization of a set of dynamic podosomes.[103] Podosomes (also known as invadopodia) are found in cells that invade tissue through matrix.[104] For example, metastatic cancer cells exhibit podosomes that are similar or identical to the podosomes that make up the actin ring.[105] The actin ring and V-ATPase-rich ruffled plasma membrane are coordinately regulated. ARF6, a regulatory protein that can control both membrane trafficking and the cytoskeleton, and which in turn can be regulated by V-ATPase acidification, may be an important player in the coordinated regulation of formation of the actin rings and ruffled plasma membranes of osteoclasts.

Osteoclasts depend upon the activity of c-Src to resorb bone.[106] It was shown that c-Src represses ARF6 activity by localizing of the Arf GTPase activating protein GIT2.[88] Repression of ARF6 activity is required for the formation of the actin rings and for bone resorption to occur.[88] Recent data suggest that ARF6/ARNO regulation that is associated with the V-ATPase may also be directly tied to glycolysis.[81] ARNO binds aldolase through a pleckstrin homology domain. A knockdown of aldolase in HeLa cells led to alterations both in changes in the distribution of acidic vesicles and cytoskeletal organization. ARNO has been shown to interact with all four isoforms of the a subunit.[81]

This provides a potential scheme in which ARF6 may be activated in response to the V-ATPase-associated vesicles and in response to V-ATPase activity, while inactivation of ARF6 may be linked to the activity of c-Src. The cycle of activation and inactivation may be required to coordinate the formation of actin rings and ruffled membranes.

The accessory proteins: ATP6AP1 and ATP6AP2 and osteoclasts

Both ATP6AP1 and ATP6AP2 are expressed in osteoclasts (Figure 5).[107–109] ATP6AP1 has been shown to have an important role in osteoclasts in vitro. Indirect evidence supports the hypothesis that local and perhaps systemic regulation of bone remodeling occurs via the renin–angiotensin system (RAS). Indeed, this may represent a tie to systemic pathologies that have bone complications. It also may well have implications for the use of pharmaceuticals that modify the RAS, like the commonly used angiotensin-converting enzyme (ACE) inhibitors used to control blood pressure. The exact nature of those effects (if any) will require better understanding of the role of ATP6AP2 in bone. Given the widespread clinical use of ACE inhibitors, we believe it to be worthwhile to investigate the potential consequence of modulating RAS on bone physiology.

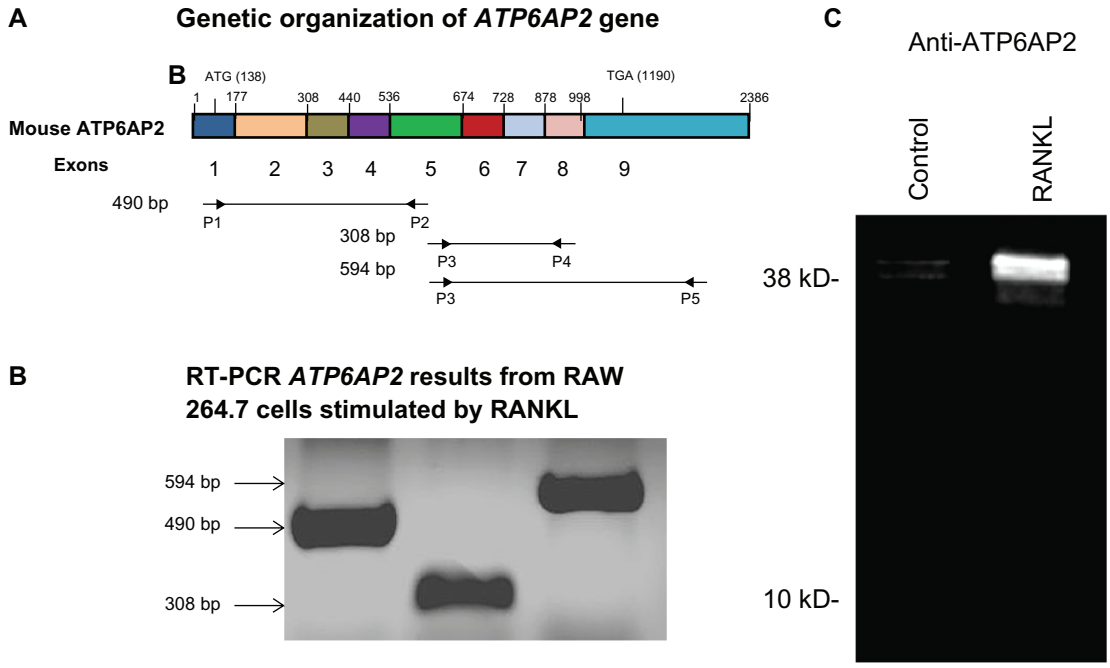

Figure 5 (**A**) The *ATP6AP2* gene. The relative positions of products of three reverse-transcription polymerase chain reaction (RT-PCR) primer pairs tested are indicated. (**B**) To determine if *ATP6AP2* is expressed in osteoclasts, we harvested inactive osteoclasts and performed RT-PCR from the two separate regions of the *ATP6AP2* mRNA indicated in A. Substantial bands of the expected sizes were detected. To confirm this result, the RNA was isolated and sequenced. As expected, the sequence was consistent with *ATP6AP2*. (**C**) Total protein from RAW 264.7 cells that were either stimulated with receptor activator of nuclear factor kappa B-ligand (RANKL) for 5 days to produce osteoclast-like cells, or left unstimulated were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to membranes, and probed using an anti-ATP6AP2 antibody against a peptide with the sequence of the C-terminal 14 amino acids of the protein.
Notes: A doublet at 38 kD was detected in RANKL-stimulated RAW 264.7 cells. We did not detect a specific band at 10 kD (the reported C-terminal product of furin cleavage). Efforts are under way to determine whether signaling triggered by attachment of osteoclasts to bone and the initiation of bone resorption stimulate the cleavage of ATP6AP2.

ATP6AP1

ATP6AP1 was first isolated from bovine chromaffin granules.[38,110] It associates with the V_0 complex. The functions of ATP6AP1 are not well understood. It has been suggested that it plays roles in rotational catalysis of the V_0 domain. The C-terminus carries a 26-amino acid sequence that contains an autonomous internalization sequence.[39] It is well established, however, that knockout of ATP6AP1 in mice results in lethality during early embryogenesis.[111] ATP6AP1 is upregulated as osteoclasts differentiate.[109] Knockdown of ATP6AP1 using virally delivered shRNAs results in less fusion of osteoclast precursors, alterations in cytoskeletal organization, and less bone resorption in vitro.[109,112] Expression of a truncated form of ATP6AP1 that lacked its 26-amino acid internalization domain impaired bone resorption.[107] Recent studies support the notion that in some manner, ATP6AP1 is involved in the regulation of unique vesicular trafficking in osteoclasts that is required for optimal development of the fully mature resorptive phenotype. One study linked ATP6AP1 to the small GTPase Rab7, which is involved in regulating vesicular trafficking.[109] If this binding interaction is vital in osteoclasts, it represents a potential candidate for novel antiresorptives. These recent results are consistent with the theme of a link between V-ATPases, vesicular trafficking, cell fusion, and bone resorption in osteoclasts, which also include the results from the d2-subunit knockout[15] and a recent study of the effects of enoxacin, an inhibitor of V-ATPase binding to microfilaments.[74] The results from ATP6AP1 knockouts could reflect that ATP6AP1 has a possible "moonlighting" role in osteoclast precursor fusion. Perhaps a simpler explanation is that the V-ATPase is a node in specialized vesicular sorting that is required for the insertion of fusogens on the osteoclast precursor cell surface.

ATP6AP2

The V-ATPase accessory protein ATP6AP2 has been shown to be a functional receptor of (pro)renin with the capacity to stimulate angiotensin signaling by activating (pro)renin and to directly stimulate extracellular signal-regulated kinase 1/2, mitogen-activated protein kinase, and phosphatidylinositide 3-kinase signaling pathways.[113–116] ATP6AP2 has been shown to be required for renin-independent scaffolding activity linking V-ATPase to the WNT-signaling pathway.[117] Because the WNT-signaling pathway is vital for bone mineralization

by osteoblasts,[118] it is reasonable to hypothesize that ATP6AP2 may play a crucial role in osteoblasts.[112] Little is known about whether ATP6AP2 might play a role in the regulation of osteoclasts. We have found that ATP6AP2 is expressed at high levels in osteoclasts and is upregulated during osteoclastogenesis (Figure 5). ATP6AP2 could provide a direct link between the RAS and osteoclasts that may be an attractive target for antiosteoporotic therapeutic agents.

The RAS is a systemic hormone system that is involved in the regulation of blood pressure and fluid balance.[119] Drugs that block the RAS are commonly used to treat hypertension.[120] However, recent data suggest that local RAS regulation also occurs in various tissues, including bone.[121–124] Artificial activation of the RAS leads to decreases in bone mass, and inhibition of RAS, using inhibitors of ACE, leads to increased bone mass.[122,125–127] These data support the idea that both systemic and local RAS regulation may affect bone remodeling and bone quality. Bone cells express elements of the RAS-signaling network, suggesting that local RAS may be vital for regulating bone remodeling.[126] Local RAS regulation could integrate with systemic regulation and/or respond to local stimuli.

When renin is bound to ATP6AP2, the enzymatic activity of renin is increased fourfold.[128] ATP6AP2 also binds and activates (pro)renin without cleaving it. Recently ATP6AP2 was shown to form a necessary, renin-independent, link between low-density lipoprotein receptor-related protein (LRP)-5/6 and Frizzled (Fz).[117] Acidification of endosomes by V-ATPase was shown to be necessary for LRP5/6 phosphorylation and activation of the WNT/β-catenin pathway, which is required for osteoblast differentiation and bone formation.

There are three different products of the *ATP6AP2* gene that have been described in cells. Full-length ATP6AP2 likely has the capacity to serve both as the (pro)renin receptor and as a V-ATPase-associated scaffolding protein.[116] Full-length ATP6AP2 can be cleaved by furin in some cell types to yield 28 KD and 10 kD (M8/9) fragments.[129] The 10 kD fragment was initially described as a V-ATPase accessory protein called M8/9.[130] In osteoclasts, we have only detected the full-length 38 kD protein (Figure 5). Interestingly, ATP6AP1 has been reported to be cleaved by furin in the pancreas[131] and in *Xenopus*,[111] but furin-cleaved ATP6AP1 has not been reported by groups studying the protein in osteoclasts.[108,109,112]

A very exciting and potentially illuminating study of ATP6AP2 recently appeared that provides evidence for the functional consequences of furin cleavage of ATP6AP2.[132] Data from this study suggest that full-length ATP6AP2 acts as a repressor of WNT signaling, probably by blocking proton-pumping activity. Cleavage of ATP6AP2 by furin releases this inhibition. It is possible that association of full-length ATP6AP2 with V-ATPase may inhibit its proton-pumping activity, and furin cleavage may be a major regulatory node in V-ATPase regulation.

Efforts to therapeutically target V-ATPases in osteoclasts

Recently, two groups have made efforts to rationally identify novel osteoclast inhibitors based on emerging information about the ruffled membrane V-ATPases. Kartner and Manolson reasoned that because a3 and d2 are found together in ruffled membrane V-ATPases, there is an element of specificity in binding between the two.[14] By using a high-throughput assay, they sought small molecules that disrupt the interaction between both a3 and B2[133] (Figure 6A) and a3 and d2[72] (Figure 6B), while not blocking interaction between other a and d isoforms. We found that a3-containing V-ATPases selectively interacted with microfilaments[74] and that mutation of the actin-binding site in the B subunit disrupted V-ATPase transport to ruffled membranes.[87] We sought small molecules that blocked this interaction (Figure 6C).[75] As described below, both groups identified novel inhibitors of osteoclast bone resorption in vitro. Efforts are underway to test these inhibitors in vivo. The success of these initial efforts also promises that better understanding of the mechanisms by which V-ATPase is transported to ruffled membranes will identify new therapeutic targets.

The primary advantage of these strategies is that they represent approaches to specific targeting of V-ATPases containing the a3 subunit. Mutations of the a3 subunit lead to a bone anabolic condition. Even though the osteoclasts cannot resorb bone, it is thought that they produce factors that stimulate osteoblasts to form bone.[35] It is possible that these new therapeutics will be selectively active against osteoclasts to block bone resorption, and leave the osteoclasts in a state where they can stimulate osteoblastic bone formation. There remain the concerns that they will affect other cells that contain a3 (pancreatic beta cells[58] and microglia[57] have been identified so far) and that the actin-binding activity of B2 has vital roles in cells not expressing a3.

Disruption of assembly of osteoclast-specific V-ATPases

Yeast two-hybrid screening identified an interaction between the a3 and B2 subunits of V-ATPase, and this interaction was confirmed using fusion proteins.[133] This finding raised the possibility of identifying small molecules that

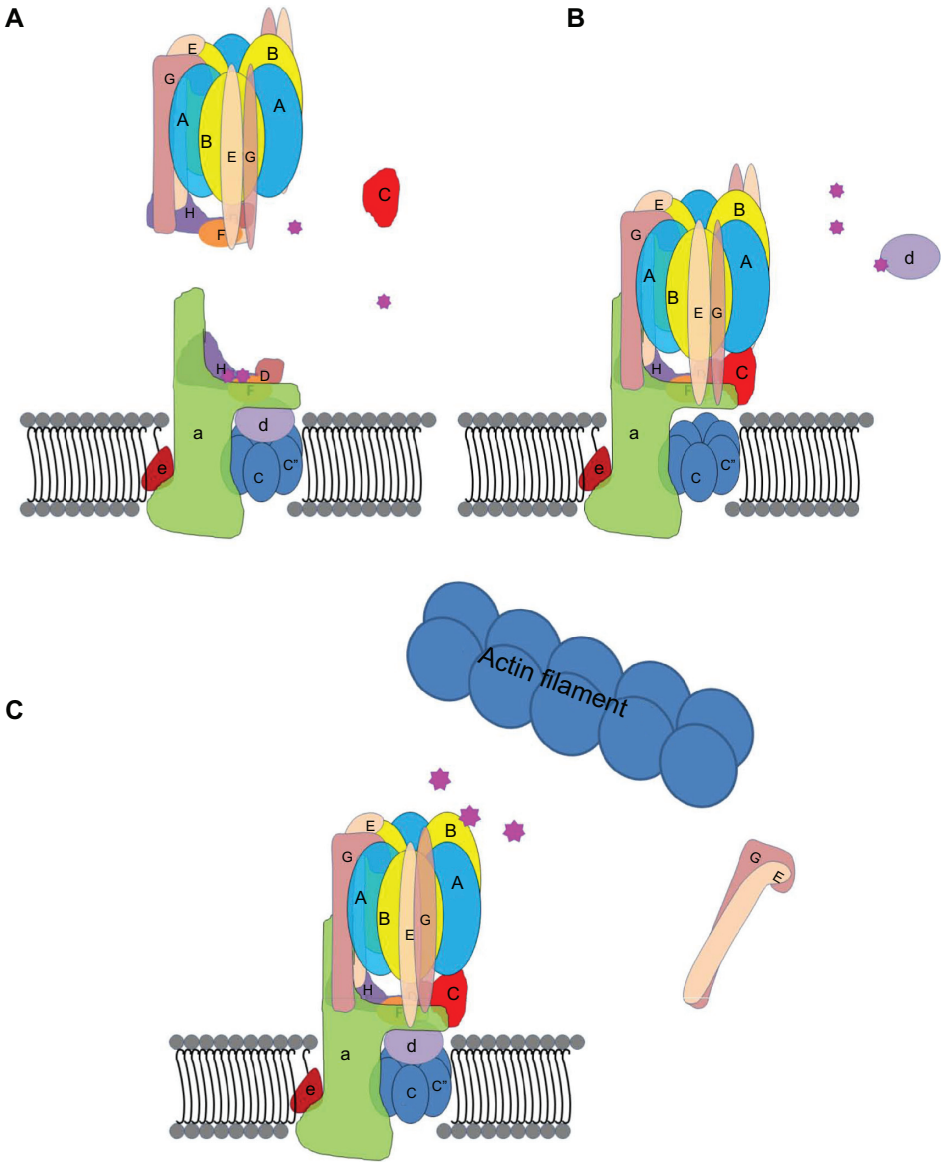

Figure 6 Rational strategies for identifying vacuolar H^+-adenosine triphosphatase (V-ATPase)-directed antiresorptive agents. (**A**) A small-molecule inhibitor of the interaction between B2 and a3 might, by binding a3, disrupt V_1/V_0 assembly of the subset of V-ATPases containing those subunits. (**B**) A small-molecule inhibitor of binding between a3 and d2 blocked d2 assembly without generally perturbing the overall V-ATPase. (**C**) A small molecule that binds the actin-binding site in the B subunit competitively inhibits the binding interactions between V-ATPases and microfilaments that are required for osteoclast bone resorption.

disrupt binding between a3 and B2, but not B2 with other a-subunit isoforms, and thus might represent a means to target the ruffled membrane V-ATPases. An enzyme-linked immunosorbent assay system was utilized to determine that in fact B2 binds all isoforms of the a subunit, but with different affinities. The basic system was adapted to use in a pilot high-throughput screen of 10,000 small molecules for 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.[133]

More recently, identification of an inhibitor of the a3–d2 interaction was also reported.[72] This molecule, 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. Luteolin is a flavonoid, and a variety of other activities have been identified, including as an inhibitor of phosphodiesterase,[134] tumor necrosis factor-α, and interleukin 6 activities,[135] and a stimulator of heme oxygenase expression.[136] Whatever the mechanism, luteolin was recently shown to reduce wear-particle osteolysis in a mouse model.[135]

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 hundreds of thousands of small molecules may yield very potent and selective osteoclast inhibitors.

Disruption of V-ATPase–microfilament interaction

Small molecules were sought that would bind to the actin-binding pocket of the B2 subunit and interfere with binding. To accomplish this, Ostrov and colleagues first generated a virtual, atomic-level model of the B2 subunit using Swiss-Prot.[137] Because the actin-binding site in B2 is related to part of the actin-interacting domain of profilin, the profilin-actin crystal[138] was also studied and used to inform regions of uncertainty in the B2 model. After the model was constructed, two pockets of the putative actin binding in the B subunit were identified, and a supercomputer-based Dock program was used to identify small molecules in a 300,000 small-molecule database from the National Cancer Institute that were predicted to interact with one of the two pockets. Of the top 40 small molecules identified in the virtual screen, enoxacin was identified as a small molecule that blocked interaction between the recombinant B subunit and microfilaments in a test tube assay. Enoxacin also inhibited osteoclast differentiation and bone resorption in vitro, but had no effect on osteoblast growth or mineralization.[137]

Enoxacin did not decrease the proliferation of osteoclast precursors or increase incidence of osteoclast apoptosis in vitro,[74] but reduced the number of multinuclear cells expressing tartrate-resistant acid phosphatase (TRAP) activity. At the molecular level, the expression of various osteoclast marker mRNAs was not altered dramatically, and indeed most protein levels measured by quantitative immunoblot were the same in vehicle-treated cultures compared with cultures treated with 50 μM enoxacin.[74] Informatively, the TRAP5b protein was expressed at high levels in the presence of enoxacin, but not converted from the proenzyme to the active form. The cytoskeletal protein L-plastin was found to have its N-terminal calcium-binding domain clipped in enoxacin-treated cultures, and dendritic cell-specific transmembrane protein (DC-STAMP), a protein linked to the fusion of osteoclast precursors to form giant cells,[139] had altered levels on the plasma membrane, although the total DC-STAMP levels were not altered. Together, these data are consistent with altered vesicular trafficking in the presence of enoxacin.[74]

At the same time that we identified enoxacin based on a screen for small molecules that block V-ATPase–microfilament binding, another group identified enoxacin as a stimulator of microRNA activity as a result of an interaction with TAR RNA-binding protein 2 (TARPB2).[140] Our data are more consistent with enoxacin inhibiting osteoclasts by blocking the V-ATPase–microfilament interaction, but we cannot exclude a role for microRNA stimulation. A potential role for enoxacin as a therapeutic was indicated by a study that showed that enoxacin inhibited human cancer growth and metastasis in a xenobiotic mouse model.[141] This elegant study provided support for stimulation of microRNAs as the mechanism for inhibiting cancer cells. Further studies of enoxacin as a potential therapeutic agent appear warranted. Because enoxacin has been used for over 20 years as an antibiotic in humans, trials in humans for anticancer or anti-bone-resorptive effects may be easier to conduct than trials of a novel molecule.

Conclusion

The V-ATPase activity in osteoclasts that is required for the resorption of bone is an attractive target for efforts to block bone resorption. In particular, accumulating data suggest that V-ATPase-targeted therapeutic agents may be bone anabolic; therefore, V-ATPase-targeted agents provide a possible route for both limiting bone loss and rebuilding bone weakened by osteoporosis. Because V-ATPase is a ubiquitous housekeeping enzyme, to date it has proven difficult to advance V-ATPase inhibitors (inhibitors of ATPase activity or proton pumping) to the clinic. The V-ATPase inhibitors that are currently known, though potent and specific, also inhibit housekeeping V-ATPases and have unacceptable side effects. More recent strategies target regulatory mechanisms that control the bone-resorptive V-ATPases in osteoclasts. Some of the potential targets identified to date include a binding interaction between V-ATPase and microfilaments,[75] binding between V-ATPase and aldolase,[76] and possible interactions with ARF6 and ARNO.[81,88,100] The accessory proteins ATP6AP1 (Ac45) and ATP6AP2 ([pro]renin receptor) are expressed in osteoclasts and also represent potential therapeutic targets.[39] Recent studies showing an interaction between ATP6AP1 and rab7 in osteoclasts[109] and regulation of bone remodeling downstream of modulators of RAS[126] offer areas of therapeutic opportunity, although much more mechanistic data is required. Efforts to make use of the emerging knowledge regarding the regulation of V-ATPases in osteoclasts to produce novel antiresorptives have begun. Although this work is in its infancy, three

novel osteoclast inhibitors have already been identified and are being tested for therapeutic value.[14,75]

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Disclosure

The authors report no conflicts of interest in this work.

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