The Journal of Experimental Biology 203, 61–70 (2000)
Printed in Great Britain © The Company of Biologists Limited 2000
JEB2347

COMPOSITION AND ASSEMBLY OF THE YEAST VACUOLAR H^+-ATPase COMPLEX

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Accepted 21 October; published on WWW 13 December 1999

Summary

The proton-translocating ATPase (H^+-ATPase) found on the membrane of the yeast vacuole is the best characterized member of the V-type ATPase family. Biochemical and genetic screens have led to the identification of 14 genes, the majority designated *VMA* (for *v*acuolar *m*embrane *A*TPase) encoding subunits of the enzyme complex. At least eight genes encode for proteins comprising the peripherally associated catalytic V_1 subcomplex, and six genes code for proteins forming the proton-translocating membrane V_o subcomplex. Several additional genes have been identified that encode proteins that are not part of the final V-ATPase complex yet are required for its assembly. These non-subunit Vma proteins function as dedicated V-ATPase assembly factors since their absence appears to inhibit assembly of the V-ATPase only. The assembly factors designated Vma12p, Vma21p and Vma22p have been localized to the membrane of the endoplasmic reticulum and aid the association of newly synthesized V-ATPase subunits translocated into the endoplasmic reticulum membrane. Two of these proteins, Vma12p and Vma22p, function together in an assembly complex that interacts directly with nascent V-ATPase subunits.

Key words: V-ATPase, yeast, *Saccharomyces cerevisiae*, vacuole, multisubunit complex, proton-translocating, assembly.

Introduction

The proton-translocating ATPase (H^+-ATPase) found on the vacuolar membrane of the yeast *Saccharomyces cerevisiae* is one of the most extensively characterized members of the V-type ATPase family (V-ATPase). V-ATPases are multisubunit complexes 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). In yeast, the V-ATPase functions to acidify the vacuole by driving the translocation of protons into the lumen of the vacuole. The hydrolysis of cytosolic ATP by the V-ATPase is required for the movement of protons from the cytosol into the interior of the vacuole. The proton gradient generated across the yeast vacuolar membrane by the V-ATPase in yeast is utilized by other membrane-bound transporters to drive the accumulation of ions and small molecules, amino acids and metabolites into the vacuole (Forgac, 1989; Jones et al., 1997). In yeast, a second V-type ATPase is also present in a nonvacuolar location, on the membranes of the Golgi and/or endosomes, serving to acidify these organelles of the secretory pathway (Manolson et al., 1994).

Like the F_1F_o-ATPase of mitochondria, the V-ATPase can be functionally divided into two structurally distinct domains; the catalytic V_1 subcomplex and the proton-translocating V_o subcomplex. Subunits comprising the catalytic V_1 subcomplex of peripherally associated proteins are localized on the cytosolic face of the vacuolar membrane. The V_1 subcomplex is assembled onto the V_o subcomplex, which is composed of both peripheral and integral membrane proteins, resulting in the formation of a functional enzyme complex (Fig. 1). The biogenesis of the multisubunit V-ATPase requires the coordinated association of V_1 subunits synthesized in the cytosol with V_o subunits that are targeted to the vacuolar membrane. We know that V-ATPase V_o subunits are transported to the vacuole *via* the secretory pathway since mutations affecting the trafficking of the soluble vacuolar hydrolase carboxypeptidase Y, *via* the secretory pathway, also affect the targeting of V_o subunits (Bryant et al., 1998).

Examination of various mutant yeast cells, lacking either a V_1 or V_o subunit, has broadened our understanding of the association and assembly of the V-ATPase. Generally, the loss of a V_1 subunit of the catalytic domain has little effect on the stability of the remaining V_1 subunits, but does prevent their assembly onto the vacuolar membrane. In cells lacking a V_1 subunit (except for Vma7p), the V_o subunits remain stable and assemble normally into a V_o subcomplex that is targeted to the vacuolar membrane. Therefore, the ability to assemble V_1 subunits onto the V_o subcomplex requires the presence of all the V_1 subunits (except the 54 kDa subunit; Ho et al., 1993b). In contrast, the loss of a V_o subunit forming the V-ATPase membrane anchor affects the stability and assembly or localization of the remaining V_o subunits. In these mutant yeast cells lacking a V_o subcomplex, the V_1 subunits are stable but

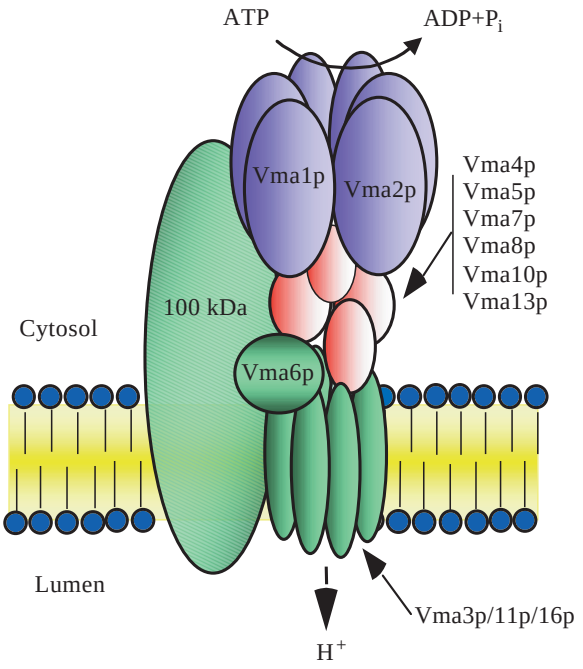

Fig. 1. Proposed composition of the yeast V-ATPase. The V-ATPase is composed of two large multisubunit subcomplexes designated V_1 (blue and red) and V_o (green). The translocation of protons into the lumen of the vacuole by the V_o subcomplex is coupled to the hydrolysis of cytosolic ATP by the V_1 subcomplex. The V_1 subcomplex is composed of peripherally associated subunits of predicted molecular mass 69 kDa (Vma1p, A), 60 kDa (Vma2p, B), 54 kDa (Vma13p, H), 42 kDa (Vma5p, C), 32 kDa (Vma8p, D), 27 kDa (Vma4p, E), 14 kDa (Vma7p, F) and 13 kDa (Vma10p, G). The catalytic subunits are shaded blue and the stalk subunits bridging the association of the catalytic subunits to the membrane are shaded red. The V_o subcomplex (green) is composed of integral and peripheral subunits, of molecular mass 100 kDa (Vph1p or Stv1p, a) and 36 kDa (Vma6p, d), and three proteolipids, 23 kDa (Vma16p, c″), 17 kDa (Vma11p, c′) and 16.5 kDa (Vma3p, c) forming the proton channel through the membrane.

present in the cytosol and are unable to assemble onto the vacuolar membrane.

Identification of genes encoding V-ATPase subunits

Biochemical analysis of a detergent-solubilized and purified yeast V-ATPase complex initially suggested that only three subunits form the functional enzyme complex (Uchida et al., 1985). Upon further analysis, the number of subunits detected by polyacrylamide electrophoresis increased to at least ten, ranging in molecular mass from 14 kDa to 100 kDa (Graham et al., 1995; 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 encoding proteins required for the assembly of a functional enzyme complex. To date, at least 17 genes have been identified that encode proteins required for the assembly of the yeast V-ATPase complex (Table 1). Of these, 14 genes encoding *bona fide* subunits of the yeast V-ATPase enzyme complex have been identified, cloned and characterized.

Yeast cells lacking V-ATPase activity due to disruption of a *VMA* gene exhibit a characteristic set of growth phenotypes. The cells lacking V-ATPase activity remain viable but demonstrate increased sensitivity to Ca^{2+} in the growth medium, an inability to grow in medium buffered to neutral pH and an inability to utilize nonfermentable sources of carbon (i.e. glycerol or ethanol). All these growth phenotypes have been successfully utilized in genetic screens aimed at identifying additional *VMA* genes (Ho et al., 1993a; Ohya et al., 1991). Genetic screens have provided the only means to identify *VMA* and other genes that code for proteins required for assembly or activity of the complex but do not function as *bona fide* V-ATPase subunits (Oluwatosin and Kane, 1997, 1998).

Composition of the V_1 subcomplex

The yeast V_1 subcomplex is composed of at least eight polypeptides of 69, 60, 54, 42, 32, 27, 16 and 14 kDa (Table 1). V_1 subunits are peripherally associated with the vacuolar membrane and can be released from the membranes by treatment with reagents such as alkaline carbonate and urea (Graham et al., 1995). In addition, V_1 subunits are released from vacuolar membranes in an ATP-dependent treatment with potassium nitrate (Graham et al., 1995). The release of V_1 subunits by nitrate occurs only in the presence of ATP, presumably reflecting a conformational change of the V_1 subcomplex due to the binding and/or hydrolysis of ATP. The ATP-induced conformational change appears to affect the physical interaction between V_1 subunits and the membrane, making them susceptible to release from the V_o subcomplex upon treatment with chaotropic reagents (e.g. 100 mmol l^{-1} 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.

Proteins homologous to all the yeast V_1 subunits have been identified in a wide range of organisms from bacteria to higher eukaryotes including humans (Stevens and Forgac, 1997). Even though we are just beginning to understand the role of these subunits, the sequence and, presumably, the structure and function have been highly conserved throughout evolution. In addition, the number of subunits required to form the V_1 subcomplex has also been conserved across a wide range of organisms.

Together, Vma1p (69 kDa subunit A; Hirata et al., 1990) and Vma2p (60 kDa subunit B; Nelson et al., 1989) form the catalytic and nucleotide-binding subunits homologous to the β and α subunits of the F_1F_o-ATPase complex, respectively. In yeast, the protein sequence of Vma1p is 48 % identical to that of Vma2p and 46–47 % identical to those of the α and β subunits of the F_1F_o-ATPase. Three copies of both Vma1p and Vma2p are present in each enzyme complex, assembling to form the catalytic ATP-hydrolyzing portion of the V-ATPase. The Vma1p is translated as a 119 kDa protein that is converted

Table 1. *Yeast genes required for V-ATPase composition and assembly*

Gene	Subunit	Apparent molecular mass (kDa)	Integral or peripheral	Proposed function
V_1				
VMA1	A	69	Peripheral	ATP-binding catalytic subunit
VMA2	B	60	Peripheral	ATP-binding subunit
VMA4	E	27	Peripheral	Interacts with Vma10p
VMA5	C	42	Peripheral	
VMA7	F	14	Peripheral	Interacts with Vma8p
VMA8	D	32	Peripheral	Interacts with Vma7p
VMA10	G	16	Peripheral	Required to stabilize Vma4p
VMA13	H	54	Peripheral	Activation of V-ATPase
V_o				
VPH1	a	100	Integral	Vacuole-specific V_o subunit
VMA3	c	16.5	Integral	Proton-translocating proteolipid DCCD binding, four TMDs
VMA6	d	36	Peripheral	Hydrophilic V_o subunit
VMA11	c′	17	Integral	Proteolipid similar to Vma3p
VMA16	c″	23	Integral	Proteolipid similar to Vma3p, possibly five TMDs
Isoform				
STV1		100	Integral	Similar to Vph1p, non-vacuolar localization
Assembly factors				
VMA12		25	Integral	Required for assembly, not a subunit
VMA21		8.5	Integral	Hydrophobic protein required for assembly, not a subunit
VMA22		21	Peripheral	Hydrophilic protein, binds to membrane *via* Vma12p

DCCD, *N*,*N*′-dicyclohexylcarbodiimide; TMD, transmembrane domain.

to a 69 kDa final product by the unique process of protein splicing involving the removal of a 50 kDa internal protein (Kane et al., 1990).

The function of the remaining V_1 subunits is unknown, but they may play a role in coupling the catalytic Vma1p and Vma2p subunits to the V_o subcomplex, analogous to the stalk subunits of the F_1F_o-ATPase. Structural analysis has revealed strong similarities between the V_1V_o-ATPase and the F_1F_o-ATPase (Svergun et al., 1998). Unfortunately, comparison of the protein sequence of the remaining yeast V_1 subunits with the sequence of the F_1F_o-ATPase stalk subunits has revealed no obvious sequence homology. 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 loss of any one of the stalk subunits prevents the assembly of either Vma1p or Vma2p onto the membranes of the vacuole, which is consistent with these proteins being essential components of the V_1 subcomplex. The 27 kDa (subunit E) and 42 kDa (subunit C) V_1 subunits are encoded by the *VMA4* (Foury, 1990) and *VMA5* genes, respectively (Ho et al., 1993a). The *VMA7* gene encodes a novel 14 kDa hydrophilic protein (subunit F), which displays characteristics of both a V_1 and V_o subunit (Graham et al., 1994; Nelson et al., 1994). In cells lacking Vma7p, the V_1 subunits are present at normal levels, but they are unable to associate with vacuolar membranes. Surprisingly, cells lacking Vma7p also show reduced levels of V_o subunits (100 kDa protein and 17 kDa proteolipid) present on the vacuolar membrane (Graham et al., 1994). Vma7p is the only V-ATPase subunit characterized that affects both the V_1 and V_o subcomplexes, suggesting its role as a *bona fide* stalk subunit bridging the two subcomplexes.

The *VMA8* gene codes for a prominent 32 kDa protein (subunit D) that was easily identified in Coomassie-stained SDS–PAGE gels of purified yeast V-ATPase complex (Graham et al., 1995; Kane et al., 1989; Nelson et al., 1995). Vma8p behaves as a classic V_1 peripheral subunit, being released from membranes by treatment with alkaline carbonate, urea and nitrate in the presence of ATP. The loss of Vma8p prevents the assembly of the other V_1 subunits onto the membrane. The results of Tomashek et al. (1997) suggest that Vma7p (subunit F) and Vma8p (subunit D) associate to form a stable complex that can be detected in the cytosol of *vma* mutant and wild-type cells. It is proposed that the Vma7p/Vma8p complex is able to associate with other V_1 subunits, eventually forming a V_1 subcomplex (Tomashek et al., 1997).

The *VMA10* gene encodes a hydrophilic protein of 13 kDa (subunit G) that migrates as a protein of slightly slower electrophoretic mobility (16 kDa), a behavior first observed in a Vma10p homologue identified in another organism, the tobacco hornworm *Manduca sexta* (Lepier et al., 1996; Supekova et al., 1995). The yeast Vma10p was originally characterized as a component of the V_o domain (Supekova et al., 1995), but recently we have demonstrated using several different criteria that Vma10p behaves as a model peripherally associated V_1 subunit (Tomashek et al., 1997), as it does in insects (Lepier et al., 1996). Interestingly, it has been observed that the loss of Vma10p (subunit G) results in an increased rate of turnover of the Vma4p (subunit E), with little effect on the stability of the other V_1 subunits (Tomashek et al., 1997). This observation, together with chemical cross-linking data, indicates that Vma10p and Vma4p form a subcomplex and probably interact directly with each other in the fully assembled V-ATPase (Tomashek et al., 1997).

VMA13 codes for a 54 kDa hydrophilic protein (subunit H), an atypical peripheral V_1 subunit that co-purifies with the V-ATPase complex (Ho et al., 1993b). Remarkably, cells lacking Vma13p are still able to assemble a V-ATPase on the vacuole, yet the assembled complex is inactive. In the absence of Vma13p, the assembled V_1 subunits appear to be more loosely associated with the membranes and can be released simply by low-salt treatment (Ho et al., 1993b). These results suggest that Vma13p may function to regulate the activity of the assembling V-ATPase complex. Unlike other V_1 subunits, Vma13p would be required only after the V_1 subcomplex has assembled onto the V_o subcomplex. The role of Vma13p would then be to activate, and possibly inactivate, the V-ATPase enzyme complex.

Assembly of the V_1 subcomplex

The loss of any single V_1 subunit prevents assembly of the remaining subunits onto the vacuolar membrane, yet they remain stable in the cytosol. Several partially assembled subcomplexes composed of V_1 subunits have been observed in the cytosol of various yeast *vma* mutants (Doherty and Kane, 1993; Tomashek et al., 1997). In mutant cells unable to form a V_o membrane anchor complex, the V_1 subunits are present in the cytosol as a large complex (approximately 550 kDa) containing at least Vma1p (subunit A), Vma2p (subunit B), Vma4p (subunit E), Vma10p (subunit G), Vma7p (subunit F) and Vma8p (subunit D; Tomashek et al., 1997). This suggests that the assembly of the V_1 subcomplex requires the presence of all V_1 subunits and that the V_1 subcomplex is capable of complete assembly prior to association with the V_o subcomplex. Where and when the V_1 subcomplex assembles with the V_o subcomplex in wild-type cells, forming a functional enzyme, is unknown.

Composition of the V_o subcomplex

The yeast V_o subcomplex is composed of at least five polypeptides of 100, 36, 23, 17 and 16.5 kDa (Table 1; Stevens and Forgac, 1997). All the V_o subunits are integral membrane proteins except for the 36 kDa protein, which is a peripheral membrane protein that is tightly associated with the V_o subcomplex (Bauerle et al., 1993). All the V_o subunits must be expressed in the cell in order to assemble a V_o subcomplex. Cells lacking any V_o subunit fail to provide a membrane anchor for the V_1 subunits, thereby prohibiting membrane association of the V-ATPase subunits. In these V_o mutant cells, the peripheral V_1 subunits, even though unable to associate with the vacuolar membrane, remain stable in the cytosol (Bauerle et al., 1993; Hirata et al., 1997). In V_1 mutant cells, the V_o subcomplex does assemble, remains stable and is successfully transported to the vacuole as in wild-type cells (Graham et al., 1995).

The 100 kDa V-ATPase subunit of the V_o subcomplex (subunit a) is a polytopic membrane protein encoded by two genes in yeast, designated *VPH1* and *STV1* (Manolson et al., 1992, 1994). The Vph1p is a subunit of the V-ATPase complex found on the vacuolar membrane, and Stv1p is a subunit of a second V-ATPase complex found on the membranes of the Golgi and/or endosome. 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 cells displaying a Vma^- growth phenotype. Loss of Vph1p results in cells possessing no detectable V-ATPase activity, yet these cells are still able to grow in media buffered to neutral pH (Manolson et al., 1992). Cells lacking Stv1p fail to display any Vma^- growth phenotype and grow like wild-type cells (Manolson et al., 1994). Only when yeast cells lack both Vph1p and Stv1p do they exhibit the full complement of Vma^- phenotypes (Manolson et al., 1994), suggesting that these proteins are functionally important. The proteins Vph1p and Stv1p are highly homologous (54 % identical) and are isoforms serving as components of V-ATPase complexes present in different cellular locations. The two V-ATPase complexes identified in yeast differ only in whether they contain Vph1p or Stv1p as the 100 kDa subunit, since both are composed of the same V_1 subunits and assemble with the same V_o subunits.

A comparison of the protein sequence of Vph1p and Stv1p reveals that both proteins are divided into a hydrophilic N-terminal half and a hydrophobic C terminus comprising several (6–9) membrane-spanning domains. Despite the similarity in sequence and in function, there is a difference between the Stv1p and Vph1p proteins resulting in the targeting of one to the vacuole and the other to an earlier compartment of the secretory pathway. It is likely that an as yet undetermined targeting sequence is responsible for the differential targeting of these two highly related proteins.

The 36 kDa hydrophilic V_o protein is encoded by the *VMA6* gene (Bauerle et al., 1993). The Vma6p (subunit d) is unusual because it is the only peripherally associated component of the membrane V_o subcomplex. Vma6p has been identified as a V_o subunit, and not a V_1 peripheral subunit, since loss of Vma6p prohibits the remaining V_o subunits from reaching the vacuole because of their inability to assemble and form a V_o

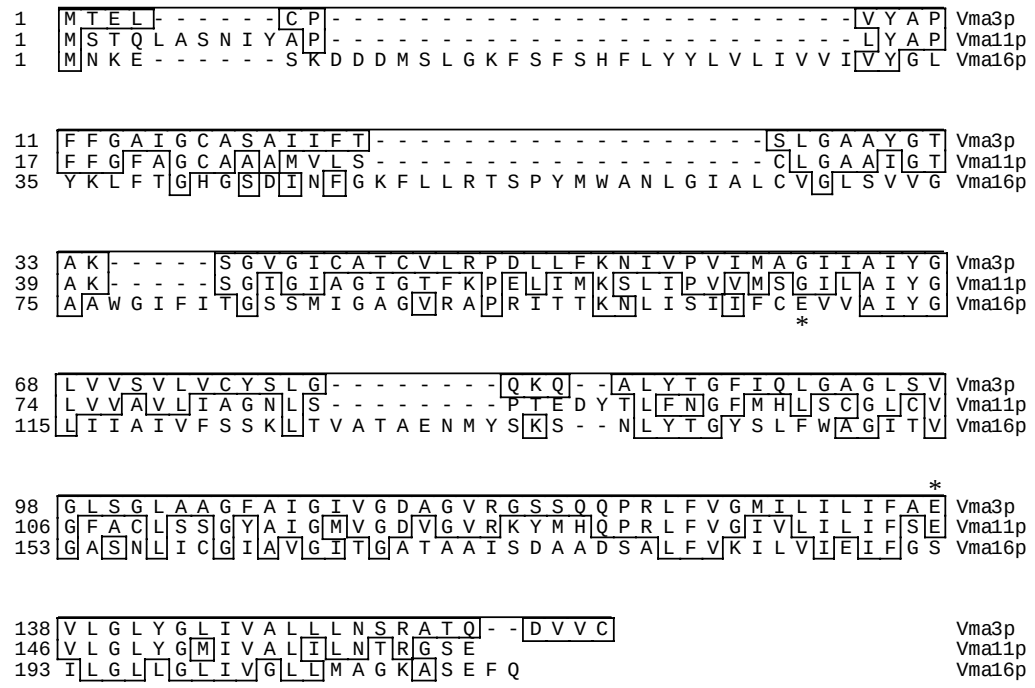

Fig. 2. Sequence comparison of yeast proteolipid V-ATPase subunits. Alignment of the amino acid sequence of subunits Vma3p, Vma11p and Vma16p generated using the MegAlign computer program. Boxed sequence indicate identities between Vma11p and/or and Vma3p. Asterisks mark the positions of key glutamic acid residues in Vma3p (Glu137), Vma11p (Glu145), and Vma16p (Glu108) that are required for proton translocation. Note the additional sequence at the N terminus of Vma16p that is predicted to form a fifth membrane-spanning domain.

subcomplex. Surprisingly, in cells lacking a V_o subunit such as Vph1p, the Vma6p remains stable in the cell yet is not found associated with membranes; instead, it is found in the cytosol. Specifically which V_o subunit serves to anchor the Vma6p to the membranes remains to be determined and is a subject of future study.

Unlike the membrane-spanning, proton-translocating channel of the F-ATPase that is formed by oligomers of the c subunit, the proton pore of the V-ATPase is formed by the assembly of at least three proteins of 16.5, 17 and 23 kDa. These proteins are encoded by the *VMA3*, *VMA11* and *VMA16* genes, respectively (Hirata et al., 1997). Vma3p (subunit c), Vma11p (subunit c′) and Vma16p (subunit c″) are referred to as proteolipids because they can be isolated from vacuolar membranes by extraction into organic solvent (Noumi et al., 1991). Like the 100 kDa proteins, the proteolipids are inserted into the membranes of the endoplasmic reticulum following synthesis, where they assemble with V_o subunits and are targeted to the membrane of the vacuole *via* the secretory pathway.

The hydrophobic 16.5 kDa protein (Vma3p) is predicted to form four membrane-spanning domains with both the N and C termini exposed to the lumen of the vacuole (Nelson and Nelson, 1989; Umemoto et al., 1990). Comparison of the amino acid sequence of the three proteolipids, Vma3p, Vma11p and Vma16p, reveals a high degree of sequence identity and presumably similar predicted membrane topography (Fig. 2). The 17 kDa proteolipid (Vma11p) is 56 % identical to the amino acid sequence of Vma3p and is also predicted to possess four membrane-spanning domains (Hirata et al., 1997; Umemoto et al., 1991). The 23 kDa proteolipid (Vma16p) is slightly larger than the other two proteins and is only 35 % identical to Vma3p (Apperson et al., 1990; Hirata et al., 1997). Unlike Vma3p and Vma11p, Vma16p is predicted to form five membrane-spanning domains.

Cells lacking Vma3p, Vma11p or Vma16p display identical phenotypes. Loss of any one of the proteolipids results in failure to assemble the V_o subcomplex and an increased rate of degradation of the 100 kDa protein Vph1p (Hirata et al., 1997). In addition, the loss of any one proteolipid prevents Vma6p from associating with the membrane V_o domain. The V_1 subunits are present at wild-type levels, but are not assembled onto the vacuolar membranes in cells lacking Vma3p, Vma11p or Vma16p. The fate of the remaining proteolipids in cells lacking any one of the proteolipid subunits has yet to be determined. We have observed that cells lacking Vma16p still contain a proteolipid of approximately 17 kDa, although in reduced amounts and not associated with vacuolar membranes (Hirata et al., 1997). It is possible that failure to assemble all three proteolipid subunits together prevents them from exiting the endoplasmic reticulum and being targeted to the vacuole.

Even though the proteolipids share a high degree of identity, they are not functionally redundant, since all three are required to assemble a functional V-ATPase. The over-expression of Vma3p in cells lacking Vma11p, or the over-expression of Vma11p in cells lacking Vma3p, does not restore the *vma*⁻ phenotype of these mutant cells to wild-type (Umemoto et al., 1991). The actual stoichiometry of Vma3p, Vma11p and Vma16p in the V_o complex remains to be determined. Direct comparison of the level of the three proteins present on vacuolar membranes by immunoblot analysis shows that Vma3p is more abundant than either Vma11p or Vma16p (Hirata et al., 1997). The presence of Vma11p and Vma16p within each V_o complex could account for the inability of the remaining proteolipids to form cross-linked species larger than

dimeric complexes (Powell, 1999). The molecular dissection of these related proteolipids to determine their stoichiometry, structure and function within the V-ATPase complex is the aim of future experiments.

Each of the proteolipids contains a glutamic acid residue (Vma3p Glu137, Vma11p Glu145, and Vma16p Glu108) that is required for proton-translocating activity (Hirata et al., 1997). In both Vma3p and Vma11p, the key glutamic acid residue is found in the fourth transmembrane domain, but in Vma16p it is in the third from last predicted transmembrane domain (Hirata et al., 1997). The glutamic acid residue of Vma3p serves as the binding site for the lipophilic compound *N, N′*-dicyclohexylcarbodiimide (DCCD). DCCD is a known inhibitor of both F-type and V-type ATPases through covalent modification of highly conserved acidic residues within Vma3p and subunit c (of F_o), thus preventing ATP-coupled proton translocation. The reaction of these conserved acidic residues in both F- and V-type ATPases with DCCD suggests that they are directly accessible to the lipid phase of the bilayer. Mutation of these key glutamic acid residues in Vma3p and subunit c prevents modification by DCCD. It has also been shown that mutation of the conserved glutamic acid residues within Vma11p and Vma16p to glutamine inactivates the V-ATPase enzyme complex, even though it appears to be fully assembled (Hirata et al., 1997). However, it is currently unknown whether both Vma11p and Vma16p are also modified by the lipophilic probe DCCD.

The inactivating mutations in Vma11p (Glu145) and Vma16p (Glu108) resulted in a higher percentage of assembled V-ATPase complexes present on the vacuolar membrane compared with membranes from wild-type cells (Hirata et al., 1997). Fractionation of detergent-solubilized vacuolar membranes results in the separation of a V_o subfraction away from the V_1V_o fully assembled V-ATPase complex. The unassembled V-ATPase complex represented by the V_o subfraction may be due to either physical removal of the V_1 subcomplex during purification or may represent a mechanism to regulate V-ATPase activity (Kane, 1995). The inability of these mutants to carry out proton translocation coupled to ATP hydrolysis directly affects the assembly and/or retention of V_1 subunits onto the vacuolar membranes.

Assembly of the V_o subcomplex

Newly synthesized 100 kDa and proteolipid proteins are inserted into the membranes of the endoplasmic reticulum, where they assemble together with Vma6p and are then targeted to the vacuole or Golgi/endosome by the secretory pathway. Failure to assemble the V_o subcomplex, because of loss of the proteolipid Vma3p or Vma6p, results in decreased cellular levels of the 100 kDa V-ATPase subunit, with little being found on the vacuolar membrane (Bauerle et al., 1993; Hirata et al., 1997). In cells lacking any one of these V_o subunits, the 100 kDa polypeptides are less stable, resulting in a decreased half-life of approximately 30 min compared with a half-life in wild-type cells in excess of 400 min (Graham et al., 1998). The stability of the 100 kDa V-ATPase subunit is increased by the ability to assemble properly into the newly forming V_o subcomplex. The increased turnover rate of the 100 kDa V-ATPase subunit in the absence of V_o assembly is not dependent on vacuolar proteases, suggesting that degradation occurs at another cellular location, such as the endoplasmic reticulum (Hill and Stevens, 1995). The degradation of the 100 kDa V-ATPase subunit in cells lacking a V_o subunit may serve to prevent transport of unassembled V_o subunits to the vacuolar membrane.

The lack of a V_o subunit, such as Vma6p, affects the localization of the proteolipid subunits since they are now prevented from reaching the vacuolar membrane (Bauerle et al., 1993). It has not been determined whether the stability, in addition to the localization, of all the proteolipids is affected by the loss of Vma6p, but we have observed no significant destabilization of the Vma3p in these mutant cells (L. Graham, unpublished results). Additional experiments are needed to determine how the assembly of these three proteolipids is coordinated, especially since they are present in different ratios within individual V-ATPase complexes.

Recall that, unlike the 100 kDa protein Vph1p, the protein Vma6p is not destabilized in cells lacking a V_o subunit, but is unable to associate with membranes (Bauerle et al., 1993). The Vma6p must assemble with the V_o subcomplex, from the cytosol, before the complex is competent to exit the endoplasmic reticulum for the vacuole. Once assembled, the Vma6p remains associated with the V_o subcomplex since it is found on vacuolar membranes even in cells unable to assemble a complete V_1 subcomplex. We have yet to identify which protein mediates the peripheral association of Vma6p with membranes. Whether it occurs *via* the 100 kDa protein or the proteolipids is the basis for future study. In our model for V_o assembly, all the V_o subunits must be present and assemble correctly in the endoplasmic reticulum prior to transport to the vacuole.

V-ATPase assembly factors

Three genes (*VMA12*, *VMA21* and *VMA22*) that have been identified through genetic analysis of *vma* mutants encode proteins required to assemble a functional V-ATPase, yet are not subunits of the enzyme complex. *VMA12* encodes a 25 kDa integral membrane protein predicted by hydropathy analysis to contain two membrane-spanning helices (Hirata et al., 1993; Ohya et al., 1991). Cells lacking Vma12p display the full complement of Vma^- phenotypes including sensitivity to high levels of Ca^{2+}, loss of V-ATPase activity and failure to grow on media buffered to pH 7.5. Biochemical analysis of cells lacking the Vma12p revealed that all the V_1 subunits were present but localized to the cytosol and thus not associated with the vacuolar membrane (Hirata et al., 1993). The V_o subunits were present in greatly reduced amounts in these mutant cells and also were not localized to vacuolar membranes, an effect similar to the loss of a V_o subunit. Surprisingly, Vma12p did not fractionate with the V-ATPase purified from vacuolar membranes, indicating that it is not a component of the final

enzyme complex. Yet, cells lacking Vma12p were unable to assemble a V_o subcomplex. Therefore, Vma12p is required for assembly of a functional V-ATPase, but is not a subunit of the enzyme complex. Cellular localization of the Vma12p to the membranes of the endoplasmic reticulum and not the vacuole suggested a role in the early assembly of the V-ATPase, specifically the V_o subunits, in the endoplasmic reticulum (Jackson and Stevens, 1997).

VMA21 and *VMA22* were identified, in a separate genetic screen, as genes whose products are also required for V-ATPase assembly (Ho et al., 1993a). Characterization of *VMA21* demonstrated that it codes for small 8.5 kDa hydrophobic protein predicted to form two membrane-spanning domains (Hill and Stevens, 1994). Cellular localization of Vma21p by immunofluorescence revealed that this protein also resides in the endoplasmic reticulum membranes, like Vma12p. Retention of this protein in the endoplasmic reticulum is dependent on a di-lysine motif at the C terminus of Vma21p since modification of this motif results in localization of the protein to the vacuolar membrane (Hill and Stevens, 1994). Cells lacking Vma21p are unable to assemble a V_o subcomplex, thus resulting in the destabilization of the 100 kDa V-ATPase subunit. A small hydrophobic protein has been identified in both the *Manduca sexta* (M9.7; Merzendorfer et al., 1999) and bovine (M9.2; Ludwig et al., 1998) chromaffin granules that displays some similarity to Vma21p. Unlike Vma21p, both M9.7 and M9.2 proteins have been proposed to be subunits of the final V-ATPase enzyme complex; however, these proteins may be present in the V-ATPase at substoichiometric levels (Ludwig et al., 1998).

VMA22 encodes a 21 kDa hydrophilic protein that is also required for the assembly of the V-ATPase (Hill and Stevens, 1995). Cells lacking Vma22p were phenotypically identical to cells lacking either Vma12p or Vma21p. Vma22p has also been localized to the membranes of the endoplasmic reticulum and not the vacuolar membrane. Subcellular fractionation analysis indicated that the membrane association of Vma22p is dependent on Vma12p, since in cells lacking Vma12p the Vma22p is found in the cytosol and not on the membrane (Hill and Stevens, 1995). A direct physical interaction between Vma12p and Vma22p was confirmed through the use of chemical crosslinking reagents under conditions that would form covalent crosslinks 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 in the absence of crosslinking reagents. Therefore, Vma12p and Vma22p appear to function together and form a stable V-ATPase assembly complex. Under similar conditions, Vma21p was not observed to be part of the Vma12p/Vma22p complex and may function separately in aiding the assembly of the V-ATPase.

Assembly of the V-ATPase

What role do the Vma21p and the Vma12p/Vma22p complex play in the assembly of the V-ATPase? One focus of our efforts has been towards identifying which of the V-ATPase subunits interact with the assembly factors. Additional crosslinking analysis revealed a direct interaction between newly synthesized 100 kDa subunit and the Vma12p/Vma22p assembly complex (Graham et al., 1998). Previous studies had shown that the 100 kDa V-ATPase subunit was inserted normally into membranes in the absence of Vma12p, thus ruling out a possible role for the Vma12p/Vma22p complex in aiding the translocation and/or folding of 100 kDa subunit in the endoplasmic reticulum membrane (Jackson and Stevens, 1997). This result suggests that the Vma12p/Vma22p complex must function at a later step aiding the assembly of the fully translocated and completely folded V-ATPase subunit into the V_o subcomplex.

Consistent with a transient interaction in the endoplasmic reticulum between the Vma12p/Vma22p assembly complex and newly synthesized 100 kDa V-ATPase subunit, kinetic analysis revealed that the interaction displayed a half-life of less than 5 min (Graham et al., 1998). The brief interaction between the Vma12p/Vma22p assembly complex and the 100 kDa V-ATPase subunit suggested that it occurs only while the newly synthesized V_o subunits are assembling in the endoplasmic reticulum. If the transport of newly synthesized proteins from the endoplasmic reticulum is blocked, by the use of a temperature-sensitive *sec* mutation, we observed a stabilization of the interaction between the Vma12p/Vma22p assembly complex and 100 kDa V-ATPase subunit (Graham et al., 1998). This stabilization suggests that continuous protein exit from the endoplasmic reticulum, specifically the packaging of the V_o subcomplex into endoplasmic-reticulum-derived vesicles, is required to disrupt the interaction between the Vma12p/Vma22p assembly complex and the V_o subunits.

We observed that the loss of Vma21p did not affect the formation of the Vma12p/Vma22p assembly complex or prevent the interaction between the assembly complex and the 100 kDa V-ATPase subunit (Graham et al., 1998). Results gathered to date suggest that Vma21p is not part of the Vma12p/Vma22p assembly complex. The hydrophobic nature of Vma21p, like that of the proteolipids, suggests that it may play a role in the assembly of the proteolipids in the V_o subcomplex. Preliminary results have revealed an interaction between Vma21p and newly synthesized proteolipids (L. Graham, P. Malkus, R. Schekman and T. Stevens, unpublished results). One role of Vma21p may be to aid in determining the correct stoichiometry of the proteolipids as they assemble into the V_o subcomplex.

Our proposed model describing V-ATPase assembly as it occurs in the endoplasmic reticulum is shown in Fig. 3. Two of the endoplasmic-reticulum-localized assembly factors, Vma12p and Vma22p, associate and appear to work together in assisting the assembly of V-ATPase subunits. Fully translocated and folded 100 kDa V-ATPase subunit interacts directly with the Vma12p/Vma22p assembly complex, possibly protecting the 100 kDa protein from degradation prior to its assembly into the V_o complex. The ability of the 100 kDa subunit to assemble into the V_o subcomplex extends its half-

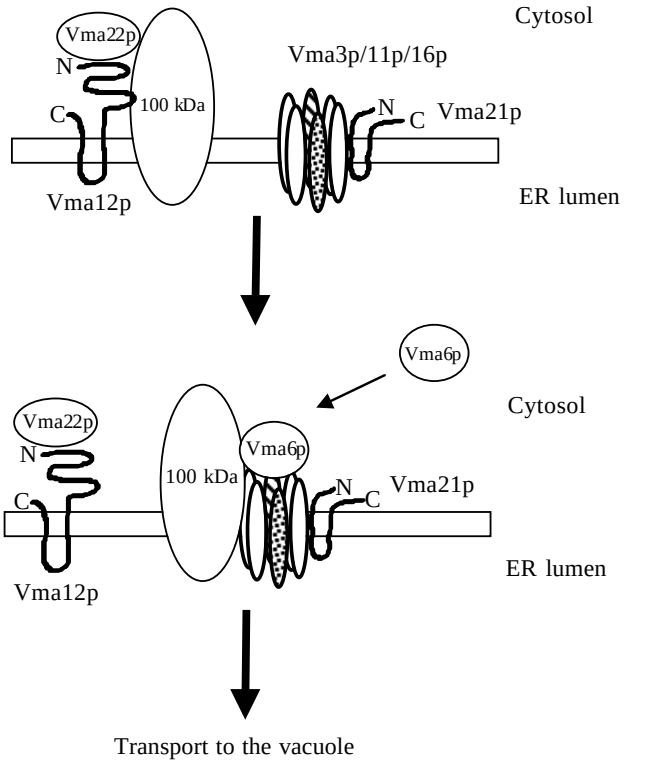

Fig. 3. Model of V_o assembly in the endoplasmic reticulum (ER). Subunits Vma12p and Vma22p interact to form a stable assembly complex in the membrane of the ER. The Vma12p/Vma22p assembly complex interacts directly but transiently with the 100 kDa protein Vph1p after it has been translated, translocated and folded correctly. The 100 kDa V-ATPase subunit would then assemble with the other V_o subunits in order for the V-ATPase to exit the ER for the vacuolar membrane. Vma21p would aid the assembly of the proteolipids (Vma3p, open; Vma11p, stippled; Vma16p, hatched) and possibly Vma6p either in parallel or downstream of the 100 kDa/Vma12p/Vma22p subcomplex. Assembly of all the V_o subunits may be followed by the dissociation of the Vma12p/Vma22p assembly factors from the V_o subcomplex prior to transport out of the ER. Vma21p may continue to interact with the V_o subcomplex following release from the Vma12p/Vma22p assembly complex and may even exit the ER with the V_o subcomplex, dissociating from the V_o subcomplex in the Golgi, followed by retrieval back to the ER.

life compared with an unassembled protein. The interaction between the 100 kDa V-ATPase subunit and the Vma12p/Vma22p assembly complex occurs even in the absence of Vma21p, and thus Vma12p/Vma22p association is independent of this third assembly factor (Graham et al., 1998).

We envisage the role of Vma21p to be one of aiding the assembly of the V-ATPase in parallel to the Vph1p/Vma12p/Vma22p interaction and involving the assembly of the proteolipids and possibly Vma6p into the V_o 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. Vma21p may serve an additional role in V-ATPase biosynthesis as shown by its di-lysine endoplasmic reticulum retention/retrieval motif. Vma21p could associate with the assembled V_o subcomplex (or the fully assembled V_1V_o complex) in the endoplasmic reticulum and escort the enzyme complex in vesicles bound for the Golgi complex (Herrmann et al., 1999). Vma21p would then dissociate from the V-ATPase early in the Golgi and be retrieved back to the endoplasmic reticulum for another cycle of assembly and transport. Vma21p may function, in addition to assembly, to load the V-ATPase complex into vesicles budding from the endoplasmic reticulum and, thus, serve as a specific 'cargo receptor' (Kuehn and Schekman, 1997). Future experiments will attempt to determine whether Vma21p is transported out of the endoplasmic reticulum with the V_o subcomplex as it is transported to the vacuole.

Future perspectives

Current investigations are now focused on determining the precise roles of the 100 kDa V-ATPase subunit and of each of the three proteolipids within the enzyme complex. We hope to determine how these polypeptides interact, their stoichiometry and their involvement in ATP-coupled proton translocation. Experiments will be designed to obtain more detailed structural information regarding specific proteolipid–proteolipid interactions. A similar approach will also be used to investigate protein–protein interactions between other V_o subunits such as Vma6p and the proteolipids. A site-directed mutagenesis approach coupled with electron microscopy studies investigating the organization of the intact V_o domain will provide a detailed understanding of the composition, arrangement and organization of the V_o domain of the yeast V-ATPase. We will continue to define the role of the assembly factors and specifically describe which V_o subunits interact directly with Vma21p.

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

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