Assembly and Targeting of Peripheral and Integral Membrane Subunits of the Yeast Vacuolar H\(^+\)-ATPase*

Patricia M. Kane†, Margery C. Kuehn, Isabelle Howald-Stevenson, and Tom H. Stevens§

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

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Previous purification and characterization of the yeast vacuolar proton-translocating ATPase (H\(^+\)-ATPase) have indicated that it is a multisubunit complex consisting of both integral and peripheral membrane subunits (Uchida, E., Ohsumi, Y., and Anraku, Y. (1985) J. Biol. Chem. 260, 1090-1095; Kane, P. M., Yamashiro, C. T., and Stevens, T. H. (1989) J. Biol. Chem. 264, 19236-19244). We have obtained monoclonal antibodies recognizing the 42- and 100-kDa polypeptides that were co-purified with vacuolar ATPase activity. Using these antibodies we provide further evidence that the 42-kDa polypeptide, a peripheral membrane protein, and the 100-kDa polypeptide, an integral membrane protein, are genuine subunits of the yeast vacuolar H\(^+\)-ATPase. The synthesis, assembly, and targeting of three of the peripheral subunits (the 69-, 60-, and 42-kDa subunits) and two of the integral membrane subunits (the 100- and 17-kDa subunits) were examined in mutant yeast cells containing chromosomal deletions in the TFP1, VAT2, or VMA3 genes, which encode the 69-, 60-, and 17-kDa subunits, respectively. The steady-state levels of the various subunits in whole cell lysates and purified vacuolar membranes were assessed by Western blotting, and the intracellular localization of the 60- and 100-kDa subunits was also examined by immunofluorescence microscopy. The results suggest that the assembly and/or the vacuolar targeting of the peripheral subunits of the yeast vacuolar H\(^+\)-ATPase depend on the presence of all three of the 69-, 60-, and 17-kDa subunits. The 100-kDa subunit can be transported to the vacuole independently of the peripheral membrane subunits as long as the 17-kDa subunit is present; but in the absence of the 17-kDa subunit, the 100-kDa subunit appears to be both unstable and incompetent for transport to the vacuole.

Vacuolar proton-translocating ATPases (H\(^+\)-ATPases)\(^1\) are found in all eukaryotic cells and acidify a number of different organelles including lysosomes, endosomes, the Golgi apparatus, secretory vesicles, and clathrin-coated vesicles (Forgac, 1989). All of the vacuolar H\(^+\)-ATPases that have been characterized are multisubunit complexes that have three subunits of ~70, 60, and 17 kDa, and one or more additional subunits. Functions have been proposed for each of the three common subunits. The 70-kDa subunit appears to contain the ATP-binding catalytic site; the 17-kDa subunit is thought to form all or part of the proton pore; and the 60-kDa subunit is proposed to be a regulatory ATP-binding subunit (Forgac, 1989). The structural genes for these three subunits have been cloned from several different species, and the predicted amino acid sequences exhibit a remarkable degree of homology among animals (Sudhof et al., 1989), plants (Manolson et al., 1988; Zimmel et al., 1988), fungi (Bowman et al., 1988a, 1988b; Hirata et al., 1990; Nelson et al., 1989; Nelson and Nelson, 1989), and archaeabacteria (Denda et al., 1988; Inatomi et al., 1989).

The yeast lysosome-like vacuole is an acidic organelle containing a vacuolar H\(^+\)-ATPase that shares many features with vacuolar H\(^+\)-ATPases from other eukaryotic cells (Klionsky et al., 1990). The yeast vacuolar H\(^+\)-ATPase was initially reported to consist of only three subunits (Uchida et al., 1985), but subsequent studies revealed that eight polypeptides, of apparent molecular masses 69, 60, 42, 36, 32, 27, and 17 kDa, co-purified with ATPase activity when the enzyme was isolated by glycerol gradient centrifugation (Kane et al., 1989). The same collection of polypeptides was immunoprecipitated by a monoclonal antibody against the 69-kDa (catalytic) subunit. The structural genes for the 69-kDa subunit (VMA1, TFP1; Hirata et al., 1990; Kane et al., 1990), 60-kDa subunit (VAT2, VMA2; Nelson et al., 1989; Yamashiro et al., 1990),\(^2\) and 17-kDa subunit (VMA3; Nelson and Nelson, 1989; Umemoto et al., 1990) have been cloned and sequenced. The cloned genes have also been mutagenized in vitro and used to replace the chromosomal copy of the structural genes, allowing an assessment of the roles of the vacuolar H\(^+\)-ATPase in vivo (Yamashiro et al., 1990; Umemoto et al., 1990; Nelson and Nelson, 1990). Mutant cells lacking any of these three subunits exhibit no ATPase activity in isolated vacuoles, fail to acidify the vacuole, grow poorly under all conditions, and fail to grow at all in medium buffered to neutral pH. An identical set of phenotypes is observed in cells in which the VMA4 gene has been disrupted (Foury, 1990), suggesting that this gene encodes another yeast vacuolar H\(^+\)-ATPase subunit. This conclusion is supported by the similarity between the predicted amino acid sequences of the VMA4 gene product

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\(^1\) The abbreviations used are: H\(^+\)-ATPase(s), proton-translocating ATPase(s); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DCCD, N,N'-dicyclohexylcarbodiimide.

\(^2\) TFP1 and VMA1 both represent the same gene, which encodes the 69-kDa subunit, and VAT2 and VMA2 are equivalent names for the gene encoding the 60-kDa subunit.
and the 31-kDa subunit of the bovine kidney vacuolar H+-ATPase (Hirsch et al., 1988).

Many studies on the vacuolar H+-ATPases have highlighted the similarities between the vacuolar and F,F,-ATPases, which are presumably based on an evolutionary relationship between these two classes of proton-translocating ATPases (Gogarten et al., 1989; Nelson and Taiz, 1989). Thus, the predicted amino acid sequences for the 70-, 60-, and 17-kDa subunits are homologous to the catalytic, regulatory, and proton pore subunits of F,F,-ATPases. This similarity at the amino acid level is reflected in the overall structure of the enzymes, both of which resemble a “ball and stick,” with ATP catalysis performed on a complex of peripheral subunits attached to a proton pore formed by several integral membrane subunits (Forgac, 1989). However, although the similarities between these two types of enzymes are indeed interesting, some of the most intriguing questions about the vacuolar H+-ATPases are rooted in the differences between the vacuolar and F,F,-ATPases. The following represent just a few of these questions.

1) Why do vacuolar H+-ATPases seem to function exclusively as proton pumps while F,F,-ATPases are rooted in the differences between the vacuolar and F,F,-ATPases? The following represent just a few of these questions.

2) Vacular ATPases are present in many subcellular compartments that are acidified to varying degrees. How is this pH regulation achieved, and do the differences in acidification reflect differences in the resident vacuolar H+-ATPases of these compartments or the action of other cellular proteins? 3) The vacuolar H+-ATPases are composed of peripheral subunits, which appear to be synthesized in the cytoplasm of the cell, and integral membrane subunits, which are presumed to enter the vacuolar network at the endoplasmic reticulum along with newly synthesized proteins destined for the vacuole (lysosome), other organelles of the secretory pathway, or the cell surface. How are subunits from these two very different environments assembled and targeted to their proper intracellular locations?

The work described here is directed toward developing the biochemical tools necessary for approaching these problems as well as beginning to address these specific issues. We provide further evidence supporting the claim that the 100- and 42-kDa polypeptides that are co-purified with vacuolar ATPase activity are indeed genuine subunits of the enzyme, a point that is particularly important because the 100-kDa subunit is present in the KNOs-stripped supernatant relative to the amount that remains with the pelleted membranes, we did identify one antibody, 1D7, that recognized the 100-kDa subunit by this procedure in addition to the three antibodies against the 69-kDa subunit described (Kane et al., 1989). Hybridoma supernatants from the second fusion were screened with the presence of immunoglobulin G by enzyme-linked immunosorbent assay using horseradish peroxidase-conjugated goat anti-mouse IgG as the second antibody. Supernatants that were strongly positive by enzyme-linked immunosorbent assay were used to probe immunoblots of the glyceral gradient-purified yeast vacuolar H+-ATPase. These subunits recognizing subunits on the immunoblots were subcloned by limiting dilution and retested. Four monoclonal antibodies were identified and cloned from this fusion. These included 1D11 and 19D7, both recognizing the 60-kDa subunit; 7A2, recognizing the 42-kDa subunit; and 7B1, recognizing the 100-kDa subunit.

**Isolation of Vacular Vesicles and the Vacular H+-ATPase**—For the biochemical studies shown in Figs. 1–3, vacular vesicles were prepared on a large scale (from a 50-litter fermentation) as described (Kane et al., 1989). Smaller scale preparations of vacuolar vesicles were done for comparison of wild-type and mutant cells, starting from 1-liter cultures as described by Roberts et al. (1991). Solubilization of vacular vesicles and glyceral gradient purification of the vacuolar H+-ATPase have been described (Kane et al., 1989). ATPase activities of vacular vesicles and glyceral gradient fractions were measured at 35 °C using the coupled enzyme assay of Lotcher et al. (1988). Protein concentration of vacular vesicles was measured by Lowry assay (Lowry et al., 1951).

**Carbohydrate Fractionation of Vacular Vesicles**—Vacular vesicles were suspended to a protein concentration of 10 mg/ml in 50 mM sodium phosphate, pH 7.5, buffer containing 0.5 mM phenylmethylsulfonyl fluoride, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml aprotinin, and 2 mM EDTA as protease inhibitors. The vesicle suspension was diluted into 100 times its volume of 100 mM sodium carbonate, pH 11.5, and incubated on ice for 30 min. The stripped
membranes were pelleted by centrifugation in a Beckman Ti-80 rotor at 175,000 × g for 3 h at 4 °C. The supernatant was precipitated with 10% trichloroacetic acid, and both the supernatant and pellet fractions were solubilized in cracking buffer for SDS-PAGE (described below).

**SDS-PAGE and Western Blotting**—Whole cell lysates were prepared by growing cells to a density of 1 × 10⁶ ml⁻¹, pelleting the cells by centrifugation, freezing the pellet at -80 °C, then solubilizing the pellets in cracking buffer (8 M urea, 5% SDS, 1 mM EDTA, 50 mM Tris-HCl, pH 6.8, 5% β-mercaptoethanol) that had been preheated to 65 °C. The cell pellet in cracking buffer was combined with an equal volume of glass beads, mixed on a vortex mixer for 30 s, heated at 65 °C for 5 min, then mixed again for 30 s. Total protein present in the lysate was measured by Lowry assay (Lowry et al., 1951) and also visualized by Coomassie staining of different cell lysates run in parallel on SDS-PAGE. Vacuolar vesicles were pelleted in a microcentrifuge and the pellets resuspended in cracking buffer followed by incubation at 70 °C for 35 min. Immunoblots were prepared and probed as described (Kane et al., 1989). Apparent molecular masses were determined relative to prestained molecular weight standards (Bethesda Research Laboratories) or Sigma low molecular weight standards.

**Extraction and Labeling of the 17-kDa Subunit**—Vacuolar vesicles were washed three times in 10 mM Tris-HCl, 1 mM EDTA, pH 7.5, buffer as described (Uchida et al., 1985) and then resuspended to a protein concentration of 2 mg/ml in the same buffer.

**[14C]DCCD** (N,N'-dicyclohexylcarbodiimide; Amersham Corp.) was added as an ethanol solution to a final concentration of 100 mM. The vesicles were incubated at ambient temperature for 90 min. Labeled vesicles were either pelleted in a microcentrifuge, solubilized, and visualized directly by SDS-PAGE and autoradiography or extracted with 2:1 CHCl₃:MeOH. The extraction was performed by pelleting the vesicles in a microcentrifuge as above, resuspending the pellet in the same volume of 2:1 CHCl₃:MeOH, and then incubating for 1 h at ambient temperature. The extract was then cleared by centrifugation, and the extracted protein present in the supernatant was precipitated by the addition of an equal volume of ice-cold diethyl ether. Both the protein precipitated from the extract and the material that was not removed into the 2:1 CHCl₃:MeOH were solubilized by addition of 20% SDS and 50 mM Tris-HCl, pH 6.8 (prewarmed to 65 °C), as described in Kane et al. (1989). The same procedure was used for extraction of unlabeled vesicles from wild-type and mutant cells, but the extracted proteins were visualized by silver staining (Wray et al., 1981) after SDS-PAGE.

**Labeling of the glycerol gradient-purified ATPase with [14C]DCCD** was accomplished by adding an ethanol solution of [14C]DCCD directly to the fraction exhibiting the maximum ATPase activity after glycerol gradient centrifugation of solubilized vacuolar vesicles. The mixture was incubated for 60 min at room temperature. The final concentration of DCCD in the mixture was 20 mM.

**Immunofluorescence Microscopy**—Cells were fixed and converted to spheroplasts as described by Roberts et al. (1991). Monoclonal antibodies 13D11 and 10D7, which recognize the 60- and 100-kDa subunits, respectively, were used at a 1:10 dilution as primary antibodies. The fluorescence signal was amplified by incubating the cells sequentially with the primary mouse monoclonal antibody, mouse anti-goat IgG polyclonal antisera, goat anti-mouse IgG antibodies, and fluorescein-conjugated mouse anti-goat IgG antiserum. Microscopy was performed with a Zeiss Axiophot microscopie equipped for Nomarski optics and epifluorescence, using a 100× oil immersion lens.

**RESULTS**

**The Yeast Vacuolar H⁺-ATPase Includes Subunits of 100 and 42 kDa**—Although partial purification of the yeast vacuolar H⁺-ATPase by two different methods indicated a subunit composition including eight different polypeptides, further analysis of the roles of these subunits in enzyme assembly and function required a means of detecting the subunits in complex mixtures. Monoclonal antibodies were prepared against washed vacuolar vesicles and screened for recognition of the glycerol gradient-purified vacuolar H⁺-ATPase on Western blots. Hybridoma supernatants recognizing the 100-, 69-, 60-, and 42-kDa subunits were identified and the cell lines cloned. We showed previously that a monoclonal antibody against the 69-kDa subunit detected this subunit in a single peak fractionating at relatively high density in a 20–50% glycerol gradient (Kane et al., 1989). This peak coincided exactly with the gradient fractions exhibiting maximum ATPase activity. The results of a similar experiment are shown in Fig. 1. EDTA-washed vacuolar vesicles were solubilized with ZW3-14, fractionated by glycerol gradient centrifugation, and the gradient fractions subjected to SDS-PAGE. Blots of the glycerol gradient profile were probed with monoclonal antibodies against the 60-kDa subunit (Fig. 1A), the 42-kDa subunit (Fig. 1B), and the 100-kDa subunit (Fig. 1C). The blots obtained with the 60- and 42-kDa subunit antibodies closely resemble those obtained with the 69-kDa subunit antibody. The position of these subunits in the gradient paralleled exactly the position of the 69-kDa subunit and corresponded to the peak of ATPase activity. The monoclonal antibodies against the 100-kDa subunit usually detected some combination of two polypeptides of apparent molecular masses 100 and 75 kDa. These polypeptides were detected in the same fractions as the 69-, 60-, and 42-kDa subunits but also exhibited a second peak at lower density (fractions 13–16 in Fig. 1C) which included fractions showing no ATPase activity. In this experiment, only the 75-kDa polypeptide is visible in the lower density peak, but in other experiments, both the 100- and 75-kDa proteins were visible. The possible implications of the lower density peak are outlined under “Discussion.” Similar results were seen with two different monoclonal antibodies against the 60-kDa subunit and two different antibodies against the 100-kDa subunit.

![Fig. 1. Detection of the 60-, 42-, and 100-kDa subunits in glycerol gradient fractions containing the vacuolar H⁺-ATPase. Solubilized vacuolar vesicles were applied to a 20–50% glycerol gradient and fractionated as described previously (Kane et al., 1989). Twenty fractions of ~600 ml each were collected from the bottom of the centrifuge tube and then precipitated with 10% trichloroacetic acid. The precipitated protein was frozen as a pellet and then solubilized with cracking buffer (see “Experimental Procedures”) and separated by SDS-PAGE (7.5% acrylamide gel). Immunoblots of gradient fractions 3–16 (where fraction 1 is at the bottom of the tube) were probed with monoclonal antibodies 13D11 (A), 7A2 (B), and 7B1 (C). A parallel immunoblot probed with monoclonal antibodies recognizing the 69-kDa subunit (11E6 and 8B1; not shown) closely resembled both A and B. Little or no cross-reacting material was detected in fractions 1, 2, or 17–20 by any of the monoclonal antibodies (not shown). Fraction numbers are indicated at the bottom of each blot; the top and bottom of the gradients are noted, and the apparent molecular masses of standards are indicated on the right.](image-url)
tween different vacuole preparations, and we believe that the 75-kDa polypeptide is a proteolytic product of the 100-kDa subunit based on several lines of evidence. 1) Higher ratios of the 75-kDa polypeptide to the 100-kDa polypeptide were seen under conditions known to encourage proteolysis, such as extended incubations at intermediate temperatures or multiple freeze-thaw cycles. Under such conditions, the 100-kDa polypeptide was gradually converted fully to the 75-kDa polypeptide (data not shown). 2) Immunoblots of whole cell lysates extended incubations at intermediate temperatures or multiple freeze-thaw cycles. Under such conditions, the 75-kDa polypeptide to the 100-kDa polypeptide were seen under conditions known to encourage proteolysis, such as intermediate temperatures or multiple freeze-thaw cycles. 3) Disulfide bonding of the 75-kDa protein to itself or other ATPase subunits to form the 100-kDa species appears unlikely since both forms persisted under strongly reducing conditions (5% β-mercaptoethanol or 1 mM dithiothreitol). 4) Controlled tryptic digestion studies of the clathrin-coated vesicle H+-ATPase revealed that the 100-kDa subunit was very sensitive to proteolysis and was converted to an 80-kDa species under very mild conditions (Adachi et al., 1990). Under conditions in which almost all of the 100-kDa subunit had been converted to the 80-kDa species, 75–80% of the ATPase activity of the detergent-solubilized or reconstituted enzyme was retained. The ratio of the 100- and 75-kDa polypeptides also appeared to have relatively little effect on ATPase activity of the yeast enzyme. Preparations of the yeast vacuole gradient-purified vacuolar H+-ATPase containing different proportions of the two species had similar levels of activity (typically 10–15 mmol of ATP min⁻¹ mg of protein⁻¹ at 35 °C). However, it was not possible to quantitate carefully any correlation between activity and proteolysis because of the difficulty of controlling the proteolytic process. Inclusion of a variety of protease inhibitors, including phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and EDTA during the glycerol gradient purification and in preparation of samples for SDS-PAGE did not eliminate the presence of the 75-kDa subunit. (However, it was difficult to maintain sufficient levels of the inhibitors throughout vacuole isolation, so some degradation may have occurred then.) The 75-kDa polypeptide was also seen in vacuoles from pep4 strains, in which all the vacuolar proteases dependent on proteasome A for activation should be inactive (Jones, 1991). Taken together, these results indicate that the 100-kDa subunit of the yeast vacuolar H+-ATPase is acutely sensitive to proteolysis leading to formation of a 75-kDa species and suggest that this may be a general feature of comparable subunits from other vacuolar H+-ATPases.

Identification of Peripheral and Integral Membrane Subunits—It was determined previously that the 69- and 60-kDa subunits of the yeast vacuolar H+-ATPase are peripheral membrane proteins by demonstrating that they are removed from the vacuolar membrane by alkaline sodium carbonate (Kane et al., 1989). A similar experiment is shown in Fig. 2. Like the 69- and 60-kDa subunits, the 42-kDa subunit was removed from the vacuolar membrane by sodium carbonate (Fig. 2A). However, the 100-kDa subunit (and the 75-kDa proteolytic product of this subunit) remained with the membrane, indicating that it is an integral membrane subunit.

The extreme hydrophobicity of the predicted amino acid sequence for the 17-kDa subunit of the yeast vacuolar H+-ATPase and its proposed role as part of the proton pore both imply that it is also an integral membrane subunit, and biochemical data from a number of sources support this hypothesis (Umemoto et al., 1990; Nomi et al., 1991). Fig. 3, A and B, shows the labeling of the 17-kDa subunit by [14C]DCCD in both the glycerol gradient-purified vacuolar H+-ATPase and vacuolar membranes. Under the conditions used here for DCCD labeling the ATPase activity of the glycerol gradient-purified ATPase was 53% inhibited, and the ATPase activity of the vacuolar vesicles was 82% inhibited. The labeled 17-kDa subunit could be extracted almost quantitatively from vacuolar vesicles into 2:1 chloroform:methanol, indicating that it is a proteolipid. Coomassie staining (Fig. 3C) or silver staining (data not shown) of the 2:1 chloroform:methanol extract and the vesicles before and after extraction revealed that the 17-kDa subunit is the predominant protein species present in the extracted material. The presence of the 17-kDa subunit in vacuolar vesicles from wild-type and ATPase mutant cells could thus be conveniently assessed by extracting the membranes with 2:1 chloroform:methanol, subjecting the extract to SDS-PAGE, and examining the Coomassie-stained (or silver-stained, see below) gel for the presence of a 17-kDa protein.

Synthesis, Localization, and Assembly of Vacuolar H+-ATPase Subunits in Deletion Mutants—The biochemical evidence provided above supports the designation of the 100- and 42-kDa subunits as genuine vacuolar H+-ATPase subunits and indicates that the 100- and 17-kDa subunits are integral vacuolar membrane proteins and the 69-, 60-, and 42-kDa subunits are peripheral membrane subunits. We next examined whether the biosynthesis, localization, and assembly of each of these subunits was affected in mutants lacking the
69-kDa subunit gene (tfplA mutant), the 60-kDa subunit gene (vat2Δ mutant), or the 17-kDa subunit gene (vma3Δ mutant).

Immunoblots of whole cell lysates from wild-type cells, tfplΔ, vat2Δ, and vma3Δ mutants were probed with antibodies recognizing the 100-, 69-, 60-, and 42-kDa subunits to determine whether deletion of one subunit gene affected synthesis or stability of other subunits. The results are shown in Fig. 4.

As reported previously (Hirata et al., 1990; Kane et al., 1990; Yamashiro et al., 1990), the 69-kDa subunit was completely missing from the tfplΔ strain (Fig. 4B, lane 2), and the 60-kDa subunit was missing from the vat2Δ strain (Fig. 4C, lane 3). However, deletion of either of these subunit genes had little or no effect on cellular levels of the other subunits (Fig. 4, A–D, lanes 2 and 3). This indicates that the synthesis and stability of the 100- and 42-kDa subunits are not dependent on the presence of the 69- and 60-kDa subunits and that the 69- and 60-kDa subunits can each be synthesized in the absence of the other subunit. The levels of the 69-, 60-, and 42-kDa subunits were also relatively unaffected in vma3Δ mutants (Fig. 4, A–D, lane 4). However, the 100-kDa subunit was present at substantially reduced levels in this mutant (Fig. 4A, lane 4).

By performing serial dilutions of the wild-type lysate, subjecting the diluted lysates to SDS-PAGE and immunoblotting, and comparing the intensity of the 100-kDa subunit staining to vma3Δ lysate samples run in adjacent lanes, we were able to estimate that the vma3Δ cells contained only about 10% as much 100-kDa subunit as the wild-type cells (data not shown). From these experiments we cannot distinguish whether the synthesis or the stability of the 100-kDa subunit is reduced in the vma3Δ mutant, but this result clearly indicates a connection between cellular levels of the 100- and 17-kDa subunits.

We addressed whether the subunits synthesized in each mutant were localized to the vacuolar membrane by examining purified vacuolar vesicles from wild-type and mutant cells. Immunoblots of vacuolar membranes from the four different cell types are shown in Fig. 5. In contrast to the results on whole cell lysates shown in Fig. 4, the 69-, 60-, and 42-kDa subunits are completely absent from the vacuolar membranes of tfplΔ, vat2Δ, and vma3Δ cells. The 100-kDa subunit and its 75-kDa proteolytic product are present at near normal levels in the tfplΔ and vat2Δ vacuoles but are completely absent from the vma3Δ vacuoles. Enough vacuolar protein was loaded on this gel for the 100-kDa subunit to be visible if it were present at similarly reduced levels in vma3Δ whole cell lysates and vma3Δ vacuoles. Therefore, we conclude that the small amount of 100-kDa subunit which is present in vma3Δ mutants is not at the vacuolar membrane. These studies were extended to examining whether the 17-kDa subunit was present in vacuoles from the wild-type and mutant strains. A silver-stained gel of 2:1 chloroform:methanol extracts of vacuolar vesicles from wild-type, tfplΔ, vat2Δ, and vma3Δ cells is shown in Fig. 6. The 17-kDa subunit is readily visible in the extracts from wild-type, tfplΔ, and vat2Δ cells (lanes 1–3) but is absent from the vma3Δ vacuoles (lane 4). This indicates that, like the 100-kDa subunit, the 17-kDa subunit can be transported to the vacuole in the absence of the 69- and 60-kDa subunits. Similar conclusions have been reported by Umemoto et al. (1990) and Nouni et al. (1991) based on [3H]DCD labeling of vacuolar membranes from wild-type and mutant cells.

The localization of the peripheral and integral membrane subunits of the yeast vacuolar H+-ATPase, represented by the
60- and 100-kDa subunits, respectively, was also analyzed by indirect immunofluorescence microscopy. As reported previously (Yamashiro et al., 1990; Umemoto et al., 1990), the vacuolar morphology of the wild-type and mutant cells was indistinguishable when viewed under Nomarski optics (Figs. 7 and 8, top panels). The vacuolar membranes of all four strains also showed comparable staining with a polyclonal antibody against alkaline phosphatase, a vacuolar membrane protein (data not shown). Clear differences were observed when wild-type and mutant cells were stained with a monoclonal antibody recognizing the 60-kDa subunit, however (Fig. 7). In wild-type cells, the 60-kDa subunit appeared to be localized to the vacuolar membrane (Fig. 7A), but in the two mutant cell lines containing the 60-kDa subunit, staining by the antibody was non-vacuolar and diffuse. This suggests that the 60-kDa subunit is present in a soluble, cytoplasmic form outside the vacuole (cf. Fig. 7B). The results from immunofluorescence microscopy also, but we discovered that the monoclonal antibodies against these subunits did not give specific staining in either the wild-type or the mutant cells.

Immunofluorescence microscopy using a monoclonal antibody recognizing the 100-kDa subunit (Fig. 8, bottom panels) yielded somewhat surprising results. Very little staining was seen in wild-type cells. (Similar levels of staining could be seen on cells from which the first (specific) antibody had been omitted and only the second antibody added, indicating that the fluorescence seen on these cells was nonspecific.) In contrast, the tps1Δ and vma3Δ cells exhibited bright, specific staining of the vacuolar membrane. This result is in agreement with the presence of the 100-kDa subunit in isolated vacuolar membranes from these mutants, but it also suggests that a cryptic epitope that is masked in wild-type cells is available for binding of the monoclonal antibody in the mutants. The vma3Δ cells exhibited no specific staining (the level of staining was essentially the same as in wild-type cells). However, this experiment alone cannot distinguish whether the epitope recognized by the antibody was unavailable in this mutant or the reduced levels of the subunit in vma3Δ cells were too low for detection under these conditions.

**DISCUSSION**

It has been suggested that vacuolar proton-translocating ATPases are composed of a "Vₐ sector" peripherally attached to a "V₀ sector" that is embedded in the membrane (Puopolo and Forgac, 1990). This proposed structure is analogous to the structure of the FₐFₒ-ATPases and is suggested by the homologies between amino acid sequences of several vacuolar and FₐFₒ-ATPase subunits (Bowman et al., 1988a, 1988b; Manolson et al., 1988; Zimniak et al., 1988; Inatomi et al., 1989; Denda et al., 1988) and by the similar subunit stoichiometries of these two classes of proton-translocating ATPases (Arai et al., 1988). The proposed structure is strongly supported by electron microscopy of the vacuolar H⁺-ATPase on Neurospora vacuolar membranes, which reveals a ball and stick structure that closely resembles the mitochondrial FₒFₒ-ATPases (Bowman et al., 1989), and by biochemical experiments indicating that a complex including several of the peripheral membrane subunits can be removed from the membrane by treatment with chaotrope anions (Puopolo and Forgac, 1990; Bowman et al., 1989; Moriyama and Nelson, 1989a, 1989b).

The structural picture that is emerging for the yeast vacuolar H⁺-ATPase indicates that this enzyme may also be well described by the VₐV₀ structural model. The 69-, 60-, and 42-kDa subunits are peripheral membrane subunits that might form part of the Vₐ sector of the enzyme. These three subunits are coordinately removed from the vacuolar membrane by treatment with KNO₃ in the presence of ATP, but the stripped subunits could not be isolated as a complex (Kane et al., 1989). However, the results shown here indicate that either the vacuolar targeting or the assembly of these subunits into the ATPase complex is highly interdependent. Thus, although the 42-kDa subunit is synthesized in yeast cells lacking the 69-kDa or the 60-kDa subunit, it does not reach the vacuolar membrane. Similarly, neither the 69-kDa nor the 60-kDa subunit is present at the vacuolar membrane in the absence of the other subunit. Although this type of experiment does not provide a direct demonstration of interactions between subunits, it does suggest strongly that these three subunits are structurally associated. The VMA4 gene is predicted to encode a hydrophilic protein with no potential transmembrane domains, so this subunit may also be part of the Vₐ domain of the yeast vacuolar H⁺-ATPase.

The 100- and 17-kDa subunits are integral membrane proteins that may form all or part of the V₀ portion of the yeast vacuolar H⁺-ATPase. The functional role of the 100-kDa
subunit in the vacuolar H⁺-ATPase complex remains unclear, and it has been suggested that not all vacuolar H⁺-ATPases contain a 100-kDa subunit. The results presented here clearly indicate that the 100-kDa subunit is part of the yeast vacuolar H⁺-ATPase complex and also suggest that this subunit could have been missed in the biochemical characterization of other vacuolar H⁺-ATPases because it is acutely sensitive to proteolysis. We showed previously that a 100-kDa subunit was isolated as part of the yeast vacuolar H⁺-ATPase complex when the enzyme was isolated by either glycerol gradient centrifugation or immunoprecipitation of the complex with an antibody recognizing the catalytic (69-kDa) subunit. Both the decreased cellular levels of the 100-kDa subunit in the \textit{uma3A} cells, which indicate that this subunit is either stabilized by the presence of the 17-kDa subunit or regulated by the presence of this subunit in some other way, and the "unmasking" of an epitope on the 100-kDa subunit only under conditions in which several of the peripheral subunits are absent from the membrane provide independent support for a structural association between the 100-kDa subunit and functionally characterized subunits of the yeast vacuolar H⁺-ATPase.

The immunoblot shown in Fig. 1 reveals that the 100-kDa subunit is present both in association with the other ATPase subunits and in a peak at lower density. One possible explanation for the lower density peak is that it represents all or part of the Vₚ portion of the vacuolar H⁺-ATPase in the absence of the Vₚ portion. The existence of this species may or may not be physiologically important. Biochemical studies of certain vacuolar H⁺-ATPases have indicated that the peripheral subunits may be removed from the membrane by a variety of treatments (Moriyama and Nelson, 1989a), so it is possible that some of the Vₚ subunits are being dissociated from the Vₚ subunits during preparation of the vacuoles or isolation of the vacuolar H⁺-ATPase. A more interesting alternative explanation is that the integral membrane subunits are present in the vacuolar membrane both in fully assembled complexes and as individual subunits or partially assembled complexes. Such partially assembled or unassembled subunits could represent intermediates in the assembly of the complex or might even play a role in regulating vacuolar acidification. The loss of the peripheral subunits during enzyme purification would appear to be more consistent with the immunofluorescence experiments, which indicate that significant amounts of the 100-kDa subunit only seem to be unmasked in mutants lacking the 69- or the 60-kDa subunits. However, it is still possible that wild-type vacuolar membranes contain the 100-kDa subunit in a partially assembled form that differs from the form present in the mutant cells and is not seen by the antibodies in immunofluorescence experiments. Some of these issues may become clearer after methods for detecting all of the yeast vacuolar H⁺-ATPase subunits have been developed and the functional roles of the subunits become better defined.

The targeting and assembly of the vacuolar H⁺-ATPases present an array of questions that we have only begun to address here. The data presented here indicate that the 100- and 17-kDa subunits can be targeted to the vacuolar membrane in the absence of either the 69- or the 60-kDa subunits but suggest that the transport of the 100-kDa subunit to the vacuole may depend on the presence of the 17-kDa subunit. Results on two vacuolar membrane proteins, dipetidyl aminopeptidase B (Roberts et al., 1989) and alkaline phosphatase (Kiontsky and Emr, 1989), demonstrated that these proteins were inserted into the endoplasmic reticulum membrane during or after translation and then traveled through the Golgi apparatus to the vacuole. Similar experiments have not been performed on the integral membrane subunits of the vacuolar H⁺-ATPase, but a number of other membrane proteins that use the secretory pathway during transport are also transported across the membrane and assembled from their component subunits in the endoplasmic reticulum (reviewed in Hurtley and Helenius, 1989). The endoplasmic reticulum is proposed to play a quality control role in the transport of these proteins, and unassembled or improperly assembled complexes are detained, and in some cases degraded, in the endoplasmic reticulum (Hurtley and Helenius, 1989, Klausner and Sitia, 1990). This type of quality control might be at work in preventing the 100-kDa subunit from reaching the vacuolar membrane in the absence of the 17-kDa subunit and might even account for the lowered levels of the 100-kDa subunit in the \textit{uma3A} cells.

The assembly of the peripheral membrane subunits presents a somewhat different set of problems. The 69-, 60-, and 42-kDa subunits are located on the cytoplasmic face of the vacuolar membrane, and neither the TFP1 nor the VAT2 gene shows any evidence of signal sequences. Therefore these subunits should never see the interior of the endoplasmic reticulum, and this might explain why the 69- and 60-kDa subunits are not necessary for transport of the integral membrane subunits to the vacuole. It is not at all clear at what point in transport these subunits might become associated with the integral membrane subunits. The total absence of the 69-, 60-, and 42-kDa subunits from the vacuolar membrane in the \textit{tfp1Δ} and \textit{vat2Δ} cells argues against the simple model in which the 17-kDa subunit, perhaps in combination with the 100-kDa subunit, acts as a template for assembly of the individual peripheral subunits (Noumi et al., 1991) and suggests instead that attachment of the peripheral subunits to the vacuolar membrane is a cooperative process requiring interactions between several subunits.

In addition, other cellular factors besides the vacuolar ATPase subunits themselves may participate in the assembly process. At least two of the vacuolar protein sorting (\textit{ups}) mutants, \textit{ups3} and \textit{ups6}, are deficient in vacuolar acidification (Rothman et al., 1989) as assessed by quinacrine accumulation, and vacuoles from these mutants exhibit reduced levels of the 69- and 60-kDa subunits at the vacuolar membrane. Neither of the genes affected in these mutants appears to correspond to any subunit of the vacuolar H⁺-ATPase (Raymond et al., 1990). It appears that the assembly and transport of the vacuolar H⁺-ATPase may be almost as complex as the assembly processes for multisubunit complexes in mitochondria, which often require the presence of not only the subunits of the complex but also a collection of accessory factors (Nobrega et al., 1990). Equipped with the biochemical characterization of the vacuolar H⁺-ATPase and the variety of tools that have been developed for study of this enzyme, we are beginning to elucidate the molecular details of the synthesis, assembly, and transport of this enzyme.

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