Role of Vacuolar Acidification in Protein Sorting and Zymogen Activation: a Genetic Analysis of the Yeast Vacuolar Proton-Translocating ATPase

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Vacuolar acidification has been proposed to play a key role in a number of cellular processes, including protein sorting, zymogen activation, and maintenance of intracellular pH. We investigated the significance of vacuolar acidification by cloning and mutagenizing the gene for the yeast vacuolar proton-translocating ATPase 60-kilodalton subunit (VAT2). Cells carrying a *vat2* null allele were viable; however, these cells were severely defective for growth in medium buffered at neutral pH. Vacuoles isolated from cells bearing the *vat2* null allele were completely devoid of vacuolar ATPase activity. The pH of the vacuolar lumen of cells bearing the *vat2* mutation was 7.1, compared with the wild-type pH of 6.1, as determined by a flow cytometric pH assay. These results indicate that the vacuolar proton-translocating ATPase complex is essential for vacuolar acidification and that the low-pH state of the vacuole is crucial for normal growth. The vacuolar acidification-defective *vat2* mutant exhibited normal zymogen activation but displayed a minor defect in vacuolar protein sorting.

Acidification of the vacuolar network has been proposed to be crucial for a number of cellular processes, including receptor-mediated endocytosis, protein sorting, and zymogen activation (16, 33, 40). The vacuolar network includes such organelles as the Golgi apparatus, endosomes, secretory vesicles, clathrin-coated vesicles, and lysosomes. The vacuolar proton-translocating ATPases (H⁺-ATPase) are a class of ion-motive ATPase thought to be responsible for the acidification of the vacuolar network (16, 40). Newly synthesized lysosomal proteins traverse at least part of this network, and as these proteins proceed toward the lysosome, they encounter environments of increasing acidity (16, 33, 40, 75). Many proteins destined for the lysosome utilize receptor-mediated transport based on the mannose 6-phosphate receptor. Ligands bind to the mannose 6-phosphate receptor at the near-neutral pH of the Golgi apparatus and are vectorially transported within vesicles to a prelysosomal compartment (19). Once the receptor-ligand complex encounters a pH at or below 5.5, the complex dissociates and the ligand proceeds on to the lysosome while the receptor recycles back to the Golgi apparatus. In mammalian systems, most lysosomal hydrolases are transported as inactive zymogens that require activation by proteolytic processing events. Some zymogens, such as pepsinogen, autoactivate when placed in an acidic environment (8, 25). This suggests that the acidic environment of the lysosome may be required for lysosomal zymogen activation.

The role of vacuolar acidification in various cellular processes has been studied by two different approaches. The first approach utilizes weak bases, such as chloroquine and NH₄Cl, which have been demonstrated to raise the intralysosomal pH of mammalian cells (44). Numerous studies have found that lysosomal protein sorting and zymogen activation were perturbed upon treatment with weak bases, although

the magnitude of the defect was dependent on the cell type and weak base used and on the lysosomal protein analyzed (reviewed in references 16, 40, 49, and 75). The second approach has been a genetic one. Mutants were selected for resistance to viruses and toxins that enter cells via receptor-mediated endocytosis. These mutants were characterized as being defective for endosomal acidification (41, 50, 51, 70) or lysosomal acidification (10). An aberrantly high level of lysosomal enzyme activity was detected in the extracellular medium during the growth of these mutants, suggesting that newly synthesized lysosomal protein was being mislocalized.

The yeast *Saccharomyces cerevisiae* has a lysosomelike vacuole that is acidic and contains a variety of hydrolases (27, 57, 58). The two approaches mentioned above for analyzing mammalian lysosomal function have also been used for assessing the functions of vacuolar acidification in yeast cells. Yeast cells treated with weak bases were defective for vacuolar acidification (77) and for localization of newly synthesized vacuolar hydrolases (59). A related study found that yeast cells treated with bafilomycin A₁, a potent inhibitor of the vacuolar class of H⁺-ATPases (6, 30), were defective for vacuolar acidification (3). Treatment of yeast spheroplasts with bafilomycin A₁ caused partially aberrant sorting and maturation of two vacuolar proteases, suggesting that vacuolar acidification may be involved in these processes (3). In genetic studies, a collection of vacuolar protein-sorting (*vps*) mutants (53, 59) was screened for vacuolar acidification defects by testing their ability to accumulate the fluorescent weak base quinacrine. A subset of *vps* mutants was identified as being defective for vacuolar acidification, and in addition to missorting vacuolar proteins, these mutants also accumulated the zymogen form of proteinase A (PrA) in the intracellular fraction (3, 59). Vacuolar acidification mutants have recently been isolated by using an assay of vacuolar pH *in vivo* (48). One such mutant (*vph1*) has a vacuolar lumen with a pH measured at 6.9, which is significantly higher than the pH of 6.2 determined for the

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TABLE 1. Strains used in this study

Strain	Genotype	Source or reference
<i>S. cerevisiae</i>		
X2180-1AL1	<i>MATa lys2 gal2 mal mel</i>	56
SEY2102	<i>MATa his4-519 leu2-3 leu2-112 ura3-52 gal2 suc2-Δ9</i>	15
JHRY99-10A	<i>MATa ade6 leu2-3 leu2-112 ura3-52 gal2</i>	J. H. Rothman
CYY7	Diploid from a cross of SEY2102 and JHRY99-10A	This study
CYY9	<i>vat2-Δ1::LEU2/VAT2</i> diploid isogenic to CYY7	This study
SF838-5A	<i>MATa ade6 leu2-3 leu2-112 ura3-52 gal2</i>	56
SF838-5AV1	<i>MATa ade6 leu2-3 leu2-112 ura3-52 gal2 vat2-Δ1::LEU2</i>	This study
SF838-1D	<i>MATa ade6 his4-519 leu2-3 leu2-112 ura3-52 pep4-3 gal2</i>	56
SF838-1DV1	<i>MATa ade6 his4-519 leu2-3 leu2-112 ura3-52 pep4-3 gal2 vat2-Δ1::LEU2</i>	This study
<i>E. coli</i>		
MC1061	<i>F⁻ hsdR hsdM⁺ araD139 (araABOIC-leu)7679 (lac)X74 galU galK rpsL</i>	9
JM103	<i>F⁺ Δ(lac pro) thi strA endA sbcB15 supE/F⁺ traD36 proAB⁺ lacI^q lacZΔM15</i>	42

wild-type vacuolar lumen. Protein sorting and zymogen activation appeared not to be perturbed in *vph1* cells (R. Preston and M. Manolson, unpublished data).

Analysis of the function of vacuolar acidification by the approaches described above has yet to provide unequivocal results, for several reasons. Results with weak bases and bafilomycin A₁ are compromised by the usual questions of the specificity of inhibitors. In the genetic studies, analysis of a small number of mutants has failed to establish causal relationships between acidification and other phenotypes. Indeed, the available data contain apparent inconsistencies concerning the importance of vacuolar acidification for protein sorting and zymogen activation.

We sought a more direct approach for studying the cellular functions mediated by the acidification of the vacuole. The yeast vacuolar H⁺-ATPase is a multisubunit complex consisting of eight polypeptides of apparent molecular masses of 100, 69, 60, 42, 36, 32, 27, and 17 kilodaltons (kDa) and can be purified to near homogeneity (30, 71). This allowed us to use a reverse genetic approach for assessing the functions of the vacuolar H⁺-ATPase. We purified the 60-kDa subunit and obtained the amino-terminal sequence. Using an appropriately designed oligonucleotide, we screened a genomic DNA library and cloned the yeast vacuolar H⁺-ATPase 60-kDa subunit gene (*VAT2*). Strains carrying a null allele of the *VAT2* gene were completely defective for vacuolar acidification. Phenotypic analysis of the null mutant provided a direct appraisal of the role of vacuolar acidity in several cellular processes.

MATERIALS AND METHODS

Strains, growth conditions, and materials. Yeast and bacterial strains used in this work are described in Table 1.

Strains were constructed by standard genetic manipulations. Unless otherwise indicated, yeast strains were grown in YEPD (yeast extract peptone), pH 5.0 (adjusted with HCl), or synthetic dextrose (SD), pH 5.0, with the appropriate supplements (64). Tetrad analysis was performed essentially as described previously (64).

Bafilomycin A₁ was a gift from Karlheinz Altendorf. Antisera to yeast phosphoglycerate kinase and PrA were described earlier (55, 66), antiserum to carboxypeptidase Y (CPY) was made against part of CPY made in *Escherichia coli* (C. Raymond and T. Stevens, unpublished data), antiserum to alkaline phosphatase was a gift from Gabrielle Pohlig, the monoclonal antibody 8B1F3 against the 69-kDa subunit was described earlier (30, 59), and fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G was from Organon Teknika. Alkaline phosphatase-conjugated goat anti-rabbit and goat anti-mouse antibodies were obtained from Promega Biotec, alkaline phosphatase color development reagents were from Bio-Rad Laboratories, and prestained protein molecular weight standards were from Bethesda Research Laboratories, Inc. Yeast extract, peptone, and agar were from Difco Laboratories. DNA restriction, sequencing, and other DNA-modifying enzymes were obtained from Bethesda Research Laboratories, Promega Biotec, Boehringer Mannheim Biochemicals, New England BioLabs, Inc., and United States Biochemicals. A yeast genomic library contained in YCp50 was a gift from Mark Rose. Fraction II lyticase was prepared as described previously (62), and Glusulase was obtained from Du Pont Co. Zymolyase 100T and Tran ³⁵S-label were from ICN Biochemicals Inc. [α -³²P]dCTP, [γ -³²P]ATP, and GeneScreen Plus were from Dupont NEN Research Products. Nitrocellulose was from Schleicher & Schuell, Inc., and IgG Sorb was from the Enzyme Center. Ultrapure urea was from IBI Biochemicals, and ultrapure sodium dodecyl sulfate (SDS) was from BDH. 6-Carboxyfluorescein diacetate (6-CFDA) was obtained from Molecular Probes. Polyvinylidene difluoride (PVDF) membrane (Immobilon) was obtained from Millipore Corp. Reagents for random priming DNA were from Amersham Corp. All reagents for oligonucleotide synthesis and amino-terminal protein sequence determinations were obtained from Applied Biosystems, Inc. All other reagents for enzymatic assays and vacuolar membrane isolation were from Sigma Chemical Co.

Purification and amino-terminal sequencing of the 60-kDa subunits. Yeast vacuolar membranes were purified from X2180-1AL1 as described earlier (30) by growing 50 liters of cells, converting the cells to spheroplasts, osmotically lysing the spheroplasts, and isolating vacuoles by two consecutive flotations on Ficoll gradients. To isolate the 60-kDa subunit of the vacuolar H⁺-ATPase, we stripped the subunit off vacuolar membranes by using nitrate (29, 30). Isolated vacuoles (10 mg) were treated with 100 mM KNO₃–5 mM ATP–5 mM MgCl₂ at 30°C for 60 min and prepared for electrophoresis as described previously (30). The separated proteins were electrotransferred onto a PVDF membrane and visualized by staining with Coomassie blue, and the protein band of interest was excised from the PVDF membrane as described by Matsudaira (37). The excised 60-kDa band was analyzed with an Applied Biosystems model 470A protein sequencer connected to an Applied Biosystems model 120A phenylthiohydantoin analyzer to obtain the amino-terminal sequence.

Isolation of the *VAT2* gene. From the amino-terminal sequence of the 60-kDa subunit, by using the yeast codon bias (11, 63), a partially degenerate oligonucleotide of 45

FIG. 1. Restriction map, sequencing strategy, and null allele for the *VAT2* gene. (A) The open box represents the reading frame of the *VAT2* gene, and the arrow beneath the box indicates the direction of translation. Restriction enzymes: C, *Cla*I; E, *Eco*RI; H, *Hind*III; G, *Bgl*II; and S, *Sal*I. (B) Depiction of the *vat2Δ::LEU2* (= *vat2-Δ1*) allele constructed as described in Materials and Methods. Open boxes represent the interrupted *VAT2* gene, and the shaded box shows the inserted *LEU2* gene with the direction of translation indicated by the arrow.

residues, 3'-CA(A/G)CGACA(A/G)CTTGTTCCAAAGTTGCA(A/G)TTCGGTTCTAACTTGATG-5', was synthesized by using an Applied Biosystems DNA synthesizer (model 380B) as described elsewhere (24). The oligonucleotide probe was radiolabeled by using T4 polynucleotide kinase with [γ -³²P]ATP and was used to screen bacterial colonies (14, 76) containing a yeast genomic library in the yeast plasmid YCp50. The probe was hybridized in an aqueous solution at 42°C for 12 h, rinsed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), washed twice in 0.2× SSC at room temperature, and washed twice in 0.2× SSC at 42°C for 20 min. Washed blots were placed into Seal-a-Meal bags and subjected to autoradiography at -80°C with XAR-5 film (Eastman Kodak Co.) with a Cronex Lightning-Plus intensifying screen (E. I. du Pont de Nemours & Co., Inc.).

Recombinant DNA methods, plasmid constructions, and DNA sequencing. Restriction endonuclease digests and ligations were performed as recommended by the suppliers. Plasmid purification, agarose gel electrophoresis, blunt ending of DNA fragments with T4 DNA polymerase, and DNA-mediated transformation of *E. coli* were done according to standard procedures (35). DNA-mediated transformation of yeast utilized the lithium acetate method (23). *E. coli* MC1061 was used for all plasmid manipulations, and JM103 was used for all M13 bacteriophage work.

Plasmid pCY37 was constructed by inserting the 5-kilobase (kb) *Cla*I-*Sal*I *VAT2* fragment (Fig. 1A), isolated from one of the original YCp50-*VAT2* clones, pCY36, into *Cla*I-*Sal*I sites of Bluescript KS⁺ (with the *Eco*RI site deleted). Nested deletions of *VAT2* for DNA sequencing utilized plasmids pCY41 and pCY42 that were constructed by inserting the 2.2-kb *Hind*III *VAT2* fragment from pCY36 in opposite orientations into the *Hind*III site of Bluescript KS⁺. For antigen production, plasmid pCY45 was constructed by inserting the 1.4-kb *Eco*RI-*Hind*III *VAT2* fragment from pCY36 into the *Eco*RI-*Hind*III site of pEXP3 that contains the *tac* promoter just upstream of the multiple cloning site (C. Raymond and T. Stevens, manuscript submitted). Plasmid pCY52 was assembled by inserting the 2.2-kb *Sal*I-*Bam*HI *VAT2* fragment of pCY41 (the *Sal*I and *Bam*HI sites are part of the Bluescript KS⁺ vector, but the construct includes the entire 2.2-kb *Hind*III *VAT2* fragment) into the *Bam*HI-*Sal*I sites of YCp50. Plasmid pTS18 has the *PEP4* gene inserted into YCp50 as described previously (1).

A chromosomal *VAT2* deletion was constructed by replacing the genomic copy with a cloned gene containing the *LEU2* gene in place of the 18-base-pair (bp) *Eco*RI fragment (Fig. 1B). Plasmid pCY37 digested with *Eco*RI was blunt

ended by T4 DNA polymerase, and a 2.1-kb *Hpa*I *LEU2* fragment was ligated to it. The resulting plasmid, pCY40, was digested with *Hind*III, and the 4.3-kb *VAT2* fragment was gel purified and used to transform *leu2* yeast strains (60). Stable Leu⁺ transformants were isolated and screened by Southern blot analysis. Yeast genomic DNA was isolated by mechanically disrupting cells with glass beads in the presence of detergent and phenol-chloroform (21). Genomic DNA was digested with *Sal*I and *Cla*I and subjected to gel electrophoresis. Separated DNA was electrotransferred onto GeneScreen Plus as recommended by the manufacturer. Hybridizations were done in aqueous solutions without dextran sulfate and were stringently washed at 65°C in a solution consisting of 40 mM NaHPO₄ (pH 7.2), 1 mM EDTA, and 1% SDS (67).

The chain termination method of Sanger et al. (61) was used for sequencing subclones of *VAT2* in M13 mp18 and mp19. Double-stranded DNA sequencing with *Taq* DNA polymerase was done according to the recommendations of the manufacturer (Promega Biotec) for *Exo*III-S₁ nuclease nested deletions (20) in the Bluescript KS⁺ plasmid system (Stratagene). DNA and amino acid sequence analyses utilized the sequence analysis software package (version 4) of the University of Wisconsin Genetics Computer Group.

Production of 60-kDa subunit antiserum and Western blotting (immunoblotting). *E. coli* cells containing pCY45 were used to produce antigen by the method described previously by Roberts et al. (52). Preparation of 60-kDa subunit-specific antiserum used 100 μg of 60-kDa subunit antigen injected into New Zealand White rabbits, followed with a boost of 100 μg of antigen 8 weeks later (73). The 60-kDa subunit antibodies were affinity purified as described previously (65).

Total yeast cell protein extracts were prepared by disrupting cells with glass beads in sample buffer with 5% SDS and 8 M urea as described elsewhere (4), except the cells and solutions were incubated at 70°C. Constant amounts of protein were loaded onto gels: 1 μg of extract for blots probed with 60-kDa subunit antibody and 25 μg of extract for the 69-kDa subunit blot. Vacuolar protein from a purified vacuole fraction was suspended in sample buffer and heated to 70°C for 15 min. Vacuolar protein (4 μg) was loaded onto the gel. Proteins separated on 10% polyacrylamide gels were electrotransferred onto nitrocellulose with a Bio-Rad Trans-Blot apparatus. Blots were blocked with 1.5% nonfat milk-0.1% Tween 20, and the primary antibody was bound as previously described (7). Secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse antibody) was used and visualized by addition of 5-bromo-

4-chloro-3-indolyl phosphate and *p*-Nitro Blue Tetrazolium chloride as specified by the manufacturer (Bio-Rad).

Fluorescence microscopy. Indirect immunofluorescence was performed with affinity-purified antibodies to the 60-kDa subunit and to alkaline phosphatase (C. J. Roberts, C. K. Raymond, C. T. Yamashiro, and T. H. Stevens, *Methods Enzymol.*, in press) that have been preadsorbed to fixed *vat2-ΔI* and *pho8Δ* cells, respectively. Analysis of vacuolar uptake of quinacrine was performed as previously described (77; Roberts et al., in press). Labeling of live yeast cells with 6-CFDA was done as described by Preston et al. (48), except that 6 μM 6-CFDA was applied to *vat2-ΔI* cells and 25 μM 6-CFDA was applied to wild-type cells to equalize the fluorescence intensities in the two cell types. Microscopy was performed with a Zeiss Axioplan photomicroscope equipped for Nomarski optics and epifluorescence with a 100× oil immersion lens. Photography was done with Kodak TMax-400 film and developer. Exposure times of 4 to 8 s were used for all the fluorescence micrographs.

Determination of vacuolar pH. In vivo assays of vacuolar pH were performed by using a flow cytometric assay of pH-dependent 6-carboxyfluorescein fluorescence as described by Preston et al. (48). Logarithmic-phase cells, at a concentration of 2×10^6 cells per ml, were labeled with 6-CFDA in YEPD containing 50 mM citric acid adjusted to pH 3.5 with HCl. Labeled cells were diluted 100-fold and analyzed on a modified FACS 440 dual argon laser flow cytometer with excitation wavelengths of 458 and 488 nm and monitoring emissions of 530 nm. Fluorescence data were acquired for 10,000 cells in each analysis. Calibration of cytometric ratios was obtained by linear regression through in vitro and in vivo ratio measurements at pHs of 6.0, 7.0, and 7.4, combined with a propagation of errors statistical analysis of the cytometric ratios. This method provides more accurate estimates of the standard error of the mean than was possible with the method described previously (48). Specificity of 6-CFDA labeling of SF838-5A (wild type) and SF838-5AV1 (*vat2-ΔI*) was determined microscopically as described above. Measurements were also performed on JHRY20-2C (*MATa his3-Δ200 leu2-3 leu2-112 ura3-52*; 55) and the isogenic *vat2-ΔI* strain with results nearly identical to those obtained for the above strains.

Enzymatic assays. Assays for vacuolar ATPase and dipeptidyl aminopeptidase B activities were performed as described by Roberts et al. (in press). ATPase activities are reported as specific activities (in units per milligram of protein), with 1 U defined as 1 μmol of phosphate liberated per min. Protein was determined by the method of Lowry et al. (34) on purified vacuoles and whole yeast cell extracts that were first solubilized in 2% SDS.

Growth curves. YEPD cultures of wild-type and *vat2-ΔI* cells were agitated overnight at 30°C to a cell density of 4×10^7 cells per ml. YEPD (50 ml) buffered with 50 mM KH_2PO_4 –50 mM succinic acid at pH values of 3.0, 4.0, 5.0, 6.0, and 7.0 (adjusted by adding HCl or KOH) was inoculated with $\sim 10^7$ cells. The cultures were agitated at 30°C, and cell density was monitored every 1.5 h for 9 h by measuring optical densities at 600 nm (when necessary, cultures were diluted to give an optical density at 600 nm of ≤ 0.2 to ensure measurement in the linear range) using a Beckman Instruments, Inc., DU-6 spectrophotometer. A final measurement was taken after 22 h of incubation, and the pH of the medium was measured. A growth curve was derived from the logarithm of the optical densities versus time. The doubling time was calculated from the part of the curve with the greatest slope.

Immunoprecipitations. Yeast cells ($\sim 5 \times 10^6$) grown in SD-Met (pH 5.0) were sedimented in a clinical centrifuge, washed once in SD-Met, and resuspended in 0.5 ml of SD-Met containing 50 mM potassium phosphate (pH 5.0) and 2 mg of carboxymethylated bovine serum albumin per ml. The cells were labeled with 100 μCi of Tran ^{35}S -label for 20 min at 30°C and chased for 30 min upon the addition of 10 μl of 5-mg/ml methionine–5-mg/ml cysteine. The pulse-chase was stopped by the addition of 5 μl of 1 M NaN_3 . The cells were converted to spheroplasts with 25 U of lyticase in 150 μl of 1.4 M sorbitol–50 mM Tris hydrochloride (pH 7.4)–2 mM MgCl_2 –10 mM NaN_3 –0.3% 2-mercaptoethanol for 30 min at 30°C. Both the pulse-chase medium and the periplasmic fraction from the spheroplasting supernatant were combined to constitute the extracellular fraction. The spheroplasts were lysed by boiling for 5 min in 775 μl of 1.3× Immuno buffer (1× Immuno buffer contains 10 mM Tris hydrochloride, pH 8.0, 0.1% SDS, 0.1% Triton X-100, and 2 mM EDTA). The extracellular fraction was boiled for 5 min after 100 μl of 10× Immuno buffer was added. Both fraction volumes were brought up to 1 ml with H_2O , preadsorbed with 50 μl of IgG Sorb for 15 min at 0°C, and pelleted in a microcentrifuge. The supernatant was incubated for 60 min at 0°C with the appropriate antibody and immunoprecipitated with 50 μl of IgG Sorb for 30 min at 0°C. The immunoprecipitate was washed twice with 1 ml of 1× Immuno buffer, suspended in sample buffer, and boiled for 5 min. The samples were analyzed by electrophoresis and prepared for fluorography as described previously (66). Quantitation of radioemissions was performed with the Ambis radioanalytic imaging system in the grid quantitation mode.

RESULTS

Cloning of the vacuolar H^+ -ATPase 60-kDa subunit gene. Our strategy for cloning the vacuolar H^+ -ATPase 60-kDa subunit gene was to purify the subunit from vacuolar membranes, determine its amino-terminal sequence, and synthesize an oligonucleotide for probing a yeast genomic library. Nitrate is a specific inhibitor of the vacuolar H^+ -ATPase (16), and its mode of inhibition is to disrupt the integrity of the ATPase complex (29, 30). In yeast cells, the 69-, 60-, and 42-kDa subunits are selectively stripped off the vacuolar membrane by nitrate in the presence of Mg-ATP (30). We used nitrate stripping of vacuolar membranes to isolate the 60-kDa subunit for amino-terminal protein sequencing (see Materials and Methods). The first two cycles of the sequencing were difficult to interpret, although the second cycle appeared to represent a valine residue. The next 14 cycles were unambiguous; therefore, the amino-terminal sequence is as follows: NH_2 -XVAVEQGFNVKPRNLNY-COOH.

By using residues 2 to 16 of the amino-terminal sequence, a partially degenerate oligonucleotide probe 45 nucleotides in length was synthesized. This oligonucleotide was then used to probe a yeast genomic library. Two positive clones were isolated from 10,000 colonies screened. Restriction mapping of the two clones revealed that these were independent clones whose inserts overlapped with each other in the region including the 5-kb *Clal*-*EcoRI* fragment (Fig. 1A).

The exonuclease III- S_1 nuclease nested deletion approach (20) was used to sequence both strands of the 2.2-kb *HindIII* fragment. The DNA sequence of that fragment contained an open reading frame that could encode a protein of 517 amino acids with a predicted molecular mass of 57,749. The amino-terminal protein sequence that was used to design the

FIG. 2. Amino and carboxy termini of the VAT2 gene product. (A) Predicted amino-terminal 36 residues of the VAT2 gene product. The amino-terminal protein sequence of the purified 60-kDa subunit is underlined, and the smaller letters show the two residues that are not identical for reasons stated in Results. (B) Predicted carboxy-terminal protein sequences of the VAT2 gene product (a), the *Neurospora vma-2* gene product (b), and the yeast 57-kDa subunit gene cloned by Nelson et al. (43) (c). Boxes denote protein sequence identities with the *Neurospora vma-2* gene product at the COOH terminus. The arrow designates the point at which the DNA sequence difference causes a change in the predicted protein sequence between the VAT2 and 57-kDa subunit gene products.

oligonucleotide probe was found near the amino-terminal end of the polypeptide preceded by a 12-amino-acid leader sequence (Fig. 2A). Neither the 12-residue leader sequence nor any other domain of the predicted polypeptide appears to represent a signal sequence or hydrophobic transmembrane domain, suggesting that the encoded protein does not enter the secretory pathway and is not an integral membrane protein. Biochemical evidence also indicates that the 60-kDa subunit is not an integral membrane protein (30). The first two residues of the predicted amino-terminal protein sequence turned out to be Lys-Lys rather than X-Val. This result was not surprising because of ambiguities in the first two cycles of the protein sequencing (see above).

The predicted amino acid sequence was compared with the 60-kDa vacuolar ATPase subunit homologs from *Neurospora crassa* (5), *Arabidopsis thaliana* (36), *Sulfolobus acidocaldarius* (12), *Methanosarcina barkeri* (22), and humans (68), and sequence identities of 82, 74, 54, 58, and 74%, respectively, were found. This is consistent with the observations of others that the 60-kDa subunit of vacuolar ATPases is highly conserved among evolutionarily distant species (5, 12, 18, 22, 36, 43, 68). This gene for the yeast 60-kDa subunit of the vacuolar H⁺-ATPase was designated VAT2.

Upon comparing the VAT2 DNA sequence against the sequence of the yeast vacuolar H⁺-ATPase 57-kDa subunit

gene described by Nelson et al. (43), we found that the sequences were identical except for two differences. First, the C at nucleotide 235 of the open reading frame (nucleotide 1000 of the sequence of Nelson et al. [43]) is a G in the VAT2 sequence, resulting in an amino acid change from Leu₇₉ to Val₇₉ of VAT2. This is a conservative amino acid change, and it is possible that this is a DNA sequence polymorphism since the two clones were from libraries derived from two different strains of *S. cerevisiae*. The other difference was an additional C in VAT2, changing the sequence starting at nucleotide 1492 of the open reading frame (nucleotide 2257 of the revised sequence of Nelson et al. [43]) from 5'-GATC CGAC-3' to 5'-GATCCCGAC-3', thus changing the reading frame at the 3' end of the gene. The nucleotide sequence of Nelson et al. (43) predicts a 515-amino-acid polypeptide that has one identity with the *Neurospora vma-2* gene product (57-kDa vacuolar H⁺-ATPase subunit; 5) in its carboxy-terminal 16 residues, whereas our DNA sequence predicts a 517-amino-acid polypeptide with six identities with the *Neurospora* 57-kDa subunit in the carboxy-terminal 18 residues (Fig. 2B).

VAT2 is a nonessential gene. To determine the phenotype of mutants deficient in the VAT2 gene product, we constructed a null allele by replacing the 18-bp fragment between the two *EcoRI* sites in the open reading frame with the *LEU2* gene (Fig. 1B). The allele *vat2Δ::LEU2* will be designated *vat2-Δ1*. A diploid strain, CYY7, was transformed such that the *vat2-Δ1* mutant allele replaced one of the chromosomal copies of VAT2. This diploid was sporulated, tetrads were dissected, and the resulting spores were scored for viability and nutritional markers. Table 2 shows the results of the tetrad dissections. Spore viability for the heterozygous diploid was almost identical to that of the isogenic wild-type diploid. Southern blots were performed on digested DNA from meiotic segregants of the heterozy-

FIG. 3. Southern blot analysis of tetrads from a *vat2-Δ1/VAT2* parent. Total yeast DNA from the parent *VAT2/VAT2* diploid strain CYY7 (lane 5), the *vat2-Δ1/VAT2* diploid strain CYY9 (lane 6), and the four progeny from each of two tetrads derived from CYY9 (lanes 1 to 4 and 7 to 10) were digested with *Clal* and *SalI* and probed with a radiolabeled 0.8-kb *EcoRI-HindIII* fragment containing part of the VAT2 gene and ~500 bp of the 5' untranslated region. The inserted *LEU2* gene contained a *SalI* site 100 bp from the end proximal to the 5' end of the VAT2 gene. The sizes and origins of the hybridizing fragments are indicated in the margins. The Leu phenotype is shown above the lanes.

TABLE 2. Tetrad analysis for viability of *vat2-Δ1* spores

Tetrad viability ^a	No. of tetrads ^b	
	<i>VAT2</i> ⁺ / <i>VAT2</i> ⁺	<i>vat2Δ::LEU2/VAT2</i> ⁺
4:0	32	52
3:1	6	10 ^c
2:2	2	3 ^d
1:3	0	0
0:4	0	0

^a Tetrad viability is designated as viable spores versus inviable spores.

^b The *VAT2*⁺/*VAT2*⁺ strain was CYY7, and the *vat2Δ::LEU2/VAT2*⁺ strain was CYY9. *vat2Δ::LEU2* is equivalent to the *vat2-Δ1* allele.

^c Of the 10 dead spores, 6 were predicted to be Leu⁺.

^d Of the 6 dead spores, 4 were predicted to be Leu⁺.

FIG. 4. Western blot analysis of the 69- and 60-kDa vacuolar H^+ -ATPase subunits from whole-cell extracts and isolated vacuolar membranes. Solubilized whole-cell extracts and purified vacuoles of wild-type (lanes 1) and *vat2-Δ1* (lanes 2) cells were loaded onto 10% polyacrylamide-SDS gels, electrophoresed, and electrotransferred onto nitrocellulose membranes. (A) Whole-cell extracts were probed with affinity-purified 60-kDa subunit antibody. Whole-cell extracts (B) and purified vacuoles (C) were probed with the 69-kDa subunit monoclonal antibody 8B1F3. The wild-type strain was SF838-5A, and the *vat2-Δ1* strain used was SF838-5AV1. kD, Kilodaltons.

gous diploid. The *vat2Δ::LEU2* allele segregated 2:2 (Fig. 3). There was no evidence of cross-hybridizing species, suggesting that no gene highly homologous to *VAT2* occurs in yeast cells. In addition, haploid strains have also been directly transformed with the *vat2Δ::LEU2* construct, with transformation frequencies similar to those observed when transformed with the *LEU2* gene alone (data not shown). The *vat2-Δ1* allele leaves intact the amino-terminal 94 residues of the protein. A deletion of the 2.2-kb *HindIII* fragment, encompassing the entire *VAT2* open reading frame (*vat2Δ::URA3 = vat2-Δ2*), was also constructed and analyzed. The *vat2-Δ2* mutant exhibited phenotypes indistinguishable from the *vat2-Δ1* mutant. These results indicate that the *VAT2* gene product is not essential for spore germination or vegetative growth of *S. cerevisiae*.

***vat2* cells contain vacuoles and are deficient for the 60-kDa H^+ -ATPase subunit.** To determine whether the *vat2-Δ1* lesion actually eliminates the 60-kDa subunit of the vacuolar H^+ -ATPase, we probed immunoblots of whole-cell lysates with antiserum against the *VAT2* gene product. Polyclonal antiserum against the carboxy-terminal 80% of the *VAT2* gene product was generated and affinity purified (see Materials and Methods). While the *VAT2* strain contained a single species of 60 kDa recognized by the antiserum (Fig. 4A, lane 1), the *vat2* strain was completely devoid of the 60-kDa antigen (lane 2). Indirect immunofluorescence microscopy studies gave results which were consistent with the immunoblot results. The vacuole was visualized as one to four obvious indentations within the cells when viewed by Nomarski optics (Fig. 5A). Vacuoles of wild-type and *vat2* cells appeared to be morphologically similar when viewed by Nomarski optics. The fluorescence micrographs (Fig. 5A) of the wild type and mutant exhibited very different staining patterns. Wild-type cells had uniform labeling around the periphery of the vacuole, consistent with the fact that the vacuolar H^+ -ATPase complex resides on the vacuolar membrane. As expected, *vat2* cells did not display any specific labeling by the 60-kDa antibody (Fig. 5A).

Even though *vat2* cells contained organelles which appeared to be morphologically similar to wild-type vacuoles, we wanted to examine the membrane surrounding these organelles for the presence of a known vacuolar membrane marker, alkaline phosphatase, by indirect immunofluorescence with a polyclonal antibody against alkaline phosphatase (32; Raymond and Stevens, submitted). We observed that the perimeters of *vat2* vacuoles, as well as those

FIG. 5. Localization of 60-kDa vacuolar H^+ -ATPase subunit and alkaline phosphatase by immunofluorescence microscopy. *VAT2* and *vat2-Δ1* cells were fixed, converted to spheroplasts, and stained with affinity-purified 60-kDa subunit antibody (A) or affinity-purified alkaline phosphatase antibody (B). Cells were viewed by Nomarski optics and fluorescence microscopy.

TABLE 3. ATPase activities of purified vacuoles from wild-type and *vat2* cells

Cells ^a	ATPase sp act (U/mg of protein) ^b	% Wild-type vacuolar ATPase sp act
VAT ⁺	2.57	100
<i>vat2-Δ1</i>	0.0273 ^c	1.1

^a The VAT⁺ strain was SF838-5A, and the *vat2-Δ1* strain was SF838-5AV1.

^b ATPase activities of isolated vacuoles are given as specific activities, with 1 U defined as 1 μmol of phosphate liberated per min. ATPase levels were measured in the presence of inhibitors of the plasma membrane and mitochondrial ATPases (100 μM sodium vanadate and 2 mM sodium azide, respectively; 71). Quantitations were from one set of vacuole preparations. Three other sets of vacuole preparations of VAT⁺ and *vat2-Δ1* strains were completed, and *vat2-Δ1* cells had 1 to 2% of the wild-type vacuolar H⁺-ATPase activity.

^c This activity was insensitive to bafilomycin A₁ (1 μM).

of the wild-type vacuoles, were specifically labeled by alkaline phosphatase antibody (Fig. 5B). This result indicated that the indentations observed by Nomarski optics in *vat2* cells indeed represented vacuoles. Similar observations have been made with antibody directed against dipeptidyl aminopeptidase B (C. K. Raymond, I. Howald, and T. H. Stevens, unpublished observations), another vacuolar membrane protein (52). These results demonstrated that vacuolar membrane biogenesis was not obviously perturbed in *vat2* cells, as was seen for some *vps* mutants (3, 54, 56). When the *vat2* mutant strains were transformed with a centromere-bearing plasmid containing the VAT2 gene (2.2-kb *Hind*III fragment; Fig. 1A), the 60-kDa subunit reappeared in immunoblots of vacuolar membranes as well as in the whole-cell lysates (data not shown). We conclude that the 2.2-kb *Hind*III fragment of VAT2 is sufficient to complement the *vat2* null mutations.

***vat2* cells are deficient for vacuolar ATPase activity.** To determine whether the 60-kDa subunit is essential for vacuolar ATPase activity, we assayed ATPase activity in vacuoles purified from a *vat2* strain. Yeast vacuoles from isogenic VAT2 and *vat2-Δ1* strains were isolated by two consecutive Ficoll flotations as described by Kakinuma et al. (28). ATPase-specific activities from those vacuoles are shown in Table 3. The specific activity of dipeptidyl aminopeptidase B was comparable between the two strains (*vat2-Δ1* activity was about 50% of that of the wild type; data not shown), whereas the ATPase activity of the mutant strain was about 1 to 2% of that of the wild type and was insensitive to bafilomycin A₁.

Functional deficiency in the vacuolar H⁺-ATPase in the *vat2* strain was correlated with an apparent absence of an intact complex of the H⁺-ATPase. Using the monoclonal antibody, 8B1F3, against the 69-kDa subunit (30) to probe an immunoblot containing whole-cell extracts of wild-type and *vat2* cells, we observed that both strains have similar amounts of the 69-kDa subunit (Fig. 4B). Because extract from an equal number of cells for each strain was applied to the immunoblot, this result suggests that there is a lack of coordinate regulation of expression of the 69-kDa subunit when the 60-kDa subunit has been eliminated. Even though the cellular level of the 69-kDa subunit in *vat2* was normal, purified vacuoles from *vat2* cells did not contain detectable levels of the 69-kDa subunit (Fig. 4C). These same membranes contained vacuolar membrane markers, dipeptidyl aminopeptidase B, and alkaline phosphatase, at levels comparable to those of wild-type vacuoles (data not shown). This result implies that without the 60-kDa subunit present, the 69-kDa subunit was synthesized and stable yet unable to

FIG. 6. 6-Carboxyfluorescein accumulation in wild-type and *vat2* vacuoles. Wild-type and *vat2-Δ1* cells treated with 6-CFDA were viewed by Nomarski optics and fluorescence microscopy.

assemble onto the vacuolar membrane. The 42-kDa subunit was affected in a manner similar to that of the 69-kDa subunit in *vat2* strains (P. Kane and T. Stevens, unpublished data); however, the effects of the *vat2-Δ1* mutation on the other subunits of the vacuolar H⁺-ATPase were not tested. Since the 69-kDa subunit contains the catalytic site of the yeast vacuolar H⁺-ATPase (30, 71, 72), its absence from the vacuole is consistent with the lack of vacuolar ATPase activity in the *vat2* mutant.

Vacuolar acidification is defective in *vat2* cells. In vitro studies with purified vacuoles demonstrated that yeast vacuolar acidification requires ATP hydrolysis (28). We wanted to determine whether the vacuolar H⁺-ATPase-deficient *vat2* mutant cells were also defective for vacuolar acidification. The lysosomotropic fluorescent dye quinacrine has been shown to accumulate within acidified vacuoles when supplied exogenously to intact yeast cells in medium buffered at an alkaline pH (77). We observed that *vat2* cells were completely defective for quinacrine accumulation in their vacuoles (data not shown). The failure of vacuoles to accumulate quinacrine suggests that there is a defect in vacuolar acidification, but it does not establish total loss of the pH gradient across the vacuolar membrane (48). To determine whether the *vat2-Δ1* mutation affected vacuolar pH in vivo, we assayed vacuolar pH by flow cytometry with the vacuole-specific, pH-sensitive vital dye 6-CF as previously described (48).

6-Carboxyfluorescein accumulation in wild-type and *vat2* cells was specific for the vacuole (Fig. 6). A strain bearing the *vat2-Δ1* allele had a vacuolar pH of 7.1 (standard error of the mean, ±0.1; n = 3), while the isogenic wild-type strain had a vacuolar pH of 6.1 (standard error of the mean, ±0.04; n = 3). Representative flow cytometric histograms for these assays are shown in Fig. 7. The *vat2* vacuolar pH was very close, if not identical, to the pH of the yeast cytoplasm, determined to be 7.1 to 7.3 by ³¹P nuclear magnetic resonance spectroscopy (13, 17). It is not known whether there is still a ΔpH across the vacuolar membrane of the *vat2* mutant strain, but it is likely to be less than 0.2 pH unit. Therefore, the vacuolar H⁺-ATPase appears to be the only enzyme capable of acidifying the yeast vacuole.

FIG. 7. pH-dependent fluorescence ratio histograms of wild-type and *vat2* cells. Cells were treated with 6-CFDA and analyzed by dual-excitation flow cytometry as described by Preston et al. (48). The abscissa is the ratio of fluorescence (emission with excitation at 488 nm/emission with excitation at 458 nm) normalized for display with units in channel numbers. Spikes in the histograms are channelization artifacts. (A) Representative calibration histograms obtained with *vat2-Δ1* cells suspended and analyzed in the presence of ionophores that equilibrated the vacuolar pH to the indicated external pH. (B) Representative histograms for wild-type and *vat2-Δ1* strains in the absence of ionophores as indicated.

Growth of *vat2* cells is impaired at neutral pH. Yeast *vps* mutants which have a vacuolar acidification defect are sensitive for growth in low-pH medium (3; C. Raymond and T. Stevens, unpublished data). This pH sensitivity suggests that proper acidification of the vacuole may be necessary for regulating the intracellular pH. We studied the growth of

TABLE 4. pH-dependent growth rates for wild-type and *vat2* strains

pH ^a	Doubling time (min) for ^b :		Ratio of doubling times for <i>vat2-Δ1</i> VAT ⁺
	VAT ⁺	<i>vat2-Δ1</i>	
3.0	98	135	1.38
4.0	84	115	1.37
5.0	83	112	1.35
6.0	84	235	2.80
7.0	100	— ^c	—

^a For the duration of the growth measurements, pH did not change by more than 0.1 U.

^b The VAT⁺ strain was SF838-5A, and the *vat2-Δ1* strain was SF838-5AV1.

^c —, Exhibited logarithmic growth with an apparent doubling time of 460 min for 1.5 generations (9 h) and failed to grow after that.

wild-type and *vat2* strains in medium that was buffered at pHs 3.0, 4.0, 5.0, 6.0, and 7.0 with 50 mM succinate–50 mM sodium phosphate (Table 4). The growth of *vat2* cells was grossly impaired relative to that of the wild type at pHs 6.0 and 7.0, and after 9 h at pH 7.0, the *vat2* cells failed to grow at all. At the lower pH values tested (3.0, 4.0, and 5.0), the *vat2* cells were slower than wild-type cells to grow, with doubling times ~35% longer. The acidification-defective *vps* mutants were sensitive to low pHs (≤ 3.5) (3; Raymond and Stevens, unpublished data); however, growth was not tested at neutral pH. One vacuolar acidification-defective mutant, *vps3*, has been observed to have significantly different growth characteristics on pH-adjusted YEPD plates relative to *vat2*. *vps3* cells grew progressively more slowly as the growth medium pH was reduced, whereas growth of *vat2* cells was unaffected from pHs 3 to 5 (C. Raymond and C. Yamashiro, unpublished observations). These results indicate that the *vat2-Δ1* mutation greatly impairs the ability of yeast to grow at neutral pH but has only a minor effect at low pHs, contrary to results obtained with certain *vps* mutants.

Vacuolar protein sorting is mildly perturbed in *vat2* cells. To test directly the role of the vacuolar H⁺-ATPase in

FIG. 8. Immunoprecipitations of CPY and PrA in *vat2* cells. Wild-type and *vat2-Δ1* cells were labeled with Tran ³⁵S-label for 20 min and chased for 30 min in the presence of 0.1-mg/ml methionine–0.1-mg/ml cysteine. Cultures were separated into two fractions (I, intracellular; E, extracellular) and immunoprecipitated with CPY antiserum (A) or affinity-purified PrA antibody (B). Positions of molecular mass standards are indicated on the right of the autoradiograms in kilodaltons (kD). proCPY indicates the position of the 69-kDa precursor form of CPY. mCPY indicates the position of the 61-kDa mature enzyme. proPrA indicates the position of the 52-kDa PrA precursor form. mPrA indicates the position of the 42-kDa mature form of PrA. The minor band below mPrA (lanes I) is of unknown origin.

protein sorting, we analyzed the effect of the *vat2-Δ1* mutation on the sorting of newly synthesized vacuolar proteins. Whole cells of wild-type and *vat2* strains were pulse-labeled with [³⁵S]methionine and [³⁵S]cysteine and then chased with unlabeled methionine and cysteine. The extracellular fraction consisted of the pulse-chase medium combined with the periplasmic fraction, and the intracellular fraction contained the cell lysate. CPY and PrA, both soluble vacuolar proteins, were then immunoprecipitated from both fractions and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography (Fig. 8). For both CPY and PrA, 20 to 25% of the newly synthesized protein chased into the extracellular fraction of labeled *vat2* cells, as compared with 4 to 8% of the extracellular CPY and PrA for pulse-chase-labeled wild-type cells. The proenzyme forms of CPY and PrA were detected in the extracellular fraction, whereas the mature forms of CPY and PrA were the dominant species detected in the intracellular fraction. Phosphoglycerate kinase, a cytosolic protein, was also immunoprecipitated from the same fractions in order to determine the level of cell lysis during the pulse-chase and cell wall removal steps of the immunoprecipitation experiment, and less than 5% extracellular phosphoglycerate kinase was detected for both wild-type and *vat2* strains (data not shown). This result, in conjunction with the observation that the mature forms of CPY and PrA were not found extracellularly, argues against the possibility that the extracellular precursor forms of CPY and PrA (proCPY and proPrA) were a result of cell lysis. Our findings suggest that the vacuolar H⁺-ATPase-dependent acidification of the vacuole is required only for highly efficient targeting of soluble vacuolar proteins.

Vacuolar acidification is not necessary for zymogen activation. The conversion of the yeast vacuolar zymogen proPrA (encoded for by the *PEP4* gene) to its mature form, PrA, has been predicted to occur via an autocatalytic mechanism that is induced by an acidic pH (1, 39, 59, 78). Immunoprecipitation of PrA (Fig. 8B) demonstrated that both wild-type and *vat2* cells accumulated mature PrA intracellularly. There was a small amount of proPrA observed in the intracellular fraction of the *vat2* immunoprecipitation (Fig. 8B); however, the level of intracellular proPrA was variable, and the majority of the intracellular proPrA appeared to chase into

the extracellular fraction with longer chase times (data not shown). This suggests that proPrA processing is essentially normal in *vat2* cells. Since mature PrA, which is responsible for processing proCPY (39, 79), resided within the *vat2* vacuole, it was not surprising to see only mature CPY in the intracellular fraction for the *vat2* mutant (Fig. 8A). This result indicates that yeast cells defective for vacuolar acidification are competent for zymogen activation independent of an acidic pH.

It was possible that preexisting mature PrA in *vat2* cells would retain the ability to activate newly synthesized proPrA, despite the neutral pH of *vat2* vacuoles. If so, the aforementioned experiment would not assess the role of acidic pH in the autocatalytic maturation of proPrA. To test whether the proPrA activation requires an acidic vacuole in the absence of preexisting mature prA, we transformed a *pep4-3* (a null mutant allele of the gene encoding PrA) strain (SF838-1D) and an isogenic *pep4-3 vat2-Δ1* strain with a centromere-containing plasmid carrying the *PEP4* gene. The transformed strains were radiolabeled as before, and CPY and PrA were immunoprecipitated. For both proteins, only the mature form was found in the intracellular fraction (data not shown). Therefore, initiation of zymogen activation does not require an acidic vacuole, even in the absence of mature PrA.

DISCUSSION

We analyzed the functions of the yeast vacuolar H⁺-ATPase by creating a null mutation in one of the vacuolar H⁺-ATPase subunit genes and studying the phenotypes associated with that mutation. Cells lacking the vacuolar H⁺-ATPase 60-kDa subunit gene were viable, but they failed to grow at neutral pH. The *vat2* mutant was shown to be deficient for vacuolar H⁺-ATPase activity and was completely defective for vacuolar acidification. Thus, the *vat2-Δ1* mutant strain was suitable for addressing the significance of the role of vacuolar acidification in vacuolar protein sorting and zymogen activation. Our data indicate that vacuolar acidification is relevant for highly efficient vacuolar protein sorting but is not a significant factor in zymogen activation.

The fact that *vat2* cells are viable suggests that the vacuolar H⁺-ATPase does not play a crucial role in the secretory pathway. The integral membrane subunits of the vacuolar H⁺-ATPase are likely to traverse the secretory pathway from the endoplasmic reticulum through to the Golgi apparatus along with other vacuolar membrane proteins (32, 52). Since vacuolar ATPase activity is unaffected in *pep4* cells (C. Yamashiro and T. Stevens, unpublished observations), it does not appear to have a zymogen form necessitating PrA-dependent activation. Vacuolar H⁺-ATPase assembly is not understood, but we are currently studying the assembly process, since it could help determine which intracellular compartments are affected by this enzyme.

Growth of the *vat2* mutant was severely compromised in neutral pH media. One possible interpretation of this result is that the vacuolar H⁺-ATPase plays a role in cytosolic pH regulation or in the maintenance of cellular membrane potential or both. Several mutant alleles of the yeast plasma membrane H⁺-ATPase gene (*pma1*) cause growth sensitivity in low-pH (pH 3.5) medium, yet they grow as well as the wild type at pH 5.5 and above (38). More recent data suggest that the *pma1* defect does not involve intracellular pH regulation but rather a membrane potential defect (47). The *pma1* cells exhibit a pH sensitivity opposite to that observed for the *vat2* cells. These pH-dependent growth defects indicate that the plasma membrane H⁺-ATPase is important for survival in low-pH conditions, while the vacuolar H⁺-ATPase is necessary for growth in neutral pH conditions. Thus, these two proton-motive ATPases appear to be required for growth of yeast cells in media over a wide pH range. The biochemical basis for these effects remains to be determined.

The viability of *vat2* cells could be formally explained by a second gene encoding the 60-kDa subunit of the vacuolar ATPase which would suppress a potentially lethal genetic lesion. A number of results and observations argue against that possibility. First, Southern blots of genomic DNA from both wild-type and *vat2* cells with the *VAT2* gene as the probe did not show any unexpected cross-hybridizing species, suggesting that no other highly homologous sequences exist. Second, our immunoblots of *vat2* whole-cell lysates did not exhibit any immunoreactive species when probed with the anti-60-kDa polyclonal antibody. Since this polyclonal antiserum was generated against the carboxy-terminal 80% of the *VAT2* gene product, which includes the most highly conserved domains of the 60-kDa subunit, it is unlikely that an isoform of this subunit exists. The *vat2-Δ1* mutation abolished vacuolar H⁺-ATPase activity in vitro and caused grossly defective vacuolar acidification in vivo.

The vacuolar H⁺-ATPase is intimately involved in the acidification of the yeast vacuole. The quinacrine accumulation defect observed for *vat2* cells suggested a defect in vacuolar acidification. We measured the luminal pH of the wild-type vacuoles to be 6.1, which is in close agreement with the pH of 6.2 determined by the same method used by Preston et al. (48). The luminal pH of *vat2* vacuoles was measured to be 7.1, which is very close, if not identical, to the pH of the cytoplasm (13, 17). These results indicate that the 60-kDa subunit is an essential component of the vacuolar H⁺-ATPase responsible for acidification of the yeast vacuole. Since no other proton-translocating enzymes have been identified in yeast vacuoles, it is likely that the vacuolar H⁺-ATPase is the only H⁺-pumping enzyme involved in vacuolar acidification. In addition, the degree of vacuolar protein missorting in yeast cells treated with weak bases (59) was essentially the same as that observed for *vat2* cells,

suggesting that the possible vacuolar H⁺-ATPase-independent acidification of other compartments within the vacuolar network does not play a role in vacuolar protein sorting.

The moderate defect in vacuolar protein sorting in *vat2* cells was surprising. A previous study of the yeast vacuolar protein-sorting mutants *vps3* and *vps6* indicated that they were defective for vacuolar acidification (3, 59). These two mutants were found to have a severe protein-sorting defect, secreting >85% of the CPY antigen and about 50% of the PrA antigen (56). We observed only 20 to 25% secretion of both CPY and PrA antigens in *vat2* cells, an amount significantly lower than that observed for *vps3* and *vps6* mutants. This suggests that the observed vacuolar-acidification defect in the *vps3* and *vps6* mutants is not responsible for the protein mislocalization phenotype. In fact, the severe protein-sorting defect in these mutants may be the cause of the vacuolar-acidification defect. The *VAT2* gene is not represented among the 40 *VPS* complementation groups (53, 54) as determined by complementation analysis (I. Howald, C. Yamashiro, and T. Stevens, unpublished data). Since *vat2* mutants are completely defective for vacuolar acidification, it is unlikely that the present collection of *VPS* complementation groups contains mutations of the vacuolar H⁺-ATPase. However, the direct screening for aberrant vacuolar-pH mutants (48) should yield mutations of the vacuolar H⁺-ATPase.

Activation of proPrA was unaffected in the vacuolar acidification-defective *vat2* cells. PrA is a member of the aspartyl protease family which includes the secreted enzyme pepsin and the lysosomal enzyme cathepsin D (69). These aspartyl proteases are initially synthesized as zymogens. Activation of pepsinogen is an intramolecular processing event induced by low pHs (8, 25). Since PrA is in the same family of proteases, the mechanism of proPrA maturation has been proposed to be similar to that of pepsinogen (1, 78). Moreover, yeast *vps* mutants defective for vacuolar acidification have been observed to accumulate increased levels of intracellular proPrA (59). We observed that activation of proPrA was unaffected by the *vat2-Δ1* mutation in both *PEP4* and *pep4-3* genetic backgrounds. These results strongly suggest that an acidic vacuole is not required for either the maintenance or the initiation of proPrA activation.

Certain cellular processes which have been postulated to directly require vacuolar acidification have been found to be only mildly affected (protein sorting) or not detectably affected (zymogen activation) in *vat2* cells, which are completely defective for vacuolar acidification. The models for these processes have been strongly motivated by studies done with mammalian cells regarding protein targeting and endocytosis; however, there are some major differences between yeast and mammalian cells. First, the ΔpH between the vacuolar lumen and cytoplasm of wild-type yeast (≈1.0) is significantly different from the ΔpH between the lysosomal lumen and cytoplasm observed for mammalian systems (≈2 to 2.5) (40). This difference in ΔpH suggests that there may be different processes or shared processes with different mechanisms mediated by vacuolar acidification in yeast and mammalian cells. A second difference is that soluble yeast vacuolar proteins do not utilize mannose 6-phosphate as a sorting determinant. Rather, the determinant in yeast appears to involve a proteinaceous domain (2, 26, 31, 74). There is indirect evidence that yeast does use a receptor-ligand system of vacuolar protein delivery (55, 66), but there must not be an absolute requirement for a low-pH environment in receptor-ligand dissociation if such a system exists in yeast cells. It should be noted that there is evidence that

certain mammalian cells utilize an alternate lysosomal protein-sorting regimen independent of mannose 6-phosphate (33), and the role of acidification in this alternate pathway is not known.

Thus far, only the growth rate of *vat2* cells was significantly perturbed, especially at pH 7.0, relative to that of wild-type cells. The basis for this growth defect is unclear, but it is possible that vacuolar acidification is important for other cellular processes that ultimately affect the growth of yeast cells. Vacuolar acidification has been postulated to play a central role in such processes as amino acid and Ca²⁺ homeostasis. In vitro studies have shown that there are amino acid and Ca²⁺ transport systems in the yeast vacuole that require a proton electrochemical gradient across the vacuolar membrane (45, 46). Since *vat2* cells are completely defective for vacuolar acidification, it is of interest to study these other cellular processes in this mutant background.

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