1102

Biochemical and functional characterization of the actin-binding activity of the B subunit of yeast vacuolar H$^+$-ATPase

Jian Zuo[1], Sandra Vergara[1], Shinya Kohno[2] and L. Shannon Holliday[1,3,*]

[1]Department of Orthodontics, University of Florida College of Dentistry, Gainesville, FL 32610, USA, [2]Department of Orthodontics and Craniofacial Developmental Biology, Hiroshima University, Hiroshima, Japan and [3]Department of Anatomy and Cell Biology, University of Florida College of Medicine, Gainesville, FL 32610, USA

*Author for correspondence (e-mail: sholliday@dental.ufl.edu)

SUMMARY

Vacuolar H$^+$-ATPase (V-ATPase) is a fundamentally important enzyme in eukaryotic cells that is responsible for acidification of endocytic compartments. The B subunits of V-ATPases from mammals and tobacco hornworm have been shown to bind actin filaments. Actin-binding activity by the B subunit is required for targeting V-ATPases to the plasma membrane of osteoclasts. Bacterially expressed B subunit from the yeast *Saccharomyces cerevisiae* bound actin filaments with a K_d of 195 nmol l^{-1}. The actin-binding domain of the B subunit was altered by mutations that reduced or eliminated the actin-binding activity. Mutants assembled properly with endogenous yeast subunits when expressed in B subunit-null yeast and bafilomycin-sensitive ATPase activity was not significantly different from yeast transformed with wild-type B subunit. Yeast containing the mutant subunits grew as well at pH 7.5 as wild-type. Screening null yeast or null yeast transformed with wild-type or mutant B subunits with sub-lethal doses of various drugs revealed that yeast containing the mutant B subunits were more sensitive to cycloheximide and wortmannin than those transformed with wild-type B subunits. These results suggest that actin-binding activity confers on the B subunit of yeast a function that is distinct from its role in the enzymatic activity of the proton pump.

Key words: V-ATPase, F-actin, *Saccharomyces cerevisiae*, actin-binding protein, cycloheximide, wortmannin, PI 3-kinase.

INTRODUCTION

Vacuolar H$^+$-ATPases (V-ATPases) are multi-subunit enzymes that play vital housekeeping roles in eukaryotic cells (Kane, 2006; Inoue et al., 2003; Harvey, 1992; Nelson and Harvey, 1999). These roles include acidifying endosomes, lysosomes and phagosomes. In some differentiated cells, including osteoclasts and renal intercalated cells, V-ATPases are expressed at high levels and play specialized roles (Gluck et al., 1998; Holliday et al., 2005; Wagner et al., 2004). Recent studies also indicate that asymmetrical targeting of V-ATPases, involving the actin cytoskeleton, plays a vital role in embryonic development (Adams et al., 2006). In all cases, V-ATPases must be delivered to the appropriate cellular destinations and activated in concert with the needs of the cell and organism. The mechanisms for targeting and activation of V-ATPases are not yet well understood.

V-ATPases bind filamentous-actin (F-actin, microfilaments) through binding sites located in the amino-terminal domain of subunit B (Holliday et al., 2000; Lee et al., 1999a; Chen et al., 2004) and in two domains of subunit C (Vitavska et al., 2003; Vitavska et al., 2005). A motif resembling the actin-binding site of mammalian profilin 1 and referred to as the profilin-like motif is vital for the actin-binding activity of the mammalian B subunits (Zuo et al., 2006; Chen et al., 2004). This actin-binding activity plays a role in the delivery of V-ATPases to the ruffled plasma membrane of osteoclasts as they activate to resorb bone (Zuo et al., 2006). In mammals, both the B2 isoform, which is expressed ubiquitously and at high levels in osteoclasts, and B1, which is expressed in kidneys and in a few other tissues, bind F-actin (Holliday et al., 2000). In addition, it was reported that subunit B

from the tobacco hornworm binds F-actin (Vitavska et al., 2003). These data indicate that the actin-binding activity of subunit B has roles in addition to its function in osteoclasts (Holliday et al., 2005).

Examination of the sequence of subunit B from the yeast *Saccharomyces cerevisiae* suggested that it might also bind actin (Holliday et al., 2005). Yeast are an advantageous model for studying V-ATPases (Kane et al., 1992; Nelson and Klionsky 1996). They are easily manipulated genetically and can be grown in quantities that allow biochemical and enzymatic studies (Kane et al., 1989; Nelson and Klionsky, 1996). Yeast grow well without V-ATPase in acidic media, but require V-ATPase activity if they are on alkaline media. This allows replacement knock-in experiments involving subunits of the V-ATPase to be readily performed (Yamashiro et al., 1990).

In this study, we report that yeast subunit B binds F-actin with a similar affinity to mammalian B subunits. We tested whether we could dissect functions of the actin-binding activity of the yeast B subunit from its known required role in the enzymatic activity of the proton pump.

MATERIALS AND METHODS
Reagents
Unless otherwise noted all reagents were obtained from Sigma Chemical Company (St Louis, MO, USA) and were of the highest available research grade. The high-fidelity Expand enzyme system was purchased from Roche Applied Science (Indianapolis, IN, USA). Restriction endonucleases and T4 DNA ligase were obtained from New England Biolabs Inc. (Beverly, MA, USA). The yeast

expression vectors YC2/CT and YES3/CT were from Invitrogen (Carlsbad, CA, USA). Monoclonal antibodies against the B subunit (13D11) and A subunit (8B1) of V-ATPase were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Zymolyase 100T was purchased from ICN (Costa Mesa, CA, USA). Bafilomycin A_1, 3-aminotriazole and isopropyl-β-D-thiogalactopyranoside (IPTG) were from Sigma.

Yeast B subunit constructs

To construct a wild-type yeast B subunit (Vma2p)-maltose-binding protein (MBP) fusion protein, we amplified by polymerase chain reaction (PCR) the DNA fragment encoding the full-length yeast B subunit from yeast DNA isolated from strain PJ69 as described previously (Lu et al., 2004). The following sense and antisense primers were used: 5′-GGCGGATCCATGGTTTTGTCTGAT-AAG-3′ and 5′-GCGCGCGTCGACTTAGATTAGAGATTC-3′; 200 pmol each of the sense and antisense primers was added to 100 ng of the DNA template in 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris-HCl pH 9.0, 1.0% Triton X-100, and 0.2 mmol l^{-1} dATP, dCTP, dGTP and dTTP. PCR was carried out for 30 cycles at 94°C for 1 min, 52°C for 1 min and 72°C for 1 min. The PCR product was ligated into the vector pMAL-c2X (New England Biolabs) at *Bam*H1/*Sal*1 restriction sites. Colonies of XL-1 Blue *Escherichia coli* (Stratagene, La Jolla, CA, USA), transformed with the ligation products, were selected for inducible expression of the fusion proteins with 0.3 mmol l^{-1} IPTG. An inducible product with a predicted size of 95 kDa was demonstrated by SDS-PAGE analysis of whole-cell extracts. The fusion protein construct was verified by dideoxynucleotide sequencing. The fusion protein was purified on amylose columns, using protocols supplied by the manufacturer (New England Biolabs). Homogeneity was demonstrated by SDS-PAGE. We replaced the profilin-like actin-binding motif in yeast B subunit using site-directed mutagenesis. 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 the sequence of the homologous stretch of the B subunit of the Archaea. The mutants, referred to as Vma2p^{Arch}- and Vma2p^{Phe}-MBP fusion proteins, were constructed using Vma2p-MBP as a template for PCR. Vma2p^{Phe}-MBP was constructed by adding a point mutation using 5′-GAAAAGGTCAAGGGCCCACGTTAC-3′ as the forward primer and 5′-GTAACGTGGGCCCTTGACCT-TTTC-3′ as the reverse primer. To generate Vma2p^{Arch} we used 5′-GGTCCATTAGTCATTGTTGCTGGAGGTGCAGGCCCAC-GTTACAACG-3′ as the forward primer and 5′-CGTTGTAA-CGTGGGCCTGCACCTCCAGCAACAATGACTAATGGACC-3′ as the reverse primer. The Expand long template PCR system (Roche Applied Science) was used to make the full-length templates. These consisted of the pMAL-c2X vector with the profilin-like actin-binding motif altered. The PCR conditions were as follows: 94°C, preheat for 2 min, 30 cycles at 94°C for 15 s, 56°C for 1 min and 72°C for 10 min, and an extra 72°C for 15 min to ensure completion of elongation steps. Sticky ends were prepared by digesting with *Bam*HI. Ligation was carried out at 15°C overnight. Bacteria were transformed with the ligation products; clones were picked and tested by restriction mapping, and constructs were confirmed by DNA sequencing. Dr Patricia Kane (Upstate Medical University, Syracuse, NY, USA) kindly provided us with the plasmid (pRS316-*VMA2*) containing Vma2p plus 150 bp upstream and 39 bp downstream. PRS316 is a yeast expression vector. The mutant versions of yeast B subunit (Vma2p^{Arch} and Vma2p^{Phe}) were made by long-range PCR using pRS316-*VMA2* as the template. In all cases, the open reading frame was sequenced following mutagenesis to confirm mutations and the absence of any additional mutations.

Actin-binding assays

Actin was prepared from rabbit muscle acetone powder by standard methods, and was further purified by two rounds of polymerization–depolymerization and gel filtration on a 2.5 cm×100 cm Sephacryl S-300 column (Amersham Pharmacia Biotech, Uppsala, Sweden) (Holliday et al., 1993). The affinity and stoichiometry of Vma2p-MBP, Vma2p^{Arch}-MBP and Vma2p^{Phe}-MBP for actin were determined by quantitative binding assays. Protein concentrations of actin and the fusion proteins were determined by BCA assay (Pierce, Rockford, IL, USA). Purified rabbit muscle actin was polymerized at a concentration of 70 μmol l^{-1} in buffer F (20 mmol l^{-1} Tris pH 8.0, 100 mmol l^{-1} NaCl, 5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} $CaCl_2$, 0.5 mmol l^{-1} ATP, 0.2 mmol l^{-1} dithiothreitol), and diluted in buffer F immediately prior to the experimental procedure. F-actin alone (0.8 μmol l^{-1}) or F-actin plus varying concentrations of fusion protein dialyzed against buffer F was incubated for 1 h at room temperature. The samples were then subjected to ultracentrifugation at 200 000 g for 45 min, and pellets and supernatants were collected, separated by SDS-PAGE, and stained with Coomassie Brilliant Blue, and the amount of fusion protein in the supernatants and pellets was determined by absorbance densitometry using a Fluorchem 8000 (Alpha Innotech, San Leandro, CA, USA) as described previously (Holliday et al., 2000).

Vma2p expression in yeast

*VMA2*Δ yeast (strain BY4743) were obtained from ATCC (American Type Culture Collection). The pRS316-Vma2p, pRS316-Vma2p^{Phe} and pRS316-Vma2p^{Arch} plasmids were transformed into *VMA2*Δ using an overnight lithium acetate transformation protocol (Ito et al., 1983) and transformants were selected on supplemented minimal medium lacking uracil (SD-uracil).

Immunoprecipitations

Yeast cells were grown in supplemented minimal medium, harvested by centrifugation at 1000 g for 5 min, resuspended in pretreatment buffer (0.1 mol l^{-1} Tris-HCl pH 9.0, 10 mmol l^{-1} dithiothreitol), and incubated for 5 min. Spheroplasts were generated by treatment with zymolyase 100T (ICN) for 20 min in SPC buffer (1% glucose, 1 mol l^{-1} sorbitol, 50 mmol l^{-1} K_2HPO_4, 16 mmol l^{-1} citric acid, pH 5.8), lysed in solubilization buffer (10 mmol l^{-1} Tris-HCl pH 7.5, 10% glycerol, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} dithiothreitol, 1% polyoxyethylene-9-lauryl ether) for 15 min and incubated with the anti-B subunit antibody 13D11 (Molecular Probes) for 60 min. After 60 min incubation with protein A-agarose beads, the immunoprecipitates were collected by centrifugation, washed three times, and incubated for 5 min at 70°C in 50 μl of cracking buffer (50 mmol l^{-1} Tris-HCl, pH 7.0, 8 mol l^{-1} urea, 5% SDS, 5% β-mercaptoethanol) for SDS-polyacrylamide gel electrophoresis and Western blot analysis. Vacuolar membrane vesicles were prepared from mid-log phase yeast cells as described previously (Lu et al., 2004). Western blotting was carried out by separating V-ATPase immunoprecipitates on 10% polyacrylamide gels as described previously (Holliday et al., 2000) using a Bio-Rad minigel apparatus (Hercules, CA, USA) followed by electrophoretic transfer to Hybond nitrocellulose membranes (Amersham Biosciences) using a Trans-Blot apparatus (Bio-Rad). To block non-specific binding, the membranes were incubated in 5% non-fat dried milk in TTBS (10 mmol l^{-1} Tris pH 8.0, 500 mmol l^{-1} NaCl and 0.05% Tween

20) with gentle agitation for 1 h at room temperature. Monoclonal antibodies against yeast A and B subunits were used to probe the blots and binding was detected using the SuperSignal West Dura substrate working solution (Pierce) according to the manufacturer's instructions.

ATPase activity

Bafilomycin A_1-sensitive ATP hydrolysis of vacuolar membranes was assayed by measuring the production of inorganic phosphate as described previously (Wang and Gluck, 1990; Lu et al., 2004). Briefly, vacuolar membrane vesicles in ATPase buffer (150 mmol l^{-1} NaCl, 2 mmol l^{-1} $MgCl_2$, 1 mmol l^{-1} vanadate, 1 mmol l^{-1} azide, pH 6.75) were preincubated for 15 min at room temperature in the presence and absence of bafilomycin A_1. The reaction was initiated by addition of ATP to a final concentration of 3 mmol l^{-1} and stopped by addition of trichloroacetic acid after 20 min. The samples were extracted with chloroform to remove lipid and detergent. After centrifugation, the upper aqueous phase was transferred to clean test tubes and incubated with buffers containing ascorbic acid and ammonium molybdate. The concentration of inorganic phosphate was measured by a spectrophotometer at 700 nm and converted to the rate of ATP hydrolysis (Wang and Gluck, 1990).

Growth assays/chemical screen

A chemical screen for drug resistance was performed as described previously (Parsons et al., 2004b). Drugs were added from concentrated stocks to autoclaved rich (YPD; 2% peptone, 1% yeast extract, 2% glucose and 2% agar) or synthetic complete (SC; 0.67% yeast nitrogen base without amino acids, 0.2% amino acid add back, 2% glucose and 2% agar) medium cooled to ~50°C. Drugs were screened and confirmed by spot assays at the concentration noted: FK506 (an immunosuppressant, 2 μg ml^{-1} in SC, AG Scientific, San Diego, CA, USA), hydroxyurea (inhibits ribonucleotide reductase, 100 mmol l^{-1} in YPD; Sigma), camptothecin (damages DNA, 15 μg ml^{-1} in YPD; AG Scientific), wortmannin (phosphatidylinositol 3-kinase inhibitor, 1.3 μg ml^{-1} in YPD; AG Scientific), sulfometuron methyl (herbicide, 3 μg ml^{-1} in SC; Sigma) and cycloheximide (translation inhibitor, 0.1 μg ml^{-1} in YPD; Sigma).

RESULTS
Yeast subunit B binds microfilaments

The *VMA2* gene encoding yeast subunit B was cloned from yeast and expressed in bacteria using the MBP expression system that we had utilized previously to characterize mammalian B subunits
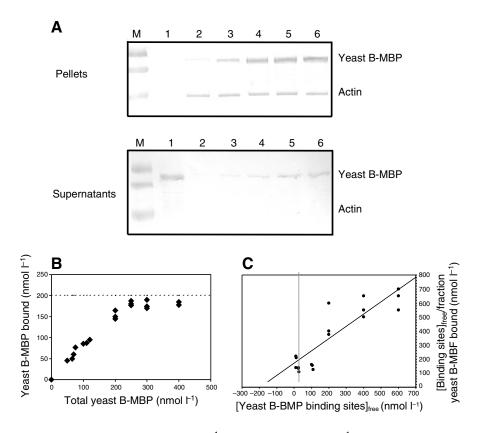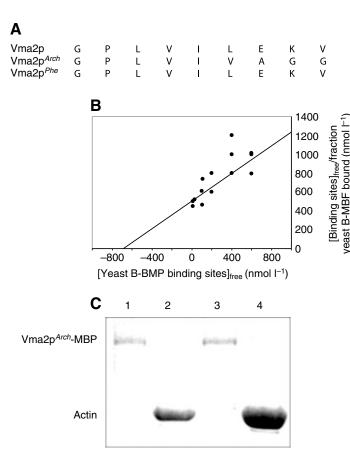

Fig. 1. Yeast B subunit binds F-actin. F-actin was diluted to 800 nmol l^{-1} and stabilized with 5 μmol l^{-1} phalloidin, mixed with various concentrations of Vma2p-MBP, and subjected to ultracentrifugation. Pellets and supernatants were collected, separated by SDS-PAGE and stained with Coomassie Brilliant Blue (A). M, markers (top to bottom; 110 kDa, 67 kDa, 43 kDa); lane 1, no actin, 2 μmol l^{-1} Vma2p-MBP; lane 2, 0.8 μmol l^{-1} F-actin, 0.4 μmol l^{-1} Vma2p-MBP; lane 3, 0.8 μmol l^{-1} F-actin, 0.8 μmol l^{-1} Vma2p-MBP; lane 4, 0.8 μmol l^{-1} F-actin, 1.2 μmol l^{-1} Vma2p-MBP; lane 5, 0.8 μmol l^{-1} F-actin, 1.6 μmol l^{-1} Vma2p-MBP; lane 6, 0.8 μmol l^{-1} F-actin, 2.0 μmol l^{-1} Vma2p-MBP. The pellets were loaded with twice the relative amount of protein compared with supernatants. (B) Actin was polymerized and diluted into solutions containing phalloidin (5 μmol l^{-1}) and yeast B-MBP as described under Materials and methods. The final actin concentration, indicated by the broken line, was 800 nmol l^{-1}. Following incubation and centrifugation, the supernatants and pellets were collected and the amount of fusion protein present was determined by densitometry of Coomassie-stained gels. (C) Varying amounts of Vma2p-MBP were added to F-actin and subjected to high speed centrifugation as described under Materials and methods. Pellets and supernatants were analyzed by SDS-PAGE and densitometry was used to determine the amount of protein in the Coomassie-stained gels. The K_d was calculated based on the finding that the fusion protein binding saturated at 1 mol of fusion protein per mol of actin filament subunit (established in A and B).

A

Vma2p	G	P	L	V	I	L	E	K	V	K	F
Vma2p*Arch*	G	P	L	V	I	V	A	G	G	A	G
Vma2p*Phe*	G	P	L	V	I	L	E	K	V	K	G

Fig. 2. Mutations in the profilin-like motif of yeast B subunit reduce or eliminate actin-binding activity. (A) Alterations in the profilin-like motif made and analyzed in the current study. Amino acids 35–45 in wild-type Vma2p, Vma2p*Phe* and Vma2p*Arch* are compared. (B) Haine's plot of Vma2p*Phe*-MBP binding to F-actin. (C) Vma2p*Arch*-MBP does not bind F-actin: Lane 1, supernatant and lane 2 pellet of 4.0 μmol l^{-1} F-actin and 1.0 μmol l^{-1} Vma2p*Arch*-MBP. Lanes 3 and 4 are the supernatant and pellet of 8.0 μmol l^{-1} F-actin and 1.0 μmol l^{-1} Vma2p*Arch*-MBP supernatant.

B

C

(Holliday et al., 2000; Chen et al., 2004). This resulting fusion protein (Vma2p-MBP) was subjected to ultracentrifugation alone, or in a mixture with F-actin. Yeast B subunit bound F-actin (Fig. 1A). As we reported previously for mammalian B subunits, we did not detect G-actin-binding activity in pulldown experiments (data not shown). As with mammalian B subunits, saturation of binding to F-actin was approached at a 1:1 stoichiometry (1 mol fusion protein:1 mol F-actin subunit; Fig. 1B). Using this stoichiometry, a dissociation constant was determined from a Haines's plot (Fig. 1C). The K_d of Vma2p-MBP was calculated to be 195 nmol l^{-1}.

Mutations in the profilin-like region of yeast B subunit reduce or eliminate the actin-binding activity of yeast subunit B

We had previously been able to eliminate the actin-binding activity of mammalian B subunits by inserting a spacer sequence derived from an Archaea (Chen et al., 2004). We made a point mutation changing phenylalanine 45 to glycine, and also made a construct with an Archaea-based spacer insert in the yeast B subunit. These constructs were expressed as MBP fusion proteins and will be referred to as Vma2p*Phe*-MBP and Vma2p*Arch*-MBP (Fig. 2A). Vma2p*Phe*-MBP bound F-actin with an affinity of 740 nmol l^{-1}, compared with 195 nmol l^{-1} for yeast B subunit-MBP (Fig. 2B). We were unable to detect binding activity in Vma2p*Arch*-MBP (Fig. 2C).

Vma2p*Arch* assembles with endogenous V-ATPase subunits in yeast and accommodates similar bafilomycin-sensitive ATPase activity compared with wild-type yeast B subunit

*VMA2*Δ yeast and *VMA2*Δ yeast transformed with Vma2p*Arch* or Vma2p were grown at pH 5.5 and immunoprecipitation experiments were performed. We found that Vma2p*Arch* and Vma2p were

expressed and assembled with yeast A subunit (Fig. 3A). Vacuolar membranes from *VMA2*Δ and *VMA2*Δ containing Vma2p*Arch* or Vma2p were isolated and probed for the presence of yeast A subunit, Vma2p and Vma2p*Arch*. Similar amounts of subunit A and the wild-type and mutant B subunits were associated with vacuoles. The vacuoles were assayed for bafilomycin-sensitive ATPase activity (Fig. 3B). No difference was detected between Vma2p*Arch* and Vma2p.

Yeast containing Vma2p*Arch* and Vma2p*Phe* grow as well at pH 7.5 as yeast containing Vma2p

*VMA2*Δ yeast or *VMA2*Δ yeast expressing Vma2p*Arch*, Vma2p*Phe* or Vma2p were grown at either pH 5.5 or pH 7.5. As expected, all grew well at pH 5.5, although the null yeast grew slightly less well than the others (Fig. 4A). The null yeast failed to grow at pH 7.5 and the Vma2p-, Vma2p*Phe*- and Vma2p*Arch*-containing yeast could not be distinguished with regard to their growth (Fig. 4A).

Vma2p*Arch* is not as protective as yeast B subunit in the presence of cycloheximide and wortmannin

It was reported that the actin cytoskeleton was not involved in glucose-dependent dissociation of V-ATPase on vacuoles (Xu and Forgac, 2001). Consistent with this result, we did not detect differences in glucose-dependent dissociation in yeast containing Vma2p and Vma2p*Arch* (data not shown).

Recently it was found that yeast V-ATPase genes, including *VMA2*, play vital roles in multidrug resistance (Parsons et al., 2004a). We tested Vma2p-, Vma2p*Phe*- and Vma2p*Arch*-containing yeast and null yeast for their ability to grow in the presence of sub-lethal concentrations of a panel of drugs. We detected no difference in growth related to the mutations in the actin-binding region when cells were exposed to camptothecin, hydroxyurea, FK506 or sulfometuron methyl. The Vma2p*Arch*-containing cells were less able to grow in the presence of cycloheximide and wortmannin (Fig. 4B).

DISCUSSION

Our data show for the first time that the B subunit from yeast V-ATPase binds F-actin. Thus, the actin-binding activity of the B subunit is found in eukaryotic cells ranging from yeast to humans. We have presented data indicating that yeast B subunit plays a role in enabling yeast growth when they are stressed by sub-lethal doses of cycloheximide or wortmannin. Previous studies identified the actin-binding activity in V-ATPases from highly specialized cells;
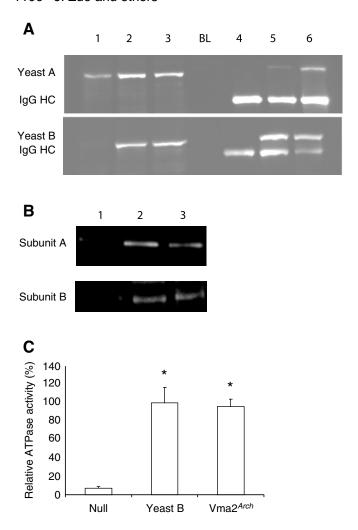
A

B

C

Fig. 3. Mutations in the profilin-like region of yeast B subunit do not affect assembly of bafilomycin-sensitive ATPase activity. (A) A yeast strain lacking yeast B subunit (*VMA2Δ*; lanes 1 and 4) was transformed with Vma2p (lanes 2 and 5) or Vma2p^{Arch} and separated by SDS-PAGE, blotted, and probed with monoclonal antibodies against yeast A subunit (top panel) or yeast B subunit (bottom panel). Lanes 1–3 are whole-cell blots; lanes 4–6 were immunoprecipitated with anti-B subunit and probed with anti-A subunit. The heavy chain (HC) of the immunoprecipitating antibody was detected in lanes 4–6. This experiment was repeated three times, the subunit A bands and heavy chain bands were analyzed by densitometry and the ratio of subunit A:heavy chain was determined. For wild-type cells the ratio was 0.94±0.02, for the Vma2p^{Arch}-transformed cells the ratio was 0.95±0.03. This indicates that very similar amounts of subunit A were immunoprecipitated with anti-B antibody from *VMA2Δ* yeast transformed with wild-type yeast B subunit or Vma2p^{Arch}. BL, blank. (B) Vacuolar membranes were prepared for assay for bafilomycin-sensitive ATPase activity as described in Materials and methods. Samples of the vacuolar membranes from *VMA2Δ* yeast (lane 1), and *VMA2Δ* yeast transformed with yeast B subunit (lane 2) or with Vma2p^{Arch} plasmids (lane 3) were subjected to SDS-PAGE and immunoblotted to demonstrate the levels of subunit A and B associated with membranes. (C) ATPase assays were performed on the remainder of the membranes. Activities are expressed relative to vacuolar membranes isolated from the *VMA2Δ* strain transformed with Vma2p, which was defined as 100% and had a specific activity of 1.11 μmol l^{-1} ATP min^{-1} mg^{-1} protein in this experiment. Vacuolar membranes from the null yeast were significantly different from those of null yeast transformed with Vma2p (Student's *t*-test, *P<0.05). The membranes from the yeast transformed with Vma2p^{Arch} were also significantly different from those of the null yeast, but not significantly different from those of the yeast transformed with Vma2p.
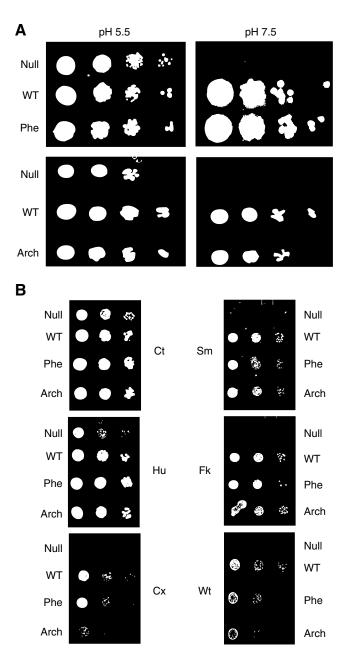
A

B

Fig. 4. Vma2p^{Arch}-containing yeast show increased sensitivity to cycloheximide and wortmannin. *VMA2Δ* yeast cells carrying no plasmid (Null) or transformed with yeast B subunit (WT), Vma2p^{Phe} (Phe) or Vma2p^{Arch} (Arch) plasmids were grown to log phase, carefully counted so that each type of cell was at the same starting concentration, subjected to serial 10-fold dilutions, and then transferred to plates with control media (A) or media containing sub-lethal doses of various drugs (B) as indicated in Materials and methods for 3 days. Ct, camptothecin; Hu, hydroxyurea; Cx, cycloheximide; Wt, wortmannin; Sm, sulfometuron methyl; Fk, FK506.

mouse osteoclasts and epithelial cells from the gut of tobacco hornworm (Holliday et al., 2000; Lee et al., 1999b; Vitavska et al., 2003). We previously demonstrated that B subunits with mutated actin-binding sites were not properly targeted in osteoclasts (Zuo et al., 2006). The results of the present study indicate that the actin-binding activity plays a role in the growth and survival of a single celled organism under specific stress conditions. We hypothesize that the specialized function of the actin-binding activity in

osteoclasts may have developed by evolutionary adaptation of an activity associated with stress responses in more generalized cells. These data also hint that the actin-binding activity may be involved in certain types of stress responses in higher organisms.

We had earlier identified a domain in mammalian B subunits that was required for actin-binding activity (Chen et al., 2004). It was similar in sequence to a known actin interaction domain found in mammalian profilin 1. We showed that subtle mutations in the 'profilin-like' region, achieved by replacing it with a spacer derived from the B subunit of an Archaea, eliminated detectable actin-binding activity. B subunits with this mutation, unlike wild-type B subunits, were not delivered to the ruffled membrane when they were expressed in osteoclasts (Zuo et al., 2006). The mutant B subunits appeared to assemble correctly with endogenous subunits (Zuo et al., 2006). However, the study of osteoclasts was technically limited and we could not eliminate endogenous B subunits or assay the ATPase activity of the proton pump containing mutant B subunits, nor could we assay the biological effects related to the presence of the mutant B subunit. Yeast have proven to be excellent model organisms for the study of V-ATPase (Nelson and Klionsky, 1996). Although yeast lacking the B subunit fail to grow at alkaline pH, they grow well in acidic media. The cells have a mechanism for uptake of acidic media and use it for necessary housekeeping acidification of compartments (Kane and Stevens, 1992). Replacement of the yeast B subunit in the $VMA2\Delta$ yeast completely restores the capacity of yeast to grow in alkaline conditions. We therefore tested whether the actin-binding activity of subunit B could be studied in yeast.

We found that yeast B subunit bound microfilaments in a similar manner to mammalian B subunits by making use of recombinant proteins expressed in a bacterial system. We found that yeast B subunit bound actin with a similar affinity to mammalian B subunits: K_d for yeast B subunit, 195 nmol l^{-1}; Human B1, 130 nmol l^{-1}; human B2, 190 nmol l^{-1} (Holliday et al., 2000).

Western blots and immunoprecipitations showed that the mutant yeast B subunits were expressed at similar levels to wild-type when $VMA2\Delta$ yeast were transformed with these constructs. The mutant B subunits contributed to equivalent levels of bafilomycin-sensitive ATPase activity. This suggested that mutations in the actin-binding site did not disrupt the ability of the mutated subunit to contribute properly to the enzymatic function of the proton pump. Yeast containing wild-type or mutated yeast B subunit grew well at pH 5.5. We were unable to detect differences in growth at pH 7.5 of yeast containing wild-type or mutant B subunits. These data are also consistent with the hypothesis that the mutations did not disrupt the enzymatic activity of the proton pump.

A previous study reported that F-actin was not required for the regulation of V-ATPase by reversible assembly in response to glucose deprivation (Xu and Forgac, 2001). Consistent with that report, we did not detect differences in the capacity of pumps containing mutant yeast B subunit to undergo assembly and disassembly in response to glucose deprivation.

Yeast V-ATPase genes are crucial for growth in the presence of high, but sub-lethal, doses of particular drugs (Parsons et al., 2004b). We performed a similar screen and found that yeast containing B subunits that lacked actin-binding activity showed reduced growth in the presence of sub-lethal levels of cycloheximide and wortmannin compared with yeast containing wild-type yeast B subunits.

Yeast living in the wild are subjected to an array of environmental challenges. Our data showing that yeast with a functional actin-binding site in their B subunit grow better in the presence of cycloheximide and wortmannin suggests, in more general terms, that the actin-binding activity of yeast B subunit provides a survival benefit to yeast encountering specific types of stressful environments. We hypothesize that this contribution to a survival advantage led to the evolution and evolutionary maintenance of the actin-binding activity. We speculate that in higher organisms, this actin-binding activity was adapted to optimize the function of certain types of highly specialized cells, including osteoclasts. In osteoclasts, the actin-binding activity of the B subunit is required to target V-ATPases to the plasma membrane (Zuo et al., 2006). However, we do not suggest that in yeast V-ATPase is targeted to the plasma membrane in response to cycloheximide or wortmannin. Rather, we propose that the common feature in yeast and osteoclasts is that the actin-binding activity is involved in vesicular sorting of V-ATPases. We hypothesize that in osteoclasts this cytoskeletal-based sorting results in a specialized population of vesicles that develop the ability to fuse with the plasma membrane to deliver V-ATPases. In yeast, this could result in acidic vesicles that are involved in the protection from cycloheximide or wortmannin challenges. As a general phenomenon, involvement of the actin cytoskeleton in vesicular sorting is well documented (Lanzetti, 2007).

We do not know the mechanism by which the actin-binding activity is involved in the protection of yeast from cycloheximide or wortmannin. It is intriguing that actin-binding activity protects yeast in the presence of wortmannin, a phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor. In osteoclasts, blocking PI 3-kinase activity leads to increased binding between V-ATPases and F-actin (Chen et al., 2004). In mammals, wortmannin inhibits both class I and class III PI 3-kinases (Carpenter and Cantley, 1996). Yeast contain only class III PI 3-kinases, which are associated with the regulation of vesicle trafficking in both yeast and mammals (Mitra et al., 2004; Carpenter and Cantley, 1996). These data suggest that the link between PI 3-kinase activity and binding between V-ATPases and F-actin may have developed early in evolution, and may be involved in the regulation of vesicle trafficking.

In summary, we have found that subunit B of V-ATPase from yeast binds F-actin in a manner similar to mammalian B subunits. Disruption of the actin-binding activity was achieved without overt consequences for the assembly and ATPase activity of the pumps in which mutant B subunits were incorporated. Our data indicate that the actin-binding activity of the B subunit is not required by yeast under normal culture conditions, but is involved in a protective response to cycloheximide and wortmannin.

REFERENCES

Adams, D. S., Robinson, K. R., Fukumoto, T., Yuan, S., Albertson, R. C., Yelick, P., Kuo, L., McSweeney, M. and Levin, M. (2006). Early, H$^+$-V-ATPase-dependent proton flux is necessary for consistent left-right patterning of non-mammalian vertebrates. *Development* **133**, 1657-1671.

Carpenter, C. L. and Cantley, L. C. (1996). Phosphoinositide kinases. *Curr. Opin. Cell Biol.* **8**, 153-158.

Chen, S. H., Bubb, M. R., Yarmola, E. G., Zuo, J., Jiang, J., Lee, B. S., Lu, M., Gluck, S. L., Hurst, I. R. and Holliday, L. S. (2004). Vacuolar H$^+$-ATPase binding to microfilaments: regulation in response to phosphatidylinositol 3-kinase activity and detailed characterization of the actin-binding site in subunit B. *J. Biol. Chem.* **279**, 7988-7998.

Gluck, S. L., Lee, B. S., Wang, S. P., Underhill, D., Nemoto, J. and Holliday, L. S. (1998). Plasma membrane V-ATPases in proton-transporting cells of the mammalian kidney and osteoclast. *Acta Physiol. Scand. Suppl.* **643**, 203-212.

Harvey, W. R. (1992). Physiology of V-ATPases. *J. Exp. Biol.* **172**, 1-17.

Holliday, L. S., Bubb, M. R. and Korn, E. D. (1993). Rabbit skeletal muscle actin behaves differently than Acanthamoeba actin when added to soluble extracts of Acanthamoeba castellanii. *Biochem. Biophys. Res. Commun.* **196**, 569-575.

Holliday, L. S., Lu, M., Lee, B. S., Nelson, R. D., Solivan, S., Zhang, L. and Gluck, S. L. (2000). The amino-terminal domain of the B subunit of vacuolar H+-ATPase contains a filamentous actin binding site. *J. Biol. Chem.* **275**, 32331-32337.

Holliday, L. S., Bubb, M. R., Jiang, J., Hurst, I. R. and Zuo, J. (2005). Interactions between vacuolar H+-ATPases and microfilaments in osteoclasts. *J. Bioenerg. Biomembr.* **37**, 419-423.

Inoue, T., Wilkens, S. and Forgac, M. (2003). Subunit structure, function, and arrangement in the yeast and coated vesicle V-ATPases. *J. Bioenerg. Biomembr.* **35**, 291-299.

Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983). Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.* **153**, 163-168.

Kane, P. M. (2006). The where, when, and how of organelle acidification by the yeast vacuolar H+-ATPase. *Microbiol. Mol. Biol. Rev.* **70**, 177-191.

Kane, P. M. and Stevens, T. H. (1992). Subunit composition, biosynthesis, and assembly of the yeast vacuolar proton-translocating ATPase. *J. Bioenerg. Biomembr.* **24**, 383-393.

Kane, P. M., Yamashiro, C. T. and Stevens, T. H. (1989). Biochemical characterization of the yeast vacuolar H+-Atpase. *J. Biol. Chem.* **264**, 19236-19244.

Kane, P. M., Kuehn, M. C., Howaldstevenson, I. and Stevens, T. H. (1992). Assembly and targeting of peripheral and integral membrane subunits of the yeast vacuolar H+-Atpase. *J. Biol. Chem.* **267**, 447-454.

Lanzetti, L. (2007). Actin in membrane trafficking. *Curr. Opin. Cell Biol.* **19**, 453-458.

Lee, B. S., Gluck, S. L. and Holliday, L. S. (1999a). Interaction between vacuolar H(+)-ATPase and microfilaments during osteoclast activation. *J. Biol. Chem.* **274**, 29164-29171.

Lee, B. S., Holliday, L. S., Krits, I. and Gluck, S. L. (1999b). Vacuolar H+-ATPase activity and expression in mouse bone marrow cultures. *J. Bone Miner. Res.* **14**, 2127-2136.

Lu, M., Sautin, Y. Y., Holliday, L. S. and Gluck, S. L. (2004). The glycolytic enzyme aldolase mediates assembly, expression, and activity of vacuolar H+-ATPase. *J. Biol. Chem.* **279**, 8732-8739.

Mitra, P., Zhang, Y., Rameh, L. E., Ivshina, M. P., McCollum, D., Nunnari, J. J., Hendricks, G. M., Kerr, M. L., Field, S. J., Cantley, L. C. et al. (2004). A novel phosphatidylinositol(3,4,5)P3 pathway in fission yeast. *J. Cell Biol.* **166**, 205-211.

Nelson, N. and Harvey, W. R. (1999). Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol. Rev.* **79**, 361-385.

Nelson, N. and Klionsky, D. J. (1996). Vacuolar H(+)-ATPase: from mammals to yeast and back. *Experientia* **52**, 1101-1110.

Parsons, A. B., Brost, R. L., Ding, H., Li, Z., Zhang, C., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R. and Boone, C. (2004a). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat. Biotechnol.* **22**, 62-69.

Parsons, A. B., Brost, R. L., Ding, H. M., Li, Z. J., Zhang, C. Y., Sheikh, B., Brown, G. W., Kane, P. M., Hughes, T. R. and Boone, C. (2004b). Integration of chemical-genetic and genetic interaction data links bioactive compounds to cellular target pathways. *Nat. Biotechnol.* **22**, 62-69.

Vitavska, O., Wieczorek, H. and Merzendorfer, H. (2003). A novel role for subunit C in mediating binding of the H+-V-ATPase to the actin cytoskeleton. *J. Biol. Chem.* **278**, 18499-18505.

Vitavska, O., Merzendorfer, H. and Wieczorek, H. (2005). The V-ATPase subunit C binds to polymeric F-actin as well as to monomeric G-actin and induces cross-linking of actin filaments. *J. Biol. Chem.* **280**, 1070-1076.

Wagner, C. A., Finberg, K. E., Breton, S., Marshansky, V., Brown, D. and Geibel, J. P. (2004). Renal vacuolar H+-ATPase. *Physiol. Rev.* **84**, 1263-1314.

Wang, Z. Q. and Gluck, S. (1990). Isolation and properties of bovine kidney brush border vacuolar H(+)-ATPase. A proton pump with enzymatic and structural differences from kidney microsomal H(+)-ATPase. *J. Biol. Chem.* **265**, 21957-21965.

Xu, T. and Forgac, M. (2001). Microtubules are involved in glucose-dependent dissociation of the yeast vacuolar [H+]-ATPase *in vivo*. *J. Biol. Chem.* **276**, 24855-24861.

Yamashiro, C. T., Kane, P. M, Wolczyk, D. F., Preston, R. A. and Stevens, T. H. (1990). Role of vacuolar acidification in protein sorting and zymogen activation - a genetic-analysis of the yeast vacuolar proton-translocating Atpase. *Mol. Cell. Biol.* **10**, 3737-3749.

Zuo, J., Jiang, J., Chen, S. H., Vergara, S., Gong, Y., Xue, J., Huang, H., Kaku, M. and Holliday, L. S. (2006). Actin binding activity of subunit B of vacuolar H(+)-ATPase is involved in its targeting to ruffled membranes of osteoclasts. *J. Bone Miner. Res.* **21**, 714-721.