Journal of Bioenergetics and Biomembranes, Vol. 31, No. 1, 1999

Assembly of the Yeast Vacuolar Proton-Translocating ATPase

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Received November 20, 1998

The yeast vacuolar proton-translocating ATPase (V-ATPase) is the best characterized member of the V-ATPase family. Biochemical and genetic screens led to the identification of a large number of genes in yeast, designated *VMA*, encoding proteins required to assemble a functional V-ATPase. A total of thirteen genes encode subunits of the final enzyme complex. In addition to subunit-encoding genes, we have identified three genes that code for proteins that are not part of the final V-ATPase complex yet required for its assembly. We refer to these nonsubunit Vma proteins as assembly factors, since their function is dedicated to assembling the V-ATPase. The assembly factors, Vma12p, Vma21p, and Vma22p are localized to the endoplasmic reticulum (ER) and aid the assembly of newly synthesized V-ATPase subunits that are translocated into the ER membrane. At least two of these proteins, Vma12p and Vma22p, function together in an assembly complex and interact directly with nascent V-ATPase subunits.

KEY WORDS: Yeast; V-ATPase; assembly; vacuoles; multisubunit; endoplasmic reticulum; membrane proteins; proton-translocating.

INTRODUCTION

The vacuole is a large multilobed organelle that plays a central role in maintaining cellular homeostasis in the yeast *Saccharomyces cerevisiae*. The vacuole is responsible for a variety of physiological processes including pH and ion regulation, amino acid storage, metal detoxification, and protein degradation (Jones *et al.*, 1997). One feature of a fully functioning vacuole is that the lumen or interior of the vacuole remains more acidic (pH $\approx$ 6) than the surrounding cytosol. In yeast, a V-type ATPase (V-ATPase) functions to acidify the vacuole by the translocation of protons into the lumen of the vacuole. The hydrolysis of ATP by the V-ATPase is required to drive the movement of protons from the cytosol into the vacuole lumen.

The V-ATPase found on the vacuolar membrane in yeast is the most extensively characterized member of the V-type ATPase family. V-ATPase complexes are present in all eukaryotic cells and are required for a range of cellular processes including receptor-mediated endocytosis, renal acidification, bone reabsorption, neurotransmitter accumulation, and activation of acid hydrolases (Stevens and Forgac, 1997). The proton gradient generated by the V-ATPase in yeast is utilized by other membrane-bound transporters to drive the accumulation of ions, amino acids, and metabolites into the vacuole (Forgac, 1989). A second V-type ATPase is also present in a nonvacuolar location within yeast cells, possibly on the membranes of the Golgi and/or endosomes, and serves to acidify these earlier organelles of the secretory pathway (Manolson *et al.*, 1994).

Yeast provide an excellent model system in which to study the structure and assembly of this multisubunit V-ATPase complex. Similar to the F_1F_0-ATPase of mitochondria, the V-ATPase is composed of a catalytic V_1 subcomplex of peripherally associated proteins localized on the cytosolic face of the vacuolar membrane. The V_1 subcomplex is assembled onto the V_0 subcomplex, which is composed of both peripheral

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0145-479X/99/0200-0039$16.00/0 © 1999 Plenum Publishing Corporation

and integral membrane proteins (Fig. 1). The assembly of the multisubunit V-ATPase occurs by a complex process involving the coordinated association of subunits synthesized in the cytosol with subunits that enter the secretory pathway upon synthesis and are targeted to the vacuole.

Through the examination of various mutant yeast cells, lacking either a V_1 or V_0 subunit, we have observed two different assembly phenotypes that describe the fate of the remaining subunits in the cell. Generally, the loss of a V_1 subunit has little or no effect on the stability of the remaining V_1 subunits, but does prevent the assembly of the remaining V_1 subunits onto the vacuolar membrane. In cells lacking a V_1 subunit, the V_0 subunits are stable, assembled normally, and present on the vacuolar membrane. The ability to assemble all the V_1 subunits onto the V_0 subcomplex does require the presence of all the V_1 subunits (except the 54-kDa subunit). In contrast, the loss of a V_0 subunit affects the stability, assembly, or localization of the remaining V_0 subunits, while the V_1 subunits are stable but present in the cytosol of cells lacking a V_0 subunit.

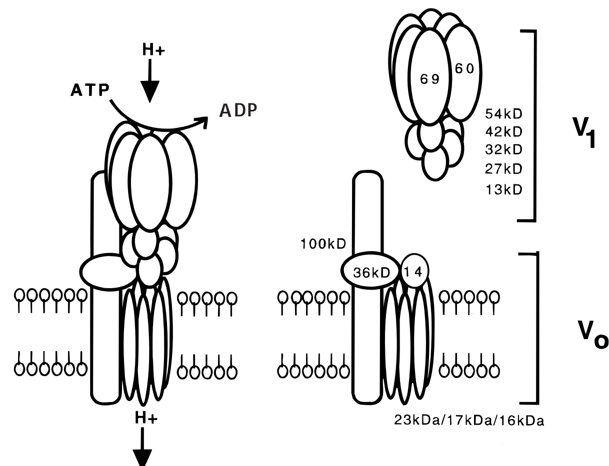

Fig. 1. Proposed structure and subunit composition of the yeast V-ATPase. The yeast V-ATPase is composed of two large multisubunit subcomplexes designated V_1 and V_0. The V_1 subcomplex of peripherally associated subunits contains proteins of predicted molecular mass 69 kDa (Vma1p), 60 kDa (Vma2p), 54 kDa (Vma13p), 42 kDa (Vma5p), 32 kDa (Vma8p), 27 kDa (Vma4p), and 13 kDa (Vma10p). The peripherally associated subunits interact with the membrane via the V_0 subcomplex of integral and peripheral subunits of molecular mass 100 kDa (Vph1p or Stv1p), 36 kDa (Vma6p), and the proteolipids 23 kDa (Vma16p), 17 kDa (Vma11p), and 16.5 kDa (Vma3p) forming the proton channel through the membrane. The 14 kDa Vma7p may interact with both the V_1 and V_0 subcomplexes (see text for details).

In this review, we will focus on the current topics regarding the assembly of the yeast V-ATPase. We will first address the expanding subunit composition of the functional enzyme complex as it is found on the membrane of the yeast vacuole. Through the analysis of mutant cells lacking a V-ATPase subunit, we can categorize subunits as being either components of the V_1 or V_0 subcomplexes and determine if they are peripherally or integrally associated with the vacuolar membrane. The analysis of mutant cells also allows us to propose a possible function for some subunits. Progress made in characterizing the structurally and functionally related F_1F_0-ATPase help guide our thinking about the roles of analogous subunits in the V-ATPase.

Second, we will summarize our work illuminating the role of three nonsubunit proteins that are required for the assembly of the yeast V-ATPase. We refer to these nonsubunit proteins as “assembly factors” since they are localized to the endoplasmic reticulum (ER) and not the vacuolar membrane and, by all criteria, their function is dedicated to the assembly of the V-ATPase. Finally, we will discuss our current models describing the early steps in V-ATPase assembly.

IDENTIFICATION OF *VMA* GENES

Purified yeast V-ATPase was isolated by fractionation of detergent solubilized vacuolar membranes on a glycerol density gradient. The V-ATPase subunits showed a biphasic distribution in the gradients representing a V_0 subunit containing fraction separated from the fully assembled and active V_1V_0 fraction (Kane *et al.*, 1992). Biochemical analysis of purified yeast V-ATPase (V_1V_0 fractions) suggested at least ten potential subunits form the enzyme complex, all ranging in molecular mass from 14 to 100 kDa (Kane *et al.*, 1989). Biochemical and genetic analyses have allowed the identification of a large number of *VMA* (*v*acuolar *m*embrane *A*TPase) genes that encode proteins required for the assembly of the yeast V-ATPase complex. Currently, at least thirteen *VMA* genes that encode subunits of the yeast V-ATPase enzyme complex have been identified, cloned, and characterized (Table I).

Yeast cells lacking V-ATPase activity remain viable, but the yeast do display several growth phenotypes. Cells lacking any V-ATPase subunit because of disruption of a *VMA* gene exhibit a characteristic set of growth phenotypes related to loss of V-ATPase activity, such as increased sensitivity to calcium in the growth

Table I. Yeast V-ATPase Subunits and Assembly Factors

Gene	Apparent Mol. wt.	Peripheral/ integral	Subcomplex
VPH1/STV1	100/102	Int.	V_0 subunit
VMA1 (*TFP1*)	69	Per.	V_1 subunit
VMA2 (*VAT2*)	60	Per.	V_1 subunit
VMA13	54	Per.	V_1 subunit
VMA5	42	Per.	V_1 subunit
VMA6	36	Per.	V_0 subunit
VMA8	32	Per.	V_1 subunit
VMA4	27	Per.	V_1 subunit
VMA16	23	Int.	V_0 subunit
VMA11	17	Int.	V_0 subunit
VMA3	16.5	Int.	V_0 subunit
VMA10	16	Per.	V_1 subunit
VMA7	14	Per.	V_1/V_0 subunit
VMA12	25	Int.	Assembly
VMA21	8	Int.	Assembly in ER
VMA22	21	Per.	Assembly

media, inability to grow in media buffered to neutral pH, and inability to utilize nonfermentable sources of carbon (i.e., glycerol or ethanol), similar to respiratory deficient cells. In this review, we will characterize the more recent additions to the list of *VMA* genes and the possible functions for the proteins they encode.

EXPANDED SUBUNIT COMPOSITION OF THE V_1 SUBCOMPLEX

Of the thirteen total V-ATPase subunits, eight polypeptides have been identified as subunits of the yeast V_1 subcomplex (Table I). The V_1 subcomplex is composed of polypeptides of 69, 60, 54, 42, 32, 27, 16, and 14 kDa. The Vma1p (69 kDa; Hirata *et al.*, 1990) and Vma2p (60 kDa; Nelson *et al.*, 1989) together form the nucleotide binding and catalytic subunits analogous to the α and β subunits of the F_1F_0-ATPase complex. Multiple copies of these subunits are present in each enzyme complex, assembling to form the catalytic ATP-hydrolyzing portion of the V-ATPase. The remaining V_1 subunits may play a role in bridging the catalytic subunits to the V_0 subunits, analogous to the stalk subunits of the F_1F_0-ATPase. However, no strong sequence similarity has been observed between the remaining V-ATPase subunits when compared to the γ, δ, and ε subunits of the F_1F_0-ATPase. Thus, it is more likely that the V-ATPase V_1 subunits share structural homology, as opposed to protein sequence homology, with the F-ATPase stalk subunits.

The 27-kDa V-ATPase subunit was found to be encoded by the *VMA4* gene (Foury, 1990). The identification of *VMA5* as encoding a subunit of the V-ATPase was a direct result of a genetic screen to isolate yeast mutants unable to grow on media buffered to a neutral pH (Ho *et al.*, 1993a). The sequence of the protein encoded by the *VMA5* gene exactly matched the peptide sequence derived from the V-ATPase 42-kDa subunit. Cells lacking either Vma5p or Vma4p prevented the assembly of either Vma1p or Vma2p onto the membranes of the vacuole, consistent with these proteins being required components of the V_1 subcomplex.

VMA13 codes for a 54-kDa hydrophilic protein that copurifies with the V-ATPase complex (Ho *et al.*, 1993b). Vma13p was shown to be a protein peripherally associated with vacuolar membranes. Remarkably, cells lacking Vma13p are still able to assemble a V-ATPase on the vacuole, although the assembled complex lacks ATPase activity. The assembled V_1 subunits appear to be more loosely associated with the membranes in the absence of Vma13p and can be released from the membranes simply by low-salt treatment (Ho *et al.*, 1993b). These results suggest a very interesting role for Vma13p, namely, functioning to regulate the activity of the assembling V-ATPase complex. Vma13p would be required only after all the V_1 subunits have assembled onto the integral membrane V_0 subunits; the association (reversible?) of Vma13p with the V-ATPase would serve to activate the enzyme complex.

The *VMA7* gene encodes a novel 14-kDa hydrophilic protein, that displays characteristics common to both V_1 and V_0 subunits (Graham *et al.*, 1994; Nelson *et. al.*, 1994). Cells lacking Vma7p have normal levels of V_1 subunits, but they fail to associate with vacuolar membranes (V_1-like). Vma7p fractionates only with the active solubilized V-ATPase complex (V_1V_0) on glycerol density gradients consistent with its V_1-like behavior (Graham *et al.*, 1994). In addition, Vma7p can be released from purified vacuolar membranes by treatment with alkaline carbonate, a condition that liberates peripherally associated membrane proteins. Surprisingly, cells lacking Vma7p also show reduced levels of V_0 subunits (100-kDa protein and 17-kDa proteolipid) on the vacuolar membrane. The stability of the 100-kDa V_0 subunit is decreased to a half-time of 45 min in the absence of Vma7p (L. Graham, unpublished results 1995) compared to a half-life in excess of 400 min in wild-type cells. Thus far, Vma7p

is the only V-ATPase subunit demonstrated to affect both the V_1 and V_0 subcomplexes, suggesting a role as a bona fide stalk subunit bridging the two subcomplexes. We are currently performing a mutational analysis of Vma7p in an attempt to separate the two functions that this protein plays in V-ATPase assembly.

A prominent 32-kDa protein was easily identified in Coomassie stained SDS-PAGE gels of purified yeast V-ATPase complex (Kane *et al.*, 1989) and was found to be encoded by the *VMA8* gene (Graham *et al.*, 1995; Nelson *et al.*, 1995). The loss of Vma8p from yeast prevents the assembly of the remaining V_1 subunits onto the vacuolar membrane. Vma8p behaves as a typical peripherally associated V_1-like subunit, which could be released from the membranes in the presence of reagents, such as alkaline carbonate and urea. In addition, Vma8p was released from vacuolar membranes in an ATP-dependent treatment with potassium nitrate. The release of Vma8p, as well as other V_1 subunits, in the presence of ATP presumably reflects a conformational change of the V_1 subcomplex due to the binding or hydrolysis of ATP. The ATP-induced conformational change appears to affect the physical interaction of V_1 subunits with the membrane now making them susceptible to release from the V_0 subcomplex upon treatment with chaotropic reagents (e.g., 100 m*M* NO_3^-). The specific release of V_1 subunits by this treatment directly reflects their role in forming the ATP hydrolyzing portion of the complex. Conversely, the lack of ATP-stimulated release of V_0 subunits is consistent with their role in proton translocation and not in forming the ATP hydrolyzing portion of the complex. Consistent with Vma8p being a V_1 subunit, cells lacking Vma8p assembled a normal V_0 subcomplex that was targeted to vacuolar membranes.

VMA10 is a member of a small subset of yeast genes whose opening reading frames are interrupted by an intron immediately following the initiating methionine (Supekova *et al.*, 1995). The *VMA10* gene encodes a hydrophilic protein of 14 kDa, about the same size as Vma7p, but Vma10p migrates as a protein of slightly slower electrophoretic mobility, a behavior observed in a Vma10p homolog identified in another organism, the tobacco hornworm (Lepier *et al.*, 1996; Supekova *et al.*, 1995). The yeast Vma10p was originally characterized as a component of the V_0 subcomplex because it remained associated with the membrane after being subjected to a cold inactivation treatment in the presence of ATP (Supekova *et al.*, 1995). More recently, we have demonstrated by several different criteria that Vma10p behaves as a model peripherally associated V_1 subunit (Tomashek *et al.*, 1997). Vma10p could be released from membranes by ATP-dependent treatment with nitrate, arguing that Vma10p is a component of the catalytic V_1 subcomplex. In yeast cells lacking Vma10p, the V_0 subcomplex assembles normally and is targeted to the vacuole, indicating that Vma10p is a V_1 subunit. Typically, the absence of a V_1 subunit has no affect on the remaining V-ATPase subunits in the cell; they are stable in the cytosol. Interestingly, it has been observed that the loss of Vma10p results in an increased rate of turnover of the Vma4p, exclusively, with little effect on the stability of the other V_1 subunits (Tomashek *et al.*, 1997). This observation together with chemical cross-linking data indicate that Vma10p and Vma4p form a subcomplex and very likely interact directly with each other in the fully assembled V-ATPase (Tomashek *et al.*, 1997).

ASSEMBLY OF THE V_0 SUBCOMPLEX

The yeast V_0 subcomplex is composed of a single 100-kDa polytopic membrane protein, a 36-kDa peripheral protein, and three extremely hydrophobic proteins collectively referred to as proteolipids of molecular mass 16.5, 17, and 23 kDa (Stevens and Forgac, 1997). Similar to the V_1 subcomplex, all V_0 subunits must be present in the cell in order to assemble the V_0 subcomplex. As previously described, the V_1 subcomplex cannot assemble on to the vacuolar membrane in the absence of the V_0 subcomplex. In contrast, the V_0 subcomplex does assemble, is stable, and successfully transported to the vacuole even in cells lacking a V_1 subunit.

The 100-kDa V-ATPase subunit of the V_0 subcomplex is encoded by two genes in yeast, designated *VPH1* and *STV1* (Manolson *et al.*, 1992, 1994). The proteins Vph1p and Stv1p are functional isoforms, but serve as components of V-ATPase complexes functioning at different locations in the cell. The Vph1p is a subunit of the V-ATPase found on the vacuolar membrane, whereas Stv1p is a subunit of a second V-ATPase found on the membranes of the Golgi and/or endosomal membranes. Neither of the genes encoding the 100-kDa proteins is designated as a *VMA* gene because loss of either Vph1p or Stv1p alone does not result in a complete Vma growth phenotype. Although vacuolar membranes from cells lacking Vph1p have less than 1% V-ATPase activity, these cells are still able to grow in media buffered to neutral pH. Only when cells lack both Vph1p and Stv1p do they exhibit

the full range of Vma phenotypes (Manolson *et al.*, 1994), suggesting that these proteins are functionally interchangeable.

The sequence of both Vph1p and Stv1p predict that the proteins are divided into a hydrophilic amino-terminal half and a hydrophobic carboxyl terminus forming several (6 or 7) membrane-spanning domains. Similar to other V_0 subunits, the newly synthesized 100-kDa proteins are inserted into the ER membranes, assembled together with other V_0 subunits and possibly V_1 subunits, and then targeted to the vacuole or Golgi/endosome by the secretory pathway. Loss of the 100-kDa proteins blocks the assembly of the V_0 subcomplex and, therefore, the V-ATPase never assembles. The two V-ATPase complexes identified in yeast appear to differ only by whether they contain Vph1p or Stv1p, yet both are composed of the same V_1 subunits and assemble with the same V_0 subunits. It is tempting to speculate that either Stv1p contains information within its protein sequence that allows it to localize to and be retained within an organelle other than the vacuole or Vph1p contains a targeting signal directing it to the vacuole, thus achieving their different cellular localization.

The stability of the 100-kDa V-ATPase subunits is determined by their ability to properly assemble into the newly forming V_0 subcomplex. In cells lacking any one of the V_0 subunits, the 100-kDa polypeptides are destabilized resulting in a half-time of turnover of approximately 30 min compared to the half-life in wild-type cells that is typically in excess of 400 min, which represents several rounds of cell division (Graham *et al.*, 1998). We have observed that the increased turnover rate of the 100-kDa V-ATPase subunit in cells lacking a V_0 subunit was not dependent on vacuolar localized proteases. Recall, the vacuole contains a collection of proteases responsible for degrading proteins once proteins have been designated for turnover. The increased degradation rate of the 100-kDa subunit was not slowed in cells also carrying a mutation in the *PEP4* gene, which encodes a key protease required for the protease activation cascade in the yeast vacuole (Hill and Stevens, 1994, 1995; Jackson and Stevens, 1997). Since the absence of vacuolar proteases did not affect the degradation of the 100-kDa subunit in cells also lacking a V_0 subunit, we conclude that the turnover must occur in another cellular location.

The stability of the 100-kDa V-ATPase subunits (Vph1p and Stv1p) is ensured by their ability to assemble with other V_0 subunits including the 36-kDa subunit encoded by the *VMA6* gene (Bauerle *et al.*, 1993). Vma6p is a hydrophilic protein lacking any apparent transmembrane domains. It is peripherally associated with the V_0 subcomplex and can be released from the membranes by treatment with alkaline carbonate or the denaturing reagent urea. Although Vma6p is a peripherally associated protein, it is not released from membranes by ATP nitrate treatment, consistent with it being a component of the V_0 proton translocating subcomplex and not the catalytic V_1 subcomplex (Tomashek *et al.*, 1997).

In the absence of Vma6p, the 100-kDa protein Vph1p is destabilized and rapidly degraded, presumably since it is unable to assemble with Vma6p (Graham *et al.*, 1998). The lack of Vma6p also affects the proteolipid subunits since they are now prevented from reaching the vacuolar membrane (Bauerle *et al.*, 1993). We have not determined if the stability of the proteolipids is also affected by the loss of Vma6p. Unlike the 100-kDa protein Vph1p, the Vma6p is not destabilized in cells lacking a V_0 subunit, such as a proteolipid. In the absence of V-ATPase assembly, Vma6p is synthesized normally, remains stable in the cytosol, but fails to assemble onto cellular membranes (Graham *et al.*, 1998). We have yet to identify what mediates the peripheral association of Vma6p with membranes and whether is occurs via the 100-kDa protein or the proteolipids is the basis for future experiments.

The membrane-spanning proton-translocating channel of the V_0 subcomplex is formed by the assembly of three related hydrophobic membrane proteins encoded by the *VMA3*, *VMA11*, and *VMA16* genes (Hirata *et al.*, 1997). These proteins are collectively referred to as proteolipids because of their ability to be isolated from vacuolar membranes by extraction into a chloroform–methanol solution. Similar to the 100-kDa proteins, the proteolipids are inserted into the membranes of the ER following synthesis, where they assemble with the V-ATPase subunits. They are then targeted to the vacuole by the secretory pathway.

All three proteolipids, Vma3p, Vma11p, and Vma16p share a high degree of amino acid sequence identity and presumably similar membrane topography. *VMA3* encodes for a protein of 16.5-kDa that is predicted to form four transmembrane-spanning domains positioning both the N- and C-terminus exposed to the lumen of the vacuole (Nelson and Nelson, 1989; Umemoto *et al.*, 1990). The 17-kDa protein, encoded by the *VMA11*, is 56% identical to the amino acid sequence of Vma3p (Hirata *et al.*, 1997; Umemoto *et al.*, 1991). *VMA16* encodes a slightly larger protein of 23-kDa and is the least similar of the three proteins,

being only 35% identical to Vma3p (Apperson *et al.*, 1990; Hirata *et al.*, 1997). An additional protein sequence at the N-terminus of Vma16p, absent in both Vma11p and Vma3p, may possibly form a fifth transmembrane domain.

The phenotypes observed in cells lacking either Vma3p, Vma11p, or Vma16p are identical to one another. Loss of any one of the proteolipids resulted in failure to assemble the V-ATPase and increased turnover of the 100-kDa protein Vph1p (Hirata *et al.*, 1997). The Vma6p is present in normal levels in cells lacking any one of the proteolipids, yet Vma6p is not associated with the membrane in these cells. The V_1 subunits were present at normal levels in proteolipid-deficient yeast, but none were found assembled onto the vacuolar membranes. It will be interesting to determine how the loss of one proteolipid affects the stability or localization of the remaining proteolipid subunits. We have observed that cells lacking Vma16p still contain a proteolipid of approximately 17 kDa (possibly the Vma3p), although in reduced amounts, however, this remaining proteolipid is not found on vacuolar membranes (Hirata *et al.*, 1997). It is possible that failure to assemble all three proteolipid subunits together prevents them from leaving the ER and being targeted to the vacuole.

Each of the proteolipids contains a highly conserved glutamic acid residue (Vma3p Glu^{137}, Vma11p Glu^{145}, and Vma16p Glu^{108}) that is required for proton-translocating activity (Hirata *et al.*, 1997). Modification of this key glutamic acid residue to glutamine inactivates the V-ATPase enzyme complex, although it appears to be fully assembled. Interestingly, it only requires the introduction of the gene encoding one of these mutant proteolipids in order to inactivate all the V-ATPase enzyme complexes in the cell. This suggests that these proteins, Vma3p, Vma11p, and Vma16p share a common, but not redundant, function in the cell.

Unlike the F_1F_0-ATPase proton channel that is formed by association of multiple copies of the c subunit, formation of the V-ATPase channel requires the presence of all three proteolipids. The actual stoichiometry of Vma3p, Vma11p, and Vma16p in the V_0 complex remains to be determined. Comparison of the three proteins localized to vacuolar membranes by immunoblot analysis clearly shows that Vma3p is more abundant than either Vma11p or Vma16p (Hirata *et al.*, 1997). In our model for V-ATPase assembly, every V_0 complex is an assembled mixture of all three proteolipids with the Vma3p present in more copies relative to either Vma11p or Vma16p. It remains a challenge to determine how the assembly of these three proteins is coordinated, especially if they are present in different ratios within the individual V-ATPase complex.

CHARACTERIZATION OF THE V-ATPase ASSEMBLY FACTORS

In addition to structural V-ATPase subunits, genetic analysis has also lead to the identification of three *VMA* genes (*VMA12*, *VMA21*, and *VMA22*) that encode proteins required to assemble a functional V-ATPase, but are not subunits of the final enzyme complex. *VMA12* was identified by a genetic screen for calcium-sensitive mutants that were also unable to grow on a nonfermentable carbon source (Ohya *et al.*, 1991). The cloned *VMA12* gene encodes a 25-kDa membrane protein predicted by hydropathy analysis to contain two membrane-spanning helices (Hirata *et al.*, 1993). Loss of the Vma12p resulted in mutant cells displaying the full set of Vma phenotypes including loss of V-ATPase activity and failure to grow on media buffered to pH 7.5. Biochemical analysis describing the fate of the remaining V-ATPase subunits in cells lacking Vma12p revealed an assembly state identical to the loss of a V_0 subunit.

Initial characterization of cells lacking the Vma12p revealed that all of the V_1 subunits were present and localized to the cytosol, but not associated with the vacuolar membrane. The V_0 subunits were present in greatly reduced amounts in these mutant cells and also were not localized to the vacuolar membranes. The surprising result was that the Vma12p did not fractionate with the purified V-ATPase, indicating it is not a component of the active complex. Yet, in the absence of Vma12p, the V_0 subcomplex was unable to assemble. Vma12p was the first protein identified that is required for functional V-ATPase assembly, but not itself a subunit of the enzyme complex.

A separate genetic screen for mutants unable to grow at pH 7.5 identified two additional genes whose products are required for V-ATPase assembly: *VMA21* and *VMA22* (Ho *et al.*, 1993a). *VMA21* encodes a small 8.5-kDa hydrophobic protein predicted to span the membrane twice (Hill and Stevens, 1994). Cellular localization of an Vma21p by immunofluorescence revealed that this protein resides in the ER membranes. Mutations in a third gene, *VMA22*, encoding a 21-kDa hydrophilic protein, also prevented the assembly of the V-ATPase (Hill and Stevens, 1995). Cells lacking Vma22p were phenotypically identical to cells lacking

Vma12p, or Vma21p. Vma22p and Vma12p have also been immunolocalized to the membranes of the ER and not the vacuolar membrane, which explains their failure to associate with purified vacuolar V-ATPase.

CHARACTERIZATION OF AN ASSEMBLY COMPLEX

Since all three of the V-ATPase assembly factors are localized to the ER, we have focused on investigating interactions between them. Vma22p and Vma12p provided the first evidence for an interaction between assembly factors and suggested the presence of an assembly complex. The ability of the hydrophilic Vma22p to associate with membranes was found to be dependent on the presence of the integral membrane protein Vma12p, suggesting that Vma12p serves either directly or indirectly in linking Vma22p and the ER membrane (Hill and Stevens, 1995). A direct physical interaction between Vma12p and Vma22p was confirmed through the use of chemical cross-linking reagents under conditions that would form covalent cross-links only between closely associated proteins (Graham *et al.*, 1998). Even after detergent solubilization of cellular membranes with 1% Triton X-100, Vma12p and Vma22p remain strongly associated in a complex. Therefore, Vma12p and Vma22p function together and form a stable V-ATPase assembly complex.

Cells lacking Vma21p clearly block the assembly of the V-ATPase and result in rapid degradation of the unassembled 100-kDa protein, Vph1p. Because of the extremely hydrophobic nature of Vma21p, it has been more difficult to determine if it is also a component of the Vma12p/Vma22p assembly complex. Under conditions of Triton X-100 solubilization of membranes, Vma21p fractionates away from the Vma12p/Vma22p complex on a density gradient (Graham *et al.*, 1998). It is entirely possible that the solubilization and fractionation conditions disrupted the interactions between Vma21p and the assembly complex. Future experiments will be directed toward identifying interactions between Vma21p and V-ATPase subunits and between Vma21p and the assembly complex.

INTERACTIONS BETWEEN THE ASSEMBLY FACTORS AND V-ATPase SUBUNITS

We were curious to identify which of the V-ATPase subunits interacted with the Vma12p/Vma22p assembly complex. An expansion of our crosslinking analysis revealed a direct interaction between the Vma12p/Vma22p assembly complex and newly synthesized 100-kDa protein, Vph1p. Protease protection studies confirmed that Vph1p was inserted normally into membranes prepared from cells lacking Vma12p (Jackson and Stevens, 1997). Thus, we were able to rule out a possible role for the Vma12p/Vma22p complex in aiding the translocation or folding of Vph1p in the ER membrane. The Vma12p/Vma22p complex must then function at a later step after these early translocation and folding events.

A kinetic analysis of the interaction between newly synthesized Vph1p and Vma12p/Vma22p revealed that the interaction was transient with a half-life of less than 5 min (Graham *et al.*, 1998). This brief interaction between the assembly complex and Vph1p suggested it occurs only while the newly synthesized V-ATPase subunits are assembling in the ER. The short half-life of this interaction also argued against the assembly complex proteins remaining associated with the V-ATPase until it reaches the vacuole. Interestingly, if the transport of newly synthesized proteins from the ER is blocked, by use of the conditional *sec12-4* mutation, we observed a stable interaction between the assembly complex and Vph1p (Graham *et al.*, 1998). Continuous protein exit from the ER, mediated by packaging of the V-ATPase complex into ER-derived vesicles appears to disrupt the association of the assembly complex with the V-ATPase subunits. This suggests a coordination between the interaction of V-ATPase subunits with ER-localized assembly factors and the vesicular movement of proteins leaving the ER.

ASSEMBLY OF THE VACUOLAR ATPase

The assembly of the yeast V-ATPase not only requires the presence of all the subunit proteins, but also the ER localized proteins, Vma12p, Vma21p, and Vma22p. These proteins play an essential role in aiding the assembly of the newly synthesized V_0 subunits. Failure to properly assemble the V_0 subcomplex, because of either loss of a V_0 subunit or assembly factor, targets the 100-kDa protein Vph1p, and possibly other V_0 subunits, for rapid degradation. In addition, our preliminary results have revealed an interaction between Vma21p and newly synthesized proteolipids (P. Malkus, R. Schekman, L. Graham, and T. Stevens, unpublished results 1998). Based on these data we can propose a model describing V-ATPase assembly (Fig.

2). The first step would involve the interaction of the completely translated and folded 100-kDa V-ATPase subunit with the Vma12p/Vma22p assembly complex, since we have observed that this interaction between the Vma12p/Vma22p assembly complex and the 100-kDa protein occurs even in the absence of Vma21p (Graham *et al.*, 1998). The role of Vma21p in aiding the assembly of the V-ATPase could then be envisioned as occurring in parallel to the Vph1p/Vma12p/Vma22p interaction and involving assembly of the proteolipids into a subcomplex. Vma21p, being a small hydrophobic protein itself, could serve as scaffolding for the assembly of the hydrophobic proteolipids, Vma3p, Vma11p, and Vma16p into a proton channel.

Because the dilysine ER-retention motif of Vma21p is required for the function and ER localization of this protein, we propose that Vma21p serves an additional role in V-ATPase biosynthesis. Vma21p could associate with the assembled V_0 subcomplex (or fully assembled V_1V_0 complex) in the ER and be transported in vesicles to the Golgi complex. In the early Golgi, Vma21p would dissociate from the V-ATPase and be retrieved back to the ER for another round of assembly/transport. Vma21p could also function to actively load the V-ATPase complex into vesicles budding from the ER, and thus serve as a specific "cargo receptor" (Kuehn and Schekman, 1997). Future experiments will be designed to test elements of the model in Fig. 2 involving Vma21p, such as whether Vma21p is transported out of the ER with the V-ATPase.

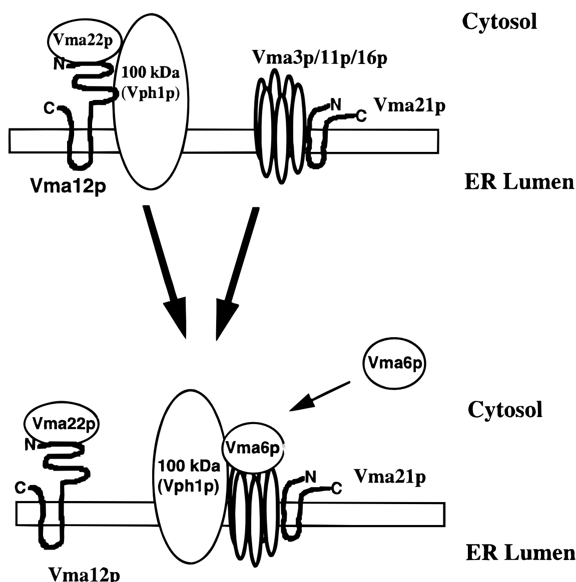

Fig. 2. Model of V-ATPase assembly in the ER. Vma12p and Vma22p interact to form a stable assembly complex in the ER membrane. The assembly complex interacts directly, but transiently, with the 100-kDa protein Vph1p after it is translated, translocated, and folded correctly. The Vph1p must then assemble with the other V_0 subunits in order for the V-ATPase to exit the ER for the vacuolar membrane. In the parallel model, Vma21p would aid the assembly of the proteolipids and possibly Vma6p with the 100-kDa/Vma12p/Vma22p complex followed by the dissociation of the Vma12p/Vma22p assembly factors from the V_0 subunits prior to transport beyond the ER. In the sequential model, Vma21p would also interact with the V_0 subcomplex following release from the Vma12p/Vma22p assembly complex and may even exit the ER with the V-ATPase complex, dissociate from the V-ATPase in the Golgi, and be retrieved back to the ER.

CONCLUSIONS

The ease of genetic manipulation, biochemical, and cell biological analyses in yeast has allowed us to expand our understanding of the assembly, structure, and function of the yeast V-ATPase. The identification of Vma proteins in yeast has, in several cases, initiated the search for homologous proteins in other organisms studied, including humans. The characterization of the three proteolipids, required for formation of the V-ATPase proton channel, introduces a new level of complexity regarding how the recognition and assembly of different ratios of these highly related proteins into the V-ATPase is carried out by the cell. Characterization of the yeast assembly factors provides the first example of an ER-localized, hetero-oligomeric assembly complex whose role is dedicated to the biogenesis of a single multisubunit enzyme complex—the yeast V-ATPase.

ACKNOWLEDGMENTS

We thank Sonja Gerrard and Kate Bowers for helpful discussions and critical reading of the manuscript. This work was supported by a grant from the National Institutes of Health (GM38006) to T.H. Stevens.

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