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Interactions Between Vacuolar H^+-ATPases and Microfilaments in Osteoclasts

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Vacuolar H^+-ATPases (V-ATPases) are transported from cytosolic compartments to the ruffled plasma membrane of osteoclasts as they activate to resorb bone. Transport of V-ATPases is essential for bone resorption, and is associated with binding interactions between V-ATPases and microfilaments that are mediated by an actin-binding site in subunit B. This site is contained within 44 amino acids in the amino terminal domain, and requires a sequence motif that resembles an actin-binding motif found in mammalian profilin 1. Small alterations in the profilin-like sequence disrupt the actin-binding activity of subunit B. The interaction between V-ATPases and microfilaments in osteoclasts is regulated in response to changes in phosphatidylinositol-3 kinase activity. During internalization of V-ATPases from the plasma membrane of osteoclasts after a cycle of resorption, V-ATPases bind microfilaments that are in podosomes, dynamic actin-based structures, also present in metastatic cancer cells. Studies are ongoing to establish the physiological role of the microfilament-binding activity of subunit B in osteoclasts and in other cells.

KEY WORDS: V-ATPase; actin; bone; ruffled membranes; membrane; trafficking; phosphatidylinositol 3-kinase; actin-related protein 2/3 complex; PI 3-kinase; cortactin.

INTRODUCTION

Vacuolar H^+-ATPases (V-ATPases) are vital enzymes in eukaryotic cells responsible for acidification of compartments of the endocytic and exocytic pathway (Nishi and Forgac, 2002). In addition, V-ATPases are expressed at high levels in cell types including osteoclasts, renal intercalated cells and neurons where they play a role in the specialized functions of the cell. The enzymatic mechanism of these enzymes is remarkable. They are large, multi-subunit mechanoenzymes that couple rotary movement driven by ATP hydrolysis to the transport of protons across an associated membrane. V-ATPases contain 12 or more different proteins and over 20 subunits, since certain proteins are expressed in multiple copies per subunit (Gluck *et al*., 1998; Nishi and Forgac, 2002; Sun-Wada *et al*., 2004; Wagner *et al*., 2004).

Another remarkable aspect of V-ATPases is the precise spatiotemporal targeting of V-ATPases to a variety of cellular compartments. In eukaryotic cells V-ATPases translocate to and acidify endosomes, lysosomes, golgi, and other intracellular compartments as required by the cell. In some specialized cells, V-ATPases are targeted to additional domains beyond those required for "housekeeping" functions. For example, V-ATPases are transported as elements of cytosolic vesicles to the apical membrane of renal intercalated cells in response to an systemic acid load (Gluck *et al*., 1996; Wagner *et al*., 2004) The precise targeting of V-ATPases to specific cytosolic compartments strongly suggests associations with cytoskeletal elements (Cao *et al*., 2005; Gundelfinger *et al*., 2003).

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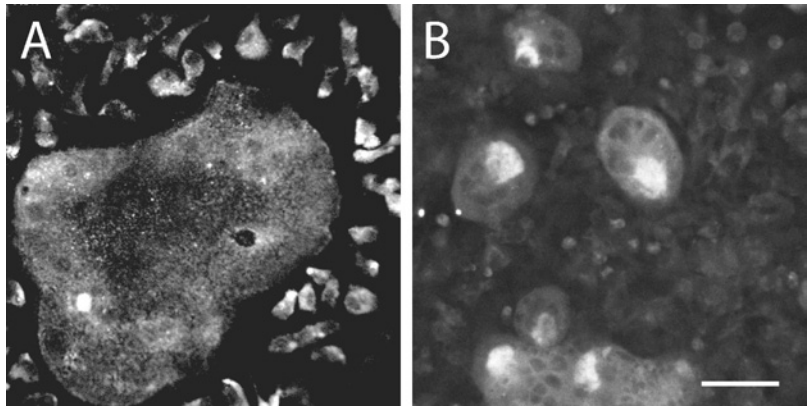

Fig. 1. V-ATPases are diffusely distributed in the cytosol of inactive osteoclasts, and concentrated into the ruffled membranes of resorbing osteoclasts. Osteoclasts were differentiated from mouse marrow and plated on a glass coverslip (A) or a bone slice (B). After 3 days, the cultures were fixed with 2% formaldehyde and stained with an anti-E subunit polyclonal antibody. In panel (A), a giant multinucleate cell is surrounded by smaller cells that are probably pre-fusion osteoclasts. Note that in all the cells, V-ATPases are diffusely distributed in the cytosol. In panel B, several resorbing osteoclasts are shown. V-ATPases in each cell are concentrated into the ruffled membrane, a specialized subdomain of the plasma membrane. Scale bar equals 30 μm.

Osteoclasts, hematopoietic cells specialized to resorb bone, express high levels of V-ATPases that are targeted to the plasma membrane (Blair *et al*., 1989). Osteoclast precursors express relatively high levels of V-ATPases (Lee *et al*., 1999b), but during osteoclastogenesis the levels of most subunits of the proton pump increases and certain tissue selective isoforms are newly expressed leading to the generation of high levels of V-ATPases with a specialized isoform composition (Lee *et al*., 1999b; Toyomura *et al*., 2000). These specialized V-ATPase are stored associated with cytosolic membrane compartments prior to osteoclast activation, and then are transported to a subdomain of the plasma membrane, known as the ruffled membrane (or ruffled border), as osteoclasts activate to resorb bone (Blair *et al*., 1989; Vaananen *et al*., 1990) (Fig. 1). After a cycle of resorption V-ATPases are endocytosed into the cytosol (Nakamura *et al*., 1997).

Unlike V-ATPases from most sources, V-ATPases immunoprecipitate from osteoclasts with microfilaments (F-actin) and myosin II (Lee *et al*., 1999a). This is due to direct binding between V-ATPases and F-actin (Lee *et al*., 1999a). V-ATPases isolated from bovine kidney bound pure rabbit skeletal muscle F-actin at a ratio of 1 V-ATPase to 8 actin filament subunits, and with an apparent affinity of 0.05 μM. Electron microscopy indicated that V-ATPases interacted with microfilaments in the region of the V-ATPase furthest from the membrane, a location suitable for allowing interactions between membrane-associated V-ATPases and microfilaments. Combined biochemical and immunocytochemical studies have provided strong circumstantial evidence that the binding interaction mediates transport of V-ATPases to and from the plasma membrane (Chen *et al*., 2004; Lee *et al*., 1999a).

THE ACTIN-BINDING ACTIVITY OF OSTEOCLAST V-ATPases REQUIRES AN ACTIN-BINDING SITE IN SUBUNIT B

Two lines of evidence initially suggested that subunit B contained actin-binding activity (Holliday *et al*., 2000). First, under conditions where isolated V-ATPases partially disassembled, subunit B was enriched in the actin-binding fractions. Second, blot overlays of isolated V-ATPase samples using F-actin identified subunit B as an actin-binding protein. After initial identification of subunit B as an actin-binding protein, we characterized the actin-binding activity in vitro using bacterially expressed truncations of mammalian subunits B1 and B2 and identified a minimal actin-binding domain that constituted amino acids 23–67 of subunit B1 (amino acids 29–73 of B2). These expressed protein bound F-actin in a 1:1 molar ratio (B subunit:actin monomer in filament) and with binding affinities of 0.130 μM for B1 and 0.190 μM for B2. Bacterially expressed subunit B fusion proteins competed with bovine kidney V-ATPases for binding to F-actin (Holliday *et al*., 2000). These data are consistent with the actin-binding activity of the B subunit being responsible for the interactions

between V-ATPases and microfilaments that we had identified.

A PROFILIN-LIKE DOMAIN IS REQUIRED FOR THE ACTIN-BINDING ACTIVITY OF SUBUNIT B

Our goal is to determine the physiological roles of the actin-binding activity of subunit B. To tease out the actin-binding activity from the enzymatic functions of V-ATPases, it is necessary to characterize the actin-binding site in sufficient detail that mutations can be made that disrupt the capacity of the B subunit to bind F-actin without effecting the capacity of subunit B to assemble with other subunits to form a fully function V-ATPases. With this in mind, we further characterized the actin-binding domain of the B subunit. A region with similar sequence to an actin-binding region of mammalian profilin 1 was identified (Chen *et al*., 2004). The 13-mer peptides derived from the profilin-like region of mammalian B1 and B2 bound actin, and replacement of a phenylalanine residue, that was found to disrupt the actin-binding activity of profilin (Schluter *et al*., 1998), also decreased the actin-binding activity of the B subunit derived peptides. Profilin and subunit B peptides were found to compete for binding to actin (Chen *et al*., 2004).

These data suggested that the profilin-like region would be vital for actin-binding activity of subunit B. To confirm this, we replaced the profilin-like sequence with a spacer with sequence derived from the B subunit of the archaea-ATPase (A-ATPase), *Pyrococcus horikoshii*. The rationale for this substitution was as follows: First, *Pyrococcus* does not have a traditional actin cytoskeleton and therefore would be unlikely to contain an actin-binding site. Second, inspection of the sequence of B subunits from archaea to humans showed that from yeast onward, the minimum actin-binding site is present and fairly conserved. For example the phenylalanine at position 65 in B2 has an equivalent phenylalanine in each organism back to archaea, but in archaea the phenylalanine is changed to glycine. Nevertheless, the basic profilin-like region is present and recognizable in *Pyrococcus*. Finally, our ultimate goal is to express a mutant B subunit that lacks actin-binding activity but retains the capacity to function as part of the pump. We reasoned that by substituting a spacer designed using the sequence from Pyrococcus B as a template; we would be less likely to disrupt the overall folding of the subunit. Based on this reasoning, we expressed fusion proteins containing the Pyrococcus-derived spacer and found that these relatively minor substitutions eliminated actin-binding activity (Chen *et al*., 2004).

IS SUBUNIT C IN OSTEOCLAST ALSO AN ACTIN-BINDING PROTEIN?

Studies of V-ATPases from *Manduca sexta* confirmed direct binding between V-ATPases and F-actin and the actin-binding activity of subunit B (Vitavska *et al*., 2003). In addition, it was found that subunit C also binds actin. Subsequently, it was shown that subunit C has two actin-binding sites and is capable of cross-linking actin filaments, and also has G-actin binding activity (Vitavska *et al*., 2005). A crystal structure of subunit C demonstrates that it is composed of two globular domains separated by a helical section (Drory *et al*., 2004). Interestingly, the globular domains have structural features in common with gelsolin. The actin-binding activity of subunit C may be associated with regulated assembly and disassembly of the proton pump (Vitavska *et al*., 2003). There are two isoforms of subunit C expressed in mammals (Wagner *et al*., 2004). C1 is ubiquitously expressed and is present at high levels in osteoclasts (Smith *et al*., 2002). Whether subunit C isoforms from mammals and other organisms bind actin is not known.

REGULATION OF THE INTERACTION BETWEEN V-ATPases AND F-ACTIN

V-ATPases from mammals have at least three relatively high-affinity actin-binding sites in the three B subunits, and potentially two more derived from subunit C. Yet V-ATPases isolated from most mammalian sources have little or no associated actin. In osteoclasts, the amount of associated actin recovered in anti-V-ATPase pull downs varies with the resorptive state of the osteoclast. These finding suggest that the V-ATPase F-actin interaction must be under physiologic control. To date, we have reported two mechanisms for altering the amount of actin bound to V-ATPases in vivo. First, we found that the amount of actin bound to V-ATPases decreased when osteoclasts activated on bone slices (Lee *et al*., 1999a). The activation process begins with patching of F-actin and V-ATPases at the site of the nascent ruffled membrane. The V-ATPases are then inserted into the plasma membrane and the actin is organized into actin rings that surround the ruffled membrane. The decrease in actin bound to V-ATPases coincided with the maturation of the mature actin ring and ruffled membrane from the initial patch (Lee *et al*., 1999a).

It is likely that signaling through $\alpha_v\beta_3$ integrin will prove to be a crucial signal in the regulation of the V-ATPase F-actin interaction. $\alpha_v\beta_3$ is the most abundant integrin on the osteoclast surface (Ross *et al*., 1993). Mice lacking β_3 integrin have dysfunctional osteoclasts

(McHugh *et al*., 2000) and mutations in $\alpha_v\beta_3$ integrin lead to Glanzmann's syndrome, in which osteoclasts are abundant but dysfunctional (Feng *et al*., 2001). We have shown that the activation of differentiated osteoclasts to resorb bone and for actin rings and ruffled membranes requires ligands of $\alpha_v\beta_3$ integrin generated by the action of interstitial collagenase (Holliday *et al*., 1997, 2003). Phosphatidylinositol 3-kinase (PI 3-kinase) activity is a likely candidate to be downstream of integrin signaling in the regulation of the V-ATPase/actin interaction (Biswas *et al*., 2004; Chellaiah *et al*., 2001; DeMali *et al*., 2003). Blocking PI 3-kinase activity in resorbing osteoclasts leads to rapid internalization of V-ATPases from the plasma membrane into cytosolic vesicles (Nakamura *et al*., 1997). We found that when activated osteoclasts were treated with wortmannin, there was an increase in binding between V-ATPases and F-actin that accompanied internalization (Chen *et al*., 2004), suggesting that regulation of PI 3-kinase activity is second mechanism for control of actin–V-ATPase interactions. Because wortmannin blocks all class I PI 3-kinases and the class III PI 3-kinase, efforts are currently underway to identify the specific PI 3-kinases involved in the regulation of the binding of V-ATPases to F-actin.

PODOSOMES AND V-ATPases

During the movement of V-ATPases to and from the osteoclast plasma membrane, V-ATPases bind F-actin that is organized into a very particular type of structure, the podosome (Teti *et al*., 1989). Podosomes are discrete actin-based structures that are organized perpendicularly to the bone substrate (King and Holtrop, 1975). Podosomes are found in a number of invasive cell types, including metastatic tumors (Linder and Aepfelbacher, 2003), but in osteoclasts, podosomes are a component of a higher order cellular structure, the actin rings. In addition to actin, podosomes contain a large number of additional actin-binding, or actin-associated proteins. Many of these are also found in focal adhesions (Vaananen *et al*., 2000). Recent studies have demonstrated that osteoclast podosomes are very dynamic. Actin filament assembly occurs with incorporation into the podosome near the plasma membrane that contacts the bone, and disassembles after actin subunits "treadmill" towards the basolateral surface (Destaing *et al*., 2003; Saltel *et al*., 2004). This sort of directed actin polymerization is often induced by the actin-related protein 2/3 complex (Arp2/3 complex) (Machesky and Bornens, 2003). We showed that Arp2/3 complex is abundant in podosomes and is required for actin ring formation (Hurst *et al*., 2004). Because the Arp2/3 complex contains two actin-related proteins (Arp2 and Arp3) that have extensive sequence and structural homology with actin, we were intrigued by the idea that these proteins might interact with V-ATPases through the B subunit, perhaps giving V-ATPases the capacity to regulate actin assembly. We were unable to detect such an interaction (I. R. Hurst and L. S. Holliday, unpublished). We nevertheless find the idea that V-ATPases may be linked to the regulation of actin polymerization and podosome formation attractive. Efforts are currently underway to test for interactions between V-ATPase subunits and podosomal proteins, including cortactin and dynamin, that have been shown to regulate Arp2/3 complex-mediated actin polymerization (McNiven *et al*., 2004; Schafer *et al*., 2002; Weaver *et al*., 2001).

FUTURE DIRECTIONS

Now that we have identified a means to disrupt the actin-binding site in subunit B by minimal alterations, we are seeking to introduce this mutant into osteoclasts and other cells. We hypothesize that the interaction between V-ATPases and F-actin in osteoclasts may be required for transport of V-ATPases to the plasma membrane surface, a highly specialized function in a specialized cell type. However, the finding that both isoforms of mammalian subunit B and subunit B from the tobacco hornworm all have actin-binding activity, suggests that the actin-binding activity of subunit B may have a more fundamental role in eukaryotic cells. Indeed, our analysis of sequences of the putative actin-binding sites in B subunits from various species suggests the possibility the actin-binding activity may be found in organisms as evolutionarily separate from mammals as yeast (Fig. 2). While this must be confirmed experimentally, the available data now indicates

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Mouse B2   MALRAMRGIV NGAAPELPVP TGGPMAGARE QALAVSRNYL SQPRLTYKTV SGVNGPLVIL DHVKFPRYAE IVHLTLPDGT KRSGQVLEVS GSKAVVQVFE GTSGIDAKKT
Bovine B2  MALRAMRGIV NGAAPELPVP TSGPLAGSRE QALAVSRNYL SQPRLTYKTV SGVNGPLVIL DHVKFPRYAE IVHLTLPDGT KRSGQVLEVS GSKAVVQVFE GTSGIDAKKT
Mouse B1         MAMEI DSRPGGLP-G SSCNLGAARE HMQAVTRNYI THPRVTYRTV CSVNGPLVVL DRVKFAQYAE IVHFTLPDGT QRSGQVLEVA GTKAIVQVFE GTSGIDARKT
Manduca B                    MAK TLSAAQANKE HVLAVSRDFI SQPRLTYKTV SGVNGPLVIL DEVKFPKFSE IVQLKLADGT HRSGQVLEVS GSKAVVQVFE GTSGIDAKNT
Yeast B                        M VLSDKELFAI NKKAVEQGFN VKPRLNYNTV SGVNGPLVIL EKVKFPRYNE IVNLTLPDGT VRQGQVLEIR GDRAIVQVFE GTSGIDVKKT
Consensus  .......... .......... .ls......e ...AV.r.%. ..PRltY.TV sgVNGPLV!L #.VKFpr%.E IV.ltLpDGT .RsGQVLE!. G.kA!VQVFE GTSGIDakkT
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Fig. 2. Comparison of amino-terminal sequence of several B subunits with known actin-binding activity along with yeast subunit B (*vma*2p). The shaded region indicates the "minimal actin-binding domain" determined from a series of truncations of the subunit B1 expressed as fusion proteins and assayed in vitro. The bottom line represents concensus sequence. (!) Anyone of IV; ($) anyone of LM; (%) anyone of FY; (#) anyone of NDQEBZ.

that interactions between V-ATPases and F-actin, two ancient, highly conserved, and fundamentally important ATPases, began early in the evolution of eukaryotic cells. Determining the physiological significance of the interactions and the means by which they are regulated promises to provide insight into the working of osteoclasts and other eukaryotic cells.

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