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Subunit Composition, Biosynthesis, and Assembly of the Yeast Vacuolar Proton-Translocating ATPase

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The yeast vacuole is acidified by a vacuolar proton-translocating ATPase (H^+-ATPase) that closely resembles the vacuolar H^+-ATPases of other fungi, animals, and plants. The yeast enzyme is purified as a complex of eight subunits, which include both integral and peripheral membrane proteins. The genes for seven of these subunits have been cloned, and mutant strains lacking each of the subunits (*vma* mutants) have been constructed. Disruption of any of the subunit genes appears to abolish the function of the vacuolar H^+-ATPase, supporting the subunit composition derived from biochemical studies. Genetic studies of vacuolar acidification have also revealed an additional set of gene products that are required for vacuolar H^+-ATPase activity, but may not be part of the final enzyme complex. The biosynthesis, assembly, and targeting of the enzyme is being elucidated by biochemical and cell biological studies of the *vma* mutants. Initial results suggest that the peripheral and integral membrane subunits may be independently assembled.

KEY WORDS: V-type ATPase; proton pump; vacuole; acidification.

BACKGROUND

The yeast vacuole is acidified by a vacuolar proton-translocating ATPase (reviewed in Klionsky *et al.*, 1990; Raymond *et al.*, 1992) that is very similar to the V-type ATPases of other fungi, plant, and animal cells (Pedersen and Carafoli, 1987). Vacuoles in yeast cells functionally resemble both mammalian lysosomes and plant vacuoles. Like the lysosome, the yeast vacuole contains a large number of proteases and other hydrolytic enzymes (Jones, 1984). Like the vacuole in plant cells, it is an important storage organelle (Matile, 1978), involved in storage of amino acids, phosphate and other metabolites, cytosolic Ca^{2+} homeostasis, and osmoregulation (reviewed in Klionsky *et al.*, 1990). The yeast vacuolar H^+-ATPase[3] plays a central role in both of these aspects of vacuolar function. Certain of the resident hydrolases of the vacuole, including proteinase A, exhibit optimal activity under acidic conditions (Jones, 1984), and the vacuolar H^+-ATPase appears to be primarily, if not solely, responsible for vacuolar acidification (Yamashiro *et al.*, 1990). In addition, the proton gradient across the vacuolar membrane established by the ATPase is used to operate a large number of antiporters for amino acids (Ohsumi and Anraku, 1981) and Ca^{2+} (Ohsumi and Anraku, 1983).

In this review, we focus on two aspects of yeast vacuolar H^+-ATPase structure and function. Biochemical and genetic studies directed at determining the subunit composition of the enzyme, including partial purification of the enzyme, assignment of peripheral and integral membrane subunits, and genetic analysis of mutants in which one of the proposed subunit genes has been disrupted, are described.

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[3]Abbreviations: H^+-ATPase, proton-translocating ATPase; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole; DCCD, *N*, *N'*-dicyclohexylcarbodiimide; AMP-PNP, adenosine 5'-(β,γ-imino)triphosphate; ER, endoplasmic reticulum; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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We also summarize the results from current experiments addressing the biosynthesis, assembly, and transport of the ATPase to the vacuole and discuss possible models for assembly and transport.

SUBUNIT COMPOSITION OF THE YEAST VACUOLAR H^+-ATPase

Partial Purification of the Yeast Vacuolar H^+-ATPase

A partial purification of the yeast vacuolar H^+-ATPase was first reported by Uchida *et al.* (1985). The enzyme was isolated by glycerol density gradient centrifugation of solubilized vacuolar membranes, and a fraction containing high specific ATPase activity was obtained that appeared to contain three polypeptides. In repeating the same purification procedure, we discovered that eight polypeptides, of relative molecular masses 100, 69, 60, 42, 36, 32, 27, and 17 kD, consistently migrated with the peak ATPase activity in density gradient centrifugation (Kane *et al.*, 1989b). This same collection of polypeptides could be immunoprecipitated from solubilized vacuolar vesicles by a monoclonal antibody recognizing the 69-kD subunit, suggesting that all of the polypeptides were part of a single complex. Based on these results we proposed that the yeast vacuolar H^+-ATPase had a more complex subunit composition than that originally proposed and, in fact, was fundamentally similar to vacuolar H^+-ATPases from bovine clathrin-coated vesicles (Arai *et al.*, 1987b; Xie and Stone, 1986), bovine chromaffin granules (Moriyama and Nelson, 1987), bovine kidney (Gluck and Caldwell, 1987), and red beet (Parry *et al.*, 1989). All of these ATPases appeared to contain three subunits of relative molecular masses about 70, 60, and 17 kD, a number of subunits of molecular mass 25–45 kD, and in most cases, a large subunit of molecular mass 100–120 kD. (The bovine kidney enzyme appeared to have no 100-kD subunit, but otherwise resembled the other enzymes.) Coomassie-blue staining of SDS-polyacrylamide gels of the partially purified yeast vacuolar H^+-ATPase suggests that the 69- and 60-kD subunits are present in roughly stoichiometric amounts and that there may be more copies of these subunits than of the 100, 42, 36, 32, and 27 kD subunits (Kane *et al.*, 1989b). This would be consistent with the stoichiometry of the bovine clathrin-coated vesicle H^+-ATPase, which contains three copies of the 69- and 60-kD subunits and six copies of the 17-kD subunit for each copy of the other subunits (Arai *et al.*, 1988).

The three subunits that were originally identified as part of the yeast vacuolar H^+-ATPase correspond to the 70-, 60-, and 17-kD subunits that have been found in all vacuolar H^+-ATPases isolated so far (reviewed in Forgac, 1989). Functions have been proposed for each of these subunits. Affinity-labeling of the 69-kD subunit with the ATP analog NBD-Cl^1 indicates that this subunit contains the catalytic site for ATP hydrolysis (Uchida *et al.*, 1988). The 17-kD subunit is labeled with *N,N'*-dicyclohexylcarbodiimide (DCCD) (Uchida *et al.*, 1985), suggesting that it forms all or part of the proton pore. The function of the 60-kD subunit in the yeast vacuolar H^+-ATPase has not been directly determined. However, the analogous 57-kD subunit from red beet vacuoles is proposed to be a regulatory ATP-binding subunit based on labeling and inhibition studies with 3'-O-(4-benzoylbenzoyl)adenosine 5'-triphosphate (Manolson *et al.*, 1985). The predicted amino acid sequence of the *VMA2* gene, which encodes the 60-kD subunit of the yeast vacuolar H^+-ATPase, also contains a potential ATP binding site (Nelson *et al.*, 1989; Yamashiro *et al.*, 1990). The functions of the 100-, 42-, 36-, 32-, and 27-kD subunits have not yet been defined, although the genetic and biochemical evidence summarized below argues strongly that at least the 100-, 42-, 36-, and 27-kD polypeptides are both purified as part of the enzyme complex and required for function of the enzyme.

Identification of Peripheral and Integral Membrane Subunits

The yeast vacuolar H^+-ATPase also resembles other V-type ATPases and the F_1F_0 (F-type)-ATPases in that it has both peripheral and integral membrane subunits (Kane *et al.*, 1989b; 1992). Both the F- and V-type ATPases consist of a complex of peripheral subunits that appears to contain the site(s) of ATP hydrolysis (called the V_1 sector of the vacuolar H^+-ATPases) attached to a complex of integral membrane subunits that forms the proton pore (the V_0 sector) (Nelson and Taiz, 1989). The 69-, 60-, and 42-kD subunits of the yeast vacuolar H^+-ATPase are removed from the vacuolar membrane by alkaline sodium carbonate, indicating that they form all or part of the peripheral complex. The 100- and 17-kD subunits behave as integral membrane subunits and remain with the membrane through alkaline sodium

carbonate treatment (Kane *et al.*, 1989b, 1992). Both the predicted amino acid sequence of the *VMA3* gene, which appears to encode the 17-kD subunit (Nelson and Nelson, 1989), and the chemical properties of this subunit indicate that it is highly hydrophobic, consistent with its proposed role as the proton pore. The 17-kD subunit could be specifically extracted from the vacuolar membrane by 2:1 chloroform:methanol, suggesting that it may be a proteolipid (Noumi *et al.*, 1991; Umemoto *et al.*, 1990). The 17-kD subunits from other vacuolar H^+-ATPases show similar properties (Arai *et al.*, 1987a; Kaestner *et al.*, 1988; Rea *et al.*, 1987; Sun *et al.*, 1987), although in no case have covalently attached lipids been identified. The membrane disposition of the 36-, 32-, and 27-kD subunits has not been biochemically determined, but the sequence of the 27-kD subunit, which is encoded by the *VMA4* gene (Foury, 1990), exhibits no potential transmembrane domains, so the 27-kD subunit may well be part of the peripheral V_1 complex.

The vacuolar H^+-ATPases are distinguished from other proton-translocating ATPases by their sensitivity to relatively low concentrations of chaotropic anions such as nitrate, isothiocyanate, and iodide (Bowman, 1983), and inhibition of ATPase activity by these agents is accompanied by removal of several of the peripheral subunits from the membrane (Adachi *et al.*, 1990; Bowman *et al.*, 1989; Kane *et al.*, 1989b). We examined the inhibition and changes in subunit associations of the yeast vacuolar H^+-ATPase in response to potassium nitrate in the presence and absence of MgATP (Kane *et al.*, 1989b). In the presence of 5 mM MgATP, the yeast enzyme showed half-maximal inhibition at 40 mM nitrate, and inhibition of ATPase activity was paralleled by removal of the 69-, 60-, and 42-kD subunits from the vacuolar membrane. The coordinate removal of the 69-, 60-, and 42-kD subunits of the yeast enzyme by treatment with nitrate provided further support for a structural association between these subunits and supports the designation of the 42-kD subunit as a genuine subunit. Interestingly, the stripping of these peripheral subunits was promoted by the presence of MgATP, the catalytic substrate, suggesting that the catalytically active enzyme may experience a conformation that is more susceptible to disruption by nitrate than the inactive enzyme. This conclusion is supported by the finding that the nonhydrolyzable ATP analog AMP-PNP did not promote subunit stripping as effectively as MgATP, and removal of the Mg^{2+} by chelation with EDTA also resulted in less subunit stripping. It was not possible to restore either ATPase activity or subunit binding to the membrane after removal of nitrate in the initial studies on the yeast vacuolar H^+-ATPase, but this may be attributed to the fact that the 69-, 60-, and 42-kD subunits appeared to dissociate into individual subunits or very small complexes under the nitrate treatment conditions used in those experiments. Work on other vacuolar H^+-ATPases has demonstrated that it is often possible to isolate the stripped peripheral subunits as a complex (Bowman *et al.*, 1989; Moriyama and Nelson, 1989; Puopolo and Forgac, 1990), although in no case is ATPase activity retained by the stripped complex, and that it may even be possible to remove the chaotrope and restore both association between the peripheral and membrane sectors and ATPase activity (Puopolo and Forgac, 1990). The earlier experiments on the yeast vacuolar H^+-ATPase are now being repeated under slightly different conditions. The peripheral subunits of a number of vacuolar H^+-ATPases can also be dissociated from the membrane by incubation at 0°C in the presence of chloride ion and MgATP (Moriyama and Nelson, 1989). Treatment of yeast vacuolar membranes with 200 mM KCl in the presence of MgATP resulted in release of at least five polypeptides (Noumi *et al.*, 1991). These may include other peripheral subunits in addition to the 69-, 60-, and 42-kD subunits, but the initial study provided no evidence that they were associated with the vacuolar H^+-ATPase. A structural model for the yeast vacuolar H^+-ATPase which incorporates both the biochemical data described above and its apparent structural similarity to other V-type ATPases and to the F-type ATPases is shown in Fig. 1.

Cloning of Subunit Genes and Analysis of Deletion Mutants

More recent studies of the subunit composition of the yeast vacuolar H^+-ATPase have relied on a combination of genetic and biochemical techniques. Several of the genes for the subunits of the yeast vacuolar H^+-ATPase have now been cloned and sequenced. The 60-kD (Nelson *et al.*, 1989; Yamashiro *et al.*, 1990) and 17-kD (Nelson and Nelson, 1989) subunit genes, designated *VMA2* (also called *VAT2*) and *VMA3*, respectively, proved to be remarkably homologous to genes for the analogous subunits in other fungi, animals, and plants. The 69-kD subunit gene, *VMA1* (also called *TFP1*), also proved to be highly homologous to the 70-kD subunit genes of

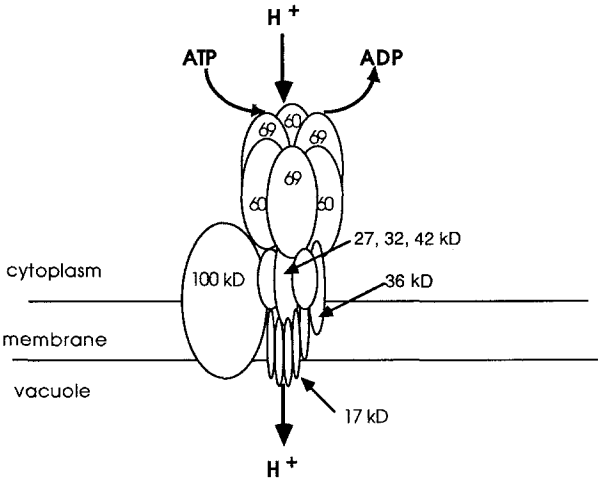

Fig. 1. *Structural model for the yeast vacuolar H^+-ATPase.* The model shown is a revision of the model originally proposed in Kane *et al.*, (1989a), and justification of several of the features is described there. This model differs somewhat in the assignment of the peripheral and integral membrane subunits; the 42 and 27 kD subunits are shown as peripheral and the 100 kD subunit is shown as transmembrane subunit, consistent with the biochemical studies described in the text, and in the case of the 27 kD subunit, the sequence of the cloned gene. Indirect evidence suggests that the biochemical behavior of the 36 kD subunit may be different from the other peripheral subunits. The membrane disposition of the 36 kD subunit is still unclear.

other vacuolar H^+-ATPases (Shih *et al.*, 1988; Hirata *et al.*, 1990), with the exception of the "spacer domain" interrupting the gene, which is described below. The identification of each of these genes with the corresponding subunit was confirmed by obtaining N-terminal and/or internal peptide sequences from the subunit, either from blots of stripped peripheral subunits after SDS-PAGE (Hirata *et al.*, 1990; Yamashiro *et al.*, 1990) or, in the case of the 17-kD subunit, from chloroform:methanol extracts of vacuolar vesicles (Anraku *et al.*, 1991).

The *VMA4* gene was identified as a gene adjacent to *MIP1* (Foury, 1990), and the *VMA4* open reading frame predicted a 27-kD hydrophilic protein with 24% amino acid identity to the bovine 31-kD subunit gene (Hirsch *et al.*, 1988). An internal peptide of the yeast 27-kD subunit (APLEEIVISNDYLNK; M. Lindorfer, M. N. Ho, and T. H. Stevens, unpublished data) matched perfectly a portion of the *VMA4* open reading frame (codons 167–181; Foury, 1990), demonstrating that the yeast *VMA4* gene encodes the 27-kD subunit of the vacuolar H^+-ATPase.

The *VMA5* gene was identified as an open reading frame adjacent to *SMY1* (M. N. Ho, S. Lillie, M. Lindorfer, and T. H. Stevens, manuscript in preparation). The *VMA5* open reading frame predicts a 42-kD hydrophilic protein, and the sequences for four internal peptides of the purified subunit are all found within this open reading frame. The predicted sequence shows an ~30% amino acid identity to the 44-kD subunit from bovine chromaffin granules (Nelson *et al.*, 1990).

The *VMA6* gene has been amplified by polymerase chain reaction using oligonucleotides corresponding to peptide sequences from the 36-kD subunit of the glycerol gradient-purified vacuolar H^+-ATPase (C. Bauerle and T. H. Stevens, unpublished data). *VMA6* is homologous to the 39-kD subunit of the bovine chromaffin granule H^+-ATPase (Wang *et al.*, 1988). The yeast gene encoding the 32-kD subunit of the yeast vacuolar H^+-ATPase has not yet been cloned.

The 100-kD subunit gene was identified in a screen for mutants defective in vacuolar acidification (*vph* mutants; Preston *et al.*, 1989). The *VPH1* gene (M. F. Manolson, D. Proteau, R. A. Preston, and E. W. Jones, personal communication) encodes a large, hydrophobic protein and is homologous to the gene for the 115-kD subunit of the bovine clathrin-coated vesicle H^+-ATPase (Perin *et al.*, 1991). The *VPH1*-encoded protein copurifies with the H^+-ATPase (M. F. Manolson and E. W. Jones, manuscript submitted), and 100-kD peptide sequences are contained within the *VPH1* open reading frame (M. N. Ho, M. Lindorfer, and T. H. Stevens, unpublished data), indicating that the *VPH1* gene encodes the 100-kD subunit of the vacuolar H^+-ATPase. The clones genes for subunits present in the glycerol gradient-purified vacuolar H^+-ATPase are matched with the corresponding subunits in Table I.

Disruption of the cloned subunit genes *in vitro* followed by replacement of the chromosomal copy of the gene with the disrupted copy has proved to be a valuable means of assessing whether the proposed subunits directly affect function of the vacuolar H^+-ATPase and has also yielded new insights into the cellular roles of vacuolar acidification. Analysis of *vma2Δ* cells revealed that the cells grew poorly under all conditions and failed to grow in medium buffered at pH 7.0, the vacuolar pH was 7.1 instead of the wild-type vacuolar pH of 6.1 (Preston *et al.*, 1989), and isolated vacuoles had no ATPase activity (Nelson and Nelson, 1990; Yamashiro *et al.*, 1990). Further analysis of the *vma2Δ* mutants provided a clearer

Table I. Summary of the Yeast Vacuolar H^+-ATPase Subunit Composition

A. Biochemically characterized subunits of vacuolar H^+-ATPase (Uchida *et al.*, 1985; Kane *et al.*, 1989)

Subunit	Gene	Membrane disposition	Proposed function	References[a]
100 kD	*VPH1*	Integral	?	1
69	*VMA1/TFP1*	Peripheral	Catalyzes ATP hydrolysis	2
60	*VMA2/VAT2*	Peripheral	Regulatory, has ATP binding site	3
42	*VMA5*	Peripheral	?	4
36	*VMA6*	?	?	5
32	?	?	?	
27	*VMA4*	Peripheral	?	6
17	*VMA3*	Integral	Proton pore, binds DCCD	7

B. Other required factors

Predicted MW	Gene	Reference
17 kD	*VMA11*	8, 9
25	*VMA12*	8
54	*VMA13*	8
?	*VMA21, 22, 23*	10

[a] References: 1. M. F. Manolson, D. Proteau, R. A. Preston, and E. W. Jones, manuscript submitted. 2. Shih *et al.* (1988); Hirata *et al.* (1990). 3. Nelson *et al.* (1989); Yamashiro *et al.* (1990). 4. M. Ho, M. Lindorfer, S. Lillie, and T. H. Stevens, manuscript in preparation. 5. C. Bauerle, M. Ho, M. Lindorfer, and T. H. Stevens, unpublished data. 6. Foury (1990); M. Ho, M. Lindorfer, and T. H. Stevens, unpublished data. 7. Nelson and Nelson (1989); Anraku *et al.* (1991); Umemoto *et al.* (1991). 8. Ohya *et al.* (1991); Y. Anraku, this volume, p. 395; 9. Umemoto *et al.* (1991). 10. K. Hill and T. H. Stevens, unpublished data.

picture of the physiological roles of vacuolar acidification. Targeting of the soluble vacuolar protease carboxypeptidase Y to the vacuole was only modestly affected in the mutants, implying that acidification of the vacuole (and possibly of the entire vacuolar network, since there appears to be no other 60-kD subunit gene in yeast cells) may not play as critical a role in protein sorting as previously proposed (Yamashiro *et al.*, 1990). Zymogen activation appeared to be unaffected by the elevated pH of the vacuole in *vma2Δ* cells; both carboxypeptidase Y and proteinase A were present in their mature, proteolytically processed form in *vma2Δ* cells (Yamashiro *et al.*, 1990). However, the severe growth defects of the vacuolar ATPase mutants indicate that other aspects of cell physiology are profoundly affected in the mutants. The exact reasons for the pH-dependent growth phenotype and the poor growth under all conditions have not been elucidated, but it may be that vacuolar acidification plays a very significant role in the regulation of cytoplasmic calcium concentrations (Hirata *et al.*, 1990; Noumi *et al.*, 1991; Ohya *et al.*, 1991) or in maintenance of the cytoplasmic pH (Banta *et al.*, 1988; Raymond *et al.*, 1990). (The various phenotypes of cells lacking vacuolar H^+-ATPase activity are summarized in more detail by Y. Anraku, this volume p. 395.) *vma2Δ*, *vma1Δ* (Kane *et al.*, 1990; Noumi *et al.*, 1991) *vma3Δ* (Nelson and Nelson, 1990; Noumi *et al.*, 1991), *vma4Δ* (Foury, 1990), and *vma5Δ* (M. Ho, M. Lindorfer, and T. H. Stevens, manuscript in preparation) cells exhibited an identical pH-dependent growth phenotype and all had no ATPase activity in isolated vacuoles. This suggests that in each case vacuolar H^+-ATPase activity was totally abolished and that each of these gene products is critical for ATPase function. As the subunit genes and deletion mutants for the other subunits become available, it will be possible to genetically assess their role in vacuolar H^+-ATPase function as well.

Besides providing strong evidence that the subunits identified biochemically are indeed necessary for function of the enzyme, genetic studies of the yeast vacuolar H^+-ATPase have also raised new questions about the subunit composition of the enzyme. In a collection of calcium-sensitive (*cls*) mutants, a number of mutants defective for vacuolar H^+-ATPase function were identified (*vma* mutants; Ohya *et al.*, 1991). In addition to mutations in the *VMA1* and *VMA3* genes, mutations were isolated in three new genes, *VMA11*, *VMA12*, and *VMA13*. Mutations in three more genes required for vacuolar H^+-ATPase function (*VMA21*, *VMA22*, and *VMA23*) were also isolated in a screen for yeast mutants incapable of growth at pH 7.5 (K. Hill and T. H. Stevens, unpublished).

These newly identified genes are summarized in the lower section of Table I. The *VMA11* gene was previously identified as the *TFP3* gene (Shih *et al.*, 1990) and is homologous to the *VMA3* gene, indicating that it may be another proteolipid subunit (Umemoto *et al.*, 1991). Interestingly, the N-terminal amino acid sequence obtained from the chloroform:methanol extract of isolated vacuolar membranes corresponded to the predicted amino acid sequence of the *VMA3* gene (Anraku *et al.*, 1991). However, the abundant 17-kD proteolipid species present in vacuolar membranes is absent from vacuoles of both *vma3*Δ (Kane *et al.*, 1992; Noumi *et al.*, 1991; Umemoto *et al.*, 1990) and *vma11*Δ (Umemoto *et al.*, 1991) cells, making it difficult to assign a function to the *VMA11* protein at this time. The *VMA12* and *VMA13* genes are predicted to encode proteins of 25 and 54 kD, respectively (Y. Anraku, this volume, pp.), but the predicted amino acid sequences of these proteins do not match any of the peptide sequences obtained from the polypeptides present in the glycerol gradient-purified vacuolar H^+-ATPase (M. Ho, M. Lindorfer, Y. Anraku, and T. H. Stevens, unpublished data). Specifically, the peptide sequences of the 27-, 32-, 36-, 42-, and 60-kD subunits in the glycerol gradient fraction, which would be the polypeptides predicted to be closest in molecular mass to the *VMA12* and *VMA13* gene products, do not correspond to the predicted amino acid sequences for these gene products.

Further studies are under way to determine whether the products of the *VMA11, 12, 13, 21, 22,* and *23* genes are part of the yeast vacuolar H^+-ATPase complex or factors required for expression of vacuolar ATPase activity that are not actually part of the final complex. It is possible that these additional *VMA*-encoded proteins are required for the biosynthesis, assembly, or targeting of the vacuolar H^+-ATPase but are not subunits of the final, assembled enzyme. Proteins of this type have been identified for a number of multisubunit complexes, including yeast cytochrome oxidase (Nobrega *et al.*, 1990) and the T-cell receptor (Bonifacino *et al.*, 1988). Alternatively, one or more of these new *VMA* gene products could be organelle-specific subunits of the vacuolar H^+-ATPase. However, this model does not explain why these genes are necessary for expression of a functional H^+-ATPase at the vacuole. It thus remains to be determined whether the products of the *VMA11–13* and *VMA21–23* genes are indeed subunits of the final H^+-ATPase complex or factors required for its function.

BIOSYNTHESIS, ASSEMBLY, AND TARGETING OF THE YEAST VACUOLAR H^+-ATPase

When and Where Is the ATPase Assembled?

The transport of both integral membrane and soluble proteins to the yeast vacuole has been studied intensively during the past few years. Soluble vacuolar proteins appear to enter the endoplasmic reticulum (ER) and travel from the ER to the Golgi apparatus along with proteins destined for secretion (Stevens *et al.*, 1982). Both sets of proteins receive Golgi-specific glycosylation (Franzusoff and Schekman, 1989), and at some point after this, vacuolar proteins are recognized and diverted to the vacuole while secreted proteins move to the cell suface by default (Johnson *et al.*, 1987; Valls *et al.*, 1987). Soluble vacuolar proteins do not rely on the mannose-6-phosphate modification that serves as a sorting determinant on many mammalian lysosomal proteins for targeting to the vacuole (Stevens *et al.*, 1982; Winther *et al.*, 1991), but do appear to contain sorting information in the form of short peptide sequences in the propeptide region (Johnson *et al.*, 1987; Klionsky *et al.*, 1988; Valls *et al.*, 1987). Vacuolar membrane proteins also traverse the initial stages of the secretory pathway in transit to the vacuole (Roberts *et al.*, 1989; Klionsky and Emr, 1989). However, there is evidence that at least one vacuolar membrane protein, dipeptidyl aminopeptidase B (DPAP-B), does not contain a positive sorting signal, but instead may be reaching the vacuole by default (C. J. Roberts, S. Nothwehr, and T. H. Stevens, manuscript submitted).

The transport and assembly of the yeast vacuolar H^+-ATPase pose some unique problems. There have been no studies of any multisubunit vacuolar proteins reported, and the structure of the yeast vacuolar H^+ATPase, with its combination of integral and peripheral membrane subunits, makes its assembly and transport pathway particularly interesting. It seems reasonable to suggest that the integral membrane subunits enter the ER and follow an intracellular path similar to other vacuolar membrane proteins, although this still does not address the question of when the various integral membrane subunits are assembled to form the V_0 sector. The assembly and transport of the peripheral subunits are even more mysterious. The cloned genes of the 69-, 60-, 42-, and 27-kD subunits contain no evidence of signal sequences or protein transmembrane domains. There are five

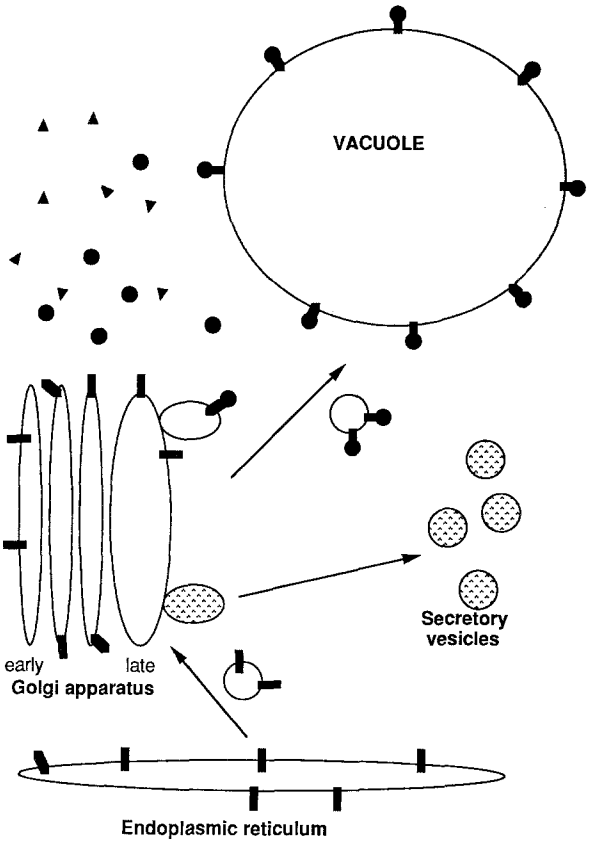

Fig. 2. *Model for targeting and assembly of the yeast vacuolar H^+-ATPase*; ●, V_1 sector of the vacuolar H^+-ATPase; ▲, unassembled subunits of the V_1 sector; ▌, V_0 sector of the vacuolar H^+-ATPase; ▛, fully assembled vacuolar H^+-ATPase. The V_1 sector is shown as partially assembling in the cytosol prior to attachment to the V_0 sector. In this diagram, the assembly of the V_1 sector on to the V_0 sector occurs in the late Golgi apparatus, but there is no experimental evidence that this is the cellular site of assembly.

potential sites for N-linked glycosylation in the predicted amino acid sequence of the 60-kD subunit and at least four in the sequence of the 69-kD subunit, but neither subunit receives N-linked glycosylation (P. M. Kane and T. H. Stevens, unpublished data). Both of these pieces of evidence suggest that the peripheral subunits that compose the V_1 sector never enter the secretory pathway, but instead remain in the cytosol prior to attachment to the integral membrane subunits. If this is true, then the possibility arises that the peripheral subunits assemble or partially assemble into the V_1 complex in the cytosol, and then become attached to the V_0 sector at some point during its transit to the vacuole. A model for assembly of the ATPase complex is shown in Fig. 2. Is has been shown tha the peripheral and integral membrane complexes of the *E. coli* F_1F_0-ATPase can be synthesized and assembled independently (Aris *et al.*, 1985; Klionsky and Simoni, 1985). We are investigating these different mechanisms for assembly and transport of the yeast vacuolar H^+-ATPase by a combination of genetic biochemical, and cell biological approaches that are described below.

Assembly of the Yeast Vacuolar H^+-ATPase in *vma* Mutants

Analysis of yeast mutants lacking one of the vacuolar H^+-ATPase subunits indicates that biosynthesis of the ATPase subunits does not depend on the presence of all of the other subunits. Immunoblots of whole cell lysates from *vma1*Δ, *vma2*Δ, *vma3*Δ, *vma4*Δ, and *vma5*Δ cells show near wild-type levels of the 69-, 60-, and 42-kD subunits, except in cases where the specific subunit genes have been deleted (Kane *et al.*, 1990, 1992; Noumi *et al.*, 1991; Umemoto *et al.*, 1990; Yamashiro *et al.*, 1990; M. N. Ho and T. H. Stevens, unpublished data). The 100-kD subunit is also at approximately wild-type levels in the *vma1*Δ and *vma2*Δ cells, but is at substantially reduced levels (about 10% of wild-type) in the *vma3*Δ cells (Kane *et al.*, 1992). (It has not been determined whether the steady-state levels of the 100-kD subunit are reduced as a result of reduced synthesis or instability of the subunit in the absence of the 17-kD subunit.) In general, these results argue against regulation of vacuolar ATPase assembly by regulation of subunit biosynthesis, and suggest instead that pools of the subunits are always present. However, until all the subunit genes have been cloned and the corresponding deletion mutants analyzed, it will not be possible to determine whether there is one subunit gene that must be expressed in order for all of the other subunits to be synthesized and stably maintained in the cell.

Further investigation of the localization of the remaining subunits in the existing deletion mutants indicates that these subunits are not properly assembled and targeted to the vacuole in the mutants. Isolated vacuolar membranes from *vma1*Δ and *vma2*Δ cells contain no 69-, 60-, or 42-kD subunit detectable on immunoblots (Kane *et al.*, 1992; Umemoto *et al.*, 1990; Yamashiro *et al.*, 1990), and in immunofluorescence micrographs of *vma1*Δ cells, the 60-kD subunit is visualized as diffuse cytoplasmic staining, rather than the vacuolar membrane staining characteristic of wild-type cells (Kane *et al.*, 1992). This result indicates that when one of the peripheral subunits is missing,

the others are not able to reach the vacuole. In contrast, immunoblots of isolated vacuolar membranes and immunofluorescence microscopy of *vma1*Δ and *vma2*Δ mutants using a monoclonal antibody against the 100-kD subunit both indicate that the 100-kD subunit is present in the vacuolar membranes of *vma1*Δ and *vma2*Δ cells (Kane *et al.*, 1992). The 17-kD subunit, visualized by DCCD-labeling and extraction into chloroform:methanol, is also present in *vma1*Δ and *vma2*Δ vacuolar membranes (Kane *et al.*, 1992; Noumi *et al.*, 1991; Umemoto *et al.*, 1990). Therefore, targeting of these integral membrane subunits to the vacuole must not require assembly with the peripheral subunits. None of the subunits for which we have a means of detection, including the 100-, 69-, 60-, 42-, and 17-kD subunits, is present in the vacuolar membranes of *vma3*Δ cells. The cellular localization of the 60-kD subunit in these cells, visualized by immunofluorescence microscopy, closely resembles the diffuse cytoplasmic distribution seen in *vma1*Δ cells (Kane *et al.*, 1992). The 69-kD subunit also showed a similar diffuse distribution in *vma3*Δ cells (Umemoto *et al.*, 1990). No specific staining of the 100-kD subunit could be seen in *vma3*Δ cells, but it was not clear from these experiments whether this was due to the reduced levels of the subunit or the masking of the epitope recognized by the monoclonal antibody used for immunofluorescence microscopy (Kane *et al.*, 1992). However, the complete absence of this subunit from immunoblots of vacuolar membranes from *vma3*Δ cells strongly indicates that the 100-kD subunit that is present in the cell is either not transported to the vacuole or else is not stable there.

Taken together, these results indicate that the integral and peripheral subunits of the yeast vacuolar H^+-ATPase may reach the vacuolar membrane by somewhat different mechanisms. The failure of the peripheral subunits to reach the vacuole in the absence of either the 69- or 60-kD subunit suggests that some assembly of these subunits either occurs prior to attachment to the vacuolar membrane (as suggested in the model in Fig. 2) or else is critical for stable association of these subunits with the integral membrane subunits. We are attempting to distinguish between these possibilities by fractionating whole cell lysates from wild-type cells and the deletion mutants. Preliminary results indicate that even in wild-type cells, a considerable portion of the 69-, and 60-kD subunits can be found in the supernatant fraction after high-speed centrifugation, and in the *vma3*Δ mutants an even higher proportion of these subunits is found in the supernatant. Further fractionation of the supernatant by glycerol gradient centrifugation indicates that the subunits are found both in a low-density fraction, where they may be present singly, and in higher-density fractions, where they may be present in higher molecular weight complexes (R. D. Doherty and P. M. Kane, unpublished data). This approach promises to be useful for isolating intermediates in assembly of the ATPase complex as well as products of arrested assembly in the deletion mutants. These experiments can be reinforced by immunoprecipitation experiments using a monoclonal antibody capable of recognizing the 69-kD subunit under both native and denaturing conditions (Kane *et al.*, 1989b).

The integral membrane subunits, represented by the 100- and 17-kD subunits, apparently do not rely on the peripheral subunits for transport to the vacuole, since both of these integral membrane subunits reach the vacuole in *vma1*Δ and *vma2*Δ mutants. However, both the reduced steady-state levels of the 100-kD subunit and the absence of the 100-kD subunit from the vacuole in *vma3*Δ cells suggest that some assembly of the integral membrane subunits may be necessary for transport to the vacuole. A number of multisubunit membrane proteins destined for the plasma membrane appear to require some degree of assembly as a prerequisite for exit from the ER, and this has been proposed as a "quality-control" step in assembly and transport of these proteins (reviewed in Hurtley and Helenius, 1989). Such a mechanism is an attractive model for transport and assembly of the integral membrane subunits of the vacuolar ATPase, but we cannot at present distinguish between this model and a model that would invoke a positive vacuolar targeting signal on the 17-kD subunit responsible for directing transport of both the 17- and 100-kD subunits. We are attempting to determine conclusively whether the integral membrane subunits of the vacuolar H^+-ATPase do not indeed follow the same route to the vacuole as other vacuolar membrane proteins by examining the transport of the 100-kD subunit in yeast mutants blocked in transport at the ER (*sec18* mutants) and the Golgi apparatus (*sec7* mutants) (Novick *et al.*, 1981). The results of these experiments may also shed light on the site of assembly of the integral membrane subunits.

Biosynthesis of the 69-kD Subunit–Protein Splicing

The *VMA1/TFP1* gene was cloned independently

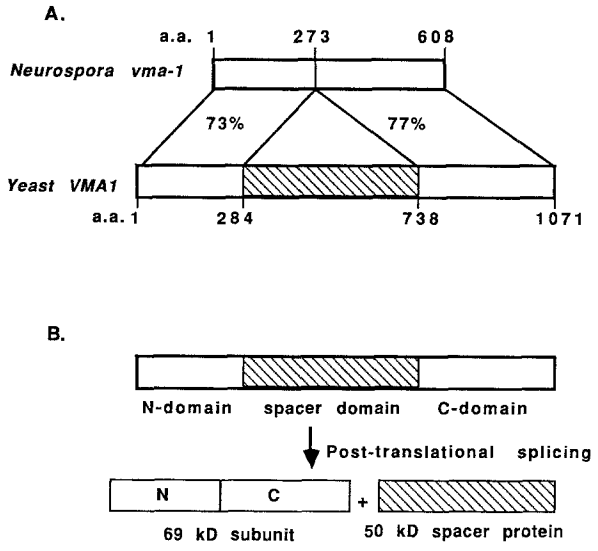

Fig. 3. *Biosynthesis of the 69 kD subunit by protein splicing.* A. Homology of the N and C-terminal thirds of the Neurospora 70 kD subunit gene (*vma-1*) to the N and C-terminal thirds of the yeast 69 kD subunits (the N and C domains). The homology is interrupted abruptly in the *VMA1* gene by the spacer domain. Percentages refer to amino acid identities between the two genes. B. The 69 kD subunit appears to be generated from the *VMA1* gene product by post-translational cleavage and splicing of a 119 kD precursor to give both the 69 kD subunit and a 50 kD spacer protein.

by Shih *et al.*, (1988) from a mutant exhibiting resistance to trifluoperazine and by Hirata *et al.*, (1990) using peptide sequences of the 69-kD subunit of the yeast vacuolar H^+-ATPase. The *VMA1* gene encodes an open reading frame of 1071 amino acids, corresponding to a protein of predicted molecular mass 119 kD. As diagrammed in Fig. 3, the N- and C-terminal thirds of the gene (designated the N and C domains) exhibit very high homology to the *Neurospora vma-1* gene (Bowman *et al.*, 1988), which encodes the 70-kD subunit of the *Neurospora* vacuolar H^+-ATPase. All of the tryptic peptides sequenced by Hirata *et al.* (1990) from the yeast vacuolar H^+-ATPase 69-kD subunit are encoded by the N or C domain, and monoclonal antibodies raised against this subunit recognize epitopes in one of those two domains (Kane *et al.*, 1990). These two domains flank an internal third of the gene that exhibits no homology to any vacuolar H^+-ATPase subunit (the spacer domain). This region does not appear to be an intron in any classical sense; Northern blots using probes exclusively against the N, spacer, or C domains all reveal a single polyadenylated mRNA of 3.7 kD, which appears to correspond to the entire open reading frame (Hirata *et al.*, 1990; Kane *et al.*, 1990). Based on these results, it was proposed that the spacer domain might be removed post-translationally by a protein splicing mechanism.

Support for protein slicing was provided by experiments with *vma1* mutants containing genetically altered spacer domains (Kane *et al.*, 1990). Wild-type cells produce approximately equal amounts of the 69-kD subunit of the vacuolar H^+-ATPase and a stable 50-kD protein (called the spacer protein) from the *VMA1* gene. Introduction of a four-base deletion in the spacer domain yielded a mutant with a *vma1*Δ phenotype (based on its pH-sensitive growth) that produced a truncated protein containing epitopes from the N and spacer domains, but not the C domain. This product was consistent with continous translation through the N and spacer domain junction and termination at a new stop codon brought into frame by the deletion. A second frameshift mutation, which corrected the reading frame before the stop codon was reached, restored ATPase function and production of the 69-kD ATPase subunit and the 50-kD spacer protein. This result argues strongly that continuous translation of the spacer domain is required for production of the 69-kD subunit. However, it does not appear that the presence of the spacer domain is necessary for synthesis of the yeast 69-kD subunit or biogenesis of the vacuolar H^+-ATPase as a whole. A complete deletion of the spacer domain, which left only the domains with homology to other vacuolar H^+-ATPase 70-kD subunits, resulted in production of a normal 69-kD subunit and a fully functional vacuolar H^+-ATPase. Thus, from the point of view of the vacuolar H^+-ATPase, the presence of the spacer domain seems to be largely incidental, although failure to properly remove this domain abolishes ATPase function.

A number of attempts to identify the predicted 119-kD precursor protein produced from the intact *VMA1* gene were unsuccessful (Kane *et al.*, 1990). Pulse-chase studies revealed only the 69- and 50-kD proteins even at pulse-labeling times as short as two minutes. *In vitro* transcription and translation experiments using a rabbit reticulocyte lysate translation system also produced only the 69-kD and 50-kD species. Even in *E. coli*, expression of the *VMA1* gene appeared to yield a properly spliced protein. These data indicate that either the cellular apparatus utilized in protein splicing is very highly conserved (existing in bacteria, fungi, and mammals) or else the splicing event is autocatalytic.

A more thorough mutagenic analysis of the

VMA1 gene promises to generate new insights into the mechanism of splicing. Preliminary experiments indicated that a three-base pair deletion near the junction of the N and spacer domains yielded a mutant with a *vma1*Δ phenotype and an apparently unspliced 119-kD protein, in addition to some smaller proteins that appear to be products of partial splicing (P. M. Kane and T. H. Stevens, unpublished data). A series of deletion and missense mutations is now under construction, and preliminary results suggest that alterations in the predicted splice junctions or the spacer domain prevent splicing, but in contrast, most of the N and C domains can be deleted without affecting splicing (A. Cooper, Y.-J. Chen, and T. H. Stevens, unpublished data). These results indicate that the spacer domain contains important imformation for protein splicing, but do not distinguish between "enzymatic information," such as formation of an active site or even a specific site recognized by another enzyme, and "conformational information," which might include the capability to fold into a conformation required for autocatalytic splicing. Drawing distinctions between these two models and developing a clear mechanism for protein splicing will once again require a combination of genetic and biochemical studies.

CONCLUSIONS

The combination of genetics, biochemistry, and cell biology available in yeast has been fruitfully applied to a number of basic questions in cell biology. This combination of techniques has also been extremely valuable in clarifying the structure, function, and biosynthesis of the yeast vacuolar H^+-ATPase. Future studies of the yeast enzyme will focus on: (1) a more complete description of the biosynthesis, assembly, and targeting of the enzyme, (2) characterization of the set of proteins that is required for ATPase function but not part of the final complex, and (3) examination of the subunit interactions and higher-order structure of the enzyme. The similarity of the yeast vacuolar H^+-ATPase to the vacuolar H^+-ATPases of other eukaryotes indicates that results obtained on the yeast enzyme will be directly applicable in other systems.

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