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Structure and Assembly of the Yeast V-ATPase

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The yeast V-ATPase belongs to a family of V-type ATPases present in all eucaryotic organisms. In *Saccharomyces cerevisiae* the V-ATPase is localized to the membrane of the vacuole as well as the Golgi complex and endosomes. The V-ATPase brings about the acidification of these organelles by the transport of protons coupled to the hydrolysis of ATP. In yeast, the V-ATPase is composed of 13 subunits consisting of a catalytic V_1 domain of peripherally associated proteins and a proton-translocating V_0 domain of integral membrane proteins. The regulatory subunit, Vma13p, was the first V-ATPase subunit to have its crystal structure determined. In addition to proteins forming the functional V-ATPase complex, three ER-localized proteins facilitate the assembly of the V_0 subunits following their translation and insertion into the membrane of the ER. Homologues of the Vma21p assembly factor have been identified in many higher eukaryotes supporting a ubiquitous assembly pathway for this important enzyme complex.

KEY WORDS: V-ATPase; yeast; membrane; assembly; vacuole; multisubunit; Golgi; ATP; proton-translocating; complex.

INTRODUCTION

The yeast V-ATPase belongs to a family of V-type ATPases present in all eukaryotic organisms. V-ATPases are large, multisubunit, membrane-associated enzyme complexes that function to acidify cellular compartments. V-ATPases carry out the active transport of protons across the membrane bilayer that is tightly coupled to the hydrolysis of ATP. The disruption of V-ATPase function is lethal in all eukaryotic organisms tested except *Saccharomyces cerevisiae*, making yeast an ideal system to study the structure, function, and assembly of this important enzyme complex (Nishi and Forgac, 2002).

In yeast, the V-ATPase is primarily localized to the limiting membrane of the vacuole. The proton gradient generated by the vacuolar V-ATPase is utilized by other membrane-bound transporters to drive the accumulation of substrates into the lumen of the vacuole. A smaller population of V-ATPase complexes can be found localized to the late or last compartment of the Golgi as well as the endosomal network, and these V-ATPases also play a role in the acidification of these organelles (Kawasaki-Nishi *et al.*, 2001; Manolson *et al.*, 1994).

The V-ATPase from yeast is the best characterized member of the V-ATPase family because of the ease of genetic and biochemical manipulation of this model organism. Currently, 13 subunits have been identified as required to form a functional enzyme complex (Fig. 1). The composition of the vacuolar localized V-ATPase is identical to the Golgi-localized complex except for one subunit that has two isoforms dictating its localization in the cell (Kawasaki-Nishi *et al.*, 2001; Manolson *et al.*, 1992, 1994).

Genes encoding subunits common to both the Golgi and vacuolar V-ATPase are called *VMA* for *v*acuolar *m*embrane *A*TPase. The difference between the Golgi and vacuolar V-ATPase is the presence of different isoforms of subunit **a**. Surprisingly, cells lacking only the Golgi-localized form of the V-ATPase display no obvious growth defects. Cells lacking only the vacuolar form of the V-ATPase are unable to acidify their vacuoles, yet they display only minimal growth impairment. The loss of both isoforms, Vph1p and Stv1p, is required

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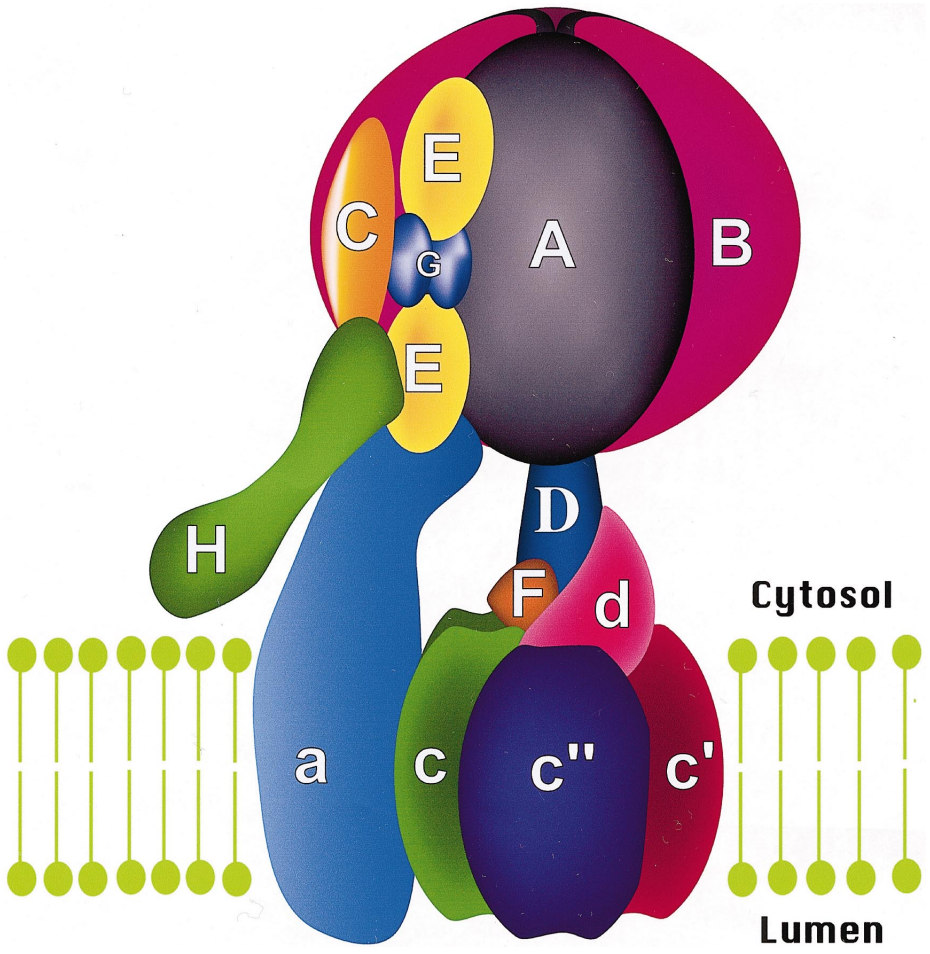

Fig. 1. Model of the yeast V-ATPase. The V_0 subunits a, c, c′, and c″ are all predicted to possess transmembrane domains, the remaining subunits are associated peripherally with the membrane. The Golgi/endosome-localized V-ATPase is identical to the vacuolar V-ATPase except for the isoforms of subunit **a**, the large 100-kDa protein. Stv1p is found associated with the Golgi and endosome localized V-ATPase and Vph1p is associated with the V-ATPase on the vacuole membrane.

before the cells display the Vma-growth phenotypes typical of cells lacking any one of the *VMA* genes (Manolson *et al.*, 1994). This suggests overlapping function between the two differentially localized V-ATPase complexes.

Prior to the complete sequencing of the yeast genome in 1997 and the generation of the yeast genomic deletion strains collection, several biochemical and genetic screens had been employed that had already identified the majority of the genes required to assemble a functional V-ATPase (Table I). Surprisingly, after rescreening the entire yeast genomic deletion collection only one previously unidentified *VMA* gene has been identified (Steinmetz and Stevens, personal communication).

The organization of the yeast V-ATPase subunits is modeled after the well-characterized F-type ATPase. V-ATPase subunits are characterized as belonging to either the V_1 subcomplex of peripherally associated subunits or to the V_0 subcomplex of membrane-associated subunits. The association of all the subunits, the V_1 with the V_0, is required for the formation of a functional enzyme complex (Nishi and Forgac, 2002). Through the analysis of yeast deletion mutants, or cells lacking a specific subunit, we have determined that the V_0 subunits can form a complex in the absence of the V_1 subunits. Similarly, most of the V_1 subunits appear to be able to assemble in the absence of the Vo subunits.

Similar to the F-ATPase, the movement or the rotation of the two subcomplexes, V_1 and V_0, relative to each other is believed to link the translocation of protons across the membrane to the hydrolysis of ATP (Capaldi and Aggeler, 2002; Noji *et al.*, 1997). The increased number of subunits forming the yeast V-ATPase, their varied

Table I. Subunits and Assembly Factors of the Yeast V-ATPase

	Gene	Molecular weights (kDa)	Apparent stoichiometry
Subunit			
V_1			
A	*VMA1*	69	3
B	*VMA2*	60	3
C	*VMA5*	42	1
D	*VMA8*	32	1
E	*VMA4*	27	2
F	*VMA7*	14	1
G	*VMA10*	13	2
H	*VMA13*	54	1
V_0			
a	*VPH1/STV1*	95/102	1
c	*VMA3*	16	4
c′	*VMA11*	17	1
c″	*VMA16*	23	1
d	*VMA6*	36	1
Assembly factors			
	VMA12	25	
	VMA21	8.5	
	VMAA22	21	

stoichiometry and arrangement adds an extra level of complexity in understanding the structure and function of the yeast V-ATPase. Recently, the rotation of the yeast V_1 domain was observed when the V_0 domain was affixed to a surface and not allowed to rotate (Hirata *et al.*, 2003). The rotation of the V_1 domain relative to the V_0 domain was dependent on ATP and could be inhibited by the presence of concanamycin, a specific inhibitor of the V-type ATPase (Hirata *et al.*, 2003).

CELLULAR FUNCTIONS RELATED TO V-ATPASE ACTIVITY

The yeast vacuole is the equivalent of the mammalian lysosome, and contains proteases required for the degradation of proteins sent to the vacuole. The vacuole is responsible for a variety of physiological processes including pH regulation, ion regulation, amino acid storage, and metal detoxification. One characteristic of the yeast vacuole is that the lumen or interior of the vacuole is more acidic (pH 6.0) compared to the surrounding cytosol. Organelle acidification has been shown in other eukaryotic systems to play a role in various cellular functions such as receptor-mediated endocytosis, renal acidification, bone reabsorption, neurotransmitter accumulation, and activation of acid hydrolases. From yeast to humans the acidification of organelles and compartments is due to the action of the V-ATPase.

The function of the yeast V-ATPase is to utilize the energy released by the hydrolysis of cytosolic ATP to drive the translocation of protons across the vacuolar or Golgi membrane. The active transport of protons by the V-ATPase results in the acidification of these organelles relative to the cytosol. The proton gradient generated by the V-ATPase is utilized by a variety of membrane bound transport proteins to maintain cellular homeostasis. As an example, the V-ATPase helps to maintain low cytosolic calcium levels (100–150 nM) even when the cells are exposed to media containing 100 times the level of calcium in the cytosol (Halachmi and Eilam, 1993).

Loss of any V-ATPase subunit results in loss of V-ATPase activity and results in a distinct set of growth phenotypes in yeast. Yeast cells lacking V-ATPase activity are unable to grow on media buffered to a neutral pH (7.5) but are able to grow on more acidic media buffered to a pH of 5.0. There is not a clear understanding of why these cells display this conditional growth phenotype but it is specific and unique to *vma* mutant yeast. Additional phenotypes, which are shared by other classes of mutants, include the inability to grow in media containing high concentrations of calcium (similar to mutants with disrupted calcium ion homeostasis; *cls* mutants) and the inability to grow on nonfermentable carbons sources (similar to mutants lacking mitochondrial function; *pet* mutants).

The only observed affect of loss of V-ATPase function on yeast cellular morphology was reported by Zhang *et al*. (1998). Yeast cells expressing a temperature-sensitive allele of the V_1 subunit Vma4p (*vma4-1*^{ts}) and shifted to the nonpermissive temperature exhibited abnormal cellular and bud morphologies. In yeast expressing this mutant protein, the cells became enlarged and the buds appeared elongated but no changes in vacuolar morphology were observed (Zhang *et al.*, 1998). Interestingly, both cells either lacking Vma4p completely or expressing the ts mutant *vma4-1* show delocalization of actin filaments suggesting a possible link between the V-ATPase and formation or maintenance of the cytoskeleton (Zhang *et al.*, 1998).

OTHER V-ATPASE-DEPENDENT CELLULAR FUNCTIONS: IS THE V_0 DOMAIN INVOLVED IN VACUOLE MEMBRANE FUSION?

The primary function of the yeast V-ATPase is to drive the active transport of protons across the Golgi or vacuolar membrane into the lumen of each organelle. However, a function completely unrelated to proton translocation has been proposed for the V_0 domain of the V-ATPase in promoting homotypic vacuole fusion in yeast (Peters *et al.*, 2001). While homotypic fusion

requires vacuole acidification (Wickner, 2002), Mayer and colleagues propose that the V_0 domain mediates vacuole membrane fusion by a "pore-based fusion mechanism" (Mayer, 2002). Mayer and colleagues have found that vacuoles from yeast cells lacking a V_0 subunit fail to undergo homotypic fusion in their *in vitro* assay, however, *in vivo* evidence for V_0 involvement is completely lacking.

To address the possible role of the V_0 domain in vacuole fusion, we have examined the morphology of vacuoles in yeast cells using a hybrid protein with GFP fused to the vacuolar integral membrane protein Pho8p, GFP-ALP (Fig. 2). Wildtype yeast cells expressing GFP-ALP revealed that many of the cells had a large vacuole seen as 1–4 lobes per cell (Fig. 2, top panel) as expected (Pringle *et al.*, 1989). By contrast, yeast cells lacking Ypt7p, a Rab GTPase protein required for homotypic vacuole fusion (Wickner, 2002), exhibit very highly fragmented vacuoles (Fig. 2, second set of panels), demonstrating the *in vivo* requirement of Ypt7p for homotypic vacuole fusion. We then investigated whether yeast cells lacking a V-ATPase subunit exhibit an *in vivo* defect in vacuole fusion using the same GFP-ALP reporter (Fig. 2, bottom sets of panels). In cells lacking a V_1 subunit such as Vma2p (in *vma2*Δ cells the V_0 complex is fully assembled and localized to the vacuole; Graham *et al.*, 1995) the vacuoles appeared as somewhat smaller multilobed organelles, but very distinct from the vacuoles seen in *ypt7*Δ cells. Interestingly, in cells lacking a V_0 subunit such as Vma3p (in which V_0 subunits never exit the ER) the vacuoles appear to be very similar to wildtype cells, and nothing like the vacuoles seen in yeast cells lacking Ypt7p.

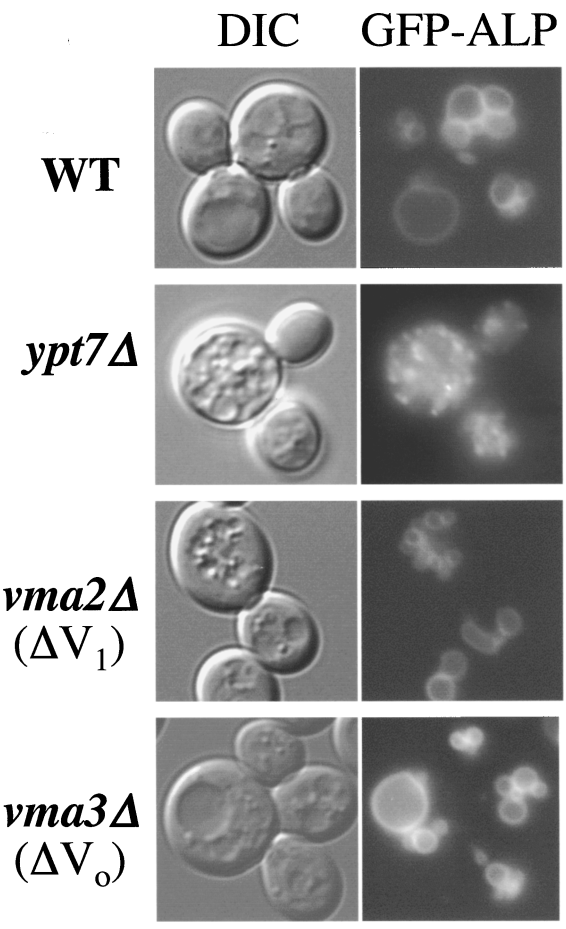

Fig. 2. Vacuolar morphology of yeast cells. Wildtype (top panels), *ykt7*Δ cells (second set of panels) and *vma2*Δ and *vma3*Δ mutants cells (bottom set of panels). All of the strains were expressing a GFP-ALP fusion protein and viewed by Nomarski optics (DIC) to observe cellular morphology and by epifluorescence microscopy (GFP-ALP) to visualize vacuolar morphology.

If the V_0 domain played an important role in vacuole–vacuole fusion one would expect that loss of V_0 subunits (*vma3*Δ cells) would prevent fusion and the vacuoles would appear highly fragmented. In addition, one would expect that disrupting the V_1 domain (*vma2*Δ mutants) and having fully exposed V_0 domains on the vacuole should actually improve vacuole–vacuole fusion according to the Mayer V_0 domain "pore-based fusion mechanism." These observations thus raise serious doubts about the proposal that the V_0 domain plays an important role in homotypic vacuole fusion.

THE V_1 SUBCOMPLEX OF THE YEAST V-ATPASE

The V_1 subcomplex possesses the sites for ATP binding and is responsible for carrying out the hydrolysis of ATP. The remaining noncatalytic V_1 subunits may participate in forming the stalk or the connection between the catalytic subunits and the V_0 subcomplex. In the yeast V-ATPase there are six possible candidates that may serve the function of the well-characterized gamma, delta, and epsilon subunits of the F-ATPase stalk. The function of the remaining noncatalytic V_1 subunits is completely unknown, except for Vma13p, which most likely plays a role in regulation of the activity of the complex. In the absence of Vma13p, yeast cells are able to assemble a 12 subunit complex that is correctly localized but functionally inactive (Ho *et al.*, 1993b; Keenan Curtis and Kane, 2002; Parra *et al.*, 2000).

COMPOSITION OF THE YEAST V-ATPASE V_1 SUBCOMPLEX

The V_1 subcomplex of both the Golgi and vacuolar localized V-ATPase in yeast is composed of the same

eight subunits Vma1p, Vma2p, Vma4p, Vma5p, Vma7p, Vma8p, Vma10p, and Vma13p (see Table I). A V-ATPase subunit is defined specifically as a peripherally associated V_1 subunit by several criteria, including its ability to be extracted from vacuolar membranes by various treatments such as alkaline carbonate or urea (Bauerle *et al.*, 1993). In the fractionation of detergent solubilized vacuolar membranes on glycerol gradients, V_1 subunits fractionate only with the fully assembled V-ATPase ($V_1 + V_0$) and are not found in a separate fraction containing the disassembled V-ATPase of only V_0 subunits (Ho *et al.*, 1993a; Kane *et al.*, 1989). In cases where yeast strains lack a V_1 subunit the remaining V_1 subunits are found in the cytosol since they are unable to correctly assemble a V_1 subcomplex (Ho *et al.*, 1993a; Tomashek *et al.*, 1996, 1997). In these same mutant cells, the V_0 subcomplex is able to assemble correctly and is localized to either the vacuole or Golgi membrane (Bauerle *et al.*, 1993).

Each V-ATPase V_1 complex contains three copies of Vma1p and three copies of Vma2p (Arai *et al.*, 1988). Biochemical evidence suggests a stoichiometry of two copies of Vma4p and two copies of Vma10p present in each V-ATPase complex (Tomashek *et al.*, 1997). The remaining V_1 subunits appear to be present at one copy per complex (Arai *et al.*, 1988).

The sequence of Vma1p (subunit A) is highly conserved across organisms and even between the V-ATPases and the F-ATPases; Vma1p shares 65–75% identity with other homologous V-ATPase subunits from a range of organisms and 26% identity with the beta subunit of the yeast F-ATPase. Interestingly, in yeast, Vma1p is one of two proteins encoded by the gene *TFP1*. Vma1p is generated from a unique process of protein splicing in which a single translation product is cleaved forming two proteins (Kane *et al.*, 1990).

Vma2p (subunit B, Vat2p) is also highly conserved in structure and function within the V-type and F-type ATPase families. In yeast the Vma2p translation product is predicted to possesses a 12-amino acid N-terminal leader sequence that is not present on the mature protein (Yamashiro *et al.*, 1990). The proposed processing of the N-terminus of Vma2p is also supported by the fact that we have been unable to successfully epitope tag Vma2p by introduction of the epitope sequence immediately after the initiating methionine. Similar to the F-ATPase alpha subunit, Vma2p is proposed to possess a nucleotide binding site that is not the catalytic site (Vasilyeva *et al.*, 2000).

The remaining noncatalytic V_1 subunits possess homologous subunits in V-ATPase complexes in other organisms but none exhibit clear homology to any F-ATPase subunits. Vma4p (subunit E) is a 27-kDa subunit of the V_1 complex of the yeast V-ATPase (Ho *et al.*, 1993a). Vma4p was the first V_1 subunit identified whose stability in the cell is dependent on the presence of another V_1 subunit (Vma10p) suggesting a direct interaction between these two subunits (Tomashek *et al.*, 1997). Crosslinking studies further support an interaction between Vma4p and Vma10p revealing that both of these proteins interact with the exterior surface of Vma2p (Arata *et al.*, 2002b). Vma5p (subunit C) is a 42 kDa subunit of the V-ATPase (Ho *et al.*, 1993a).

Vma7p (subunit F) behaves as a typical peripherally associated V_1 subunit since it can be stripped from the membrane by alkaline carbonate treatment and fractionates only with the assembled V-ATPase complex. Interestingly, cells lacking Vma7p also affect the stability of the V_0 complex, unlike the deletion of any other V_1 subunit (Graham *et al.*, 1994). Vma8p (subunit D) is a 32-kDa V_1 subunit that can be crosslinked to Vma7p indicating physical association between these two subunits (Graham *et al.*, 1995; Tomashek *et al.*, 1997). Vma8p can also be crosslinked to the interior face of Vma2p suggesting both Vma8p and Vma7p may form part of the stalk connecting the catalytic subunits to the V_0 subcomplex (Arata *et al.*, 2002a,b).

Vma10p (subunit G) is required to maintain stable levels of Vma4p in the cell. The gene encoding Vma10p is unusual for yeast genes because it possesses an intron immediately following the initiating methionine. Stable crosslinked species can be formed between Vma10p and Vma4p, further supporting their close proximity in the V-ATPase complex. The calculated mass of the Vma10p-Vma4p subcomplex identified by native gel electrophoresis is twice the size of the Vma10p-Vma4p crosslinked product suggesting that two copies of each protein (E_2G_2) may be present in each V-ATPase complex (Tomashek *et al.*, 1997).

Vma13p (subunit H) is thought to play a role in the regulation of the V-ATPase complex. Vma13p is the only subunit not needed for assembly of the complex, but is instead required for activation (Ho *et al.*, 1993b). V-ATPase complexes isolated from *vma13*Δ strains can have V-ATPase and proton-pumping activity restored upon addition of recombinantly expressed Vma13p (Flannery and Stevens, unpublished data). Interestingly when Vma13p was first identified, it was shown that only the C-terminal 299 amino acids (out of 478) are needed for a functional V-ATPase. In subsequent studies, the N-terminus of Vma13p has been implicated in interactions with other proteins and may have other functions independent of its role in the V-ATPase (Zhong *et al.*, 2000). Vma13p may also play a role in inhibiting the ATPase activity of V_1 subcomplexes that are not associated with

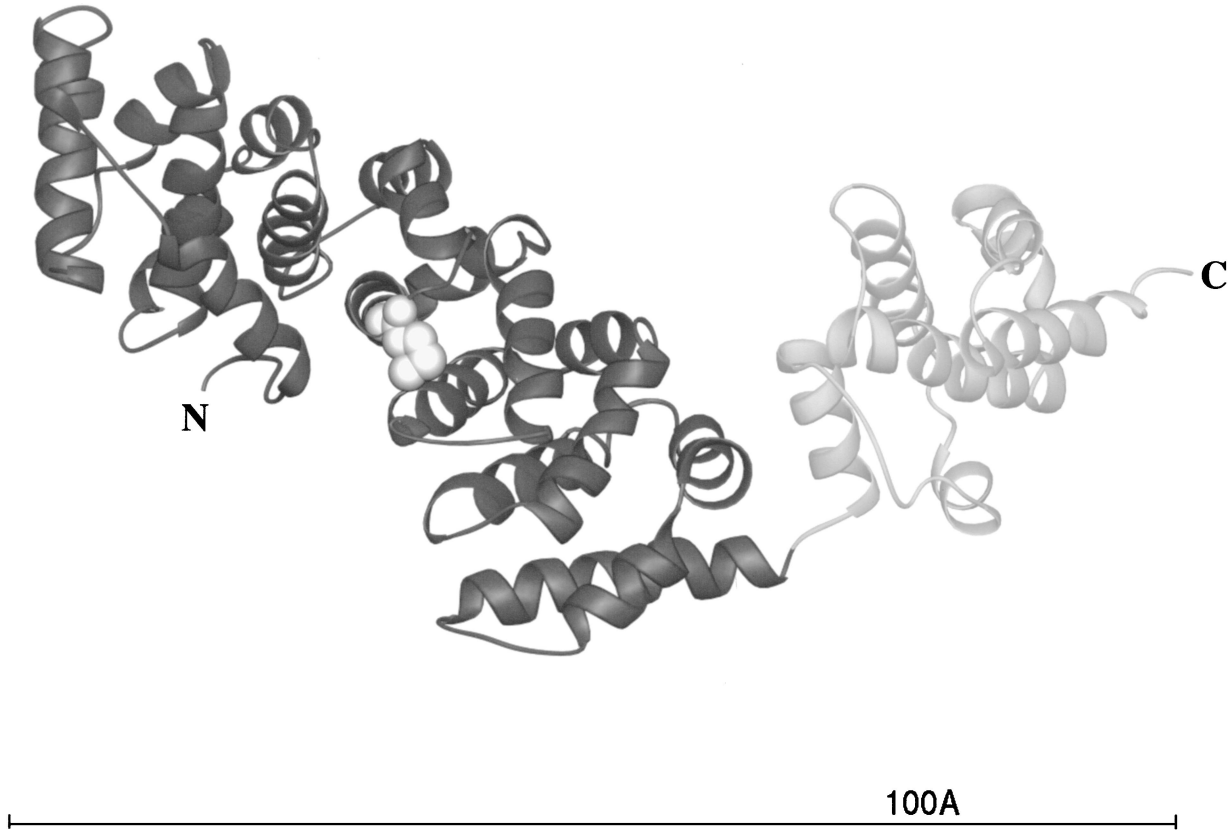

Fig. 3. Structure of Vma13p. Model of the 4.2 Å resolution structure of Vma13p (PDB ID 1HO8) with the N-terminal domain is depicted in dark grey, and the C-terminal domain in light grey. Methionine 180 is highlighted using space filling, because the truncated Vma13p lacking the first 179 amino acids is fully functional.

the membrane but are found in the cytosol (Parra *et al.*, 2000).

Vma13p has recently become the first V-ATPase subunit to have a crystal structure solved (Sagermann *et al.*, 2001). The data reveals that the protein structure is mainly alpha helical and contains two domains (Fig. 3). The N-terminal domain (amino acids 2-352) consists of 17 consecutive alpha helices that stack upon one another in a right-handed spiral to form a superhelix. The C-terminal domain (353–478) has a similar overall structure as the N-terminal domain, however the alpha helices are less regularly arranged. A flexible linker creating an interface between the domains connects the two domains. Electrostatic analysis indicates that this interface may be a possible site for binding Vma13p to the V-ATPase (Sagermann *et al.*, 2001). There is also a shallow groove in the N-terminal domain that, in the crystal structure, has the first 10 amino acids bound. A structural homology search revealed that the peptide interaction is similar to that seen in the importin family of proteins, where the binding of the peptide, in importins, provides a regulatory switch for the binding affinity of other peptides. Since this region can be deleted without affecting the function of the V-ATPase, it may play a role in regulating some other functions not related to the complex.

ASSEMBLY OF THE YEAST V-ATPASE V_1 SUBUNITS

The loss of a V_1 subunit prevented the association of the remaining V_1 subunits with the V_0 subcomplex, yet these polypeptides remain stable in the cytosol. Biochemical analysis of various deletion mutants allowed the identification of specific V_1 subunit complex intermediates in the assembly of the V_1 subcomplex. The largest cytosolic complex observed included Vma1p, Vma2p, Vma4p, Vma7p, Vma8p, and Vma10p (Tomashek *et al.*, 1997). Strong associations were observed in several mutant backgrounds between Vma7p and Vma8p and a second subcomplex between Vma4p and Vma10p. As mentioned previously, association of Vma4p with Vma10p ensures its stability in the cell. Unfortunately, a more accurate view

of the arrangement of the noncatalytic V_1 subunits relative to Vma1p, Vma2p, and the V_0 subunits must await a crystal structure of the entire V_1V_0 complex.

THE V_0 SUBCOMPLEX OF THE YEAST V-ATPASE

Subunits of the V_0 subcomplex play a key role in the translocation of protons from the yeast cytosol into the lumen of the vacuole or the Golgi. The translocation of protons does not occur through a pore or a channel formed by the V_0 subunits, but utilizes charged residues buried within the transmembrane domains of several of these subunits. The rotation of the V_0 subcomplex is required for the translation of protons across the membrane and is driven by the hydrolysis of ATP. The subunits Vma3p, Vma11p, and Vma16p are believed to form a hexameric ring, similar to the ring formed by multiple copies of subunit c in the F-ATPase. Vma3p, Vma11p, and Vma16p each contain a charged glutamic acid residue that is proposed to accept protons from the cytosol, and then releases the protons into the lumen of the vacuole.

COMPOSITION OF THE YEAST V-ATPASE V_0 SUBCOMPLEX

The V_0 complex is composed of five subunits, Vma3p, Vma11p, Vma16, Vma6p, and Vph1p or Stv1p depending on whether the complex is localized to the vacuole or Golgi. Vma3p (subunit c) is a small 16-kDa hydrophobic subunit predicted to possess four transmembrane spanning domains. Vma11p (subunit c′) is very similar to Vma3p in both amino acid sequence (50% identical) and structure, and is also predicted to span the membrane four times. Both Vma3p and Vma11p have a highly conserved glutamic acid residue located near the center of the fourth transmembrane domain that is essential for proton translocation. Protease digestion experiments of epitope tagged Vma11p and Vma3p support a lumenal orientation for both the N-terminus and C-terminus of these proteins (Flannery and Stevens, unpublished results).

Vma16p (subunit c″) is a 23-kDa membrane protein similar to Vma3p and Vma11p, but it is predicted to contain five transmembrane domains (Hirata *et al.*, 1997). Vma16p has two glutamic acid residues located in the third and fifth transmembrane domains that could participate in proton translocation. Mutational analysis of each glutamic acid residue in Vma16p revealed that the glutamate residue in the third transmembrane domain is required for proton transport activity. To inactivate the V-ATPase requires the mutation of only one of the critical glutamic acid residues in either Vma3p, Vma11p, or Vma16p suggesting a high level of cooperativity between these subunits in their role in proton translocation (Hirata *et al.*, 1997).

The topology of Vma16p is currently being actively investigated. Transmembrane modeling programs predict that there are five transmembrane regions for the protein (Fig. 4). We have shown that with protease digestion of epitope tags, there are an odd number of transmembrane

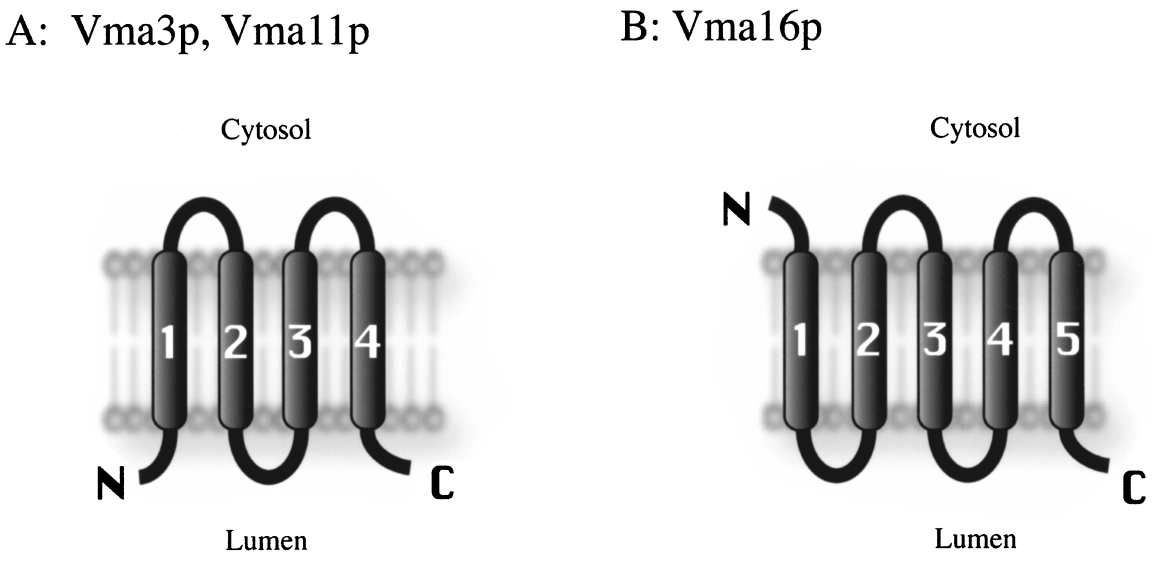

Fig. 4. Topology of the V_0 subunits Vma3p, Vma11p, and Vma16p. A. Vma3p and Vma11p are predicted to form four membrane-spanning domains with both the C-terminus and the N-terminus oriented toward the organelle. B. Vma16p is predicted to have five transmembrane helices, and recent data place the C-terminus in the lumen and the N-terminus in the cytosol.

regions and that the C-terminus resides in the lumen of the vacuole, while the N-terminus resides in the cytosol (Flannery and Steven, unpublished results). Our proposed topology for Vma16p is the opposite of that proposed by Nichi *et al.* (2003). In their model Vma16p has a topology opposite of the topology of either Vma3p or Vma11p. Our topological model for Vma16p is further supported by the work of Kim *et al*. (2003) who experimentally determined the topology of several membrane proteins encoded by *VMA* genes including Vma16p. Using reporter proteins that were fused to the C-terminus of Vma16p these researchers determined a lumenal orientation for the C-terminus. Based on the homology to Vma3p and Vma11p, and predicted packing arrangement, the first helix of Vma16p would be in the center of the proteolipid ring allowing it to be in position to interact with one of the stalk subunits.

Several deletions have been constructed to determine if the first transmembrane helix of Vma16p is required for V-ATPase function. Deleting amino acids 12-55 of Vma16p disrupted the function of the V-ATPase since the cells carrying this truncated Vma16p exhibited slowed growth on media buffered to neutral pH, reflecting a role for the first TMD in the function of the complex (Graham and Stevens, unpublished results; Gibson *et al.*, 2002). However, if only the region of amino acids predicted to be the first transmembrane helix (amino acids 12–38) were deleted, this form of Vma16p resulted in a completely assembled complex with only slightly reduced ATPase activity, and the cells exhibited no growth defects. A Vma16p/Vma11p chimera, where the first 38 amino acids of Vma16p were placed on the N-terminus of Vma11p, was stable when expressed in yeast cells. Although this Vma16p/Vma11p chimera could function in place of Vma11p, it could not replace Vma16p or restore normal growth and V-ATPase activity in *vma16*Δ cells (Flannery and Stevens, unpublished results). Experiments are underway to determine which V-ATPase subunits interact with the Vma16p N-terminus.

Vma6p (subunit d) is the only V_0 subunit that is not predicted to span the membrane bilayer, yet it consistently fractionates with the other V_0 subunits (Bauerle *et al.*, 1993). In cells unable to assemble a V_0 subcomplex, such as *vma3*Δ, Vma6p is no longer associated with the membrane but is found in the cytosol. There also appeared to be a reduction of the levels of Vma6p in *vma3*Δ cells suggesting the protein was destabilized in absence of a V_0 subcomplex (Bauerle *et al.*, 1993). We have yet to determine which specific V_0 subunit interacts with Vma6p mediating its association with the membrane.

Either Vph1p or Stv1p can function as the subunit **a** in the V_0 subcomplex depending on where the V-ATPase complex is localized. Subunit **a** is a large membrane protein possessing a hydrophilic N-terminal domain (amino acids 1-419 in Vph1p and amino acids 1-465 in Stv1p) and a polytopic transmembrane spanning C-terminal domain (Manolson *et al.*, 1992, 1994). Analysis of chimeric subunit **a** proteins created by swapping the N-terminal domains of Vph1p with that of Stv1p revealed that the information determining the localization of these proteins is present entirely in the N-terminus (Kawasaki-Nishi *et al.*, 2001). Additional mutational analysis will be aimed toward identifying the specific residues required to localize the Stv1p-containing V-ATPase to the Golgi.

STRUCTURE OF THE YEAST V-ATPASE V_0 SUBCOMPLEX

A feature very unique to the V-ATPases is the presence of three hydrophobic subunits, Vma3p, Vma11p, and Vma16 (c,c′, and c″) that form a hexameric proteolipid ring. Recall, each subunit c, c′, and c″ contains a glutamic acid residue directly involved in proton translocation. Biochemical and genetic analyses confirm that all three proteins, Vma3p, Vma11p, and Vma16p are required for proton translocation. In the structurally similar F-ATPase multiple copies (10–14) of a single hydrophobic polypeptide (subunit c), each contains only two transmembrane domains, forms the proteolipid ring.

Using a biochemical approach we addressed the question of the stoichiometry of Vma3p, Vma11p, and Vma16 in the yeast V_0 subcomplex (Powell *et al.*, 2000). Immunoprecipitation of Vo subunits from yeast cells expressing epitope tagged Vma3p, Vma11p, and Vma16p confirmed that each V_0 subcomplex contained all three proteins. Further, immunoprecipitation experiments from cells expressing two different epitope tagged copies of the same subunit (e.g. Vma11p-c-*myc* and Vma11p-HA) revealed that only one copy of Vma11p and one copy of Vma16p are present in a single V_0 subcomplex but multiple copies of Vma3p are present in each V-ATPase complex. The exact number of copies of Vma3p in each yeast V-ATPase complex is yet to be determined, but based on earlier subunit stoichiometry studies (Arai *et al.*, 1988), the best estimate is four copies of Vma3p per V_0 subcomplex (Stevens and Forgac, 1997).

ASSEMBLY OF THE YEAST V-ATPASE V_0 SUBCOMPLEX

Assembly of the V_0 subcomplex requires the presence of the subunits Vma3p, Vma6p, Vma11p, Vma16p,

and either Vph1p or Stv1p or both. The newly synthesized integral membrane V_0 subunits are inserted into the ER membrane, and they are assembled into the V_0 subcomplex aided by three ER localized assembly factors (see the following sections). Only a fully and correctly assembled V_0 subcomplex is able to exit the ER for the Golgi or vacuolar membrane.

Failure to assemble a V_0 subcomplex results in rapid turnover of Vph1p or Stv1p by a process referred to as ER quality control (ERQC; Graham *et al.*, 1998; Hill and Cooper, 2000). The association of Vma6p with the membrane is dependent on the presence of the V_0 subunits. In yeast cells lacking an integral membrane V_0 subunit, such as Vma3p, Vma6p is found not on the membrane but instead in the cytosol. Initial analysis of the fate of the remaining subunits in cells suggests that failure to assemble a V_0 complex blocks their exit from the ER.

V-ATPASE ASSEMBLY FACTORS

Genetic screens have identified not only the genes encoding subunits of the yeast V-ATPase but also genes encoding non-subunit proteins required for the assembly, stability, and/or targeting of the V-ATPase complex. Three proteins have been identified, Vma12p, Vma21p, and Vma22p, that are required for the formation of a functional V-ATPase in yeast. All three proteins have been localized to the membrane of the ER suggesting they function very early in the biosynthesis, insertion, and assembly of the V-ATPase. Cells lacking in any one of these three non-subunit proteins prevents the assembly the V_0 subunits, thus blocking the exit of the V_0 subunits from the ER. Since Vma12p, Vma21p, and Vma22p are required for the proper assembly of the V_0 in the ER, we refer to them as V-ATPase assembly factors. Cells lacking Vma12p, Vma22p, or Vma21p do not display general defects in the processing and targeting of other vacuolar or general membrane proteins. The function of these assembly factors seems to be dedicated solely to the assembly of the yeast V-ATPase.

Vma12p is a 25-kDa integral membrane protein predicted to have two transmembrane spanning domains (Hirata *et al.*, 1993). The topology of Vma12p was determined by protease digestion studies and is consistent with a model placing both the N-terminus and the C-terminus on the cytosolic face of the ER membrane (Jackson and Stevens, 1997). Cells lacking Vma12p, or any of the other assembly factors, display the same set of phenotypes as cells lacking a V_0 subunit. In the absence of Vma12p, the yeast cells are unable to grow at neutral pH or in the presence of high calcium (Hirata *et al.*, 1993). In addition, the loss of Vma12p results in the rapid turnover of Vph1p in a process that is independent of vacuolar proteases but instead involves the ERQC pathway (Hill and Cooper, 2000). Protease digestion experiments of Vph1p have ruled out the possibility that the increased rate of degradation of Vph1p in cells lacking Vma12p was due to improper insertion of Vph1p in to the ER membrane (Jackson and Stevens, 1997). The accessibility of Vph1p to proteases in wildtype cells as compared to cells lacking Vma12p was identical, suggesting that the topology of Vph1p in the ER membrane was identical in wildtype and *vma12*Δ cells.

Vma22p is a 21-kDa hydrophilic protein with no predicted transmembrane spanning domains. The loss of Vma22p results in an identical phenotype to cells lacking Vma12p. Vma22p was localized to the membranes of the ER except in cells lacking Vma12p, which suggests Vma12p serves as the anchor for Vma22p to the ER membrane (Hill and Stevens, 1995). Vma12p and Vma22p form a complex in the ER that has been shown to interact directly but transiently with the V-ATPase subunit a in the ER membrane (Graham *et al.*, 1998).

The first V-ATPase assembly factor characterized was Vma21p, a small 77 amino acid (8.5 kDa) hydrophobic protein predicted to possess two membrane-spanning domains (Fig. 5; Hill and Stevens, 1994). Cells lacking Vma21p are unable to assemble the V_0 subcomplex and display phenotypes indistinguishable from cells lacking Vma12p or Vma22p. Fractionation of detergent solubilized membranes showed that Vma21p was not part of the Vma12p-Vma22p complex suggesting Vma21p plays a different role in V-ATPase assembly in the ER (Graham *et al.*, 1998).

The carboxy-terminal four residues of Vma21p contain a dilysine motif ($-3,-4$) that is required to maintain Vma21p in the ER. Mutation of the dilysine (KK) motif to diglutamine (QQ) resulted in a steady state vacuolar localization of Vma21p (Hill and Stevens, 1994). Coatomer subunits of COPI vesicles have been shown to bind directly to dilysine motifs in proteins and that binding is required for retrograde transport, in this case the return of Vma21p to the ER.

The presence of an ER retrieval signal in the C-terminal tail of Vma21p suggests the protein may exit the ER with the V-ATPase and be retrieved back from a post Golgi compartment. Using an *in vitro* ER vesicle budding assay Vma21p, together with V_0 subunits, was found to be enriched in the transport vesicles compared to Vma12p, supporting an additional role for Vma21p in the transport of the V-ATPase out of the ER (Malkus and Schekman, unpublished results). We have begun a detailed mutational analysis of Vma21p to identify both

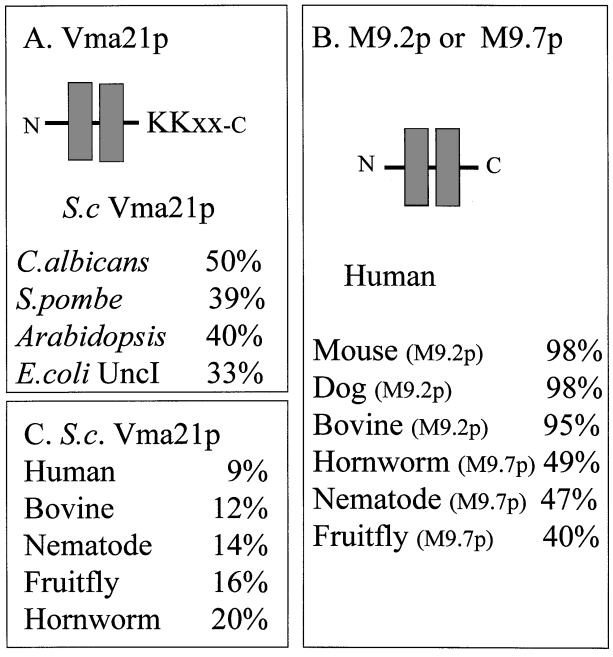

Fig. 5. Sequence comparison between Vma21p-like proteins. Cartoon characterizing features of the family of small Vma21p-like proteins. Shaded rectangles represent sequences predicted to form a transmembrane domain. A. Sequence homology between *Saccharomyces cerevisiae* (*S.c*) Vma21p-like proteins. Only Vma21p from yeast, *C. albicans* and *Arabidopsis* possess a C-terminal dilysine motif. B. Sequence homology between M9 proteins. Higher eukaryotes possess a single Vma21p-like protein described as either M9.2p or M9.7p. C. Sequence comparison of the *S.c.* Vma21p to M9.2 or M9.7 proteins. Numbers shown represent percent identities compared to either the yeast Vma21p or human Vma21-like M9.2 protein.

the residues critical for V_0 assembly and the signals required for packaging and transport of the V-ATPase out of the ER.

MODEL FOR ASSEMBLY OF THE YEAST V-ATPASE IN THE ER

The assembly of a functional V-ATPase in yeast not only requires that all the structural subunits are present, but also requires the three assembly factors Vma12p, Vma21p, and Vma22p (Fig. 6). Vma12p and Vma22p form an assembly complex that functions separately from the assembly role of Vma21p. The Vma12p-Vma22p has been shown to interact directly with the V-ATPase subunit **a**, playing a role in assembly after the subunit is synthesized and inserted into the ER membrane. The interaction between Vma12p-Vma22p and subunit **a** occurs transiently. The Vma12p-Vma22p complex dissociates from the V-ATPase as the V-ATPase exits the ER.

Vma21p is not part of the Vma12p-Vma22p assembly complex, which suggests it has a different role in V-ATPase assembly. Since Vma21p is a small hydrophobic membrane protein similar to the V_0 subunits Vma3p, Vma11p, and Vma16p it may play a role in the assembly of these proteins. Recall, each V-ATPase contains single copies of Vma11p and Vma16p but multiple copies of Vma3p. One function of Vma21p may be to ensure the correct stoichiometry of the V-ATPase subunits as they assemble to form a V_0 subcomplex. The presence of the dilysine ER retrieval motif in the C-termimal tail of Vma21p strongly suggests it cycles between the ER and Golgi compartments. In addition to its role in assembly, Vma21p may serve to escort the assembled V-ATPase complex out of the ER (Herrmann *et al.*, 1999). Vma21p would separate from the V-ATPase in the Golgi complex and be retrieved back to the ER for another round of V-ATPase assembly.

Have the V-ATPase assembly factors been conserved across species? Homologues of the V-ATPase structural subunits can easily be identified in a range of eukaryotic organisms. One would expect that the cellular machinery required to assemble this important multisubunit complex would also be conserved across species. A search of various genome databases revealed that currently there are only two hypothetical proteins that appear homologous to yeast Vma12p; one in *Candida albicans* (CaVph2p; 35% identical) and one from *S. pombe* (16% identity). A similar database search for Vma22p homologous proteins has identified a hypothetical protein in *C. albicans* (CaVma22p; 20% identical) and a hypothetical human protein (MGC12981) sharing 25% identity. It may be that the overall structure and function of Vma12p and Vma22p are conserved across species but not the primary sequence, making it more challenging to identify homologous proteins.

An extensive group of small hydrophobic proteins of molecular mass 9.2–9.4 kDa that are similar to Vma21p and predicted to span the membrane twice have been identified in many higher eukaryotic organisms (Fig. 5). Unlike Vma21p, these proteins lack a dilysine ER retrieval motif and in some cases have been found to purify along with the V-ATPase complex isolated from bovine chromaffin granules (M9.2) and tobacco horn worm (Ludwig *et al.*, 1998 , Merzendorfer *et al.*, 1999). A Vma21p-like protein has been identified from the sequencing of the *C. albicans* (CaVma21p; 50% identical) and *Arabidopsis* (Atlg05780; 40% identical) genomes. Unlike the M9.2 and M9.7 proteins the AtVma21p and the CaVma21p have a dilysine motif (−3,−4) suggesting they are also ER localized and may function in V-ATPase assembly similar to the yeast Vma21p.

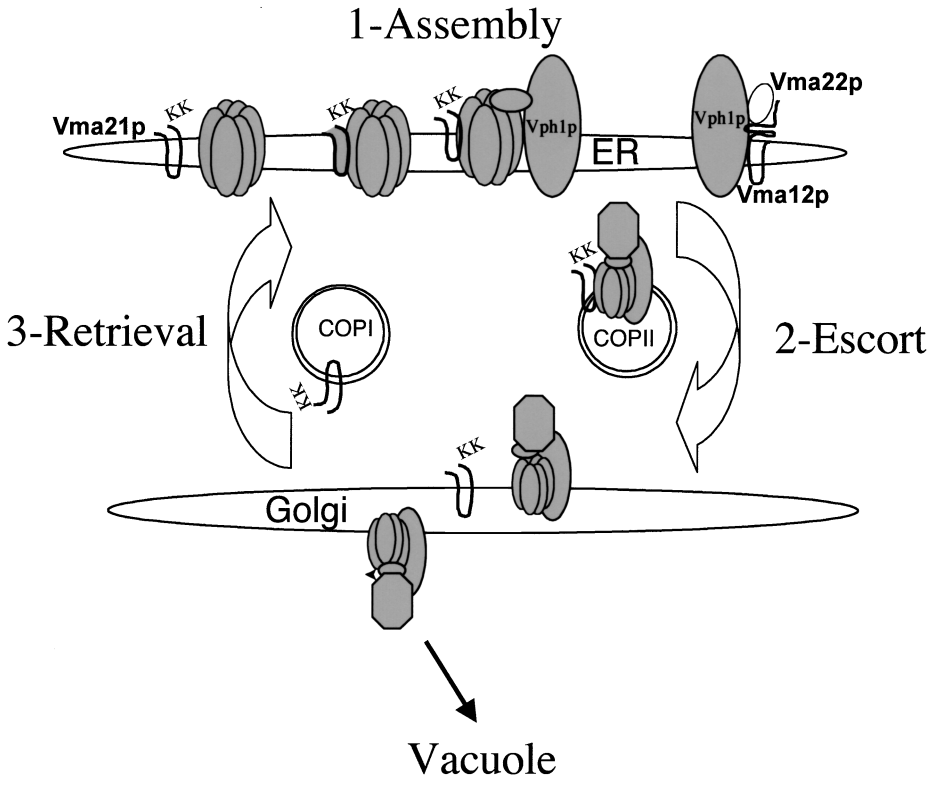

Fig. 6. Roles of Vma21p in the assembly of the yeast V-ATPase. (1) Assembly-Vma21p aids the assembly of the proteolipids in the ER. Vma12p/Vma22p forms a complex that interacts directly with the 100-kDa subunit **a** in the ER. (2) Escort-Vma21p remains associated with the V-ATPase to escort the complex out of the ER. (3) Retrieval-Vma21p separates from the V-ATPase in the Golgi and is retrieved for another round of assembly and escort. The Vph1p V-ATPase is trafficked on to the membrane of the vacuole.

CONCLUSION

Extensive biochemical and genetic screening has identified a large collection of proteins required to form a functional V-ATPase in yeast. Except for the catalytic and nucleotide binding subunits (Vma1p and Vma2p) the function of the majority of the peripherally associated V_1 subunits is unknown. By comparison to the structurally and functionally related F-ATPases, some subunits are likely to play a structural role, linking the catalytic domain to the membrane V_0 domain. The remaining V_1 subunits may serve to transfer the energy released from ATP hydrolysis to drive the translocation of protons across the membrane bilayer. The structural studies of Vma13p are beginning to shed some light on how this protein functions in the complex. Lacking details of the structure, the actual arrangement of any of the individual subunits within the V_1 subcomplex is unknown.

The stiochiometry and topology of the V_0 subunits has become an active area of interest. The conclusion from our results supports a model of the V-ATPase complex containing a single copy each of Vma11p and Vma16p, but multiple copies of Vma3p per complex. One role of the assembly factor proteins may be to regulate the stiochiometry and arrangement of the V_0 subunits as they assemble in the ER. Vma3p, Vma11p, and Vma16 each contain a charged residue within a transmembrane domain that is intimately involved in the translocation of protons across the membrane, and these three subunits are proposed to assemble to form a "proteolipid" ring. We have found that the topology of Vma16p is the same as Vma3p and Vma11p, with the C-terminus of Vma16p facing the lumen and the extra transmembrane domain of Vma16p spanning the membrane. The precise arrangement of these hydrophobic proteins within the V_0 subcomplex must await future investigations.

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REFERENCES

Arai, H., Terres, G., Pink, S., and Forgac, M. (1988). *J. Biol. Chem.* **263**, 8796–8802.

Arata, Y., Baleja, J. D., and Forgac, M. (2002a). *J. Biol. Chem.* **277**, 3357–3363.

Arata, Y., Baleja, J. D., and Forgac, M. (2002b). *Biochemistry* **41**, 11301–11307.

Bauerle, C., Ho, M. N., Lindorfer, M. A., and Stevens, T. H. (1993). *J. Biol. Chem.* **268**, 12749–12757.

Capaldi, R. A., and Aggeler, R. (2002). *Trends Biochem. Sci.* **27**, 154–160.

Gibson, L. C., Cadwallader, G., and Finbow, M. E. (2002). *Biochem. J.* **366**, 911–919.

Graham, L. A., Hill, K. H., and Stevens, T. H. (1994). *J. Biol. Chem.* **269**, 25974–25977.

Graham, L. A., Hill, K. J., and Stevens, T. H. (1995). *J. Biol. Chem.* **270**, 15037–15044.

Graham, L. A., Hill, K. J., and Stevens, T. H. (1998). *J. Cell Biol.* **142**, 39–49.

Halachmi, D., and Eilam, Y. (1993). *FEBS Lett.* **316**, 73–78.

Herrmann, J. M., Malkus, P., and Schekman, R. (1999). *Trends Cell Biol.* **9**, 5–7.

Hill, K., and Cooper, A. A. (2000). *EMBO J.* **19**, 550–561.

Hill, K. J., and Stevens, T. H. (1994). *Mol. Biol. Cell* **5**, 1039–1050.

Hill, K. J., and Stevens, T. H. (1995). *J. Biol. Chem.* **270**, 22329–22336.

Hirata, R., Graham, L. A., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1997). *J. Biol. Chem.* **272**, 4795–4803.

Hirata, T., Iwamoto-Kihara, A., Sun-Wada, G. H., Okajima, T., Wada, Y., and Futai, M. (2003). *J. Biol. Chem.* **278**, 23714-23719.

Hirata, R., Umemoto, N., Ho, M. N., Ohya, Y., Stevens, T. H., and Anraku, Y. (1993). *J. Biol. Chem.* **268**, 961–967.

Ho, M. N., Hill, K. J., Lindorfer, M. A., and Stevens, T. H. (1993a). *J. Biol. Chem.* **268**, 221–227.

Ho, M. N., Hirata, R., Umemoto, N., Ohya, Y., Takatsuki, A., Stevens, T. H., and Anraku, Y. (1993b). *J. Biol. Chem.* **268**, 18286–18292.

Jackson, D. D., and Stevens, T. H. (1997). *J. Biol. Chem.* **272**, 25928–25934.

Kane, P. M., Yamashiro, C. T., and Stevens, T. H. (1989). *J. Biol. Chem.* **264**, 19236–19244.

Kane, P. M., Yamashiro, C. T., Wolczyk, D. F., Neff, N., Goebl, M., and Stevens, T. H. (1990). *Science* **250**, 651–657.

Kawasaki-Nishi, S., Bowers, K., Nishi, T., Forgac, M., and Stevens, T. H. (2001). *J. Biol. Chem.* **276**, 47411–47420.

Keenan Curtis, K., and Kane, P. M. (2002). *J. Biol. Chem.* **277**, 2716–2724.

Kim, H., Melen, K., and Von Heijne, G. (2003). *J. Biol. Chem.* **278**, 10208–10213.

Ludwig, J., Kerscher, S., Brandt, U., Pfeiffer, K., Getlawi, F., Apps, D. K., and Schägger, H. (1998). *J. Biol. Chem.* **273**, 10939–10947.

Manolson, M. F., Proteau, D., Preston, R. A., Stenbit, A., Roberts, B. T., Hoyt, M. A., Preuss, D., Mulholland, J., Botstein, D., and Jones, E. W. (1992). *J. Biol. Chem.* **267**, 14294–14303.

Manolson, M. F., Wu, B., Proteau, D., Taillon, B. E., Roberts, B. T., Hoyt, M. A., and Jones, E. W. (1994). *J. Biol. Chem.* **269**, 14064–14074.

Mayer, A. (2002). *Annu. Rev. Cell Dev. Biol.* **18**, 289–314.

Merzendorfer, H., Huss, M., Schmid, R., Harvey, W. R., and Wieczorek, H. (1999). *J. Biol. Chem.* **274**, 17372–17378.

Nishi, T., and Forgac, M. (2002). *Nat. Rev. Mol. Cell Biol.* **3**, 94–103.

Nishi, T., and Kawasaki-Nishi, S., and Forgac, M. (2003). *J. Biol. Chem.* **278**, 5821–5827.

Noji, H., Yasuda, R., Yoshida, M., and Kinosita, K., Jr. (1997). *Nature* **386**, 299–302.

Parra, K. J., Keenan, K. L., and Kane, P. M. (2000). *J. Biol. Chem.* **275**, 21761–21767.

Peters, C., Bayer, M. J., Buhler, S., Andersen, J. S., Mann, M., and Mayer, A. (2001). *Nature* **409**, 581–588.

Powell, B., Graham, L. A., and Stevens, T. H. (2000). *J. Biol. Chem.* **275**, 23654–23660.

Pringle, J. R., Preston, R. A., Adams, A. E., Stearns, T., Drubin, D. G., Haarer, B. K., and Jones, E. W. (1989). *Methods Cell Biol.* **31**, 357–435.

Sagermann, M., Stevens, T. H., and Matthews, B. W. (2001). *Proc. Natl. Acad. Sci. U. S. A.* **98**, 7134–7139.

Stevens, T. H., and Forgac, M. (1997). *Annu. Rev. Cell Dev. Biol.* **13**, 779–808.

Tomashek, J. J., Graham, L. A., Hutchins, M. U., Stevens, T. H., and Klionsky, D. J. (1997). *J. Biol. Chem.* **272**, 26787–26793.

Tomashek, J. J., Sonnenburg, J. L., Artimovich, J. M., and Klionsky, D. J. (1996). *J. Biol. Chem.* **271**, 10397–10404.

Vasilyeva, E., Liu, Q., MacLeod, K. J., Baleja, J. D., and Forgac, M. (2000). *J. Biol. Chem.* **275**, 255–260.

Wickner, W. (2002). *EMBO J.* **21**, 1241–1247.

Yamashiro, C. T., Kane, P. M., Wolczyk, D. F., Preston, R. A., and Stevens, T. H. (1990). *Mol. Cell. Biol.* **10**, 3737–3749.

Zhang, J. W., Parra, K. J., Liu, J., and Kane, P. M. (1998). *J. Biol. Chem.* **273**, 18470–18480.

Zhong, X., Malhotra, R., and Guidotti, G. (2000). *J. Biol. Chem.* **275**, 35592–35599.