

Acidification of the Lysosome-like Vacuole and the Vacuolar H⁺-ATPase Are Deficient in Two Yeast Mutants That Fail to Sort Vacuolar Proteins

Joel H. Rothman, Carl T. Yamashiro, Christopher K. Raymond, Patricia M. Kane, and Tom H. Stevens

Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403

Abstract. Organelle acidification plays a demonstrable role in intracellular protein processing, transport, and sorting in animal cells. We investigated the relationship between acidification and protein sorting in yeast by treating yeast cells with ammonium chloride and found that this lysosomotropic agent caused the mislocalization of a substantial fraction of the newly synthesized vacuolar (lysosomal) enzyme proteinase A (PrA) to the cell surface. We have also determined that a subset of the *vpl* mutants, which are deficient in sorting of vacuolar proteins (Rothman, J. H., and T. H. Stevens. 1986. *Cell*. 47:1041-1051; Rothman, J. H., I. Howald, and T. H. Stevens. *EMBO [Eur. Mol. Biol. Organ.] J.* In press), failed to accumulate the lysosomotropic fluorescent dye quinacrine within their vacuoles, mimicking the phenotype of wild-type

cells treated with ammonium. The acidification defect of *vpl3* and *vpl6* mutants correlated with a marked deficiency in vacuolar ATPase activity, diminished levels of two immunoreactive subunits of the proton-translocating ATPase (H⁺-ATPase) in purified vacuolar membranes, and accumulation of the intracellular portion of PrA as the precursor species. Therefore, some of the *VPL* genes are required for the normal function of the yeast vacuolar H⁺-ATPase complex and may encode either subunits of the enzyme or components required for its assembly and targeting. Collectively, these findings implicate a critical role for acidification in vacuolar protein sorting and zymogen activation in yeast, and suggest that components of the yeast vacuolar acidification system may be identified by examining mutants defective in sorting of vacuolar proteins.

A number of intracellular protein transport and processing reactions occur within the acidic interiors of the organelles that mediate these processes in eukaryotic cells (Mellman et al., 1986; Bowman and Bowman, 1986). These organelles, including the lysosome and components of the endocytic and exocytic pathways, comprise the organellar system known as the vacuolar network. The participation of a low luminal pH in intracellular sorting of proteins secreted via the constitutive and regulated exocytic pathways (Moore et al., 1983), ligands internalized by endocytosis (Mellman et al., 1986), proteins delivered to compositionally distinct plasma membranes of polarized epithelial cells (Caplan et al., 1987), and newly synthesized lysosomal proteins (von Figura and Hasilik, 1986), has been implicated from the effects of "lysosomotropic" agents that inhibit acidification of this vacuolar network. The importance of organellar acidification is also evident from studies of several Chinese hamster mutant cell lines that are defective in endosomal acidification (Merion et al., 1983; Marnell et al., 1984; Robbins et al., 1983). Among the numerous phenotypes attributed to the acidification defect is the failure of these cells to properly

localize newly synthesized lysosomal proteins (Robbins et al., 1984). However, the precise molecular defects leading to the failure in acidification are unknown (Timchak et al., 1986; Stone et al., 1987).

Acidification also appears to play a role in triggering proteolytic maturation of precursor proteins during transport. For example, proteolytic processing of proinsulin has been correlated with acidification of the secretory granules that transport the prohormone to the cell surface (Orci et al., 1987). The acidic environment of the lysosome is required for the activity of hydrolases that are sequestered within it, and it has been suggested that these hydrolases exhibit a low pH optimum to ensure that they are inactivated if released from the lysosome into the more basic cytoplasm (Mellman et al., 1986). The acidic state of vacuolar network organelles thus appears to be critical for many of the normal activities of eukaryotic cells.

The yeast vacuole is an acidic organelle containing hydrolytic enzymes, and is considered to be equivalent to the lysosome of animal cells (Wiemken et al., 1979; Rothman and Stevens, 1988). Sorting of proteins to the yeast vacuole has been shown to follow a pathway that is similar to that followed by lysosomal proteins in animal cells (Stevens et al., 1982). Genes encoding molecular components required for

J. H. Rothman's present address is Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, U. K.

Table 1. Yeast Strains

Strain	Genotype	Source
JHRY20-2C	<i>MATa, his3-Δ200, ura3-52, leu2-3, leu2-112</i>	Rothman et al., 1986
JHRY20-2C <i>vpl8-Δ1</i>	<i>MATa, his3-Δ200, ura3-52, leu2-3, leu2-112, vpl8-Δ1::URA3</i>	Derived from JHRY20-2C
JHRY61-1B	<i>MATa, his4-519, leu2-3, leu2-112, vpl3-2</i>	Rothman and Stevens, 1986
JHRY64-5B	<i>MATα, his4-519, ura3-52, leu2-3, leu2-112, lys2, vpl6-2</i>	Rothman and Stevens, 1986
SF838-1D	<i>MATα, ade6, his4-519, ura3-52, leu2-3, leu2-112, pep4-3, gal</i>	Rothman and Stevens, 1986
SF838-1D <i>vpl3-Δ1</i>	<i>MATα, ade6, his4-519, ura3-52, leu2-3, leu2-112, pep4-3, gal, vpl3-Δ1::LEU2</i>	Derived from SF838-1D
SF838-1Dm220	<i>MATα, ade6, his4-519, ura3-52, leu2-3, leu2-112, pep4-3, gal, vpl6-11</i>	Rothman and Stevens, 1986
SF838-1Dm108	<i>MATα, ade6, his4-519, ura3-52, leu2-3, leu2-112, pep4-3, gal, vpl8-10</i>	Rothman and Stevens, 1986
SF838-9DR2L1	<i>MATa, his4-519, ura3-52, leu2-3, leu2-112, lys2, pep4-3, gal</i>	Rothman and Stevens, 1986
SF838-9DR2L1 <i>vpl3-Δ1</i>	<i>MATa, his4-519, ura3-52, leu2-3, leu2-112, lys2, pep4-3, gal, vpl3-Δ1::LEU2</i>	Derived from SF838-9DR2L1
SF838-9DR2L1m1038	<i>MATa, his4-519, ura3-52, leu2-3, leu2-112, lys2, pep4-3, gal, vpl6-2</i>	Rothman and Stevens, 1986
SF838-9DR2L1m1057	<i>MATa, his4-519, ura3-52, leu2-3, leu2-112, lys2, pep4-3, gal, vpl8-3</i>	Rothman and Stevens, 1986
X2180-1B	<i>MATα, mal, mel, gal2</i>	Yeast Genetic Stock Center

The *vpl6-2*, *vpl6-11*, *vpl8-3*, and the *vpl8-10* strains were the original isolates of the indicated *vpl* alleles and are isogenic to strain SF838-1D or SF838-9DR2L1. The *PEP4⁺* *vpl3-2* and *vpl6-2* strains are outcrosses of the indicated *vpl* alleles (Rothman and Stevens, 1986).

protein sorting (Rothman and Stevens, 1986; Bankaitis et al., 1986), as well as sorting determinants residing on vacuolar proteins (Valls et al., 1987; Johnson et al., 1987; Klionsky et al., 1988), have been identified in yeast. Thus, yeast provides a simple system for dissecting the mechanisms by which newly synthesized proteins are sorted to the vacuole and for examining the role of acidification of the vacuolar network in protein sorting.

The acidic pH of the yeast vacuolar lumen appears to be generated and maintained by a proton-translocating ATPase (H^+ -ATPase) located in the vacuolar membrane (Uchida et al., 1985). This H^+ -ATPase complex has been purified from yeast vacuoles and is comprised of at least three (Uchida et al., 1985), and probably more (Kane et al., 1989) distinct polypeptides. The function of each of these subunits is not understood, nor is it known whether this complex is capable of translocating protons across the vacuolar membrane in the absence of other components.

Although it is clear that the yeast vacuole maintains a lower internal pH than that of the cytoplasm (Navon et al., 1979; Makarow and Nevalainen, 1987), it has not been demonstrated that this acidic environment is essential for delivery of newly synthesized proteins into the vacuole. Isolation of mutations in the genes encoding the vacuolar H^+ -ATPase subunits or other proteins involved in acidification of the vacuolar system would allow a direct test of the role of acidification in protein sorting. In this report, we provide evidence suggesting that vacuolar network acidification is required for vacuolar protein sorting and vacuolar zymogen activation. We also demonstrate that a limited subset of the mutants that are defective in vacuolar protein sorting are deficient in vacuolar acidification and ATPase activity at the vacuolar membrane. The genes represented by these acidification-defective mutants may encode subunits of the vacuolar H^+ -ATPase or components required for proper assembly and localization of this enzyme in the vacuolar membrane.

1. *Abbreviations used in this paper:* H^+ -ATPase, proton-translocating ATPase; PrA, proteinase A; proPrA, precursor form of PrA.

Materials and Methods

Yeast Strains

The yeast strains used in this study were constructed by standard genetic manipulations. The genotypes of these strains are indicated in Table 1. The *vpl3-Δ1* allele carries a substitution of the *LEU2* gene within the *VPL3* open reading frame, and the *vpl8-Δ1* allele carries a substitution of the *URA3* within the *VPL8* gene. These deletion constructs were integrated into the yeast genome to replace the wild-type chromosomal copies of these genes by standard techniques (Rothstein, 1983). Haploid strains carrying either the *vpl3-Δ1* or the *vpl8-Δ1* alleles were viable at all temperatures and displayed a Vpl^- phenotype (C. Raymond, unpublished observations).

Materials

Carrier-free [^{35}S]H₂SO₄ and zymolyase 100T were from ICN Biomedicals, Inc. (Irvine, CA). Fraction II lyticase was prepared as described previously (Scott and Schekman, 1980). [^{125}I]protein A was from Amersham (Arlington Heights, IL), nitrocellulose was from Schleicher and Schuell, Inc. (Keene, NH), IgG Sorb was from the Enzyme Center (Boston, MA), and SDS was from BDH Biochemicals Ltd. (Poole, UK). Acetylated BSA used in radiolabeling experiments was from Bethesda Research Laboratories (Bethesda, MD), and ZW3-14 used in vacuolar H^+ -ATPase solubilization was from Calbiochem-Behring Corp. (San Diego, CA). Quinacrine and all other reagents used for enzymatic and protein assays were obtained from Sigma Chemical Co. (St. Louis, MO).

Antibodies to yeast carboxypeptidase Y, proteinase A and phosphoglycerate kinase were described in earlier communications (Rothman et al., 1986; Stevens et al., 1986). Antiserum prepared against the 57-kD subunit of the beet H^+ -ATPase (Manolson et al., 1987) was a gift of M. Manolson and R. Poole. The monoclonal antibody (8B1F3) specific for the yeast 69-kD H^+ -ATPase subunit was generated by immunizing and boosting mice with washed vacuolar membranes (prepared as described in Uchida et al., 1985), followed by a final boost with H^+ -ATPase subunits obtained by KNO₃ stripping of vacuolar membranes (Kane et al., 1989). The anti-69-kD monoclonal antibody reacted with a unique 69-kD protein band in immunoblots of the purified H^+ -ATPase complex, solubilized vacuolar membranes, or total yeast cell extracts.

Immunoprecipitation and Fluorography

Cultures of midlog phase yeast cells growing at 30°C were pulse labeled with [^{35}S]H₂SO₄ (100–1,000 μ Ci) in MV-pro medium containing 50 mM potassium phosphate (pH 5.7) and 0.5 mg/ml BSA, and chased as previously described (Stevens et al., 1986). In the experiments performed in the presence of ammonium, the growth medium contained 50 mM potassium phosphate, pH 7.7. The chase period was initiated by the addition of 10 mM Na₂SO₄. The pulse and chase periods were as indicated in the figure legends. Cultures were separated into intracellular (Fig. 1, lanes *f*; spheroplast pellet)

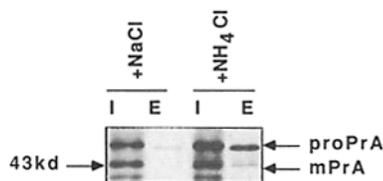


Figure 1. Effects of a lysosomotropic agent on sorting of PrA. A culture of strain X2180-1B was treated with 400 mM sodium chloride (NaCl) or 400 mM ammonium chloride (NH₄Cl) for 30 min at 30°C, subsequently labeled for 30 min and then chased for 60 min in the presence of the same concentrations of these compounds. *I*, intracellular fraction; *E*, extracellular fraction obtained by pooling the periplasmic and medium fractions before addition of antiserum. The positions of migration of the proPrA and mature PrA (mPrA) and molecular mass standards (in kilodaltons) are noted.

and extracellular (Fig. 1, lane *E*; spheroplast supernatant plus medium) fractions and immunoprecipitated (Stevens et al., 1986). The immunoprecipitated proteinase A was solubilized in sample buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.1% bromophenol blue), and electrophoresed on 10% polyacrylamide SDS gels (Stevens et al., 1986). After electrophoresis, gels were fixed, permeated with sodium salicylate for fluorography (Chamberlain, 1979), dried and exposed to film at -80°C (XAR-5; Eastman Kodak Co., Rochester, NY).

Quinacrine Staining and Fluorescence Microscopy

Analysis of cells for vacuolar uptake of quinacrine was performed as described by Weisman et al., (1987). Cells of the indicated genotype were incubated for 5 min at 25°C in the presence of 200 μM quinacrine in YEPD buffered to pH 7.7 with 50 mM potassium phosphate, washed once in the same medium without the dye, and prepared for Nomarski optics and fluorescence microscopy as described by Weisman et al. (1987). Microscopy was performed using a microscope (Axioplan; Carl Zeiss, Inc., Thornwood, NY) equipped for Nomarski optics and epifluorescence with a 100× oil-immersion objective.

Isolation of Vacuolar Membranes and Enzymatic Assays

Yeast vacuolar membranes were purified by spheroplasting cells, lysing the cells osmotically, and floating vacuoles over two consecutive Ficoll gradients as described by Kakinuma et al. (1981). ATPase activity of the isolated vacuolar membranes was determined using a coupled assay and an ATP-regeneration system (Lotscher et al., 1984). ATPase activities are reported as specific activity (U/mg), with one unit defined as 1 μmol phosphate liberated · min⁻¹ · mg⁻¹. Protein was determined by the method of Lowry (Lowry et al., 1951) on purified vacuoles that were first solubilized in 2% SDS.

Western Blotting

Vacuolar proteins from a purified vacuole fraction were solubilized in sample buffer and incubated at 70°C for 15 min. A constant amount of vacuolar material, 10 μg of vacuolar protein/lane, was loaded onto a 10% polyacrylamide SDS gel and electrophoresed (Laemmli, 1970). Total yeast cell protein extracts were prepared by vortexing yeast cells with glass beads at 65°C in protein sample buffer containing 8 M urea and 5% SDS. A constant amount of protein, equivalent to 1 × 10⁷ cells (~50 μg total protein), was loaded on each lane of a 10% polyacrylamide SDS gel. After electrophoresis, proteins were electroblotted onto nitrocellulose and H⁺-ATPase polypeptides were detected with monoclonal antibody 8BIF3 following the procedure supplied with the immune-blot assay kit from Bio-Rad Laboratories (Cambridge, MA), except that nonfat dry milk (1%) was used as nonspecific protein instead of gelatin. Bound antibody was subsequently decorated with [¹²⁵I]protein A and detected by autoradiography (Burnette, 1981).

Results

Lysosomotropic Agents Perturb Vacuolar Protein Sorting

To investigate the role of vacuolar acidification in protein targeting in yeast, we analyzed the effects of lysosomotropic agents on sorting of newly synthesized proteins to the vacuole. Wild-type yeast cells were treated with ammonium chloride, labeled with [³⁵S]H₂SO₄, and fractionated into intracellular and extracellular fractions. Proteinase A (PrA), a soluble vacuolar protein, was then immunoprecipitated from these fractions and analyzed by fluorography as shown in Fig. 1. Control cells that had been treated with sodium chloride mislocalized only low levels of a precursor form of PrA (proPrA) to the extracellular fraction, whereas cells treated with ammonium chloride misdirected a much higher proportion of the total newly synthesized proPrA to the cell surface. Similar results were obtained when cells were treated with the lysosomotropic agent neutral red, or when another vacuolar enzyme, carboxypeptidase Y, was immunoprecipitated from fractions of cells treated in the same way (not shown). The appearance of extracellular PrA from cells treated with these agents was not a result of cell lysis since (a) no mature PrA was observed in this fraction although mature PrA was found intracellularly, and (b) the cytoplasmic protein phosphoglycerate kinase was not found in the extracellular fractions (not shown). These observations suggest that neutralization of the vacuolar network in yeast results in the secretion of newly synthesized vacuolar proteins.

Some *vpl* Mutants Fail to Accumulate Quinacrine within Their Vacuoles

The fluorescent dye, quinacrine, has been shown to accumulate within vacuoles when supplied exogenously to intact yeast cells in medium buffered at alkaline pH (Weisman et al., 1987) (Fig. 2). When the luminal pH of the vacuole is raised by addition of 200 mM ammonium to the growth medium (Makarow and Nevalainen, 1987), quinacrine fails to accumulate within the vacuole (Weisman et al., 1987) (Fig. 2), indicating that concentration of the dye within the vacuole is dependent on the acidic state of this organelle. To test whether any of the *vpl* mutants were defective for vacuolar acidification, we exposed representative mutants from each of the 19 *VPL* complementation groups (Rothman and Stevens, 1986; Rothman et al., 1989a) to quinacrine and followed its uptake by fluorescence microscopy. In these studies the location of the vacuole was determined by Nomarski optics microscopy. Most of the mutants accumulated only slightly lower levels of quinacrine within their vacuoles than did isogenic wild-type cells (e.g., Fig. 2, *vpl8-10*). In contrast, although the *vpl3* and *vpl6* mutants contained mostly normal looking vacuoles as visualized by Nomarski optics, these cells were exceptionally deficient in quinacrine accumulation (Fig. 2). These findings indicate that the *VPL3* and *VPL6* gene products are required for the establishment or maintenance of a low vacuolar pH.

vpl3 and *vpl6* Mutants Are Deficient in Vacuolar ATPase Activity

To determine whether the apparent deficiency in vacuolar

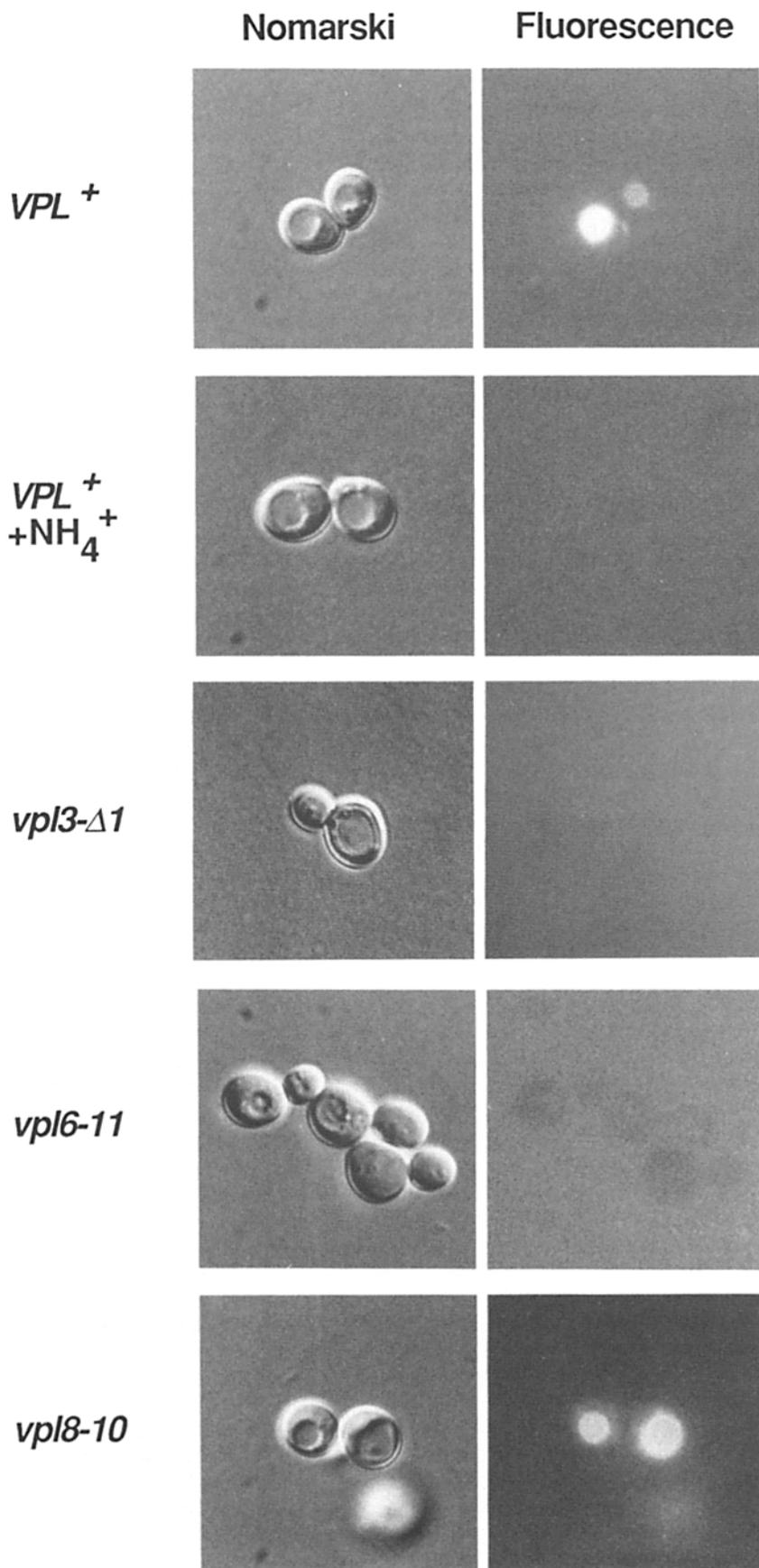


Figure 2. Vacuolar quinacrine accumulation is blocked in certain *vpl* mutants. Quinacrine-treated cells were viewed by Nomarski optics (*left*) and fluorescence (*right*) microscopy. The *VPL*⁺ strain was SF838-1D, and each of the mutants noted in the figure was an isogenic mutant carrying the indicated *vpl* allele. In the second pair of micrographs, strain SF838-1D was treated with quinacrine in the presence of 200 mM ammonium acetate (*VPL*⁺ + *NH*₄⁺).

Table II. Acidification in Wild-type and Selected *vpl* Mutant Yeast Strains

<i>vpl</i> allele	H ⁺ -ATPase specific activity (U/mg)	% vacuolar ATPase specific activity	Quinacrine staining
<i>VPL</i> ⁺	1.12	100	+
<i>vpl3-Δ1</i>	0.071	6.3	—
<i>vpl6-2</i>	0.068	6.1	—
<i>vpl8-3</i>	0.81	72	+

ATPase activities of isolated vacuoles are given as specific activities and percent of wild-type specific activity. The vacuolar ATPase activities were the same for cells carrying different alleles of each *vpl* complementation group. ATPase levels (Lotscher et al., 1984) were determined in the absence and presence of inhibitors of the plasma membrane and mitochondrial ATPases (100 μM sodium vanadate and 2 mM sodium azide respectively; Uchida et al., 1985). In all cases, these nonvacuolar ATPases together accounted for <5% of the total ATPase activity of the isolated vacuoles. The reported ATPase values represent the average of at least two vacuolar isolations of a given mutant. The *VPL*⁺ strain was SF838-9DR2L1; the *vpl3* strain was SF838-9DR2L1 *vpl3-Δ1*; the *vpl6* strain was SF838-9DR2L1m1038; and the *vpl8* strain was SF838-9DR2L1m1057.

acidification in the *vpl3* and *vpl6* mutants was reflected in reduced levels of the vacuolar H⁺-ATPase, we purified vacuoles from wild-type and isogenic *vpl* mutant yeast cells by the method of Kakinuma et al. (1981), and assayed the isolated vacuolar membranes for ATP hydrolysis. It was necessary to perform these analyses on isolated vacuoles since crude extracts of yeast contain high levels of nonvacuolar ATPase activity even in the presence of inhibitors of the mitochondrial and plasma membrane enzymes (our unpublished observations). In these experiments, neither sodium vanadate, which inhibits the plasma membrane ATPase nor sodium azide, an inhibitor of the mitochondrial enzyme, was found to reduce the ATPase activity in the purified vacuoles significantly (<5% inhibition), indicating that the vacuolar fractions were not substantially contaminated with either of these enzymes (Uchida et al., 1985; Bowman and Bowman, 1986). However, >90% of the ATPase activity of purified vacuolar membranes from wild-type cells was inhibited by 10 nM bafilomycin A₁, a specific inhibitor of vacuolar ATPases from different sources (Bowman et al., 1988; Kane et al., manuscript in preparation). The results of some of these analyses, performed on wild-type cells and three representative *vpl* mutants, are presented in Table II. Whereas those mutants that labeled normally with quinacrine, such as *vpl8*, contained specific activity levels for the vacuolar ATPase that were only slightly lower than that of wild-type cells, *vpl3* and *vpl6* mutant vacuoles contained very low ATPase levels (Table II). The residual ATPase activity in *vpl3* and *vpl6* mutant vacuolar membranes was further reduced by 10 nM bafilomycin A₁, suggesting that the residual ATPase activity in these membranes is attributable to the vacuolar H⁺-ATPase. The greatly reduced vacuolar ATPase activities in *vpl3* and *vpl6* mutants indicate that these cells are defective in the vacuolar membrane H⁺-ATPase.

Two Subunits of the Vacuolar H⁺-ATPase Are at Diminished Levels in the Vacuolar Membranes of *vpl3* and *vpl6*

To clarify the nature of the defect in the vacuolar H⁺-

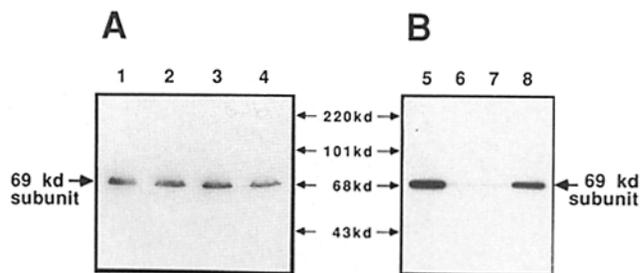


Figure 3. Western blot analysis of the 69-kD H⁺-ATPase subunit from whole cell extracts and isolated vacuolar membranes. Solubilized protein extracts from whole cells (**A**) or purified vacuolar membranes (**B**) from a *VPL*⁺ strain (lanes 1 and 5), *vpl3* strain (lanes 2 and 6), *vpl6* strain (lanes 3 and 7), and a *vpl8* strain (lanes 4 and 8) were loaded on a 10% polyacrylamide SDS gel and electrophoresed. The gels were electroblotted onto nitrocellulose membranes and probed with anti-69-kD monoclonal antibody 8B1F3. Approximately 50 μg of total yeast protein was loaded in each lane of **A**, and 10 μg of protein from a purified vacuole fraction was loaded in each lane of **B**. The *VPL*⁺ strain was SF838-9DR2L1, the *vpl3* strain was SF838-9DR2L1 *vpl3-Δ1*, the *vpl6* strain was SF838-9DR2L1m1038, and the *vpl8* strain was SF838-9DR2L1m1057. The position of the 69-kD H⁺-ATPase subunit is shown relative to protein standards.

ATPase of *vpl3* and *vpl6* mutants, we analyzed vacuolar membranes isolated from these mutants for immunoreactive subunits of the H⁺-ATPase complex. Uchida et al. (1985) have reported that the H⁺-ATPase complex consists of three subunits of molecular masses 89, 64, and 19.5 kD. Following the same purification procedures, we have found a somewhat different molecular mass distribution for these three subunits of 69, 60, and 17 kD, and identified several other proteins that copurify with these subunits (Kane et al., 1989). Based on studies with covalent modifying reagents directed at the ATP binding site, it has been suggested that the 69-kD polypeptide is the ATP-binding catalytic subunit of the complex (Uchida et al., 1988).

We examined the levels of the 69-kD subunit in isolated vacuolar membranes from *vpl* and wild-type strains by probing a Western blot of vacuolar membrane proteins with a monoclonal antibody (8B1F3) that specifically recognizes this subunit. Fig. 3 **B** shows the results of such an immunoblot. It is apparent that the 69-kD H⁺-ATPase subunit was at greatly diminished levels (Fig. 3, lanes 6 and 7) in the two mutants (*vpl3* and *vpl6*) that exhibited severe deficiencies in vacuolar ATPase specific activity. The level of the 69-kD H⁺-ATPase subunit in *vpl8* vacuolar membranes was only slightly diminished relative to *VPL*⁺ (Fig. 3, lanes 5 and 8), in accord with the ATPase activities for this mutant (Table II). The monoclonal antibody against the 69-kD subunit was also used to probe Western blots of whole cell extracts from the same *vpl3*, *vpl6*, *vpl8*, and wild-type strains. As shown in Fig. 3 **A**, all of the mutants had steady-state levels of the 69-kD subunit equivalent to wild type. This result indicates that *vpl3* and *6* mutants contain normal levels of the 69-kD polypeptide, yet only a small portion of the subunit reaches the vacuolar membrane.

These studies were extended to examine the effects of the *vpl3* and *vpl6* mutations on the levels of the 60-kD subunit

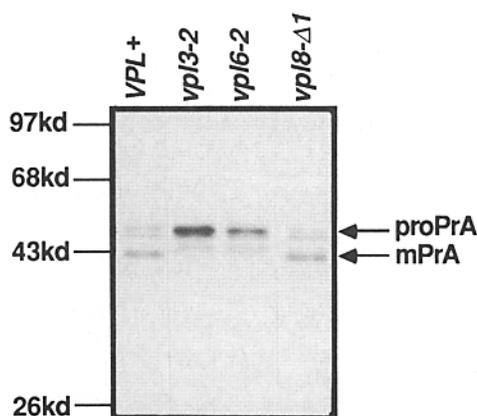


Figure 4. Intracellular proPrA accumulation in acidification-defective mutants. Cultures of cells of the indicated genotype were pulse labeled for 15 min, chased for an additional 15 min, and intracellular extracts of the labeled cells were prepared from spheroplasts and immunoprecipitated with PrA antiserum as described in the legend to Fig. 1. The *VPL*⁺ strain was JHRY20-2C, the *vpl3* strain was JHRY61-1B, the *vpl6* strain was JHRY64-5B, and the *vpl8* strain was JHRY20-2C *vpl8-Δ1*. Symbols are as noted in Fig. 1.

of the H⁺-ATPase complex in the vacuolar membrane. We used a polyclonal antibody generated against the 57-kD subunit of the beet tonoplast H⁺-ATPase complex (Manolson et al., 1989). This antibody has been found to cross react with the 60-kD subunit of the yeast vacuolar H⁺-ATPase (Kane et al., 1989), and thus serves as a useful reagent for analyzing this polypeptide. Using this antibody, similar results were obtained as for the 69-kD subunit; i.e., *vpl3* and *vpl6* mutants were found to contain much lower levels of this polypeptide in their vacuolar membranes than in wild-type or *vpl8* cells (not shown). Unfortunately, the specificity of the red beet polyclonal antibody against the 60-kD subunit was too poor to permit quantitation of this subunit in Western blots of whole cell lysates. Our findings demonstrate that the vacuolar membranes of *vpl3* and *vpl6* mutants, but not *vpl8* mutants, are deficient in at least two subunits of the vacuolar H⁺-ATPase.

Deficiencies in Vacuolar Acidification Correlate with Intracellular Accumulation of proPrA

It has been proposed that conversion of the precursor form of the vacuolar zymogen proPrA to its mature form PrA occurs by an autocatalytic mechanism that is strongly favored at an acidic pH (Ammerer et al., 1986; Woolford et al., 1986; Mechler et al., 1987). If this hypothesis is correct, it would be expected that mutants defective in vacuolar acidification should fail to convert the intracellular portion of the enzyme to its mature form. By immunoprecipitating PrA antigen from radiolabeled *vpl3* and *vpl6* mutants, we determined that a major fraction of the intracellular portion of PrA accumulated as the 52-kD proPrA species, whereas in other *vpl* mutants (e.g., *vpl8-Δ1*) most appeared as the proteolytically processed 42-kD species (Fig. 4). These observations correlate with the results of quinacrine labeling described above, and are consistent with the hypothesis that PrA activation is promoted by an acidic environment.

Discussion

In this paper, we have presented four lines of evidence that the yeast vacuolar H⁺-ATPase and vacuolar acidification participate in protein sorting and proteolytic zymogen activation: (a) a lysosomotropic agent that has been shown to raise the pH of the vacuole (Makarow and Nevalainen, 1987; Weisman et al., 1987) leads to substantially increased secretion of two vacuolar proteins; (b) some mutants defective in sorting of vacuolar proteins are also defective in vacuolar acidification; (c) vacuoles isolated from acidification-defective *vpl* mutants are deficient in ATPase activity and contain reduced levels of two H⁺-ATPase subunits; and (d) in mutants that fail to acidify their vacuoles, the intracellular portion of proPrA is not efficiently processed to the mature species. In support of these data, Banta et al. (1988) have reported that a yeast *vpl3* mutant (allelic to *vpl6*) also fails to accumulate quinacrine in its vacuole. In addition, these investigators reported that bafilomycin A₁, which is known to inhibit specifically and potently vacuolar H⁺-ATPases from many sources (Bowman et al., 1988), causes wild-type yeast cells to fail to accumulate quinacrine in their vacuoles and to mislocalize newly synthesized vacuolar proteins to the cell surface. Taken together, the above results implicate a direct role for acidification of the vacuolar network in vacuolar protein sorting.

The observation that *vpl3* and *vpl6* mutants are deficient in vacuolar acidification makes it possible to examine the role of the vacuolar membrane pH gradient in a number of cellular processes. For example, we have shown that *vpl3* and *vpl6* mutants accumulate intracellular proPrA, suggesting that low pH triggers maturation of proPrA. Consistent with these findings, PrA-related proteins from other organisms have been shown to autoactivate at low pH (Bustin and Conway-Jacobs, 1971; James and Sielecki, 1986). In addition to zymogen activation, the pH gradient across the vacuolar membrane is thought to be exploited by several transporters that act to concentrate small molecules (e.g., Ca²⁺ and amino acids) in the vacuole (Ohsumi and Anraku, 1981, 1983). The acidification defective *vpl* mutants should allow a determination of the *in vivo* role of an acidic vacuolar pH in accumulation of these small molecules.

Although our findings suggest that the low pH environment of the yeast vacuole is required for protein sorting and zymogen activation, we have not proven a causal relationship between the increased pH of the vacuolar lumen in *vpl3* and *vpl6* mutants and the failure to sort and activate soluble vacuolar proteins. Indeed, we cannot rule out the possibility that these mutants are deficient in vacuolar ATPase activity and acidification resulting from an overall defect in vacuolar biogenesis (i.e., that the *Vpl*⁻ phenotype is the cause of the acidification defect rather than its effect). However, mutants such as *vpl8* mislocalize proportions of carboxypeptidase Y and PrA similar to *vpl3* and *vpl6* mutants (Rothman and Stevens, 1986), yet are not markedly deficient in vacuolar acidification, ATPase activity, or H⁺-ATPase subunit composition (Table II and Fig. 3). Clearly, mislocalization of a high percentage of newly synthesized PrA and carboxypeptidase Y is not obligatorily associated with acidification defects. Thus, it is possible that the *VPL3* and *VPL6* gene products are required directly for the localization, assembly, or function of the vacuolar H⁺-ATPase. These observations

support the model that a failure to acidify the vacuolar network results in a vacuolar protein sorting defect.

Vacuolar acidification might function in promoting dissociation of vacuolar proteins from their sorting receptors (Rothman et al., 1989b), in analogy to low pH-induced release of endocytosed ligands from their cell surface receptors (Mellman et al., 1986) or to uncoupling of lysosomal proteins from mannose-6-phosphate receptors (von Figura and Hasilik, 1986). This model will be directly testable when the functions of the *VPL3* and *VPL6* gene products are understood, or when the genes encoding the vacuolar H⁺-ATPase subunits have been identified and disrupted.

The fact that vacuolar membrane ATPase activity is substantially reduced in *vpl3* and *vpl6* mutants and the observation that immunoreactive H⁺-ATPase subunits are similarly diminished suggests that these mutants could carry lesions in genes encoding subunits of the complex itself. The presence of normal levels of the 69-kD subunit in whole cell lysates from a *vpl3Δ* strain indicates that this gene does not encode the 69-kD subunit, but our results do not rule out the possibility that the *VPL6* gene encodes the 69-kD subunit. The normal levels of the 69-kD subunit in whole cell lysates of *vpl3* and *vpl6* mutants also indicate that the mutations do not affect the level of expression of the 69-kD subunit gene. Although we could not perform similar tests of the whole cell lysates using the polyclonal antibodies against the 60-kD subunit, we now have cloned the 60-kD subunit gene, as well as the *VPL3* and *VPL6* genes, and have confirmed that the three genes are different (C. Yamashiro, C. Raymond, and T. Stevens, unpublished results). There are several other possibilities, however. The lowered levels of both the 69- and 60-kD subunits in vacuolar membranes of *vpl3* and *vpl6* mutants may indicate that the absence of one subunit of the H⁺-ATPase (caused by a mutation in its structural gene) results in reduced levels of the others in the vacuolar membrane because of cooperative assembly of the complex. We now have evidence that the vacuolar H⁺-ATPase complex contains four to six other polypeptides in addition to the 69- and 60-kD subunits (Kane et al., 1989), and mutations in any of these polypeptides could potentially disrupt the structure or assembly of the complex. Alternatively, the *VPL3* and *VPL6* gene products may be accessory proteins required for assembly of the H⁺-ATPase complex or its transport to the vacuole. At present we cannot distinguish between the various possibilities.

In light of our findings, it is possible that most or all of the genes encoding the vacuolar H⁺-ATPase complex, the components required for its assembly and localization, and the proteins carrying our accessory functions required for acidification could be identified using procedures for isolating yeast mutants defective in protein sorting (Rothman and Stevens, 1986; Bankaitis et al., 1986; Robinson et al., 1988; Rothman et al., 1989a). Molecular clones of such genes will prove useful in understanding the functional roles that their products play in vacuolar acidification and protein sorting. To this end, we are using the cloned *VPL3* and *VPL6* genes to assess the structure, localization, and mechanism of action of their products.

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