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Close-Up and Genomic Views of the Yeast Vacuolar H^+-ATPase

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The yeast V-ATPase has emerged as an excellent model for other eukaryotic V-ATPases. In this review, recent biochemical and genomic studies of the yeast V-ATPase are described, with a focus on: 1) the role of V_1 subunit H in coupling ATP hydrolysis and proton pumping and 2) identification of the full set of yeast haploid deletion mutants that exhibit the pH and calcium-sensitive growth characteristic of loss of V-ATPase activity. The combination of "close-up" biochemical views of V-ATPase structure and mechanism and "geomic" views of its functional reach promises to provide new insights into the physiological of V-ATPases.

KEY WORDS: vacuole; yeast; ATPase; proton pump; genomic.

INTRODUCTION

V-ATPases are ATP-driven proton pumps that acidify organelles such as the lysosome, Golgi apparatus, and endosomes in all eukaryotic cells (Nishi and Forgac, 2002). The yeast V-ATPase has proven to be an excellent model for all eukaryotic V-ATPases. Not only are the overall subunit composition and individual subunit sequences very similar between the yeast V-ATPase and those of higher eukaryotes, but the yeast V-ATPase also shares common regulatory mechanisms with V-ATPases of other systems. We are interested in both a "close-up" view of the V-ATPases, directed toward understanding the subunit structure, function, and mechanism of this enzyme and a "genomic" view of the yeast V-ATPase, directed toward placing this versatile and widespread pump in its cellular context. This review describes recent results on both of these fronts, specifically, mutational analysis of subunit H and a genomic screen for new *vma* mutants.

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Subunit H: A Bridge Between V_1 and V_0 Sectors of V-ATPases?

V-ATPases share a rotational catalytic mechanism with F-type ATPases/ATP synthases(Cross, 2000; Hirata *et al*., 2003; Imamura *et al*., 2003). Both V- and F-ATPases physically separate the ATP-hydrolyzing catalytic subunits and the proton pore subunits (Fig. 1). Conformational changes in one domain of the enzyme are transmitted to the other by rotation of a set of rotor stalk subunits that couples rotation between the catalytic sites to formation of a proton channel at the interface of the ring of membrane-bound c subunits and the a subunit (Cross, 2000; Fillingame *et al*., 2002). One requirement of rotational catalysis is one or more stators, which fix the link between the catalytic subunits and the membrane-bound a subunits, thus allowing the relative rotation of the rotor that is essential for productive catalysis (Dunn *et al*., 2000).

The *E. coli* F-ATP synthase relies on only two different subunits to achieve this stator function: a dimer of integral membrane b subunits, and the δ subunit, a soluble subunit that binds near the top of the F_1 catalytic headgroup (Dunn *et al*., 2000). The structural differences between the V- and F-ATPases appear to be most pronounced

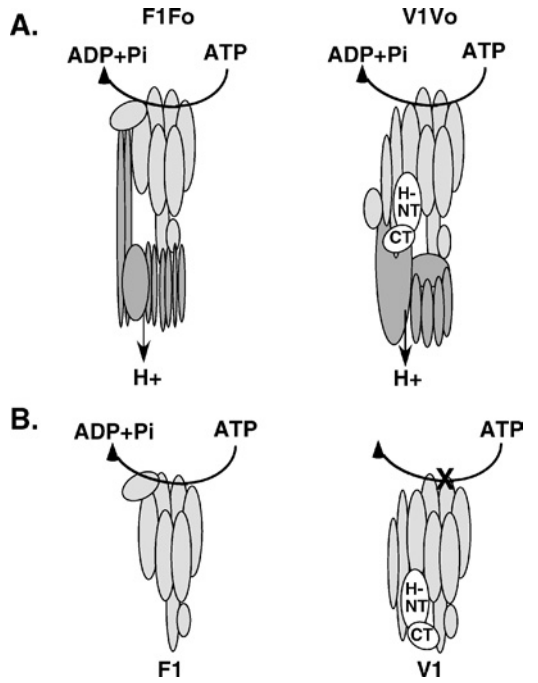

Fig. 1. Comparison of structural models for F- and V-type ATPases. A. *Comparison of E. coli F_1F_0 and yeast V_1V_0 complexes*. F_1 and V_1 subunits are shown in light gray, except for the H subunit of V_1 which is shown in white to highlight the two domains, NT and CT, that were separated in the mutagenesis experiments described in the text. F_0 and V_0 subunits are shown in dark grey. The F_1F_0 complex is shown as operating in the ATP hydrolysis direction, but also operates in the reverse direction, using a proton gradient to synthesize ATP; V_1V_0 only operates in the ATP hydrolysis direction shown. B. *Comparison of E. coli F_1 and yeast V_1 complexes detached from V_0*. F_1 complexes are highly active ATPases when detached from the membrane, but free F_1 complexes are rarely found *in vivo*. V_1 complexes are found *in vivo*, but they do not hydrolyze ATP under physiological conditions. Subunit H helps to inhibit the ATPase activity of cytosolic V_1 complexes, and one potential mechanism for this would be tethering of the peripheral and central stalks of the enzyme as shown.

in the stator stalks (Margolles-Clark *et al*., 1999; Nishi and Forgac, 2002). A proposed structure of the yeast V-ATPase is compared to that of the *E. coli* F-ATPase in Fig. 1(A). There are at least six different subunits that form part of the stator stalk in V-ATPases. Only one of these, the G subunit, shows limited homology with the b subunits of F-ATPases (Supekova *et al*., 1996; Hunt and Bowman, 1997), while the others are conserved among eukaryotic V-ATPases, but have no equivalent in F-ATPases. In addition to the G subunit, the C, E, H, soluble N-terminal domain of subunit a, and non-homologous domain of catalytic subunit A are all believed to be part of one or more peripheral, stator stalks (Shao and Forgac, 2004; Venzke *et al*., 2005). Both the number of subunits implicated in stator stalk function and electron micrographs of V-ATPases (Wilkens *et al*., 2004; Venzke *et al*., 2005) indicate that the peripheral stalk(s) of V-ATPases are more complex than those of F-ATPases. (It should be noted that very recent data suggests that V-ATPases may even have two peripheral stalks (Venzke *et al*., 2005).)

We are particularly interested in the structure and function of subunit H. Yeast mutants lacking subunit H (*vma13*Δ mutants) lose all V-ATPase activity and organelle acidification, indicating that it is an essential subunit of the yeast V-ATPase (Ho *et al*., 1993). However, the remaining subunits of the V-ATPase in the *vma13*Δ mutant assemble, and inactive and unstable V_1V_0 complexes are found at the vacuole (Ho *et al*., 1993). Subunit H overexpression to high levels is lethal and to modest levels results in uncoupling of ATP hydrolysis and proton transport (Curtis and Kane, 2001). These data indicate that subunit H is important for functional attachment of V_1 to V_0. This conclusion is further supported by electron microscopy and crosslinking data that place the H subunit near the interface of V_1 and V_0 (Wilkens *et al*., 2004).

The H subunit was the first V-ATPase subunit to be crystallized. The structure reported by Sagermann *et al*. (Sagermann *et al*., 2001) revealed a relatively extended protein comprised of two rather independent domains, a 348 amino acid N-terminal domain (H-NT) and a 126 amino acid C-terminal domain (H-CT). We reasoned that the H subunit might "bridge" V_1 and V_0 sectors by binding to different parts of the two sectors via its two domains. To test this model, we expressed the two domains of the H subunit individually and together in yeast cells lacking the H subunit (Liu *et al*., 2005). Both domains were stably expressed, and surprisingly, we found that the N-terminal domain alone supported some V-ATPase function *in vivo*, based on its ability to partially complement the growth defects of a mutant lacking the H subunit. Better growth could be achieved by expressing both domains of the subunit together, suggesting that covalent attachment between the two domains is not essential for function. Further analysis of the mutants in isolated vacuoles revealed a number of novel features. Although the H-NT and H-CT domains were found at the vacuole, there was no ATPase activity or proton pumping in the mutant containing only H-CT. In contrast, the mutant containing H-NT had considerable ATPase activity (25% of wild-type), but this activity was relatively insensitive to the V-ATPase inhibitor concanamycin A and was almost completely uncoupled from proton transport. The ATPase activity in the H-NT vacuoles did come from the V-ATPase, because it was largely inhibited by *N*-ethyl maleimide (NEM), which inhibits V-ATPases at low concentrations by alkylating a critical catalytic site cysteine

(Feng and Forgac, 1992). The difference in inhibition by concanamycin and NEM might be accounted for by their different modes of action; concanamycin binds to the c subunits of the V_0 sector (Bowman and Bowman, 2002; Huss *et al*., 2002) and inhibits the ATPase by virtue of the tight coupling of the enzyme, and NEM directly inhibits ATP hydrolysis. Expression of the H-CT domain with the NT domain resulted in increased ATPase activity in vacuolar membranes with greater sensitivity to concanamycin A, and greatly improved coupling of ATP hydrolysis and proton pumping (Liu *et al*., 2005). Taken together, these results partially support our initial vision of subunit H as a bridge between the V_1 and V_0 sectors; the NT-domain appears to be capable of activating ATP hydrolysis in the V_1 sector, but does not establish proper connections to V_0, resulting in loss of concanamycin sensitivity and coupling of proton transport to ATP hydrolysis. The CT domain appears to complete the functional bridging to some extent, possibly through interactions with the V_0 sector, even when it is not covalently attached to the N-terminal domain.

As described above, subunit H can be viewed as an activator of V_1V_0 complexes, but this is not its only role. In yeast, *M. sexta*, and likely in other cells as well, assembled V_1V_0 complexes are in dynamic equilibrium with free (cytosolic) V_1 and V_0 sectors (Kane, 2000; Wieczorek *et al*., 2000). This equilibrium can be rapidly shifted in response to growth conditions, and reversible disassembly is believed to be an important regulatory mechanism for V-ATPases. It is therefore important that the ATPase activity of free, cytosolic V_1 sectors be "silenced" to prevent unproductive consumption of cytosolic ATP stores, and the H subunit is critical to this silencing (Parra *et al*., 2000) (Fig. 1B). V_1 sectors isolated from wild-type cells contain subunits A, B, D, E, F, G, and H, along with a substoichiometric amount of C subunit, and do not hydrolyze MgATP (Zhang *et al*., 2003). (Most of the C subunit is released from both the V_1 and V_0 sectors when the V-ATPase disassembles (Kane, 1995).) V_1 sectors lacking subunit H have uninhibited MgATPase activity, although this activity slows within a few minutes, perhaps as a result of additional inhibitory mechanisms (Parra *et al*., 2000). We have hypothesized that the H subunit may switch between bridging V_1 and V_0 in the intact enzyme, in order to activate ATP-driven proton transport, to bridging the peripheral and stator stalks in free V_1 upon disassembly of the enzyme, in order to halt rotational catalysis (Kane and Smardon, 2003). In this context, it will be very interesting to see if either or both of the two domains of the H subunit are competent to inhibit the ATPase activity of free V_1 sectors, and these experiments are underway.

A Genomic View of V-ATPase Function in Yeast

The development of deletion mutant arrays, in which each individual non-essential yeast gene has been deleted (Winzeler *et al*., 1999), has provided an unprecedented opportunity for understanding V-ATPase function on a genomic scale and identifying all gene products that produce a similar phenotype to the mutants lacking V-ATPase subunits. A number of genetic screens have identified defects in the V-ATPase deletion mutants (*vma* mutants) that had not been appreciated previously (Davis-Kaplan *et al*., 2004; Parsons *et al*., 2004; Serrano *et al*., 2004; Lesuisse *et al*., 2005; Outten *et al*., 2005). These defects, including hypersensitivity to many different drugs and iron-dependent growth, suggest new functional connections to V-ATPase activity.

We chose to use the deletion mutant array to screen for mutants that share the pH-and calcium-sensitive growth defects of the *vma* mutants (Sambade *et al*., 2005). Mutants lacking any of the V-ATPase subunits show a distinctive set of growth characteristics, collectively known as the Vma^- phenotype (Nelson and Nelson, 1990; Ohya *et al*., 1991). These characteristics include optimal growth at pH 5, inability to grow at pH 7.5, inability to grow at elevated extracellular calcium concentrations, and little or no growth on nonfermentable carbon sources. We compared growth of all the mutants of the deletion mutant array on plates buffered to pH 5 and plates buffered to pH 7.5 containing 60 mM $CaCl_2$ (Sambade *et al*., 2005). Approximately 65 mutants grew at pH 5 but either grew poorly or failed to grow at high pH and calcium. Among the gene products mutated in these strains were 10 of the 13 known V-ATPase subunits and three previously identified V-ATPase assembly factors. The remaining mutants were screened for other features characteristic of the Vma^- growth phenotype, including poor growth on non-fermentable carbon sources and 4 mM Zn^{2+} (Manolson *et al*., 1994), dependence on the calcineurin for growth (Garrett-Engele *et al*., 1995; Tanida *et al*., 1995), and decreased uptake of the fluorescent lysosomotropic amine quinacrine into the vacuole.

A number of mutants shared multiple characteristics with the known *vma* mutants, but only one, the *cwh36*Δ mutant, had a phenotype identical to the V-ATPase subunit mutants (Sambade *et al*., 2005). This mutant contained a deletion in a previously uncharacterized gene that was annotated as a "dubious open reading frame" based on comparison of the *S. cerevisiae* genome to genomes of closely related fungi (Kellis *et al*., 2003). This same comparison, however, suggested that there might be an unannotated open reading frame containing two introns on the

opposite strand. Deletion of *CWH36* would delete much of this open reading frame as well. We addressed which open reading frame was responsible for the mutant phenotype of the deletion strain by introducing premature stops into both (Sambade and Kane, 2004). We found that a premature stop codon in the *CWH36* open reading frame resulted in no change in growth at high pH and calcium, but introduction of a premature stop into the open reading on the opposite strand, now named *VMA9*, reproduced the entire set of growth phenotypes of the deletion mutant. A similar conclusion was reached by others independently (Davis-Kaplan *et al*., 2004).

This result strongly implicated the *VMA9* gene product in V-ATPase activity. This was supported by weak homology to a small, hydrophobic protein that associated with the V-ATPase of *Manduca sexta* and bovine chromaffin granules (Ludwig *et al*., 1998; Merzendorfer *et al*., 1999), but had not been demonstrated to be necessary for V-ATPase activity. A C-terminal epitope tag was introduced into Vma9p (Sambade and Kane, 2004). The tagged protein was isolated with vacuolar membranes and shown to be associated the intact V-ATPase in wild-type cells and with the V_0 sector in vacuoles isolated from a mutant lacking V_1 sectors. Thus, Vma9p, also known as subunit e, is a genuine subunit of the V-ATPase that is essential for its activity. What is the functional role of subunit e in the V-ATPase? This is still unknown. As with other V_0 subunits, disruption of *VMA9* results in destabilization of the V_0 a subunit. This suggests that subunit e may assemble with the rest of the V_0 subunits at the endoplasmic reticulum, since destabilization of the a subunit generally arises from ER-associated degradation (Hill and Cooper, 2000). There have been no direct biochemical tests of its placement in the V_0 sector, but the structure and mechanism of the V-ATPase offers some intrinsic constraints on where an additional subunit could be located. Subunit e has no intermembrane acidic residue that could participate in proton transport, and because the ring of c rings must sequentially rotate against the a subunit for proton transport to occur (Fillingame *et al*., 2002; Aksimentiev *et al*., 2004), it seems unlikely that the e subunit would be outside of the c ring. There may be room for this subunit in the interior of the c ring, because both electron micrographs of eukaryotic V-ATPases (Wilkens and Forgac, 2001) and the recent crystal structure of the c ring from *E. hirae* (Murata *et al*., 2005) suggest that the V-ATPase c ring may be considerably larger than the c ring of F1Fo-ATPases . It could also be associated with the a subunit, which has seven to nine transmembrane domains (Leng *et al*., 1999), only one of which participates directly in proton transport in most models. Neither of these locations provides any insight into its function, so e subunit function is one of many V_0 sector features that is not yet understood.

A number of other mutants exhibited a relatively strong Vma^- growth phenotype in the genomic screen. Several of these also exhibited defects in vacuolar acidification, but quinacrine staining was not completely absent as in the *cwh36*Δ mutant (Sambade *et al*., 2005). Among these was a small subset, including *vps34*Δ, *vps15*Δ, *vps45*Δ, and *vps16*Δ, of the more than 60 *vps* (vacuolar protein sorting) mutants. The *vps34*Δ and *vps15*Δ mutations disrupt the sole phosphatidylinositol 3-kinase in yeast (Schu *et al*., 1993; Stack *et al*., 1995). These mutations had been shown previously to compromise vacuolar acidification (Raymond *et al*., 1992), but the biochemical basis of their effects on the V-ATPase are not understood. We observed greatly reduced V-ATPase activity in isolated vacuolar membranes in these two mutants (15–17% of wild-type) and although the enzyme was present at somewhat lower levels, the remaining V-ATPase still appeared to be less active than wild-type. Vps45p is part of a complex that recognizes phosphatidyl inositol 3-phosphate in the endosome and participates in transport of proteins to the vacuole (Cowles *et al*., 1994; Peterson *et al*., 1999); its impact on the V-ATPase may overlap that of Vps34p/Vps15p. In contrast, the *vps16*Δ mutation greatly disrupts vacuolar structure (Raymond *et al*., 1992). Although the mutant has a strong Vma^- phenotype, we observed considerable quinacrine uptake into small vesicles that contained a GFP-tagged subunit of V_1. Therefore, we believe that this mutant does not have any dramatic effect on the V-ATPase itself, but instead disrupts vacuolar function. We are still in the process of determining which of the mutations identified directly affect the V-ATPase itself, and which may mimic the Vma^- phenotype for other reasons. However, the beauty of this genomic screen is that it has given us: (1) a much more complete picture of the genes essential for V-ATPase activity, (2) a number of potential V-ATPase regulators, and (3) a broader picture of the physiological basis of the Vma^- phenotype. Following up on these results will occupy others and us for some time to come !

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