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

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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 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 organelar system known as the vacuolar network. The participation of a low lumenal 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 organelar 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
deficient in vacuolar acidification and ATPase activity at the activation. We also demonstrate that a limited subset of the evidence suggesting that vacuolar network acidification is required for vacuolar protein sorting and vacuolar zymogen activation of mutations in the genes encoding the vacuolar H⁺-ATPase subunits or other proteins involved in 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 subunits (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. The function of each of these subunits is a simple system for dissecting the mechanisms by which newly synthesized proteins are sorted to the vacuole within the yeast genome to replace the wild-type chromosomal copies of these genes by standard techniques (Rothstein, 1983). Haploid strains carrying either the vpl-Δ1 allele or the vpl8-Δ1 alleles were viable at all temperatures and displayed a Vpl⁻ phenotype (C. Raymond, unpublished observations).

### 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 vpl-Δ1 or the vpl8-Δ1 alleles were viable at all temperatures and displayed a Vpl⁻ phenotype (C. Raymond, unpublished observations).

#### Materials

Carrier-free [³⁵S]H₂SO₄ and zymolyase 100T were from ICN Biomedicals, Inc. (Irvine, CA). Fraction II lyticase was prepared as described previously (Scott and Scheckman, 1980). [³⁵S]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 ZW-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., 1985; Stevens et al., 1986). Antiserum prepared against the 57-kD subunit of the beef 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 [³⁵S]H₂SO₄ (100-1,000 μCi) 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 I) and cell wall fractions (Fig. 1, lanes II).
and extracellular (Fig. 1, lane E; spheroplast supernatant plus medium) frac-
tions and immunoprecipitated (Stevens et al., 1986). The immunoprecipi-
tated proteinase A was solubilized in sample buffer (50 mM Tris-HCl, pH
6.8, 10% glycerol, 2% SDS, 2% ~-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 de-
scribed by Weisman et al. (1987). Cells of the indicated genotype were in-
cubated for 5 min at 25°C in the presence of 200 #M 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). Micros-
copy was performed using a microscope (Axioplan; Carl Zeiss, Inc.,
Thornwood, NY) equipped for Nomarski optics and epifluorescence with a
100x 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 gra-
dients as described by Kakinuma et al. (1981). ATPase activity of the iso-
lated vacuolar membranes was determined using a coupled assay and an
ATP-regeneration system (Lotsher et al., 1984). ATPase activities are
reported as specific activity (U/mg), with one unit defined as 1 #mol phos-
phate liberated · min	extsuperscript{-1} · mg	extsuperscript{-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 sam-
ple buffer and incubated at 70°C for 15 min. A constant amount of vacuolar
material, 10 #g of vacuolar protein/lane, was loaded onto a 10% polyacryl-
amide SDS gel and electrophoresed (Laemmli, 1970). Total yeast cell pro-
tein 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 x 10	extsuperscript{7} cells (~50 #g total protein), was
loaded on each lane of a 10% polyacrylamide SDS gel. After electrophore-
sis, proteins were electroblotted onto nitrocellulose and #H	extsuperscript{-}ATPase poly-
peptides were detected with monoclonal antibody 8BIF3 following the pro-
cedure 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
Lysoosomal Agents Perturb Vacuolar
Protein Sorting
To investigate the role of vacuolar acidification in protein tar-
getting in yeast, we analyzed the effects of lysoosomal agents on sorting of newly synthesized proteins to the vacu-
ole. Wild-type yeast cells were treated with ammonium chloride, labeled with [35S]H2SO4, and fractionated into intra-
cellular 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 propor-
tion of the total newly synthesized proPrA to the cell surface. Similar results were obtained when cells were treated with
the lysoosomal 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
with these agents was not a result of cell lysis since (a) no mature PrA was observed in this fraction although ma-
ture 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 accumu-
late 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 vacu-
ole 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., 1989) to quinacrine and followed its
uptake by fluorescence microscopy. In these studies the loca-
tion of the vacuole was determined by Nomarski optics mi-
croscopy. Most of the mutants accumulated only slightly
lower levels of quinacrine within their vacuoles than did iso-
genic wild-type cells (e.g., Fig. 2, vpl8-10). In contrast, al-
though the vpl3 and vpl6 mutants contained mostly normal
looking vacuoles as visualized by Nomarski optics, these
cells were exceptionally deficient in quinacrine accumula-
tion (Fig. 2). These findings indicate that the VPL3 and VPL6
gene products are required for the establishment or main-
tenance of a low vacuolar pH.

vpl3 and vpl6 Mutants Are Deficient in Vacular
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* + NH₄⁺).
Acidification in the vpl3 and vpl6 mutants was reflected in reduced levels of the vacuolar H^+-ATPase, 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 mM 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 mM 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.

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

| vpl allele | H^+-ATPase specific activity (U/mg) | % vacuolar ATPase specific activity | Quinacrine staining |
|------------|-------------------------------------|------------------------------------|---------------------|
| VPL^+      | 1.12                                | 100                                | +                   |
| vpl3-Δl    | 0.071                               | 6.3                                | -                   |
| vpl6-2     | 0.068                               | 6.1                                | -                   |
| vpl8-3     | 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-Δl; the vpl6 strain was SF838-9DR2L1vpl8; and the vpl8 strain was SF838-9DR2L1vpl8.

![Figure 3](image)

**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^+ lane (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 an anti-69-kD monoclonal antibody 8BIF3. 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-Δl, the vpl6 strain was SF838-9DR2L1vpl8, and the vpl8 strain was SF838-9DR2L1vpl8. The position of the 69-kD H^+-ATPase subunit is shown relative to protein standards.
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 reactivity is observed with the 60-kD subunit of the yeast vacuolar H+-ATPase complex in the vacuolar membrane. We have shown that the red beet polyclonal antibody against the 57-kD subunit of the H+-ATPase complex in the vacuolar membrane. We have shown that the red beet polyclonal antibody against the 57-kD subunit of the H+-ATPase complex in the vacuolar membrane.

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 (Ammerein 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. 5). 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 mannos-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 vpl3a 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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