Interaction between Vacuolar H\textsuperscript{+}-ATPase and Microfilaments during Osteoclast Activation\textsuperscript{*}

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Vacuolar H\textsuperscript{+}-ATPases (V-ATPases) are multisubunit enzymes that acidify compartments of the vacuolar system of all eukaryotic cells. In osteoclasts, the cells that degrade bone, V-ATPases, are recruited from intracellular membrane compartments to the ruffled membrane, a specialized domain of the plasma membrane, where they are maintained at high densities, serving to acidify the resorption bay at the osteoclast attachment site on bone (Blair, H. C., Teitelbaum, S. L., Ghiselli, R., and Gluck, S. L. (1989) Science 249, 855–857). Here, we describe a new mechanism involved in controlling the activity of the bone-resorptive cell. V-ATPase in osteoclasts cultured in vitro was found to form a detergent-insoluble complex with actin and myosin II through direct binding of V-ATPase to actin filaments. Plating bone marrow cells onto dentine slices, a physiologic stimulus that activates osteoclast resorption, produced a profound change in the association of the V-ATPase with actin, assayed by coimmunoprecipitation and immunocytochemical colocalization of actin filaments and V-ATPase in osteoclasts. Mouse marrow and bovine kidney V-ATPase bound rabbit muscle F-actin directly with a maximum stoichiometry of 1 mol of V-ATPase per 8 mol of F-actin and an apparent affinity of 0.05 μM. Electron microscopy of negatively stained samples confirmed the binding interaction. These findings link transport of V-ATPase to reorganization of the actin cytoskeleton during osteoclast activation.

Normal bone remodeling requires precise control over the rates of bone formation by osteoblasts and degradation by osteoclasts. Bone degradation entails the activation of osteoclasts to a highly polarized state, with formation of a specialized ruffled membrane at their bone attachment site that confers the ability to resorb bone. The osteoclast ruffled membrane is bounded by a ring of actin filaments and associated proteins on the cytoplasmic face of the plasma membrane, localized to the zone on the cell surface that is adherent to bone (2–4). The adherence zone forms a tightly sealed extracellular compartment that is acidified by densely packed proton-transporting V-ATPases\textsuperscript{1} in the ruffled membrane (1, 5); acidification of this compartment promotes dissolution of bone mineral and degradation of bone matrix protein by cysteine proteinases secreted by the osteoclast (6–8).

In osteoclasts actively resorbing bone, most of the cellular V-ATPase is polarized to the ruffled membrane (1). Polarization of V-ATPases to discrete plasmalemmal domains in epithelial cells is dependent on the microtubules in the cytoskeleton (9). The involvement of actin filaments in polarization is not yet clear, although actin filament binding to V-ATPase-dense vesicles has been observed in proton-transporting epithelial cells of toad urinary bladder (10).

When cultured in medium containing 1,25-dihydroxyvitamin D\textsubscript{3}, mouse marrow cells develop into osteoclasts (11, 12) that contain most of the V-ATPase in the cultures (13). Osteoclasts from 1,25-(OH)\textsubscript{2}D\textsubscript{3}-treated plates plated on bone or dentine slices undergo a transformation to the activated phenotype, in which actin rings form, V-ATPase is polarized to the ruffled membrane, and bone is resorbed (1, 2, 5). In contrast, osteoclasts from identical cultures placed on glass coverslips fail to form ruffled membranes and do not have physiologically detectable plasma membrane V-ATPase (14, 15).

Recent studies suggest that V-ATPase is associated with the cytoskeleton in osteoclasts cultured in vitro (16, 17). V-ATPase was found in a detergent-insoluble (cytoskeletal) fraction of osteoclast-containing bone marrow cultures from normal mice (16). In contrast, in osteoclasts from osteosclerotic (oc\textsuperscript{−} / oc\textsuperscript{−}) mice, which are unable to form ruffled membranes, the amount of V-ATPase in the detergent-insoluble fraction was reduced (16), prompting speculation that V-ATPase binding to the cytoskeleton might be involved in ruffled membrane formation. In addition, genetic studies from yeast point toward an association between V-ATPase and the actin cytoskeleton (18). These authors (18) postulated that alterations in the actin cytoskeleton and cytoskeletal processes caused by mutation of V-ATPase subunits are the result of indirect effects resulting, perhaps, from changes in intracellular pH affecting cytoskeletal organization.

Here, we examine the interaction between the Triton-insoluble cytoskeleton and V-ATPase in mouse marrow osteoclasts. We show that V-ATPase binds actin filaments directly and that the interaction varies in response to physiologic stimuli, implicating a role for the actin-V-ATPase complex in controlling the transport of V-ATPase to the ruffled membrane.

MATERIALS AND METHODS

Reagents—All materials were obtained from the Sigma unless otherwise noted.

Mouse Marrow Cultures—Osteoclasts were generated from primary mouse marrow cultures (12). 8–20 g Swiss-Webster mice were killed by (OH\textsubscript{2})\textsubscript{2}D\textsubscript{3}, 1, 25-dihydroxyvitamin D\textsubscript{3}; αMEM, α-minimum Eagle’s medium; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.

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\textsuperscript{1} The abbreviations used are: V-ATPase, vacuolar H\textsuperscript{+}-ATPases; 1, 25-(OH)\textsubscript{2}D\textsubscript{3}, 1, 25-dihydroxyvitamin D\textsubscript{3}; αMEM, α-minimum Eagle’s medium; BSA, bovine serum albumin; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate.

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cervical dislocation, and femora and tibia were dissected free from adherent tissue. Marrow was removed by cutting both bone ends, inserting a syringe with a 25-gauge needle, and flushing the marrow using αMEM plus 10% fetal bovine serum (αMEM D10). The marrow was washed twice with αMEM D10 and then plated at a density of 1 × 10^6 cells/cm² on tissue culture plates in αMEM D10, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. After 5 days in culture, multinucleated osteoclasts appeared. The osteoclast phenotype was verified by demonstrating staining of the multinucleated cells for tartrate-resistant acid phosphatase activity, high level of V-ATPase expression, and bone-resorptive capacity.

Immunohistochemistry—Mouse marrow cultures were grown for 5 days on tissue culture plates in αMEM D10 plus 10^-8 M 1,25-dihydroxyvitamin D₃. On day 6, the cells were scraped from the tissue culture plates with a tissue culture scraper and plated on dentine slices (a kind gift of the U. S. Department of Fisheries, San Diego, CA). After incubation for varying lengths of time in αMEM D10 plus 10^-8 M 1,25-dihydroxyvitamin D₃, the cells were fixed in 2% formaldehyde in Buffer C (30 mM HEPES, pH 7.4, 100 mM NaCl, 2 mM CaCl₂) for 20 min, permeabilized with HENAC plus 0.1% Triton X-100 for 15 min, and incubated overnight with HENAC plus 10% bovine serum albumin (BSA) and 5 mM sodium azide (blocking solution) at 4 °C to block nonspecific binding. The slices were then incubated for 2 h in the anti-V-ATPase monoclonal antibody E11 (20) in HENAC plus 1% Triton X-100, washed 3 times with HENAC, and incubated for 1 h in Texas Red rhodamine isothiocyanate-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Park, PA), diluted 1:500 in HENAC plus 10% BSA. After an overnight wash in HENAC, the slices were stained with either rhodamine or fluorescein-conjugated phalloidin (5 μg/ml) in HENAC plus 10% BSA for 10 min and washed three times in HENAC. Samples were examined within 1 h using a Nikon epifluorescence microscope or Bio-Rad and Zeiss scanning laser confocal microscopes.

Detergent Extraction—Mouse marrow cultures were grown as described above, and mature osteoclast-containing cultures were scraped and plated on coverslips or dentine slices. After 2 days, cells were permeabilized with HENAC plus 0.5% Triton X-100 and 0.01 mg/ml rhodamine phalloidin. After 10 min on ice, the extracted remnants were fixed in ice-cold 200 mM HgCl₂, 90 mM sodium acetate, and 3.7% formaldehyde for 20 min, quenched with Lugol’s solution for 2 min, washed twice with sodium thiocyanate and then 3 times in HENAC. Samples were then incubated in blocking solution followed by E11 and fluorescein isothiocyanate-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Park, PA) diluted 1:500 in HENAC plus 10% BSA. After an overnight wash in HENAC, the slices were stained with either rhodamine or fluorescein-conjugated phalloidin (5 μg/ml) in HENAC plus 10% BSA for 10 min and washed three times in HENAC. Samples were examined within 1 h using a Nikon epifluorescence microscope or Bio-Rad and Zeiss scanning laser confocal microscopes.

Immunoprecipitation and Immunoblots—Six- to eight-day-old mouse marrow cultures were labeled overnight in 90% methionine- and cysteine-free culture medium containing 10% dialyzed fetal bovine serum and 50 μCi/ml TranSCAN®-S-label (ICN). Cells were then washed in phosphate-buffered saline and solubilized in Triton X-100 buffer (1% Triton X-100, 20 mM Tris-Cl, pH 7.4, 1 mM EDTA, 1 mM diethiothreitol, 35 μg/ml aprotinin, 20 mM sucrose, 1 mM NaN₃, 0.5 mM sodium azide, 50 mM Tris-HCl, pH 7.4, 8 M urea, 5 mM dithiothreitol, 0.6% CHAPS, 1.5% Triton X-100, and protease inhibitors). Protein purification of V-ATPase from bovine kidney membranes prepared as described previously (21) was collected. Immunopurification of V-ATPase from bovine kidney was performed exactly as described (21). The molar concentration of V-ATPase was estimated based on the initial concentration of V-ATPase determined by BCA assay (Pierce) and estimating the molecular weight of the V-ATPase to be 578 kDa.

Actin was purified from rabbit muscle actin powder by standard methods (22, 23). Actin was labeled with [γ-^32P]ATP and subjected to autoradiography and/or phosphorimetry. Immunoblots of actin, and A, B, and E subunits of the V-ATPase were excised and PAGE was performed, and gels were stained with Coomassie Blue. The proportions of actin in the supernatant and pellet were determined by the method of Fenner et al. (25) except that the amount of 25% pyridine used to extract bands was reduced to 0.4 ml. Tissue-cultured-stained actin bands were cut out and extracted for 2 days in 25% pyridine, and the absorbance of the extract at 590 nm was determined.

To determine whether gelsolin-shortened F-actin bound V-ATPase as well as long actin filaments (26, 28), actin (70 μM), was polymerized in buffer G plus 100 mM NaNCl and 5 mM MgCl₂ in the presence of gelsolin (Sigma). The proportions of actin in the supernatant and pellet were determined by the method of Fenner et al. (25) except that the amount of 25% pyridine used to extract bands was reduced to 0.4 ml. To determine whether gelsolin-stained actin bands were cut out and extracted for 2 days in 25% pyridine, and the absorbance of the extract at 590 nm was determined.

Determination of the stoichiometry of V-ATPase binding to F-actin required that actin concentrations below the critical concentration be used. To accommodate this, actin (70 μM) was polymerized in buffer G plus 100 mM NaNCl and 5 mM MgCl₂, then diluted to 200 nM in the same buffer, and 5 μM V-ATPase in buffer G was added (gelsolin filaments), and 1 mg/ml bovine serum albumin (to reduce nonspecific binding). After 2 h at room temperature and ultracentrifugation at 200,000 × g for 45 min, pellets and supernatants were collected, subjected to SDS-PAGE, and the gels were stained with Coomassie Blue. The proportions of actin in the supernatant and pellet were determined by the method of Fenner et al. (25) except that the amount of 25% pyridine used to extract bands was reduced to 0.4 ml. Transmission electron microscopy (Bio-Rad), and Zeiss transmission electron microscope operated at 80 kV.

Transmission Electron Microscopy—Rabbit muscle actin (70 μM) was polymerized in 20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.5 mM ATP, 0.2 mM CaCl₂, 0.1% Triton X-100 and diluted into the same buffer to 2.5 μM plus or minus 0.05 μM V-ATPase. The samples were then spun at 200,000 × g for 45 min, and the pellets were collected in the same buffer except with no detergent. 400 mesh Formvar/carbon-coated nickel grids were used to collect samples that were negatively stained with 1% uranyl acetate (28). The specimens were examined using a Zeiss transmission electron microscope operated at 80 kV.
RESULTS

To study the role of the actin cytoskeleton in regulating V-ATPase distribution during osteoclast activation, we examined the distribution of F-actin and V-ATPase in osteoclasts plated on coverslips and dentine slices using labeled phalloidin to detect actin filaments, and monoclonal antibody E11, against the E subunit, to detect V-ATPase (20). In inactive osteoclasts on glass, V-ATPase colocalized with the loose network of actin filaments at the cell periphery and was concentrated in the actin ring (Fig. 1, A–C). The actin ring that forms in inactive osteoclasts is not associated with ruffled membrane formation and occurs at the periphery of cells. In contrast, in osteoclasts plated on dentine, the distribution of actin and V-ATPase varied with the state of the resorptive cycle of the cells. At the initial stage of the resorption cycle, before the formation of actin rings, actin patches formed near the cell surface, in which V-ATPase and actin were localized (Fig. 1, D–F, large arrow). At a later stage of the cycle, rings of actin formed surrounding the patches, and extensive colocalization of actin and V-ATPase occurred within the newly formed rings (Fig. 1, D–F, small arrow). In fully activated osteoclasts, most of the actin in the central patches had dissipated, leaving the actin rings intact, but V-ATPase remained localized in the interior of the ring (Fig. 1, G–I).

To determine if the V-ATPase was associated with the Triton-insoluble actin-based cytoskeleton, we extracted osteoclasts on coverslips and dentine slices with 0.5% Triton X-100 containing fluorescein-phalloidin to stabilize microfilaments, and we fixed the samples for examination of V-ATPase distribution by immunocytochemistry. In osteoclasts on coverslips, V-ATPase staining was present throughout the cell in a distribution similar to that of the loose actin filament network (Fig. 2, A–C). The actin rings of the inactive osteoclasts were quite labile under these conditions and were never preserved. V-ATPase did not remain associated with actin from stromal cells in the culture. In osteoclasts plated on dentine slices, the true actin rings sometimes survived detergent extraction. Although the V-ATPase in the ruffled membranes was extracted, V-ATPase that colocalized with the loose filaments internal to the ring remained (Fig. 2, D–F).

These observations suggested that the V-ATPase associates directly with the Triton-insoluble cytoskeleton during the process of osteoclast activation. To examine this possibility, we prepared detergent extracts of osteoclast-containing mouse marrow cultures, centrifuged the extracts at low speed (20,000 × g for 10 min) or high speed (200,000 × g for 1 h), then...
electron blotted and probed them for the presence of V-ATPase (Fig. 3A). In these experiments, the low speed supernatant contained cytoskeletal actin filaments and associated proteins, as well as detergent-soluble proteins; the high speed pellet removed all of the actin filaments and any actin-bound proteins. Although solubilized V-ATPase usually does not pellet under the conditions of the high speed centrifugation (21), half of the total E subunit was found in the high speed pellets (Fig. 3A), suggesting that the enzyme was present in a large molecular weight complex.

To explore this further, radiolabeled V-ATPase was immunoprecipitated from the low speed supernatant (LSS), the high speed supernatant, and the high speed pellet (HSP) using Sepharose beads coated with antibody E11, and the proteins were subjected to SDS-PAGE and detected by autoradiography (Fig. 3B).

The A, B, and E subunits of the V-ATPase were readily detected. Additional polypeptides with molecular masses of 205, 20, and 20 kDa also were recovered in immunoprecipitates from both the low speed supernatant and high speed pellet (Fig. 3B). To determine if these proteins were bound specifically to the V-ATPase on the antibody beads, we tested whether the E11 cognate peptide (20) inhibited binding of both the V-ATPase and the additional proteins to E11 beads (Fig. 3C). E11 immunoprecipitates were incubated with peptide, washed, and analyzed by SDS-PAGE. Incubation of the immunoprecipitates with cognate peptide removed nearly 100% of the bound V-ATPase. The remaining supernatant is shown in Fig. 3A, demonstrating that depolymerization of actin was sufficient to dissociate the V-ATPase from the actin filaments. The remaining supernatant was subjected to SDS-PAGE (Fig. 5). Neither actin nor myosin II were associated with the V-ATPase after this treatment, indicating that depolymerization of actin was sufficient to dissociate the V-ATPase from the actin filaments.

Since the unidentified 205-, 42-, and 20-kDa polypeptides corresponded in size to the cytoskeletal elements myosin heavy chain, actin, and myosin light chain, respectively, we analyzed unlabeled immunoprecipitates by immunoblot analysis, and we confirmed that the 205 and 42 kDa were myosin II heavy chain and actin (Fig. 3D).

As discussed above, osteoclasts have a quiescent phenotype on glass coverslips but become actively resorptive cells when plated on bone slices. To determine whether the interaction of V-ATPase with the actin cytoskeleton might have a role in osteoclast activation, we examined whether the V-ATPase-actin complex differed in osteoclasts plated on glass coverslips or bone. In mature 1,25-(OH)2D3-stimulated osteoclast-containing mouse marrow cultures plated on glass coverslips, 44.5% (range 37.0–54%) of the V-ATPase remained associated with the cytoskeleton, whereas only 12.7% (range 9.9–14.5%) of the V-ATPase was complexed with actin in the cultures on dentine slices (Fig. 4A). To confirm that osteoclasts on dentine were resorptive, slices used in the immunoprecipitation experiment were examined for the presence of resorption lacunae. Fig. 4, B and C, shows representative fields from a control dentine slice and a slice used for osteoclast attachment, demonstrating large numbers of resorption pits produced by osteoclasts in this experiment.

To determine whether the V-ATPase binds to actin or myosin in the complex, we examined the effect of actin depolymerization on V-ATPase association with the cytoskeleton. Immunoprecipitations were performed on the low speed supernatants from 1,25-(OH)2D3-stimulated mouse marrow cultures, and the complexes were treated overnight with buffer G (23) and DNase I, a potent actin monomer-sequestering protein (29). After an overnight incubation, the beads were washed free of DNase I and any other proteins that had dissociated overnight; the V-ATPase was eluted with the E11 cognate peptide, and the samples were subjected to SDS-PAGE (Fig. 5). Neither actin nor myosin II were associated with the V-ATPase after this treatment, indicating that depolymerization of actin was sufficient to dissociate the myosin II from V-ATPase, suggesting that myosin binds to V-ATPase indirectly through actin filaments.

To confirm that V-ATPase bound actin rather than myosin II, solubilized mouse marrow cultures were subjected to high speed centrifugation, and E11 immunoprecipitations were performed from the supernatant. The beads were eluted with the E11 cognate peptide, and the samples were dialyzed overnight against buffer G. The dialyzed samples (Fig. 6, lane 1) were centrifuged to remove any residual filamentous actin or aggregated V-ATPase. The remaining supernatant is shown in Fig.

**Fig. 2. Association of V-ATPase with the detergent-insoluble cytoskeleton of osteoclasts.** Day 5 mouse bone marrow cultures containing osteoclasts were scraped and replated on either glass coverslips (A–C) or dentine slices (D–F). After 2 days, cells were extracted with Triton X-100 and then fixed and examined for actin and V-ATPase localization as described under ‘Materials and Methods.’ Green staining (A and D) represents fluorescein-phalloidin; red staining (C and F) is E11 (anti-V-ATPase) with a Texas-Red-conjugated secondary antibody. B and E are merged images. Samples were viewed by scanning laser confocal microscopy. A–C, the dotted line demarcates the lower edge of a single osteoclast. Cytoskeletal remnants from other cells are seen below the dotted line, but V-ATPase was retained only by the osteoclast. The scale bar (C) is equivalent to 15 μm. D–F, a single resorption site is visible. The scale bar (see F) is equivalent to 4 μm.
V-ATPase Binds F-actin in Osteoclasts

Fig. 3. Interaction of V-ATPase with the actin cytoskeleton. A, 1,25(OH)2D3-treated mouse bone marrow cultures were washed and solubilized in Triton X-100 buffer as described under “Materials and Methods.” Samples were centrifuged at low speed (20,000 × g) at 4 °C for 10 min and then at high speed (200,000 × g) at 4 °C for 1 h. Supernatants and pellets from the low and high speed centrifugation were electrobotted and probed with E11. B, 1,25(OH)2D3-treated mouse bone marrow cultures were radiolabeled, then washed, solubilized, and centrifuged at low speed as in A. 30 × 106 cpm from the supernatant were used for immunoprecipitation (low speed supernatant, LSS). An equal number of cpm was centrifuged at high speed as in (A), and the high speed supernatant (HSS) and high speed pellet (HSP) were recovered. All samples were immunoprecipitated with E11 as under “Materials and Methods,” and samples were separated by SDS-PAGE and subjected to autoradiography. C, marrow cultures were labeled, solubilized, and immunoprecipitated as in B but were treated additionally as follows. The antigen-antibody bead complexes were washed with NET-GEL buffer, and samples were either suspended in SDS-PAGE sample buffer (control) or mixed with 5 mg/ml of E11 cognate peptide (20), or an irrelevant peptide derived from osteopontin (Ost). Following treatment with the peptides, beads were washed with NET-GEL buffer and analyzed by SDS-PAGE and fluorography as in B. D, whole marrow culture extracts (WC) or immunoprecipitations from low and high speed supernatants (S) and pellets (P) were subjected to SDS-PAGE and immunoblotted either with E11, with anti-non-muscle myosin II polyclonal antibody (Cortex, San Leandro, CA), or with a monoclonal anti-actin antibody (Sigma).

6, lane 2. 4.5 μM purified rabbit skeletal muscle G-actin was added and was then either polymerized by addition of 5 mM MgCl2 at room temperature or left unpolymerized, and the samples were pelleted by high speed centrifugation. V-ATPase was found complexed to the added actin in the pellet under conditions promoting actin polymerization (Fig. 6, lanes 3 and 4) but remained in the supernatant under conditions in which actin was left unpolymerized (Fig. 6, lanes 5 and 6). No myosin II was detected in these samples after the initial centrifugation, suggesting that the V-ATPase binds to actin filaments rather than to myosin.

Since V-ATPase from osteoclasts (30) has similar properties to V-ATPase from bovine kidney microsomes (21), we tested whether the bovine kidney V-ATPase binds directly to purified rabbit muscle actin. Kidney V-ATPase was mixed with purified rabbit G-actin, and the mixture was centrifuged to remove any filamentous actin or aggregated V-ATPase. The sample was divided and incubated in the presence or absence of Mg2+. The samples were centrifuged again at 200,000 × g, and the supernatants and pellets were electrobotted and probed with E11. As shown in Fig. 7, V-ATPase pelleted under conditions in which the actin polymerized (+Mg2+) but remained in the supernatant when the actin remained monomeric (−Mg2+).

To ensure that cosedimentation of V-ATPase with F-actin was not the result of nonspecific trapping of V-ATPase in networks of long actin filaments, we next performed pelleting assays using gelsolin-shortened filaments. V-ATPase pelleted equally well in the presence of actin filaments formed with gelsolin at molar ratios of 1:20 and 1:40 (gelsolin:actin) as with full-length filaments (Fig. 8).

As further evidence of V-ATPase-actin interaction, kidney V-ATPase was subjected to two cycles of actin polymerization and depolymerization, a standard method for detecting F-actin-binding proteins (31). Purified G-actin and kidney V-ATPase were mixed and centrifuged; actin was polymerized by addition of magnesium, and the sample was centrifuged again. The pellet was dialyzed against buffer G overnight to depolymerize the actin, centrifuged to remove remaining filamentous actin and insoluble aggregates, and polymerized again with magnesium. The sample was centrifuged, and the final supernatant and pellet were collected. Samples from each step were subjected to SDS-PAGE, and the resulting gel was silver-stained. V-ATPase was recovered in the pellet bound to filamentous actin in both polymerization cycles, further confirming that the V-ATPase complex contains an actin-binding domain (Fig. 9).

Phalloidin-stabilized F-actin was used to determine the molar ratio where V-ATPase approached saturation of the F-actin-binding sites. F-actin (200 nm) was incubated with different concentrations of V-ATPase, and the amount of V-ATPase induced to pellet at high speeds was determined by densitometry of E11 immunoblots (Fig. 10). Finding that V-ATPase binding to F-actin approached saturation at a ratio of about 1 mol of V-ATPase per 8 mol of F-actin monomer, we estimated the molarity of free binding sites to be 1/8 the molar concentration of F-actin. Pelletting assays were performed, and the amount of V-ATPase pelleting was determined based on pyridine extraction of V-ATPase bands from Coomassie-stained gels. These data were used to construct the Haines plot shown in Fig. 11, from which we obtained an apparent Kd for the interaction of 50 nm.

To confirm binding further, we directly visualized V-ATPase bound to actin filaments. V-ATPase were clearly detected bound to the sides of F-actin (Fig. 12). Greater than 95% of the V-ATPases were associated with filaments (data not shown). V-ATPase appeared to interact with microfilaments through the thick head of the V1 domain, rather than through the stalk.
DISCUSSION

Previous studies suggested that V-ATPase interacted with the detergent-insoluble cytoskeleton of osteoclasts (16, 17) and that this interaction is crucial for osteoclast function (16). Here, we demonstrate that V-ATPase binds directly to actin filaments. Although immunoprecipitation experiments showed that both actin and myosin II were associated with V-ATPase recovered from osteoclasts, purified V-ATPase pelleted with exogenous rabbit muscle actin, even in fractions containing no detectable myosin II. Finally, immunoaffinity-purified V-ATPase from bovine kidneys bound highly purified rabbit muscle actin in pelleting assays. The estimated stoichiometry of binding was 1 V-ATPase per 8 actin monomers. Based on this stoichiometry we determined the dissociation constant for the V-ATPase-actin interaction to be 50 nM.

The ability to bind actin filaments appears to be a general characteristic of mammalian V-ATPases, since V-ATPase derived from both osteoclasts and bovine kidney bound microfilaments made from rabbit muscle actin. In osteoclasts the amount of V-ATPase bound to F-actin changed with the state of activation of the cell. The protein composition of the V-ATPase was not altered during activation, indicating that binding of...
V-ATPase to microfilaments, in osteoclasts, is under regulated control. Because V-ATPases from various sources share many common subunits, it is likely that the osteoclast V-ATPase does not contain a unique component that confers the ability to bind microfilaments. However, in coimmunoprecipitation experiments not shown here, we found little or no actin associated with V-ATPase from kidney cell-derived detergent extracts.2

2 B. S. Lee, L. S. Holliday, I. Krits, and S. L. Gluck, unpublished data.
Thus, although kidney V-ATPase binds to purified actin very tightly in vitro, it does not always do so in vivo. This also is consistent with the hypothesis that V-ATPase binding to F-actin is regulated in cells.

The interaction of V-ATPase with the actin cytoskeleton varied with the state of activity of osteoclasts, suggesting that the interaction may have a role in regulating the transport of V-ATPase from cytoplasmic stores to the ruffled membrane during osteoclast activation. Cytoplasmic vesicles are known to be transported by molecular motors that travel along microfilaments or microtubules (32, 33). The V-ATPase in the osteoclast appears not to fit that paradigm. V-ATPase attaches directly to microfilaments and indirectly to myosin II, a two-headed molecular motor associated with cytoskeletal contraction but not with vesicle transport along filaments.

We propose the following model to explain V-ATPase polarization in osteoclasts. In inactive osteoclasts, V-ATPase is distributed diffusely in the cell (13, 16), bound to the cortical cytoskeleton by direct interaction with actin. Cytoskeletal contraction upon osteoclast activation, powered by myosin II force generation, could impel actin filaments to form the patches observed at osteoclast attachment sites (2). The cytoskeletal contraction we envision is similar to the contraction of contractile rings in cytokinesis (34, 35) or the capping of cell-surface receptors (36, 37). We showed that V-ATPase colocalized with actin in the initial patches, early in osteoclast activation, but that later V-ATPase was present in the ruffled membrane and did not colocalize extensively with actin filaments, suggesting that formation of the ruffled membrane occurs after the release of V-ATPase from actin binding. Cytochemistry of detergent-extracted osteoclast remnants confirmed that V-ATPase interacted with the detergent-insoluble cytoskeleton in areas where V-ATPase and microfilaments colocalized. Immunoprecipitation experiments supported these findings by showing that the amount of V-ATPase complexed with actin was reduced significantly in osteoclasts following activating stimuli. After formation of the ruffled membrane, actin filaments remained in the actin ring and dissipated in the regions adjacent to the ruffled membrane.

In prior studies, osteoclasts microinjected with inhibiting antibodies to myosin II showed a marked reduction in bone resorptive activity (38). Also consistent with our hypothesis are genetic studies in yeast which demonstrate that defects in the resorptive activity (38). Also consistent with our hypothesis are genetic studies in yeast which demonstrate that defects in the actin ring and dissipation following activating stimuli. After formation of the ruffled membrane, actin filaments remained in the actin ring and dissipated in the regions adjacent to the ruffled membrane.

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