Vacuolar H\(^+\)-ATPase (V-ATPase) binds microfilaments, and that interaction may be mediated by an actin binding domain in subunit B of the enzyme. To test for possible physiologic functions of the actin binding activity of V-ATPase, early responses of resorbing osteoclasts to inhibition of phosphatidylinositol 3-kinase activity by wortmannin and LY294002 were examined. Rapid co-localization between V-ATPase and F-actin was demonstrated by immunocytochemistry, and corresponding association between V-ATPase and F-actin in immunoprecipitations and pelleting assays was detected. This response was reversed as osteoclasts recovered resorptive activity after inhibitors were removed. By expressing and characterizing fusion proteins containing segments of the actin-binding amino-terminal regions of the B subunits of V-ATPase, we mapped the actin-binding site to a 44-amino acid domain. An 11-amino acid segment with a sequence similar to the actin-binding site of human profilin I was detected within this region. 13-Mers containing these profilin-like segments bound actin in fluorescent anisotropy studies and competed with profilin for binding to actin. Using site-directed mutagenesis, the 11-amino acid profilin-like actin-binding motifs (amino acids 49–59 of B1 and 55–65 of B2) were replaced with an 11-amino acid spacer with a sequence based on the homologous sequence from subunit B of Pyrococcus horikoshii, an organism that lacks an actin cytoskeleton. These substitutions eliminated the actin-binding activity of the B subunit fusion proteins. In summary, binding between V-ATPase and F-actin in osteoclasts occurs in response to blocking phosphatidylinositol 3-kinase activity. This response was fully reversible. The actin binding activities of the B subunits of V-ATPase required 11-amino acid actin-binding motifs that are similar in sequence to the actin-binding site of mammalian profilin I.

Interplay between cytoskeletal elements and components of cellular regulatory and effector systems allow eukaryotic cells to achieve functional polarity and compartmentalization (1, 2). Fine, spatiotemporal regulation of acidification of the Golgi apparatus, of endocytic and phagocytic vesicles, of compartments for uncoupling receptor and ligand, and of lysosomes is vital to most eukaryotic cells (3–6). As the enzyme primarily responsible for the acidification of these compartments, as well as the polarized secretion of protons in certain specialized cell types, such as osteoclasts and intercalated cells of the kidney, the vacuolar H\(^+\)-ATPase (V-ATPase) would appear a likely candidate for linkage to the cytoskeleton (7). Recent studies have implicated interactions between V-ATPase and the microtubule-based or microfilament-based cytoskeletons as being important for regulating the specialized functions of osteoclasts and intercalated cells (7–15). No direct or indirect mechanism for linking V-ATPase to microtubules has yet been recognized, but three linkages to microfilaments have been identified, two involving the B subunit of V-ATPase (8, 14) and a third mediated by the C subunit (9).

V-ATPase is a complex enzyme containing at least 13 different subunits, some of which are present in multiple copies per holoenzyme (16, 17). As a close relative to the ATP synthase, V-ATPase is a rotary motor (18, 19). The A subunit is the site of ATP hydrolysis and forms an alternating hexagon with the B subunit. Together, the A and B subunits alter conformation in response to ATP hydrolysis to power turning of a central stalk. This is coupled to proton transport across an associated membrane. The physical location of the B subunit within the enzyme, exposed in a position farthest removed from the associated membrane, makes it an attractive candidate to mediate interactions between V-ATPase and cytoskeletal elements (8).

There are two isoforms of the B subunit. The B2, or “brain” isoform, is expressed ubiquitously, at low levels in most cells and at high levels in cells including osteoclasts, macrophages, and neurons (20, 21). The B1 or “kidney” isoform is much more restricted in its distribution. It is found at high levels in intercalated cells of the kidney and in certain cells of the eyes, ears, epididymus, and placenta (20–25). Recently, a PDZ-binding domain was identified in the carboxyl terminus of B1, and data were presented suggesting that B1 interacts with Na\(^+\)/H\(^+\) ex-
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change regulatory co-factor, which in turn binds ezrin/radixin/moesin class actin-binding proteins, thus providing a potential link between V-ATPase and the actin cytoskeleton (14).

More directly, our group has shown that both B subunit isoforms contain high affinity actin-binding sites that mediate direct interaction between V-ATPase and F-actin (8). These binding sites were previously narrowed to amino acids 1–106 of B1 and 1–112 of B2. We have provided evidence that this interaction is involved in V-ATPase transport in osteoclasts and have proposed that the interaction between V-ATPase and microfilaments is required for efficient bone resorption (7). The fact that the B1 isoform, which is not found in osteoclasts, also binds actin suggests that interaction between V-ATPase and F-actin is important in cell types other than osteoclasts.

Additional evidence for linkage of V-ATPase to the actin cytoskeleton was produced in studies of V-ATPase from *Manduca sexta*. Isolated *Manduca* V-ATPase was found to bind F-actin with a similar affinity to that of bovine kidney and mouse marrow V-ATPase. Interaction between subunit B and microfilaments was confirmed. In addition, the C subunit was reported to bind microfilaments (9).

Phosphatidylinositol 3-kinase (PI 3-kinase) activity is a crucial general mechanism for establishing cell polarity. Numerous studies indicate that PI 3-kinase activity is involved in regulating the formation of actin rings and ruffled membranes of resorptive osteoclasts (26–33). Src homology 2-containing inositol-5-phosphatase, a phosphatase that coordinates with class I PI 3-kinases to regulate phosphatidylinositol signaling, is required for normal osteoclast activity (34).

We previously found that the actin binding activity of the B subunit was located in the amino-terminal 106 amino acids of B1 and 112 amino acids of B2 (8). Fusion proteins containing these segments competed with V-ATPase enzyme for binding to microfilaments. Additionally, in unactivated osteoclasts, much of the V-ATPase was bound to F-actin (7). In contrast, in active osteoclasts, little binding between V-ATPase and F-actin was detected.

When osteoclasts are treated with wortmannin, internalization of V-ATPase from the ruffled membrane to cytosolic vesicles occurs synchronously over a period of 40 min (35). The early response of resorbing osteoclasts to blocking PI 3-kinase activity with respect to the interaction between V-ATPase and F-actin was examined. Rapid association between V-ATPase and microfilaments was detected, indicating direct or indirect regulation of the binding interaction by PI 3-kinase activity. This interaction was rapidly reversible. We sought to precisely map the actin-binding sites in the mammalian B subunits and to identify amino acid residues that are crucial for the interaction.

We demonstrate that the binding sites are in 44-amino acid sections of the B subunits and that a portion of the binding sites, which is similar in sequence to the actin-binding site of mammalian profilin I, is required for the actin binding activities of the B subunits.

**EXPERIMENTAL PROCEDURES**

**Materials**—Rabbit skeletal muscle actin was either obtained commercially (Cytoskeleton, Inc., Denver, CO) or used in pelleting assays or prepared from frozen muscle (Pel-Freez, Rogers, AR) in Buffer G (5 mM Tris-HCl, pH 7.0, 0.2 mM ATP, 0.2 mM dithiothreitol, 100 mM NaCl, and 0.01% sodium azide, pH 7.8) (35). Human recombinant profilin I was purified as described previously (36). *Escherichia coli* strain TB1 was obtained from New England BioLabs (Beverly, MA). Unless otherwise noted, other reagents were obtained from the Sigma.

**Generation of Osteoclasts**—Mouse marrow osteoclasts were generated as described previously (37). C3H/HeJ-B10.D2–/–J23-G Webster mice were killed by cervical dislocation; femora and tibia were dissected from adherent tissue; and marrow was removed by cutting both bone ends, inserting a syringe with a 25-gauge needle, and flushing the marrow using a-MEM plus 10% fetal bovine serum (a-MEM D10). The marrow was washed twice with α-MEM D10 and then plated at a density of 1 × 10⁶ cells/cm² on tissue culture plates for 5 days in α-MEM D10 plus 10⁻⁵ µM 1,25-dihydroxyvitamin D₃. Cultures were fed on day 3 by replacing half of the medium per plate and adding fresh 1,25-dihydroxyvitamin D₃. After 5 days in culture, osteoclasts appeared. These were detected as giant cells that stained positive for tartrate-resistant acid phosphatase activity (TRAP; a marker for mouse osteoclasts) or overexpressed V-ATPase subunits (38, 39).

**Antibodies**—The monoclonal antibody E11, directed against the E subunit of V-ATPase (40), was used in this study. New polyclonal antibodies were produced using the amino-terminal 20 amino acids of the human B2 subunit, the human α3 (amino acids 660–676), the carboxyl-terminal 20 amino acids of the human H subunit (SF/SD), and the bacterially produced 20 amino acids of human subunit B2. These antibodies were generated commercially (ResGen, Huntsville, AL). The anti-actin monoclonal antibody AC-40 was obtained from Sigma.

**Histochemistry, Immunohistochemistry, and Tabulation of Actin Rings, V-ATPase Patches, and TRAP Cells**—Mouse marrow cultures were grown for 5 days on tissue culture plates in α-MEM D10 plus 10⁻⁵ µM 1,25-dihydroxyvitamin D₃. On day 6, the cells were scraped free from the tissue culture plates and loaded onto dentine slices. For the indicated times and durations, cultures were treated with wortmannin (100 nM), an irreversible inhibitor of PI 3-kinase activity (34); LY294002 (50 µM), a competitive inhibitor of PI 3-kinase activity (34); jasplakinolide (1 µM), a membrane-stable marine toxin that promotes actin depolymerization (35); or Me₂SO as vehicle. After the time indicated, the cells were fixed in 4% formaldehyde in PBS, pH 7.4, for 20 min. Cells were permeabilized with PBS plus 0.2% Triton X-100 for 15 min, blocked overnight with PBS plus 10% bovine serum albumin and 5 mM sodium azide at 4 °C. For E subunit and B subunit staining, slices were incubated for 2 h in E11 (64 µg/ml) or polyclonal anti-B2 antibody (diluted 1:1000) in PBS plus 10% bovine serum albumin, washed three times with HENAC, and incubated for 1 h in Texas Red or Cy2-conjugated anti-mouse antibody (Jackson Immunoresearch Laboratories, West Park, PA) both diluted 1:500 in PBS plus 10% bovine serum albumin. After an overnight wash in HENAC, the slices were examined for fluorescence staining. For rhodamine staining, cells were stained with rhodamine-conjugated phalloidin (5 µg/ml) in PBS plus 10% bovine serum albumin for 10 min prior to examination, washed three times in PBS, and examined immediately. After actin rings or V-ATPase patches had been determined, slices were then stained for tartrate-resistant acid phosphatase activity, and the numbers and morphology or TRAP cells were counted. Counters who were blinded to the trial conditions performed scoring of actin rings, V-ATPase patches, and TRAP cells. Total numbers of actin rings were determined, and some osteoclasts were observed to contain two or three actin rings or V-ATPase patches. Cells were examined using a Zeiss Axioslide II fluorescence microscope (Zeiss, Thornwood, NY) or a Bio-Rad MRC 1024 laser-scanning confocal microscope.

**Immunoprecipitations**—Immunoprecipitations were performed as described previously (7). Osteoclasts were generated from primary mouse marrow cultures. Cells were washed in PBS and solubilized in Triton X-100 buffer (1% Triton X-100, 20 mM Tris, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 0.1% SDS, 10% glycerol, 5 mM sodium azide, and protease inhibitors). Following a centrifugation at 20,000 × g for 10 min to remove insoluble material, the extracts were incubated for 1 h at 4 °C with 20 µg of E11 or a 1:200 dilution of anti-B2. 50 µl of protein A beads (Sigma) was added, and the mixture was incubated for 1 h at 4 °C with rocking. The protein A beads were collected by centrifugation at 10,000 × g for 15 s at 4 °C and washed three times with the Triton X-100 buffer. The wash buffers were removed by aspiration with a bent 23-gauge needle, and Laemmli sample buffer was added. The samples were heated at 85 °C for 10 min, cooled to room temperature, and centrifuged at 10,000 × g for 1 min, and the supernatants were applied to SDS-polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies as described in the legends of Figs. 3 and 4. Fusion proteins—DNA fragments representing 35% of the coding region of the human B1 and B2 genes were generated by PCR using full-length B1 and B2 subunit genes in pCE4 as templates. We found that constructs made using the B2 sequence as a template were particularly susceptible to degradation by endogenous bacterial proteases. We were unable to overcome this problem using low temperature incubation strategies and protease-deficient bacteria. Because both B1 and B2 express endogenous proteases, we focused our efforts on the B1-based constructs. The sense and antisense primers used for the various constructs were as follows: MBP-B1-23–106, sense (5′-CGCGATATCCGAGAACACTGTC-AGG-3′) and antisense (5′-GCTCTAGACTAGAAGTGCTTC-3′);
Actin rings were rapidly and reversibly disrupted by PI 3-kinase inhibitors. A, mouse marrow osteoclasts were incubated on dentine slices for 2 days and then treated with 100 nM wortmannin. Cells were fixed at time points and stained with phalloidin. The numbers of actin rings and total osteoclasts (TRAP

multinucleate cells) were counted. The asterisk indicates p < 0.05. B, top panels, confocal micrograph of typical osteoclasts at time 0 (Control) or after a 10-min treatment with wortmannin (Wortmannin). Note that actin rings were no longer present after inhibition of PI 3-kinase activity, and one side of the ring appeared to have "folded" into the ruffled membrane area. This was typical, although in a minority of cells, actin rings were replaced with multiple patches or scattered podosomes. Middle panels, inverted images of the top panels give a better perspective on the podosomal nature of the structures. Bottom panels, enlargements of the small sections of actin structures pointed to by arrows and boxed in the middle panels. These show the podosomal nature of the structures before and after inhibition of PI 3-kinase activity. Podosomes appear as F-actin-rich dots with less enriched areas surrounding. The small arrows point to podosomes. The dots are consistent with the 0.3-μm diameter size reported previously for podosomes (42). The bottom panels were optimized to reveal podosomes. Scale bars, 10 μm in the top two panels and 0.5 μm in the bottom two panels. Images were analyzed at a similar level of resolution to collect data for C. C, somewhat smaller numbers of podosomes were detected per cell after treatment with wortmannin for 10 min compared with vehicle-treated active osteoclasts. The asterisk indicates p < 0.05. D, the number of actin rings expressed by cultures treated with 100 nM wortmannin for 10 min returned to pretreatment levels 2 h after wortmannin was washed out. The asterisk indicates p < 0.05. E, the number of actin rings expressed after 30 min of treatment with 50 μM LY294002 returned to pretreatment levels 30 min after LY294002 was washed out. The asterisk indicates p < 0.05.
Expression of Fusion Proteins—Preliminary experiments were performed inducing bacterial expression of fusion proteins with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside for various times to determine the optimal induction time for each fusion protein. For larger scale preparations, bacteria were grown from an overnight culture inoculated into 1 liter of LB medium (250 ml to 2 liters). Bacteria were grown at 37 °C with shaking to an absorbance of 0.5 at 600 nm. Bacteria were then induced by adding isopropyl-1-thio-β-D-galactopyranoside at 0.3 mM for the optimal time, harvested by centrifugation at 8,000 × g for 20 min, broken by sonication, and subjected to centrifugation at 40,000 × g for 1 h, and the supernatant was collected. Purification was accomplished by am- 

Nucleotide Exchange—To remove excess free ATP, actin was dialyzed against a buffer containing an amount of ATP equal to the actin concentration. Actin (1.5 μM) and B subunit peptide were incubated in a 300-μl glass cuvette with buffer G without ATP. After 10 min, eATP was added to a final concentration of 15 μM to start the reaction. After mixing, samples were placed in a spectrophotometer, and a time course of fluorescence changes was recorded. Exchange rates were determined by fitting to a single exponential algorithm as described previously (41). Microcal Origin 5.0 Professional Edition (Northampton, MA) was used for fitting the experimental data.

Site-directed Mutagenesis—We replaced the profilin-like actin binding motif in MBP-B1-(1–106) and MBP-B2-(1–112) using site-directed mutagenesis. The knock-out was performed making use of long range mutagenesis. The knock-out was performed making use of long range mutagenesis in MBP-B1-(1–106) and MBP-B2-(1–112) inserts as templates. Primers were designed to prime PCR of the whole plasmid beginning in the region of the profilin-like motif. The 5′ ends of the primers incorporated nucleotides that did not match the template but instead coded for sequence of the homologous stretch of MBP-B1-(1–106) or MBP-B2-(1–112) inserts.
the B subunit of the Archaebacteria, Pyrococcus horikoshii. However, in order to facilitate ligatation of PCR product after the PCR was complete, two amino acids were changed in the Pyrococcus sequence to insert a BamHI site to make overhanging ends. These changes from the Pyrococcus sequence were methionine to glycine at position 4 of the spacer and isoleucine to serine at position 5 of the spacer. In addition, the valine at position 9 was changed to glycine in order to introduce a restriction enzyme site to use to select clones. The primers used were as follows: MBP-B1 (1–106, spacer 49–59) sense (5′-CGGGATCCGGTGAAGGAGTGGCCAGGATCATGAAATGTC-3′) and antisense (5′-CGCGGATCCCTGGAACGCGGATCATGAAATGTC-3′) and anti-sense (5′-CGGGATCCGGTGAAGGAGTGGCCAGGATCATGAAATGTC-3′) and anti-sense (5′-CGGGATCCGGTGAAGGAGTGGCCAGGATCATGAAATGTC-3′).

Blocking PI 3-Kinase Activity in Resorptive Osteoclasts Triggers Rapid Binding between V-ATPase and Microfilaments—Mouse bone marrow cultures were grown to maturity and then scraped and loaded onto sperm whale dentine slices. After 2 days, from 74.1 to 91.4% of the TRAP⁺ cells were active as defined by having at least 1 actin ring and ruffled membrane complex (data not shown). Cultures at this stage were treated with wortmannin or Me₂SO as the vehicle control. Osteoclasts were fixed after 1, 3, 5, and 10 min of treatment and stained with phalloidin. The number of actin rings per total number of osteoclasts was determined (Fig. 1A). The proportion of osteoclasts with actin rings decreased from 91 ± 4 to 15 ± 2% after 10 min. The actin rings lost their shape and formed patches in response to blocking PI 3-kinase activity occurred as a result of podosomes becoming present in great numbers in the area of the ruffled membrane. The appearance was generally as if the actin ring had collapsed inward. At time points as early as 3 min, significant reorganizations of the actin rings and co-localization of the actin cytoskeleton (43, 44), the binding and co-localization of V-ATPase and F-actin were detected. In merged images of F-actin and V-ATPase staining, at early time points, there was little yellow staining, indicating little close association between the two. Instead, F-actin staining was intercalated within discrete areas of V-ATPase staining. With time, the staining pattern progressed to the point where very precise and extensive co-localization of the F-actin and V-ATPase was present in merged images after 10 min. This resulted in extensive regions of yellow staining in the merged images (Fig. 2).

To determine whether co-localization between V-ATPase and F-actin was indicative of binding between the two, V-ATPase was immunoprecipitated from detergent extracts of the cells at time points after PI 3-kinase inhibition. Increasing amounts of F-actin and V-ATPase staining, at early time points, there was little yellow staining, indicating little close association between the two. Instead, F-actin staining was intercalated within discrete areas of V-ATPase staining. With time, the staining pattern progressed to the point where very precise and extensive co-localization of the F-actin and V-ATPase was present in merged images after 10 min. This resulted in extensive regions of yellow staining in the merged images (Fig. 2).

Because PI 3-kinase activity is tightly linked to the organization of the actin cytoskeleton (43, 44), the binding and co-localization between V-ATPase and F-actin that was observed after treatment with PI 3-kinase inhibitors could have been a nonspecific consequence of the disruption of the organization of the actin cytoskeleton. To control for this possibility, we tested the marine toxin jasplakinolide, a membrane-permeable agent that prevents F-actin from depolymerizing (45). Prior to these experiments, we found that jasplakinolide (which binds the sides of actin filaments and could therefore potentially directly
block binding by V-ATPase) did not alter the binding of isolated vacuolar H\textsuperscript+-ATPase to F-actin (data not shown). Like treatment with PI 3-kinase inhibitors, treatment with this reagent led to disruption of a significant portion of the actin rings in osteoclasts (Fig. 4, A and B). Treatment with jasplakinolide resulted in the formation of F-actin patches that were similar in appearance to those that occurred when PI 3-kinase was inhibited. Unlike treatment with PI 3-kinase inhibitors, V-ATPase diffused from the site of the ruffled membrane and did not co-localize with F-actin (Fig. 4B). Osteoclasts were treated for 30 min with jasplakinolide or wortmannin for 20 min, and the osteoclasts were detergent-solubilized and immunoprecipitated with anti-E subunit antibody. The amount of actin associated with V-ATPase was unaffected by jasplakinolide treatment but was markedly increased by treatment with wortmannin (Fig. 4C).

Recovery of Osteoclasts from Treatment with LY294002—V-ATPase association with F-actin was followed as the PI 3-kinase inhibitor LY294002 was added at time 0, and then after a 10-min treatment the inhibitor was removed, and osteoclasts were allowed to recover. As with inhibition with wortmannin, 10 min after inhibition of PI 3-kinase activity, V-ATPase and F-actin were highly co-localized (Fig. 5A). 10 min after washout of LY294002, the V-ATPase and F-actin remained co-localized, but after 40 min, most osteoclasts had regained the resorptive phenotype with segregated actin rings and ruffled membranes. As expected, little V-ATPase was detected associated with the detergent-insoluble cytoskeleton at time 0. After the 10-min treatment with LY294002, much more V-ATPase was cytoskeletonally associated. 50 min after removal of the inhibitor, only a trace amount of V-ATPase was detected in the detergent-insoluble cytoskeletal fraction (Fig. 5B).

Mapping and Characterization of the Actin Binding Domain in the B Subunit—A series of fusion proteins containing short regions of the N-terminal domain of the B1 and the B2 subunits were tested for their ability to bind F-actin in pelleting assays (Fig. 6, A and B). These experiments identified sequence between amino acids 23 and 67 in B1 as being responsible for the actin binding activity. The various constructs and their actin binding activities are depicted schematically (Fig. 6C).

The B Subunit Actin-binding Site Contains a Region That Is Similar to the Actin-binding Site of Profilin 1 and Independently Binds Actin—A high confidence model of the B subunit was generated (3D-PSSM; Imperial College, London) relying on...
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The Region That Is Similar to Profilin in the B Subunits Is Required for the Actin Binding Activity of the Fusion Proteins—

Fusion proteins with the profilin-like motifs altered were generated by site-directed mutagenesis. We replaced the 11-amino acid profilin-like region in MBP-B1(1–106) and MBP-B2(1–112) with a spacer based on the corresponding 11 amino acids from the B subunit of the archaeabacteria, *P. horikoshii*, an organism that lacks an actin cytoskeleton (Fig. 5A). These constructs were expressed, purified, and tested in actin binding assays and found to have completely lost their capacity to bind microfilaments (Fig. 8B).

**DISCUSSION**

Blocking PI 3-kinase activity induces rapid (40-min) internalization of V-ATPase from ruffled membranes (33). The early events associated with that internalization were examined. Inhibition of PI 3-kinase activity rapidly disrupted the structure of the actin ring. This involved a small decrease in the number of podosomes per cell but primarily involved disruption of the organization of podosomes so that they no longer formed an actin ring. Instead, podosomes were detected in the ruffled membrane area, which in resorbing cells normally excludes podosomes. Podosomes are transient (half-life about 2 min) and remain stationary with respect to the substrate. Changes in the shape of structures composed of podosomes involve altering the relative position of the new podosome formation (42). Concurrent with the presence of abundant F-actin in the ruffled membrane, we detected co-localization and binding of the F-actin to V-ATPase of the ruffled membrane. This shows that blocking PI 3-kinase activity triggers a change in the relative location of F-actin in cells. Because F-actin in the ruffled membrane area appears to be organized into podosomes, it suggests that PI 3-kinase activity alters the relative location of the new podosome formation.

V-ATPase binds F-actin with high affinity (*Kₐ = 55–100 nM*) (7, 9). Data suggest that this interaction is mediated, at least in part, by actin-binding sites located in the amino-terminal domain of both isoforms of the mammalian B subunit (8). Despite their capacity to bind F-actin with high affinity, V-ATPase is normally not recovered from most cells with bound F-actin. In unactivated osteoclasts, a high portion of V-ATPase is bound to microfilaments (Fig. 5), cultures were detergent-extracted with Triton X-100 at the start of the experiment and 15 min after the beginning of LY294002 treatment (5 min after the inhibitor was washed out) and 60 min after the start of LY294002 treatment (50 min after the inhibitors was washed out). The samples were subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with an anti-B2 antibody.

![Figure 5](image)

**Figure 5.** V-ATPase and F-actin reversibly associate in response to treatment and washout with LY294002 in unison with disruption and reformation of ruffled membranes and actin rings. A, osteoclasts were treated with 50 μM LY294002 for 10 min, after which the inhibitor was washed out and cells were allowed to recover. Typical cells are depicted from cultures fixed at the indicated time points in minutes. V-ATPase was stained with E11 (red); F-actin was stained with phalloidin (green). Areas of co-localization appear in yellow. Bar, 5 μm. B, cultures were detergent-extracted with Triton X-100 at the start of the experiment and 15 min after the beginning of LY294002 treatment (5 min after the inhibitor was washed out) and 60 min after the start of LY294002 treatment (50 min after the inhibitors was washed out). The samples were subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with an anti-B2 antibody.

the sequence similarity between B subunit and the α and β subunits of the ATP synthase. Two surface-exposed segments were identified, separated by an intervening stretch that is predicted to be buried in the B subunit structure (data not shown). One of the regions that is predicted to be exposed on the surface of the B subunit was contained by a stretch of 11 amino acids with sequence that is similar to a portion of the actin-binding site of mammalian profilin I (Fig. 7A). Based on this similarity and the structural prediction of the B subunit, we synthesized and tested peptides containing this site for their capacity to bind actin with high affinity, V-ATPase is normally not recovered from most cells with bound F-actin. In unactivated osteoclasts, a high portion of V-ATPase is bound to microfilaments (Fig. 5), cultures were detergent-extracted with Triton X-100 at the start of the experiment and 15 min after the beginning of LY294002 treatment (5 min after the inhibitor was washed out) and 60 min after the start of LY294002 treatment (50 min after the inhibitors was washed out). The samples were subjected to SDS-PAGE, electroblotted to nitrocellulose, and probed with an anti-B2 antibody. The peptides tested were the profilin homology sequences from both B1 and B2 (with two additional amino acids, amino acids 60 and 61 of B1 and amino acids 66 and 67 of B2 added to the C-terminal end as a spacer). The B2 peptide bound actin with an affinity of 21 ± 5 μM; B1 bound with an affinity of 71 ± 15 μM. Both bound actin specifically with affinities in a range consistent with the actin binding activity of similarly sized peptides derived from other actin-binding proteins (Fig. 7B). As a control, a peptide identical to the B2 peptide tested above, except with the phenylalanine at position 11 of the 13-mer peptide changed to an alanine (Fig. 7A), was tested. Studies of the profilin actin-binding site (46–48) suggested that this substitution would decrease the binding affinity of the peptide to actin. As predicted, the alanine for phenylalanine decreased the binding affinity of the peptide to actin by 71-fold less well than the unsubstituted peptide (Fig. 7B). These peptides competed with human profilin I for binding to actin, indicating that they were binding the same location on actin as profilin (Fig. 7C). Although the peptides bound actin at the same site as profilin, they had different effects on the exchange rate of nucleotides bound to actin. Profilin increased the rate of nucleotide exchange at the high affinity nucleotide-binding site of actin (49). In contrast, the B subunit-derived peptides decreased the nucleotide exchange rate (Fig. 7D).
There is evidence that class I PI 3-kinase is involved in the regulation of the polarized structures of resorbing osteoclasts (33). Nothing is currently known about whether a class III PI 3-kinase is important for bone resorption by osteoclasts. It is intriguing to note that both class I and class III PI 3-kinases are required for phagocytosis by macrophages, a process that is thought to be closely related to bone resorption by osteoclasts (54). Class I PI 3-kinase activity is required for the reorganizations of the actin cytoskeleton that are required for phagocytosis, whereas class III activity is involved in phagosomal maturation (54). We suspect that a similar scheme may be in place in osteoclasts.

Our goal is to understand the mechanism by which V-ATPase interacts with F-actin in cells, the physiologic reason for the interaction, and the means by which it is regulated. To accomplish these tasks, it is crucial to characterize the actin binding activity in sufficient detail to allow the design of mutated versions of subunit B that no longer bind F actin yet retain their capacity to serve in the enzymatic and proton pumping activities of the enzyme. We had previously narrowed the actin-binding site in the B subunit to the amino-terminal 106 amino acids in B1 and 112 amino acids in B2. Here we mapped the minimal F-actin-binding site in the B subunit of the human V-ATPase to amino acids 23–67 in B1 (homologous to amino acids 29–73 in B2). Within that region, an actin-binding motif that is similar to a portion of the actin-binding site of human profilin I was identified. We showed that this profilin-like motif is required for the binding of the B subunit to F-actin.

Quite a lot is known about the version of the actin-binding motif that appears in profilin. Certain point mutations within the motif cause dramatic decreases in the affinity of the mutated profilin for actin (47). Key residues, like phenylalanine 59, were predicted to be crucial based on the crystal structure of the profilactin complex (48). These predictions were confirmed experimentally. Phenylalanine 59 in B1 and phenylalanine 65 in B2 appear to occupy homologous positions to phenylalanine 59 in profilin I. Consistent with this, a 13-mer peptide from the B2 profilin homology motif, which had the phenylalanine in question changed to alanine, displayed dramatically less actin binding activity than the parent peptide. In addition, the spacer sequence from \( P. horikoshii \), an organism that lacks actin, does not have a phenylalanine in the key position in the homologous stretch of the B subunit. Organisms that have actin, ranging from humans to yeast, appear to have this key phenylalanine as well as many other conserved residues within the actin binding region. We have not tested whether B subunits from nonmammalian organisms bind actin. The finding...
that V-ATPase from *Manduca* binds actin suggests that V-ATPases from many species may have the capacity to directly interact with microfilaments.

We used the technique of long range PCR (55) to replace the 11-amino acid profilin-like motifs with homologous sequence from the archaeabacteria, *P. horikoshii*. We replaced the profilin-like motifs with spacers rather than simply deleting them, because deletion would probably disrupt the overall structure of the proteins and make interpretation of the data obtained with the deletion mutants impossible. The spacers chosen were based on sequence from *P. horikoshii*, an organism lacking actin, and thus, one that would not have a physiologically relevant actin-binding site. They retain significant portions of the mammalian sequence, but certain key residues are altered. Consistent with the hypothesis that the profilin-like motifs are crucial to the actin binding activities of B subunits, no actin binding activity was detected in either MBP-B1-(1–106, spacer 49–59) or MBP-B2-(1–112, spacer 55–65), despite the fact that 6 of the 11 amino acids in the spacers were identical or conserved compared with the B subunit sequences (Fig. 8A). These data provide strong evidence that the profilin-like motifs are essential for the actin binding activities of the B subunits.

Profilin is a G-actin-binding protein, and profilin-like 13-mers derived from subunit B bound G-actin well but bound F-actin poorly (48). One contact site includes the sequence discussed in this report. The sum of these multiple contact sites results in G-actin binding activity, as revealed by x-ray crystallography of the profilactin complex (48). One contact site includes the sequence discussed in this report. The sum of these multiple contact sites results in G-actin binding activity.
were separated by SDS-PAGE, and gels were stained with Coomassie Blue.

of Florida College of Medicine) for use of the miniultracentrifuge and for

sites, three B subunits, and a C subunit. Elucidating this

like spectrin and ankyrin, V-ATPase has its own actin-binding

which interact with the cytoskeleton through adapter proteins

neatly within that scheme. In contrast to other ion channels,

to the cytoskeleton (57). V-ATPase in osteoclasts appears to fit

and microfilaments.

not sufficient for high affinity interaction between subunit B

subunit B. Thus, the profilin-like sequence is necessary but

actin-binding domain that favors interaction with F-actin is

additional elements provide additional contact sites with F-

whether they enforce a specific conformation on the

actin-binding domain that favors interaction with F-actin is

known. Importantly, the profilin-like region was required

for the high affinity F-actin binding activity of subunit B. Thus, the profilin-like sequence is necessary but

not sufficient for high affinity interaction between subunit B and microfilaments.

A recent theme in cell biology is the finding that ion channels

and transporters are frequently bound and functionally linked
to the cytoskeleton (57). V-ATPase in osteoclasts appears to fit

neatly within that scheme. In contrast to other ion channels,

which interact with the cytoskeleton through adapter proteins

like spectrin and ankyrin, V-ATPase has its own actin-binding

sites, three B subunits, and a C subunit. Elucidating this

unique interaction with the cytoskeleton seems to us to be

important to understanding pH regulation in cells.

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REFERENCES

1. Chung, C. Y., Funamoto, S., and Firtel, R. A. (2001) Trends Biochem. Sci. 26, 557–566

2. Lelièvre, S. A., and Bissell, M. J. (1998) J. Cell. Biochem. Suppl. 30, 250–263

3. Nelson, N., and Harvey, W. R. (1999) J. Biol. Chem. 274, 961–968

4. Ichimura, K., and Moore, H. P. (2001) J. Biol. Chem. 276, 18489–18505

5. Lowry, O. H., and Firtel, R. A. (2001) J. Biol. Chem. 276, 18489–18505

6. Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

7. Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

8. Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

9. Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

10. Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

11. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

12. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

13. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

14. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

15. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

16. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

17. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

18. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

19. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505

20. Wiederhold, T., Breton, S., and Gluck, S. L. (2003) J. Biol. Chem. 278, 18489–18505
V-ATPase Interaction with Microfilaments

S. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3541–3545
21. Puopolo, K., Kumamoto, C., Adachi, I., Magner, R., and Forgac, M. (1992) J. Biol. Chem. 267, 3696–3706
22. Wurzburg, B., Wurzburg, T., Kupper, B., Nelson, N., Brown, D., and Gluck, S. L. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6752–6757
23. Karet, F. E., Finberg, K. E., Nelson, R. D., Nayir, A., Sanjad, S. A., Rodriguez-Surian, J., Santos, F., Cremers, C. W., Di Pietro, A., Hoffbrand, B. I., Winiarz, J., Bakaloglu, A., Ozen, S., Dusunsel, R., Goodyer, P., Hulten, S. A., Wu, D. K., Skvorak, A. B., Morton, C. C., Cunningham, M. J., Jha, Y., and Lifton, R. P. (1999) Nat. Genet. 21, 84–90
24. Breton, S., Smith, P. J., Lui, B., and Brown, D. (1996) Nat. Med. 2, 470–472
25. Skirven, M. A., MacLaren, L. A., and Wildeman, A. G. (1999) J. Histochem. Cytochem. 47, 1247–1254
26. Lee, Z. H., Lee, S. E., Kim, C. W., Lee, S. H., Kim, S. W., Kwack, K., Walsh, K., and Kim, H. H. (2002) J. Biochem. (Tokyo) 131, 161–166
27. Chellaiah, M., and Hruska, K. (1996) Mol. Biol. Cell 7, 743–753
28. Chellaiah, M. A., Biwa, R. S., Yuen, D., Alvarez, U. M., and Hruska, K. A. (2001) J. Biol. Chem. 276, 47434–47444
29. Lakkakorpi, P. T., Wesolowski, G., Zimolo, Z., Rodan, G. A., and Rodan, S. B. (1997) Exp. Cell Res. 237, 296–306
30. Arron, J. R., Velogolokhai, M., Wong, B. R., Naramura, M., Kim, N., Gu, H., and Choi, Y. (2001) J. Biol. Chem. 276, 30011–30017
31. Trilling, M. F., Sims, S. M., and Dixon, S. J. (2001) J. Bone Miner. Res. 16, 1237–1247
32. Pilkington, M. F., Sims, S. M., and Dixon, S. J. (1998) J. Bone Miner. Res. 13, 688–694
33. Nakamura, I., Sasaki, T., Tanaka, S., Takahashi, N., Jimi, F., Kurokawa, T., Kita, Y., Iharai, S., Suda, T., and Fukai, Y. (1997) J. Cell. Physiol. 172, 230–239
34. Takeshita, S., Namba, N., Hase, J. J., Jiang, Y., Gerant, H. K., Silva, M. J., Brodt, M. D., Helgason, C. D., Kalesnikoff, J., Rauh, M. J., Humphries, R. K., Krystal, G., Teitelbaum, S. L., and Ross, F. P. (2002) Nat. Med. 8, 943–949
35. Kang, F., Laine, B. O., Bub, M. R., Southwick, F. S., and Purich, D. L. (1997) Biochemistry 36, 8384–8392
36. Yarmola, E. G., Somasundaram, T., Boring, T. A., Specter, I., and Bub, M. R. (2000) J. Biol. Chem. 275, 28120–28127
37. Holliday, L. S., Dean, A. D., Greenwald, J. E., and Glucks, S. L. (1995) J. Biol. Chem. 270, 18981–18989
38. Holliday, L. S., Welgus, H. G., Filszar, C. J., Veith, G. M., Jeffrey, J. J., and Gluck, S. L. (1997) J. Biol. Chem. 272, 22053–22058
39. Lee, B. S., Holliday, L. S., Krits, I., and Gluck, S. L. (1999) J. Bone Miner. Res. 14, 2127–2130
40. Hembred, P., Guo, X. L., Wang, Z. Q., Zhang, K., and Gluck, S. (1992) J. Biol. Chem. 267, 9948–9957
41. Yarmola, E. G., Edison, A. S., Lenox, R. H., and Bub, M. R. (2001) J. Biol. Chem. 276, 22551–22558
42. DeStaing, O., Saltel, F., Geminard, J. C., Jurdie, P., and Baud, F. (2003) Mol. Biol. Cell 14, 407–416
43. Wang, F., Herzmark, P., Weiner, O. D., Sarin, S. R., Servant, G., and Bourne, H. R. (2002) Nat. Cell Biol. 4, 515–518
44. Weiner, O. D., Neilsen, P. O., Prestwich, G. D., Kirschner, M. W., Cantley, L. C., and Bourne, H. R. (2003) Nat. Cell Biol. 4, 509–513
45. Bubb, M. R., Senderowicz, A. M., Sausville, E. A., Duncan, K. L., and Korn, E. D. (1994) J. Biol. Chem. 269, 14869–14871
46. Van dencker, J. S., Kaiser, D. A., and Pollard, T. D. (1989) J. Cell Biol. 109, 619–626
47. Schluter, K., Schleicher, M., and Jockusch, B. M. (1998) J. Cell Sci. 111, 3261–3273
48. Schutt, C. F., Myslik, J. C., Rozycki, M. D., Goonasekere, N. C., and Lindberg, U. (1993) Nature 365, 810–816
49. Mockr, S. C., and Korn, E. D. (1980) Biochemistry 19, 5359–5362
50. Chung, C. Y., Potikyan, G., and Pertel, R. A. (2001) Mol. Cell 7, 937–947
51. Rickert, P., Weiner, O. D., Wang, F., Bourne, H. R., and Servant, G. (2000) Trends Cell Biol. 10, 466–473
52. Nakamura, I., Takahashi, N., Sasaki, T., Tanaka, S., Udagawa, N., Murakami, H., Kimura, K., Kabuyama, Y., Kurokawa, T., Suda, T., and Fukai, Y. (1995) FEBS Lett. 361, 79–84
53. Ti, M., Okada, T., Hanaki, K., and Hazei, O. (1995) Trends Biochem. Sci. 20, 363–367
54. Vieira, O. V., Botelho, R. J., Rameh, L. Brachmann, S. M., Matsuo, T., Davidson, H. W., Schreiber, A., Backer, J., M. Cantley, L. C., and Grinstein, S. (2001) J. Cell Biol. 155, 19–25
55. Barnes, W. M. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2216–2220
56. Pring, M., Weber, A., and Bub, M. R. (1992) Biochemistry 31, 1827–1836
57. Bennett, V., and Barnes, A. J. (2001) Physiol. Rev. 81, 1353–1392
58. Cano, M. L., Lauffenburger, D. A., and Zymon, S. H. (1991) J. Cell Biol. 115, 677–687