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Assembly and Regulation of the Yeast Vacuolar H^+-ATPase

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The yeast vacuolar proton-translocating ATPase (V-ATPase) is an excellent model for V-ATPases in all eukaryotic cells. Activity of the yeast V-ATPase is reversibly down-regulated by disassembly of the peripheral (V_1) sector, which contains the ATP-binding sites, from the membrane (V_0) sector, which contains the proton pore. A similar regulatory mechanism has been found in *Manduca sexta* and is believed to operate in other eukaryotes. We are interested in the mechanism of reversible disassembly and its implications for V-ATPase structure. In this review, we focus on (1) characterization of the yeast V-ATPase stalk subunits, which form the interface between V_1 and V_0, (2) potential mechanisms of silencing ATP hydrolytic activity in disassembled V_1 sectors, and (3) the structure and function of RAVE, a recently discovered complex that regulates V-ATPase assembly.

KEY WORDS: V-ATPase; vacuole; acidification; vma mutant; proton pump; RAVE; yeast; stalk.

INTRODUCTION

V-ATPases are present in every eukaryotic cell where they are responsible for acidification of lysosomes, the Golgi apparatus, and endosomes and involved in protein sorting, pH and calcium homeostasis, and zymogen activation (Nelson and Harvey, 1999; Nishi and Forgac, 2002). Our laboratory has a long-term interest in elucidating subunit structure and function in V-ATPases and in defining cellular mechanisms for regulating these multifaceted proton pumps. The yeast vacuolar proton-translocating ATPase has emerged as an excellent model for V-ATPases and has been our system of choice.

There are several reasons why the yeast V-ATPase is a particularly attractive model. All of the known subunits have been cloned and sequenced, and most of the subunit sequences are remarkably conserved among eukaryotes. This conservation extends to subunit structure as well; even the least conserved yeast subunit, subunit H, can be functionally replaced by the human homologue (Lu *et al.*, 1998), as can many of the other V-ATPase subunits. With one exception, deletion of any V-ATPase subunit gene leads to a well-defined Vma-phenotype, characterized by a reduced growth rate under all conditions, optimal growth at pH 5, and failure to grow at elevated pH and/or calcium concentrations (Nelson and Nelson, 1990; Ohya *et al.*, 1991; Yamashiro *et al.*, 1990). This phenotype has allowed both the identification of new subunit genes and rapid assessment of V-ATPase function in new mutants. Only one yeast V-ATPase subunit, the a subunit, is encoded by two organelle-specific isoforms; both isoforms must be deleted to generate the full Vma-phenotype (Manolson *et al.*, 1994).

V-ATPases appear to be highly regulated proton pumps, and at least some aspects of their regulation are preserved in yeast as well. All V-ATPases are composed of a peripheral V_1 complex attached to a membrane-bound V_0 complex (see below). Several years ago, we found that after its initial assembly, the yeast V-ATPase could rapidly and reversibly disassemble into free cytosolic V_1 sectors and membrane-bound V_0 sectors in response to a brief (2–5 min) glucose deprivation (Kane, 1995). The Wieczorek lab independently discovered a very similar dissociation of the Manduca sexta V-ATPase under conditions of nutrient deprivation (Sumner *et al.*, 1995). The existence of reversible dissociation in yeast and insects suggests that it is a general mechanism of regulating V-ATPase activity. This initial work has been reviewed

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previously (Kane, 2000; Kane and Parra, 2000; Wieczorek *et al.*, 2000). Recently, we have pursued the mechanisms underlying this mode of regulation as well as the implications for V-ATPase structure. In this review, we focus on recent data addressing these issues, particularly functional characterization of the V-ATPase stalk subunits, mechanisms of silencing ATP hydrolytic activity in free V_1 sectors, and the function of the RAVE complex.

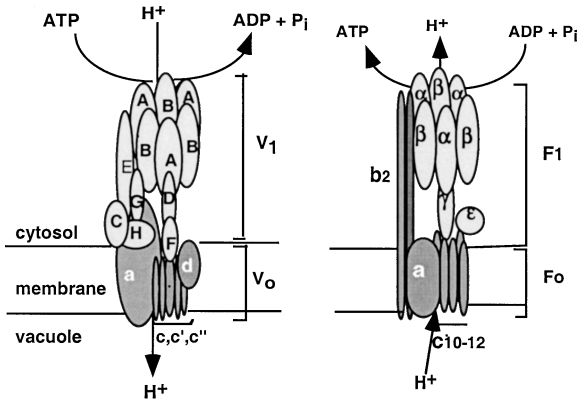
STALK SUBUNITS OF THE YEAST V-ATPase

The discovery that V-ATPases are organized into a peripheral membrane complex (V_1) attached to an integral membrane complex (V_0) occurred at approximately the same time as the cloning and sequencing of the first V-ATPase subunits (reviewed by Bowman *et al.*, 1992; Nelson and Taiz, 1989). Both of these advances pointed to fundamental similarities between V-ATPases and the F-ATP synthases, and the much more extensive characterization of the F-ATPases suggested a framework for further studies of V-ATPases. A number of aspects of V-ATPase structure and function proved to fit this framework (reviewed in Forgac, 1999; Nelson and Harvey, 1999; Nishi and Forgac, 2002): (1) the catalytic (A) and regulatory (B) ATP binding subunits and the proteolipid subunits (c, c′, and c″) showed clear sequence homology with their F_1F_0 counterparts, (2) electron micrographs of V-ATPases showed a "ball and stick" structure for V-ATPase that was subsequently resolved into at least two "stalk" structures connecting the V_1 headpiece containing the ATP-binding subunits and the V_0 sector containing the proton translocating subunits (Boekema *et al.*, 1997), and (3) mechanistic studies indicated positive cooperativity between ATP hydrolytic sites in V-ATPases that was consistent with that observed in F-ATPases and supportive of a rotational catalytic mechanism. Rotational catalysis was very recently observed in a V-type ATPase from thermophilic bacteria (Imamura *et al.*, 2003).

Clear differences between F- and V-ATPases also began to emerge, however. As the V-ATPase stalk subunit genes were cloned and sequenced, it became clear that they were quite conserved among V-ATPases at both the amino acid sequence and structural level, but, in most cases, were not readily comparable to the F-ATPase stalk subunits of prokaryotes or eukaryotes (Grüber *et al.*, 2001; Margolles-Clark *et al.*, 1999). Many biochemical studies indicated that unlike F_1 sectors, which are highly active ATPases, free V_1 sectors are not active as ATP hydrolases, and free V_0 sectors are not open proton pores. Free V_1 and V_0 sectors were observed along with assembled V_1V_0 complexes in many eukaryotic cells (reviewed in Kane and Parra, 2000). The discovery that the yeast and *Manduca sexta* V-ATPases disassembled in vivo suggested that the free complexes could arise from a similar process in other eukaryotic cells and provided a physiological logic for silencing ATP hydrolysis in free V_1 sectors; specifically, protection of cytosolic ATP stores from cytosolic V_1 sectors that were not bound to V_0 and thus incapable of ATP-driven proton transport. All of these results also presented a paradox in the comparison to F-ATPases. Recent models for F-ATPase structure assign the stalk subunits to either a rotor stalk or a stator stalk (Cross, 2000). The rotor stalk is responsible for rotating between catalytic subunits and thus transmitting conformational changes arising from ATP hydrolysis to a portion of the proton pore, and the stator stalk is responsible for stably attaching the ATP-binding subunits to the remainder of the proton pore so that rotation can be productive. If V-ATPases also work by rotational catalysis, they must have structural equivalents of the rotor and stator stalks (Boekema *et al.*, 1997), but they must also retain the ability to rapidly and reversibly dissociate V_1 from V_0. This suggests that the stator stalk in V-ATPases must balance the stability needed for productive catalysis with the instability needed for regulation by reversible disassembly.

Assignment of specific subunits to V-ATPase stalks has been difficult and is still a work in progress (Arata *et al.*, 2002a,b; Grüber *et al.*, 2001; Rizzo *et al.*, 2003; Xu *et al.*, 1999). Figure 1 shows a working model for the yeast V-ATPase. In this model, we place the D and F subunits in the central, rotor stalk, based primarily on the work of others (Arata *et al.*, 2002a,b). This places the E, G, C, and H subunits of V_1 and the cytosolic portion of the V_0 a subunit in the peripheral, stator stalk. We have pursued structural and functional aspects of the V-ATPase stalk subunits using a variety of approaches in yeast. Recently, we have focused on the structure and function of the G, C, and H subunits.

Supekova *et al.* (1996) were the first to identify a limited homology between the V-ATPase G subunit and the F_0 b subunit. In *Escherichia coli*, a dimer of b subunits is believed to form an extended coiled-coil reaching through the membrane to the top of the α/β hexamer that functions as the core of the stator stalk (Dunn *et al.*, 2000). V-ATPase G subunits have no transmembrane domain, but by comparing helical wheel projections of the N-terminal half of the G subunit and the b subunit sequence immediately after the transmembrane domain, Hunt and Bowman showed that the conserved amino acids fell predominantly on one face of the helix (Hunt and Bowman, 1997). We tested this model by site-directed mutagenesis of the yeast G subunit (Charsky *et al.*, 2000). Changing conserved charged residues to alanine in this region of the protein yielded a number of different phenotypes. Among



Gene Product		MW	Letter name	F-ATPase homolog?
V1:	Vma1	69 kD	A	β
	Vma2	60	B	a
	Vma13	54	H	
	Vma5	42	C	
	Vma8	32	D	
	Vma4	27	E	
	Vma10	16	G	b?
	Vma7	14	F	
Vo:	Vph1/Stv1	100	a	a?
	Vma6	36	d	
	Vma16	19	c''	c
	Vma11	16	c'	c
	Vma3	16	c	c

Fig. 1. Subunit composition and structural model for the yeast V-ATPase. Our working model for the yeast V-ATPase (left) is compared to a model of the the *E. coli* F-ATPase (right). V_1 and F_1 subunits are shown in light gray; V_0 and F_0 subunits are shown in dark gray. Nomenclature for the yeast subunits is defined below.

the most interesting were two mutations, R25A and R25L. Both mutant strains contained apparently wild-type levels of V-ATPase subunits, but had higher levels of ATPase activity and assembled V_1 subunits in isolated vacuoles. These mutants also showed lower levels of disassembly when cells were deprived of glucose. Previous results had shown that inhibition of V-ATPase activity could inhibit V-ATPase disassembly (Parra and Kane, 1998), but this was the first indication that a fully active V-ATPase could be stabilized against disassembly in response to glucose deprivation. This indicates that rather small changes in the stalk subunits could significantly perturb the balance between V-ATPase stability and instability described above. Other mutations, including several point mutations in the nonhomologous regions of the A subunit identified recently (Shao *et al.*, 1993), have resulted in a similar combination of effects (little disassembly, despite good activity in isolated vesicles). We hypothesize that this class of mutations may stabilize the stator stalk.

Sorgen *et al.* (1998, 1999) demonstrated that function of the *E. coli* F_0 b subunit was remarkably resistant to insertions or deletions in the region showing homology to the V_1 G subunits. We made two to four amino acid deletions and additions in two areas within this region of the yeast G subunit (Charsky *et al.*, 2000). Only one of the mutations (ΔQ29D30) allowed any activity in isolated vacuoles, but most of the mutations did allow assembly of V_1 with V_0, suggesting that the mutations exerted a specific effect on catalysis. The observation that a two amino acid deletion (ΔQ29D30) permitted some function, while a four amino acid deletion (ΔR28-K31) (which should better preserve the conserved face of the putative alpha helix) did not, suggests that the initial model on which we based our mutagenesis might be oversimplified. Subsequent work has suggested that the F_0 b subunit, which is a dimer in *E. coli*, may form an unusual coiled-coil structure (Del Rizzo *et al.*, 2002); ability to form this type of structure could not be easily assessed from a simple helical wheel analysis. In addition, there are many questions still remaining about the G subunit: (1) How many copies of the G subunit are present in each yeast V-ATPase complex? (2) If it does form a coiled-coil, does the G subunit coil with itself or another V_1 subunit? (3) Any stator structure containing the G subunit must still be tethered to the V_0 sector, since the G subunit has no transmembrane domain. Which subunits provide this tether? Answering these questions could provide important insights into stator structure and function in V-ATPases.

There is substantial genetic and biochemical evidence indicating that the E and G subunits interact. These subunits have been chemically crosslinked in vitro and observed to form a complex in vivo (either in multiple copies or in combination with one or more additional subunits) (Tomashek *et al.*, 1997; Xu *et al.*, 1999). Genetic deletion of the G subunit destabilizes the E subunit significantly (Tomashek *et al.*, 1997); this is quite unique among the V_1 subunits, and suggests a functionally important interaction between these subunits. We have also found that these two subunits interact strongly and specifically in a Far-western (overlay) blot and by two-hybrid assay, suggesting that their interaction is direct (Charsky *et al.*, manuscript in preparation). In addition to the G subunit, the E subunit has been shown to crosslink to many other V_1 subunits and the V_0 a subunit. Recently the E subunit was crosslinked to several locations on the B subunit predicted by modelling to lie on the outer surface of the A/B headpiece of V_1 (Arata *et al.*, 2002a). Taken together, this evidence may suggest that the E subunit forms the "core" of the V-ATPase stator stalk, perhaps in combination with the G subunit (Arata *et al.*, 2002a,b).

In contrast to the G and E subunits, which have credible functional parallels in F-ATPases, the C and H subunits are found in all V-ATPases, but have no apparent equivalent in the F-ATPases. Both subunits have been assigned

to the peripheral, stator stalk of the V-ATPase based primarily on crosslinking and in vitro binding studies (Lu *et al.*, 2002; Xu *et al.*, 1999). Mutants lacking the C or H subunit (vma5Δ or vma13Δ mutants, respectively) are different from most other vma mutants in that the remaining subunits are extensively assembled into free V_1 sectors, free V_0 sectors, and even some V_1V_0 complexes (Doherty and Kane, 1993; Ho *et al.*, 1993a,b; Kane *et al.*, 1999). However, both mutants exhibit the full range of Vma-phenotypes, indicating that the complexes formed are not functional, and V_1V_0 complexes isolated from the mutants are unstable (Ho *et al.*, 1993a,b). These results suggest that the C and H subunits play a critical role in bridging the V_1 and V_0 sectors and in coupling ATP hydrolysis and proton transport. They may also be important in stabilizing the V-ATPase stator stalk.

We probed the structure and function of subunit C by random and site-directed mutagenesis (Curtis *et al.*, 2002). Overall, the function of subunit C was remarkably resistant to mutations (based on the ability of mutant subunit genes to complement the Vma-growth phenotypes of a strain lacking subunit C). However, a number of mutations had significant effects on V-ATPase activity in isolated vacuoles. In particular, a conserved 8 amino acid region very near the C-terminus of the subunit appears to be very important for stable assembly of V_1V_0; mutations in this region reduced V-ATPase activity in vitro by up to 85% because of loss of V_1 subunits from the vacuolar membrane during vacuole isolation. The three most interesting mutations changed three conserved aromatic amino acids of the C subunit gene to alanine (F260A, Y262A, and F385A). Remarkably, isolated vacuolar vesicles from these mutants contained wild-type or near wild-type activity, but achieved this activity with much lower levels of V_1 subunits, and at least in one case (F260A), almost no C subunit. We concluded that these mutations resulted in an unstable V-ATPase with a much higher kcat (at least threefold) for ATP hydrolysis and proton pumping. Taken together, these results indicate that the C subunit is critical for stabilizing association between the V_1 and V_0 sectors and suggest that this stabilization may be achieved at some cost to the overall rate of ATP-driven proton pumping.

Overexpression of the wild-type H subunit in yeast highlighted its importance in coupling ATP hydrolysis and proton transport. High level overexpression of either the C or H subunit is lethal to yeast cells; low level (threefold or less) overexpression of either subunit results in a Vma-growth phenotype, indicating that V-ATPase activity has been compromised (Curtis and Kane, 2002). Surprisingly, vacuolar vesicles isolated from a yeast strain overexpressing the wild-type H subunit exhibited an ATP hydrolysis rate 72% of that in wild-type cells, but supported almost no proton transport. This was the first example of a fully uncoupled V-ATPase complex, and it highlights the importance of the H subunit in establishing a functional connection between V_1 and V_0. A high resolution structure of the yeast H subunit is now available (Sagermann *et al.*, 2001) and is the first such structure for any V-ATPase subunit. This structure provides an unprecedented opportunity for structure-directed mutagenesis and should be very valuable for further examining H subunit function.

STRUCTURAL AND FUNCTIONAL FEATURES OF FREE V_1 SECTORS

We envision disassembly of fully assembled V-ATPase complexes into free V_1 and V_0 sectors as a mechanism for downregulating V-ATPase activity when energy is limited. In this scheme, it is critical that the ATPase activity of V_1 be silenced when it is released from the membrane (Kane and Parra, 2000; Wieczorek *et al.*, 2000). As described above, silencing of ATP hydrolysis in V_1 sectors is an apparent difference from most F_1 sectors. We examined the structure and activity of wild-type and mutant V_1 sectors in order to address the mechanism underlying silencing of ATPase activity in free V_1 sectors (Parra *et al.*, 2000). We developed a purification of V_1 sectors based in part on a purification protocol for the *M. sexta* V_1 (Gräf *et al.*, 1996) and proceeded to isolate V_1 sectors from several different yeast strains. In yeast cells grown in glucose, their preferred carbon source, 30–40% of the total V_1 sectors are cytosolic, but in yeast cells deprived of glucose for as little as 5 min, 75–85% of the total V_1 sectors are cytosolic because of disassembly of previously assembled V_1V_0 complexes (Kane, 1995). Yeast cells lacking one of the V_0 subunits assemble V_1 subunits only into cytosolic V_1 sectors (Doherty and Kane, 1993; Tomashek *et al.*, 1996). We compared the subunit composition of cytosolic V_1 complexes isolated from wild-type and vma3Δ mutant cells, with and without glucose deprivation prior to lysis (Parra *et al.*, 2000). In all cases, the purified V_1 sectors had the same subunit composition, which included all of the V_1 subunits except subunit C. When the isolated V_1 sectors were assayed for ATP hydrolysis, they showed no Mg^{2+}-dependent ATPase (MgATPase) activity, as expected from previous results, but they did exhibit some Ca^{2+}-dependent ATP hydrolysis (CaATPase activity), which slowed and ended within a few minutes. V_1 sectors from *M. sexta* also exhibited CaATPase, but not MgATPase activity (Gräf *et al.*, 1996). In both cases, the concentrations of calcium required for activity were far higher than those ever encountered in the cytosol, indicating that the ATPase activity of free V_1 sectors is effectively silenced in vivo.

In order to probe the mechanism of silencing in more detail, we isolated V_1 sectors from a vma13Δ mutant strain (Parra *et al.*, 2000). As described above, subunit H (Vma13p) is not required for assembly of V_1V_0 complexes, and we found that free V_1 sectors from the vma13Δ strain contained all of the V_1 subunits at an apparently normal stoichiometry except for subunits C and H. The enzymatic properties of the cytosolic V_1 complexes were markedly different in the absence of subunit H. The initial velocity of CaATPase activity was much greater in the absence of subunit H and the rate of hydrolysis was linear for up to 20 min. Remarkably, the V_1 sectors lacking subunit H also had a significant initial velocity of MgATPase activity, although the velocity decayed until there was no further ATP hydrolysis within 5 min of ATP addition. These results indicate that subunit H, which appears to be an activator of the membrane-bound V-ATPase, is an inhibitor of free V_1 sectors.

This result has several implications. Subunit H must be involved in the conformational change in V_1 that accompanies release from V_0 in a functionally significant manner. One intriguing possibility is shown in Fig. 2. As described above, we would place the H subunit in the stator of the intact V_1V_0 complex, and there is evidence that it is near the interface of V_1 and V_0 (Landolt-Marticorena *et al.*, 2000). In this context, the H subunit could inhibit ATPase activity in V_1 by bridging the stator and rotor stalks after it is released from contact with V_0, and thus prevent rotation of the rotor stalk. Suzuki *et al.* (2000) have artificially created a similar situation in an F_1-ATPase by adding the soluble portion of the b subunit to F_1 and forming a crosslink between a cysteine introduced at the tip of the b subunit and the γ subunit of the rotor stalk. If such a similar, noncovalent, structure forms when V_1 is released from the membrane, it would also fit the symmetrical appearance of *M. sexta* V_1, in which the stalk subunits appear as a central mass below the A/B headpiece (Radermacher *et al.*, 1999, 2001; Svergün *et al.*, 1998). Our results also suggest that there are additional mechanisms of inhibiting cytosolic V_1 sectors, however, because the V_1 sectors from the vma13 mutants do not sustain their MgATPase activity. The source of this additional inhibition is not known, but could include product inhibition at the catalytic site in the free V_1 sectors or loss of one of more subunits during catalysis.

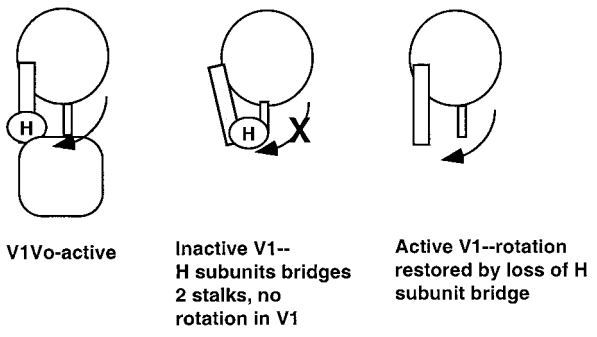


Fig. 2. A possible mechanism for inhibition of ATPase activity in free V_1 sectors by the H subunits. Left: The A/B headpiece of the V-ATPase is attached to the V_0 sector via a central stalk that rotates to give productive catalysis in the intact enzyme and a peripheral stalk containing subunit H. Center: We propose that release of V_1 from V_0 allows the H subunit to bridge the central and peripheral stalks, inhibiting ATPase activity by preventing rotation. Right: Loss of the H subunit may activate MgATPase activity in V_1 by freeing the central stalk, so that it is able to rotate.

Efficient inhibition of MgATPase activity in cytosolic V_1 is likely to be physiologically important, because we would predict that sustained ATP hydrolysis by cytosolic V_1 sectors in vivo would be damaging to cells. The vma13Δ mutant shows no additional growth defects beyond the characteristic Vma-growth defect, possibly because of the additional mechanisms for inhibiting MgATPase activity. As described briefly above, we have seen a deleterious gain of function in cells overexpressing the H or C subunit, however, and it is possible that the additional damaging function is activation of MgATPase activity in free V_1 sectors (Curtis and Kane, 2002). Interestingly, the additional growth defect arising from C and H subunits overexpression occurs only under conditions where cytosolic V_1 sectors can assemble, consistent with the hypothesis that the overexpressed subunits are activating V_1 (Curtis and Kane, 2002). The severe growth defect of these cells has made further biochemical analysis difficult, but we have found that subunit C binds to cytosolic V_1 sectors when it is overexpressed, and it is possible that this binding activates V_1-ATPase activity.

Disassembly of the V-ATPase in response to glucose deprivation implies that changes in extracellular carbon source can be communicated to V-ATPase complexes present on intracellular membranes. As yet, the nature of this signal is not clear (Parra and Kane, 1998). We had anticipated that the subunit composition of cytosolic V_1 complexes isolated from glucose-deprived cells might differ from that of glucose-grown cells, or that additional proteins might coisolate with V_1 complexes in a glucose-dependent manner. We observed neither of these in our initial purifications of V_1 (Parra *et al.*, 2000), but we did observe a few proteins that copurified with the V-ATPase regardless of whether glucose was present. Three of these later proved to belong to the RAVE complex.

THE RAVE COMPLEX

The RAVE complex was identified by the Deshaies lab through a proteomics approach aimed at identifying

new binding partners for the versatile adaptor protein Skp1p (Seol *et al.*, 2001). Skp1p is a very highly conserved protein that is best known as a subunit of the SCF (Skp1-cullin-F-box) class of E3 ubiquitin ligases which are involved in targeting proteins for degradation (Deshaies, 1999). In the SCF ubiquitin ligases, Skp1p bridges one of several F-box proteins to a complex containing a cullin (Cdc53p) and a RING H2-containing protein (Hrt1p) (Deshaies, 1999). The RING-containing protein is directly responsible for transfer of ubiquitin to a substrate targeted for degradation. The F-box proteins all contain an "F-box motif" (Bai *et al.*, 1996) that is directly responsible for binding to Skp1p (Orlicky *et al.*, 2003; Zheng *et al.*, 2002) and provide specificity to SCF complexes by recognizing the protein substrate for ubiquitination in a phosphorylation-dependent manner (Bai *et al.*, 1996; Deshaies, 1999; Willems *et al.*, 1999). Although the SCF complexes are better characterized by far, it is important to note that Skp1p is also present in a number of other nonproteolytic complexes (Kaplan *et al.*, 1997; Wiederkehr *et al.*, 2000). The functional mechanism of many of the these complexes is poorly understood, but some of them are linked to ubiquitination, even if their function is not solely to target proteins for proteosomal degradation (Kuras *et al.*, 2002).

Seol *et al.* (2001) used yeast Skp1p tagged with a myc9 epitope to affinity-purify Skp1p-binding partners from a yeast cell lysate, and then identified the partners by mass spectrometry. Among a large number of binding partners, two previously uncharacterized proteins, Rav1p and Rav2p, were shown to form a cytosolic complex with Skp1p and several V_1 subunits, but significantly, this complex did not contain other SCF complex members essential for ubiquitin ligase activity. The functional significance of the RAVE complex for the V-ATPase was demonstrated by the fact that mutants lacking Rav1p and Rav2p (rav1Δ and rav2Δ mutants) exhibited a partial Vma-phenotype and partial defects in vacuolar acidification. (SKP1 is an essential gene because of the diverse functions of SCF ubiquitin ligases in the cell cycle, and so it is more difficult to confirm that it directly affects the V-ATPase.) Still more intriguing, the rav1Δ mutant showed a kinetic delay in reassembly of V-ATPase complexes after readdition of glucose to a glucose-deprived culture. Taken together, these results strongly suggested that the RAVE complex helps cytosolic V_1 complexes reassemble with V_0 at the membrane.

We hypothesized that glucose-dependent interaction of the RAVE complex and V_1 in the cytosol might help to explain the effect of Rav1p on reassembly (Smardon *et al.*, 2002). Comparison of cytosolic fractions from yeast cells grown in glucose, deprived of glucose for a short time, or deprived of glucose followed by glucose restoration confirmed that the RAVE complex bound to V_1 complexes that appeared in the cytosol after glucose deprivation and then released these complexes upon glucose V_1 readdition. However, in analyzing RAVE–V_1 binding in a strain where V_1 is constitutively cytosolic because one of the V_0 subunits is missing (vma3Δ), we found constant levels of RAVE binding to V_1 regardless of extracellular glucose concentration. This result indicates that RAVE–V_1 binding is not inherently glucose-dependent, but that RAVE binds V_1 complexes whenever they are in the cytosol. This might suggest a more general role for RAVE beyond glucose-dependent reassembly. Consistent with this, we found that vacuoles isolated from rav1Δ and rav2Δ mutant cells had very low levels of V-ATPase activity and correspondingly low levels of V_1 subunits assembled at the membrane, even when the cells were grown in glucose prior to vacuole isolation. This result was somewhat surprising given the observation of Seol *et al.* that rav mutants exhibited only a partial Vma-growth phenotype and appeared to retain some V_1 subunits at the vacuole in vivo, at least at 30°C (Seol *et al.*, 2001). We believe, however, that the RAVE complex is essential for stable assembly of the V-ATPase under all conditions, and that assembly of a population of unstable but functional V-ATPase complexes in the absence of RAVE may account for the partial acidification and V-ATPase assembly observed in vivo.

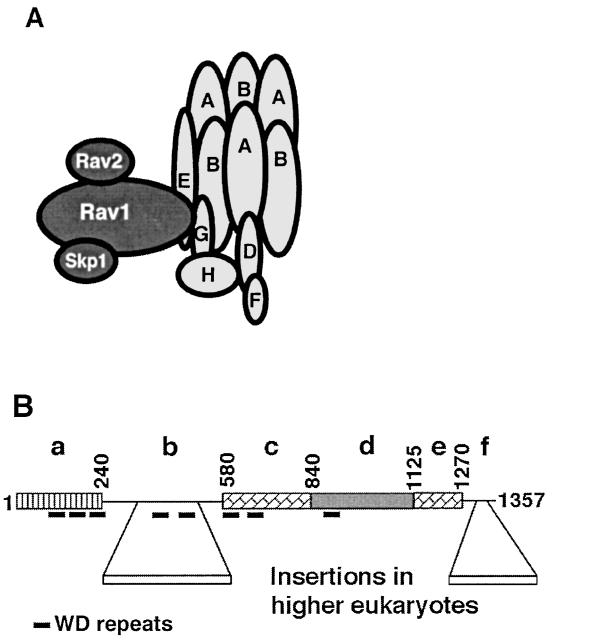
Independent assembly of preassembled V_1 and V_0 subcomplexes is not the predominant biosynthetic pathway for formation of V_1V_0 complexes. Instead, wild-type cells grown under optimal conditions appear to use a concerted assembly pathway in which V_1 and V_0 subunits associate at very early times and subunits of both sectors are gradually added (Kane *et al.*, 1999). This raises the question of whether RAVE has a general impact on V-ATPase assembly by functioning in the V-ATPase biosynthetic pathway, or perturbs a dynamic equilibrium between assembled V_1V_0 and free V_1 and V_0 complexes that is established after the initial biosynthetic assembly of the V-ATPase. To address this question, we asked whether rav mutants could still affect the level of V-ATPase assembly in a mutant that does not release V_1 from V_0 in response to glucose deprivation (vma11-E145L mutant; Parra and Kane, 1998). Even in the vma11-E145L mutant, deletion of RAV1 resulted in much higher levels of free V_1 and V_0 sectors (Smardon *et al.*, 2002). This indicates that RAVE intervenes in both biosynthetic assembly of the V-ATPase and in reassembly of disassembled V_1 and V_0 subcomplexes.

Seol *et al.* (2001) proposed a topology for the RAVE complex, based on partial complexes formed in

the absence of one of the subunits. Their model places Rav1p at the center of the RAVE complex and indicates that Rav1p is also responsible for binding to V_1. We sought to narrow down the site of RAVE binding to V_1 by measuring RAVE–V_1 binding in the absence of individual V_1 subunits (Smardon *et al.*, 2002). Binding of RAVE to V_1 was almost completely lost in the vma4Δ and vma10Δ mutants, which lack subunits E and G, respectively. The E subunit is very unstable in absence of subunit G (Tomashek *et al.*, 1997), and so the vma10Δ mutant effectively lacks both subunits E and G. On the basis of these results, we hypothesized that RAVE binds to V_1 via subunits E and/or G, and proposed the topology for RAVE-V_1 binding shown in Fig. 3(A). Consistent with the model in Fig. 3(A), we have subsequently found that Rav1p interacts with Rav2p, Skp1p, and Vma4p (subunit E) in a two-hybrid assay, but Rav2p and Skp1p interact neither with each other nor with Vma4p (Smardon and Kane, unpublished data).

The data summarized in Fig. 3(A) indicate that Rav1p is at the "heart" of the RAVE complex and its interaction with V_1. For this reason, we are particularly interested in understanding the structure of Rav1p. Yeast Rav1p is a 155 kDa protein, and sequence analysis programs (Andrade *et al.*, 2000) indicate that it contains eight WD repeats. X-ray structures available for a number of WD repeat proteins indicate that the WD repeats assemble with each other to form seven to eight blades of a β-propeller that offers at least two surfaces that can potentially support protein–protein interaction (Orlicky *et al.*, 2003). The WD repeats in RAV1 are noncontiguous, but could still assemble to form a β-propeller structure with multiple faces available for protein–protein interactions. Equally significant is the observation that RAV1 has no F-box sequence. As described above, many Skp1-binding proteins, including proteins involved in nonproteolytic Skp1p complexes, bind to Skp1p via an F-box sequence motif (Craig and Tyers, 1999; Skowyra *et al.*, 1997). While there are certainly other proteins that lack an F-box but still bind to Skp1p, such as the cullins (yeast Cdc53p), the absence of any F-box-containing protein in the RAVE complex is rather novel. We are currently using a combination of methods to delineate the regions of Rav1p responsible for interacting with Skp1p, Rav2p, and V_1; these studies promise to give us a better understanding of RAVE function as well.

Is there a RAVE complex or equivalent affecting V-ATPase assembly in higher eukaryotes? No functional equivalent of the yeast RAVE complex has yet been identified biochemically. However, Skp1p is a very highly conserved protein that would be available for assembly into a RAVE-like complex in any eukaryotic cell.



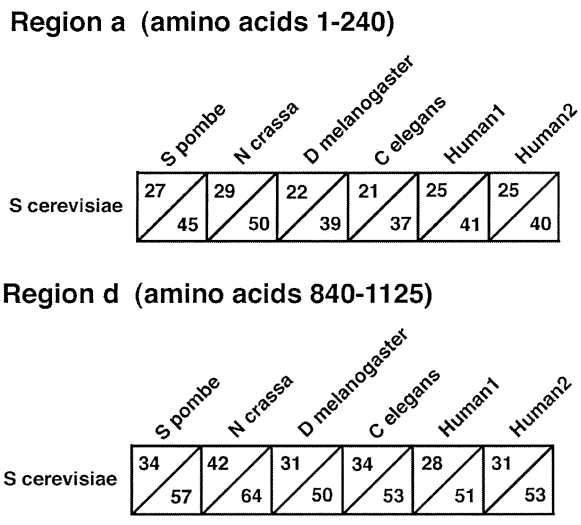


Fig. 3. Rav1p is a conserved protein that is central to the RAVE–V_1 complex. (A) Model for Rav1p binding to Skp1p, Rav2p, and V_1. V_1 subunits are shown in light gray; RAVE subunits are shown in dark gray. (B) Structural model of RAV1 from sequence comparison. The blocks marked a–f represent regions of yeast RAV1 showing differing degrees of homology with RAV1 from other eukaryotes. Regions a and d are the most conserved, regions c and e show limited conservation, except between fungi, and regions b and f show very little conservation. The positions of the eight WD repeats in yeast RAV1 are indicated. The percentage of amino acids identical (top) or conserved (bottom) between *S. cerevisiae* RAV1 regions a (amino acids 1–240) and d (amino acids 840–1125) and the corresponding regions of other eukaryotic RAV1 homologs is also shown. Humans have two potential homologues of RAV1.

BLAST searches also reveal that virtually all eukaryotes have apparent homologues of RAV1 (Fig. 3(B)). Two regions of RAV1, corresponding to amino acids 1–240 and 840–1125 in the yeast RAV1 sequence are particularly conserved. The N-terminal sequence (a in Fig. 3(A), amino acids 1–240) contains three of the WD repeats, but the sequence conservation includes the region preceding the WD repeats as well as the repeats. The other conserved sequence (d in Fig. 3(A), amino acids 840–1125) includes the last WD repeat of the yeast RAV1 sequences, but the highest levels of sequence identity are in the region immediately following this WD repeat. Curiously, although all of the potential RAV1 homologues contain multiple WD repeats and can be aligned in these two conserved regions, they can be quite divergent outside these two regions. The fungal RAV1 homologues are comparable in size to *S. cerevisiae* RAV1 and can be aligned throughout their sequences, although the two regions indicated as a and d in Fig. 3(B) are still the most highly conserved. The human, *Caenorhabdtis elegans*, and *Drosophila* RAV1 homologues are almost twice as large as the yeast RAV1. Much of this difference in size can be attributed to large insertions in two regions, as shown in Fig. 3(B). These insertions contain even more WD repeats; in the most extreme example, the *Drosophila* DmX protein is predicted to have at least 30 WD repeats (Kraemer *et al.*, 1998). These comparisons raise a number of questions, some of which may become clearer as functionally important regions of yeast RAV1 are identified and characterized. In addition, it is notable that BLAST searches have identified no homolog of *S. cerevisiae* RAV2, even in other fungi. It is possible that the functions of Rav2p can be performed by proteins with a very low level of sequence identity or that some of the large insertions into the apparent RAV1 homologues of higher eukaryotes perform the function of Rav2p in yeast.

There are clearly a number of important questions remaining about the RAVE complex. First, it is not at all clear how this complex is able to influence V-ATPase assembly. Does it catalyze a posttranslational modification in free V_1 sectors that has not yet been characterized, or does it have a more general chaperone-like role? Second, the presence of Skp1p in RAVE is intriguing, because it raises the possibility of crosstalk between the V-ATPase and the many functions linked to Skp1p through its SCF ubiquitin ligase functions. Finally, does ubiquitination play a role in RAVE function, as it does in many other Skp1p-containing complexes? These questions open new areas for investigation of V-ATPase assembly and regulation.

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REFERENCES

Andrade, M. A., Ponting, C. P., Gibson, T. J., and Bork, P. (2000). *J. Mol. Biol.* **298**, 521–537.
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.
Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J. W., and Elledge, S. J. (1996). *Cell* **86**, 263–274.
Boekema, E. J., Ubbink-Kok, T., Lolkema, J. S., Brisson, A., and Konings, W. N. (1997). *Proc. Natl. Acad. Sci. U.S.A.* **94**, 14291–14293.
Bowman, B. J., Vazquez-Laslop, N., and Bowman, E. J. (1992). *J. Bioenerg. Biomembr.* **24**, 361–370.
Charsky, C., Liu, J., Smardon, J., Tarsio, M., Kane, P. M. (manuscript in preparation).
Charsky, C. M., Schumann, N. J., and Kane, P. M. (2000). *J. Biol. Chem.* **275**, 37232–37239.
Craig, K. L., and Tyers, M. (1999). *Prog. Biophys. Mol. Biol.* **72**, 299–328.
Cross, R. L. (2000). *Biochim. Biophys. Acta* **1458**, 270–275
Curtis, K. K., Francis, S. A., Oluwatosin, Y., and Kane, P. M. (2002). *J. Biol. Chem.* **277**, 8979–8988.
Curtis, K. K., and Kane, P. M. (2002). *J. Biol. Chem.* **277**, 2716–2724.
Del Rizzo, P. A., Bi, Y., Dunn, S. D., and Shilton, B. H. (2002). *Biochemistry* **41**, 6875–6884.
Deshaies, R. J. (1999). *Annu. Rev. Cell Dev. Biol.* **15**, 435–467.
Doherty, R. D., and Kane, P. M. (1993). *J. Biol. Chem.* **268**, 16845–16851.
Dunn, S. D., McLachlin, D. T., and Revington, M. (2000). *Biochim. Biophys. Acta* **1458**, 356–363.
Forgac, M. (1999). *J. Biol. Chem.* **274**, 12951–12954.
Gräf, R., Harvey, W. R., and Wieczorek, H. (1996). *J. Biol. Chem.* **271**, 20908–20913.
Grüber, G., Wieczorek, H., Harvey, W. R., and Müller, V. (2001). *J. Exp. Biol.* **204**, 2597–2605.
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.
Hunt, I. E., and Bowman, B. J. (1997). *J. Bioenerg. Biomembr.* **29**, 533–540.
Imamura, H., Nakano, M., Nohi, H., Muneyuki, E., Ohkuma, S., Yoshida, M., and Yokoyama, K. (2003). *Proc. Natl. Acad. Sci. U.S.A.* **100**, 2312–2315.
Kane, P. M. (1995). *J. Biol. Chem.* **270**, 17025–17032.
Kane, P. M. (2000). *FEBS Lett.* **469**, 137–141.
Kane, P. M., and Parra, K. J. (2000). *J. Exp. Biol.* **203** (Pt 1), 81–87.
Kane, P. M., Tarsio, M., and Liu, J. (1999). *J. Biol. Chem.* **274**, 17275–17283.
Kaplan, K. B., Hyman, A. A., and Sorger, P. K. (1997). *Cell* **91**, 491–500.
Kraemer, C., Weil, B., Christmann, M., and Schmidt, E. R. (1998). *Gene* **216**, 267–276.
Kuras, L., Rouillon, A., Lee, T., Barbey, R., Tyers, M., and Thomas, D. (2002). *Mol. Cell* **10**, 69–80.

Landolt-Marticorena, C., Williams, K. M., Correa, J., Chen, W., and Manolson, M. F. (2000). *J. Biol. Chem.* **275**, 15449–15457.
Lu, M., Vergara, S., Zhang, L., Holliday, L. S., Aris, J., and Gluck, S. L. (2002). *J. Biol. Chem.* **277**, 38409–38415.
Lu, X., Yu, H., Liu, S. H., Brodsky, F. M., and Peterlin, B. M. (1998). *Immunity* **8**, 647–656.
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.
Margolles-Clark, E., Tenney, K., Bowman, E. J., and Bowman, B. J. (1999). *J. Bioenerg. Biomembr.* **31**, 29–37.
Nelson, H., and Nelson, N. (1990). *Proc. Natl. Acad. Sci. U.S.A.* **87**, 3503–3507.
Nelson, N., and Harvey, W. R. (1999). *Physiol. Rev.* **79**, 361–385.
Nelson, N., and Taiz, L. (1989). *Trends Biochem. Sci.* **14**, 113–116.
Nishi, T., and Forgac, M. (2002). *Nat. Rev. Mol. Cell. Biol.* **3**, 94–103.
Ohya, Y., Umemoto, N., Tanida, I., Ohta, A., Iida, H., and Anraku, Y. (1991). *J. Biol. Chem.* **266**, 13971–13977.
Orlicky, S., Tang, X., Willems, A., Tyers, M., and Sicheri, F. (2003). *Cell* **112**, 243–256.
Parra, K. J., and Kane, P. M. (1998). *Mol. Cell Biol.* **18**, 7064–7074.
Parra, K. J., Keenan, K. L., and Kane, P. M. (2000). *J. Biol. Chem.* **275**, 21761–21767.
Radermacher, M., Ruiz, T., Harvey, W. R., Wieczorek, H., and Grüber, G. (1999). *FEBS Lett.* **453**, 383–386.
Radermacher, M., Ruiz, T., Wieczorek, H., and Grüber, G. (2001). *J. Struct. Biol.* **135**, 26–37.
Rizzo, V. F., Coskun, Ü., Radermacher, M., Ruiz, T., Armbrüster, A., and Grüber, G. (2003). *J. Biol. Chem.* **278**, 270–275.
Sagermann, M., Stevens, T. H., and Matthews, B. W. (2001). *Proc. Natl. Acad. Sci. U.S.A.* **98**, 7134–7139.
Seol, J. H., Shevchenko, A., and Deshaies, R. J. (2001). *Nat. Cell Biol.* **3**, 384–391.
Shao, E., Nishi, T., Kawasaki-Nishi, S., and Forgac, M. (2003). *J. Biol. Chem.* **278**, 12985–12991.
Skowyra, D., Craig, K. L., Tyers, M., Elledge, S. J., and Harper, J. W. (1997). *Cell* **91**, 209–219.
Smardon, A. M., Tarsio, M., and Kane, P. M. (2002). *J. Biol. Chem.* **277**, 13831–13839.
Sorgen, P. L., Bubb, M. R., and Cain, B. D. (1999). *J. Biol. Chem.* **274**, 36261–36266.
Sorgen, P. L., Caviston, T. L., Perry, R. C., and Cain, B. D. (1998). *J. Biol. Chem.* **273**, 27873–27878.
Sumner, J. P., Dow, J. A., Earley, F. G., Klein, U., Jäger, D., and Wieczorek, H. (1995). *J. Biol. Chem.* **270**, 5649–5653.
Supekova, L., Sbia, M., Supek, F., Ma, Y., and Nelson, N. (1996). *J. Exp. Biol.* **199**, 1147–1156.
Suzuki, T., Suzuki, J., Mitome, N., Ueno, H., and Yoshida, M. (2000). *J. Biol. Chem.* **275**, 37902–37906.
Svergun, D. I., Konrad, S., Huss, M., Koch, M. H., Wieczorek, H., Altendorf, K., Volkov, V. V., and Grüber, G. (1998). *Biochemistry* **37**, 17659–17663.
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.
Wieczorek, H., Grüber, G., Harvey, W. R., Huss, M., Merzendorfer, H., and Zeiske, W. (2000). *J. Exp. Biol.* **203** (Pt 1), 127–135.
Wiederkehr, A., Avaro, S., Prescianotto-Baschong, C., Haguenauer-Tsapis, R., and Riezman, H. (2000). *J. Cell Biol.* **149**, 397–410.
Willems, A. R., Goh, T., Taylor, L., Chernushevich, I., Shevchenko, A., and Tyers, M. (1999). *Philos. Trans. R. Soc. Lond. B: Biol. Sci.* **354**, 1533–1550.
Xu, T., Vasilyeva, E., and Forgac, M. (1999). *J. Biol. Chem.* **274**, 28909–28915.
Yamashiro, C. T., Kane, P. M., Wolczyk, D. F., Preston, R. A., and Stevens, T. H. (1990). *Mol. Cell Biol.* **10**, 3737–3749.
Zheng, N., Schulman, B. A., Song, L., Miller, J. J., Jeffrey, P. D., Wang, P., Chu, C., Koepp, D. M., Elledge, S. J., Pagano, M., Conaway, R. C., Conaway, J. W., Harper, J. W., and Pavletich, N. P. (2002). *Nature* **416**, 703–709.