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

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The yeast V-ATPase is highly similar to V-ATPases of higher organisms and has proved to be a biochemically and genetically accessible model for many aspects of V-ATPase function. Like other V-ATPases, the yeast enzyme consists of a complex of peripheral membrane proteins, the V_1 sector, attached to a complex of integral membrane subunits, the V_0 sector. Multiple pathways for biosynthetic assembly of the enzyme appear to be available to cells containing a full complement of subunits and enzyme activity may be further controlled during biosynthesis by a protease activity localized to the late Golgi apparatus. Surprisingly, the assembled V-ATPase is not a static structure. Instead, fully assembled V_1V_0 complexes appear to exist in a dynamic equilibrium with inactive cytosolic V_1 and membrane-bound V_0 complexes and this equilibrium can be rapidly shifted in response to changes in carbon source. The reversible disassembly of the yeast V-ATPase may be a novel regulatory mechanism, common to V-ATPases, that works *in vivo* in coordination with many other regulatory mechanisms.

KEY WORDS:

BACKGROUND

The *Saccharomyces cerevisiae* vacuolar proton-translocating ATPase was one of the first V-type ATPases to be isolated (Uchida *et al.*, 1985). The primary biochemical source of the yeast V-ATPase is the vacuole. The fungal vacuole is both a storage and a degradative organelle that resembles the vacuole of plant cells and the lysosome of mammalian cells. The yeast V-ATPase acidifies the vacuole to a pH of approximately 6.0 and drives secondary transport of Ca^{2+}, amino acids, and other nutrients. V-ATPases also reside in other intracellular compartments in yeast (Manolson *et al.*, 1994), but there is presently no evidence that they reside at the plasma membrane. Thus, the functional role of the yeast V-ATPase is analogous to the "constitutive" roles of V-ATPases in intracellular compartments of all eukaryotic cells.

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In the past few years, yeast has emerged as an excellent model system for study of V-ATPases because of the combination of genetic, biochemical, molecular, and cell biological approaches available. All V-ATPases are comprised of a complex of peripheral subunits containing the nucleotide binding subunits, the V_1 sector, attached to a complex of integral membrane subunits containing the proton pore, the V_0 sector. In yeast cells, the V_1 sector includes eight subunits; the V_0 sector includes five subunits. The genes for all of these subunits have been cloned. Disruption of any of these genes, with the exception of the individual *STV1* and *VPH1* genes, which encode functionally redundant homologs of one of the subunits (Manolson *et al.*, 1994), is conditionally lethal. Yeast cells lacking a V-ATPase subunit can grow in medium buffered to pH 5, but fail to grow in one buffered to pH 7.5, a medium containing elevated calcium concentrations, or one containing standard concentrations of nonfermentable carbon sources (reviewed in Nelson and Klionsky, 1996; Stevens and Forgac, 1997). Together these characteristics constitute the Vma^-

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phenotype. This phenotype has proved to be a highly specific indicator of loss of V-ATPase activity and has provided both a means of identifying new subunit genes and a stringent test of whether gene products were essential for ATPase activity. Furthermore, the yeast V-ATPase is highly similar to V-ATPases of other fungi, plants, and animals. Subunits originally identified in yeast have invariably proved to be components of other V-ATPases as well. The subunit composition of the yeast V-ATPase and the identification of each of the subunit genes was recently reviewed by Stevens and Forgac (1997). A structural model of the yeast V-ATPase is shown in Fig. 1.

In the past several years, we have focused on a number of aspects of the structure, function, assembly, and regulation of the yeast V-ATPase that should be generally relevant to V-ATPases of other eukaryotes. Progress in examining the biosynthesis and assembly of the yeast V-ATPase, mechanisms of regulating the ATPase *in vivo*, and the immediate cellular consequences of loss of V-ATPase activity will be reviewed. This review is not comprehensive. For further information on the yeast V-ATPase, the reader is referred to recent reviews by Stevens and Forgac (1997), Nelson and Klionsky (1996), and Graham and Stevens (this issue).

BIOSYNTHESIS OF THE YEAST V-ATPase

Dual Pathways for V-ATPase Assembly in Wild-Type Cells?

Biosynthesis of V-ATPases poses a fascinating set of cell biological problems. V-ATPases are comprised of subunits derived from cytosol that never enter the secretory pathway and integral membrane subunits that must traverse at least part of the vacuolar network. They do not have a single intracellular destination; instead, V-ATPases must be distributed among several intracellular compartments that ultimately have different internal pH values. These problems have been approached from a number of different angles.

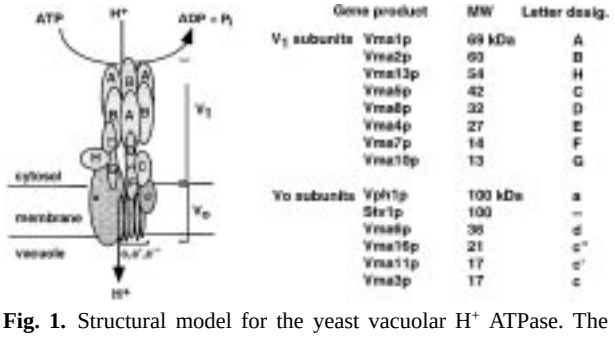

Fig. 1. Structural model for the yeast vacuolar H^+ ATPase. The nomenclature proposed by Stevens and Forgac (1997) is used here. V_1 subunits are indicated in light gray and V_0 subunits are indicated in dark gray.

Isolation of partial complexes from mutant cells lacking one subunit of the enzyme has provided initial clues about assembly and subunit–subunit interactions in the enzyme complex. We initially examined effects on assembly in five different mutant strains by immunoprecipitation of partially assembled complexes and isolation of cytoplasmic complexes by density gradient centrifugation (Doherty and Kane, 1993). These experiments indicated that the V_1 and V_0 sectors of the V-ATPase could assemble independently. Mutants lacking a single V_1 subunit were able to assemble V_0 complexes and target these complexes to the vacuole; mutants lacking a single V_0 subunit were able to assemble cytoplasmic V_1 complexes. Partially assembled V_1 complexes were also observed in mutants lacking single V_1 subunits, both by immunoprecipitation (Doherty and Kane, 1993; Kane *et al.*, submitted) and by native gel electrophoresis (Tomashek *et al.*, 1996; 1997), suggesting a hierarchy of interactions in formation of V_1 Although these studies provided important information about available subunit–subunit interactions, they do not necessarily define the assembly pathway of the wild-type V-ATPase, which has all the subunits available at the time of assembly.

Pulse-chase studies of enzyme assembly in wild-type cells revealed the pathways of assembly when all subunits are present (Kane *et al.*, submitted). Yeast cells were biosynthetically labeled in a brief pulse, the labeled subunits were allowed to assemble for varied periods of chase, and then fully and partially assembled subcomplexes were immunoprecipitated with subunit-specific monoclonal antibodies. If the ATPase were assembled from V_1 and V_0 sectors formed independently (Fig. 2), as suggested from results with the deletion mutants, then we would anticipate that an antibody against a V_1 subunit would immunoprecipitate fully assembled V_1 complexes first, followed by V_1V_0 complexes. This was not what was observed. Instead, the fully assembled V_1V_0 complexes that were formed over a 60-min chase appeared to be assembled by the "concerted" assembly pathway shown in Fig. 2. Specifically, very early interactions (within 2 min of chase) were detected between the 100-kDa V_0 subunit and several V_1 subunits, followed by association

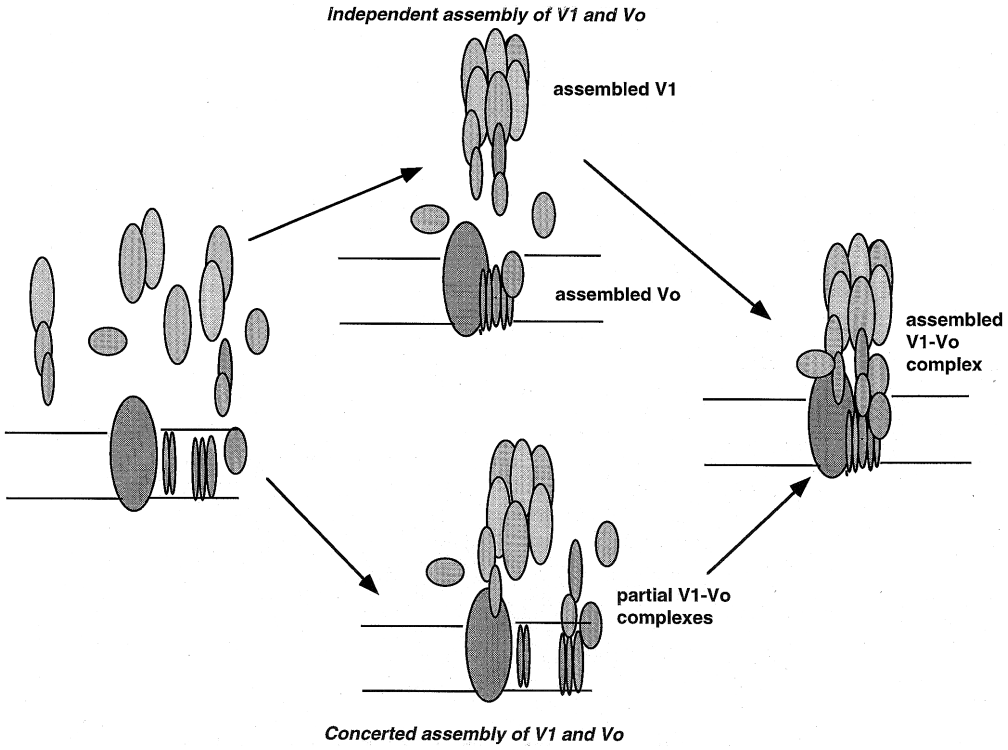

Fig. 2. Models for biosynthetic assembly of the yeast V-ATPase.

with the 17- and 36-kDa V_0 subunits over the next 5–15 min. Although we were able to detect a pool of V_0 sectors that appeared to assemble independently, we did not see this pool disappear into V_1 V_0 complexes over a 60–90-min chase period.

Further support for an early assembly intermediate containing the 100-kDa subunit in association with the V_1 subunits was obtained in experiments with yeast *sec* mutants, which are blocked at various stages in transport through the secretory pathway (reviewed in Rothblatt *et al.*, 1994). The *sec12-4* and *sec18-1* mutants are "early-acting" mutants, which are blocked in transport of proteins from the endoplasmic reticulum at 37°C, but allow normal transport at 25°. Both mutants produced normal V_1 V_0 complexes when incubated at 25°C. When these two mutants were biosynthetically labeled at 37°C for 30 min, however, they accumulated an intermediate that contained several V_1 subunits associated with the 100-kDa subunit (Kane et al, submitted). This intermediate complex lacked the 17-kDa V_0 subunit at 37°C and was similar to the intermediate complex immunoprecipitated from wild-type cells within 5 min of labeling. We were able to show that the complex present at 37°C was a true intermediate by allowing it to accumulate at 37°C and then shifting the *sec* mutants back to 25°. Under these conditions, transport from the endoplasmic reticulum resumed in the mutants and the accumulated intermediate became associated with the labeled 17-kDa protein to form fully assembled V_1V_0 complexes. Taken together, these results argue that when all subunits of the V-ATPase are present: (1) assembly involves early associations between the V_1 subunits and the 100-kDa V_0 subunit; (2) these V_1V_0 associations precede assembly of the other V_0 subunits with the 100-kDa V_0 subunit; and (3) initial V_1–V_0 interactions must occur in the endoplasmic reticulum.

The concerted assembly pathway does not appear to be exclusive, however. The results in the deletion mutants described above, as well as the presence of free V_1 and V_0 sectors in wild-type cells, argue that independent assembly of V_1 and V_0 sectors can also occur. Future experiments will address how individual subunits of the V-ATPase are directed into different assembly pathways and will also attempt to integrate recent information on V_0 subunit assembly (Graham *et al.*, 1998) into the V_1V_0 assembly models shown in Fig. 1. Tomashek *et al.* (1997) determined that assembly of the V-ATPase *in vitro* was regulated by nucleotide binding to the catalytic subunit and we have found

that cells grown in a poor carbon source contain higher levels of free V_1 and V_0 sectors than cells grown in glucose (Kane, 1995). These results raise the intriguing possibility that the cell can manipulate the biosynthesis of the V-ATPase to achieve a distribution of free V_1 and V_0 sectors and fully assembled complexes appropriate for the existing growth conditions. However, final interpretation of these data must incorporate both biosynthetic assembly of the enzyme and reversible disassembly of V-ATPase complexes (discussed below).

A Role for the Kex2p Protease in Yeast Vacuolar H^+-ATPase Activity

In an effort to identify the full set of gene products necessary for vacuolar H^+-ATPase activity, we chemically mutagenized yeast cells, enriched for mutants that grew more dense during overnight growth in nonfermentable carbon sources and then screened for the traditional set of Vma^- phenotypes, including pH-and Ca^{2+}-sensitive growth and failure to grow on nonfermentable carbon sources (Oluwatosin and Kane, 1997). In addition to several new alleles of existing *VMA* genes, we identified mutants falling into five new complementation groups (designated *VMA41–VMA45*). The affected gene in the *vma45-1* mutant was cloned by complementation and shown to encode Kex2p, a serine endoprotease localized in the late Golgi apparatus (Fuller *et al.*, 1989). Deletion of the *KEX2* gene generated the full range of Vma^- phenotypes and *kex2* mutant cells could not accumulate the lysosomotropic amine quinacrine into the vacuole *in vivo*, indicating an acidification defect. The pH-dependent growth defects of the *kex2* mutant strain could be partially suppressed by overexpression of a different protease with overlapping substrate specificity (Mkc7p; Komano and Fuller, 1995), suggesting that the protease activity of Kex2p played an essential role in vacuolar acidification. Therefore, we were surprised to find that the V-ATPase isolated from *kex2* mutant vacuoles exhibited wild-type levels of ATPase activity and proton pumping and a subunit composition indistinguishable from the enzyme isolated from wild-type cells. This is the first time that a mutant of this type has been isolated; all other mutants that displayed the full range of Vma^- phenotypes have proved to have a "biochemically defective" V-ATPase, even when the affected gene did not encode an ATPase subunit (reviewed by Stevens and Forgac, 1997; Graham and Stevens, this volume).

It may be that mutations in *KEX2* indirectly prevent vacuolar acidification in a manner so effective that they mimic a total loss of V-ATPase activity. Alternatively, it may be that the V-ATPase in *kex2* mutants is inactive *in vivo*, consistent with the Vma^- phenotype, but becomes activated *in vitro*. One explanation consistent with the latter possibility is that the V-ATPase is associated with an inhibitor protein in the early stages of its biogenesis and transport to the vacuole and this inhibitor is a Kex2p substrate. In this model, the V-ATPase would normally be present in fully assembled but inhibited state until it reached the late Golgi apparatus and was activated by Kex2p; in the *kex2*Δ mutant, the inhibitor would not be cleaved and released in the Golgi apparatus and would, therefore, be transported with the V-ATPase to the vacuole. The inhibitor could, however, be lost during cell lysis, giving *in vitro* activation of the V-ATPase. We plan to explore this model further by a genetic approach.

REGULATION OF V-ATPase ACTIVITY *In Vivo*

Disassembly and Reassembly of the Yeast Vacuolar H^+-ATPase *In Vivo*

In our initial studies of biosynthesis and assembly of the yeast V-ATPase, we regarded production of an assembled V_1V_0 complex as a stable endpoint. To our surprise, we discovered that the V-ATPase is a much more dynamic structure than we expected and, in fact, it can be reversibly disassembled as a mechanism of regulating activity in response to changing extracellular conditions (Kane, 1995). Furthermore, the parallel discovery of a similar process in *Manduca sexta* (Sumner *et al.*, 1995) suggests that reversible disassembly of V-ATPases may be a general regulatory mechanism.

In yeast, quantitation of immunoprecipitated V_1V_0 and free V_0 complexes indicates that 60–70% of the total V_0 complexes are assembled with V_1 sectors (Kane, 1995; Parra and Kane, 1998). Independent experiments had also identified a population of free V_1 sectors in yeast cytosol (Doherty and Kane, 1993). We believed these free V_1 sectors were assembly intermediates until we discovered that a brief (2 min) glucose deprivation could result in disassembly of up to 75% of previously assembled V_1V_0 complexes into free V_1 and V_0 complexes. Readdition of glucose, for

times as short as 2 min, stimulated quantitative reassembly of the disassembled complexes (Kane, 1995), as diagrammed in Fig. 3. The disassembled V_0 complex contained at least the 100-, 36-, and 17-kDa proteolipid (a, d, and c) subunits, and the V_1 complex contained, minimally, the 69-, 60-, 32-, 27-, and 16-kDa (A, B, D, E, and G) subunits. The 42-kDa C subunit was lost from both sectors, but reassembled with glucose readdition, and the assembly behavior of the 54-kDa H and 14-kDa F subunits of the V_1 sectors has not yet been established. Both disassembly and reassembly occurred in the presence of cycloheximide, indicating that neither process requires new protein synthesis. Interestingly, regulation of V-ATPase assembly in response to carbon source appears to be both a short- and a long-term process. An abrupt shift of the cells from glucose, the preferred carbon source, to other less-preferred carbon sources, such as raffinose, galactose, and glycerol-ethanol stimulated disassembly of the enzyme as effectively as depriving the cells of any carbon source. In addition, long-term (overnight) growth of cells in raffinose or glycerol-ethanol resulted in a higher percentage of disassembled V_1 and V_0 subcomplexes. These disassembled complexes remained competent for rapid reassembly when glucose was added to the cells, suggesting that even over long periods of growth in a poor carbon source, the cells remain poised to recruit free V_1 subcomplexes into active V_1V_0 complexes when growth conditions improve. Disassembly and reassembly are not artifacts of rapid shifts in carbon sources generated in the laboratory. When cells are allowed to fully metabolize the glucose in the medium and shift from fermentative to respiratory metabolism, disassembly of the ATPase also occurs (Parra and Kane, 1998).

How does an *extracellular* change in nutrients generate a major structural change in V-ATPase complexes present on *internal* organelles? We do not yet have a complete picture, but we have some clues (Parra and Kane, 1998). The glucose "signal" for maintaining or restoring assembly of the V-ATPase does not involve many of the standard glucose signaling pathways that have been characterized in yeast. Both disassembly and reassembly occur normally in mutants lacking critical components of the ras-cAMP, major glucose repression and derepression, and protein kinase C pathways. Glucose metabolism beyond formation of glucose 6-phosphate appears to be essential. A mutant lacking phosphoglucoisomerase (*pgi1* mutant) accumulates glucose 6-phosphate when grown in the presence of glucose but disassembles the V-ATPase complex. Reassembly of the V-ATPase is observed in *pgi1* mutants when fructose, which enters glycolysis beyond the Pgi1p-requiring step, is added to the cells. Cellular ATP levels drop with glucose deprivation on a timescale consistent with enzyme disassembly, but restoration of ATP levels does not correlate with enzyme reassembly. Finally, disassembly appears to require an active V-ATPase; concanamycin A treatment can partially inhibit disassembly of the wild-type enzyme and two different mutations in V_0 sector subunits that allow assembly but abolish ATP hydrolysis also inhibit disassembly (Parra and Kane, 1998).

We hypothesize that disassembly of the yeast V-ATPase effectively "turns off" both ATP hydrolysis and proton translocation, so that disassembly and reassembly of the enzyme *in vivo* is a genuine mechanism of enzyme regulation. There is abundant *in vitro* evidence that in eukaryotic systems, the disassembled V_1 sector is inactive as an ATPase, at least under near-physiological conditions, and free V_0 sectors are not open proton pores (Bowman *et al.*, 1989; Kane *et al.*, 1989, Puopolo and Forgac, 1990; Ward *et al.*, 1991; Zhang *et al.*, 1992). A large number of experiments have shown that V_1 subunits can be dissociated from membrane-bound V_0 sectors *in vitro* by treatment of membranes with low concentrations of chaotropic anions in the presence of MgATP and that the dissociated enzyme retains no ATP hydrolytic activity. Removal of the chaotrope was able to restore enzyme function in a number of cases, but restoration of activity required reassembly of the complex (Puopolo and Forgac, 1990; Ward *et al.*, 1991). We developed a system of *in vitro* disassembly and reassembly of the yeast V-ATPase in which the enzyme present in isolated vacuolar vesicles was dissociated by chaotropic anion (Fig. 3; Parra and Kane, 1996). Disassembly of the yeast enzyme at relatively low chaotrope concentrations required both the presence of the substrate,

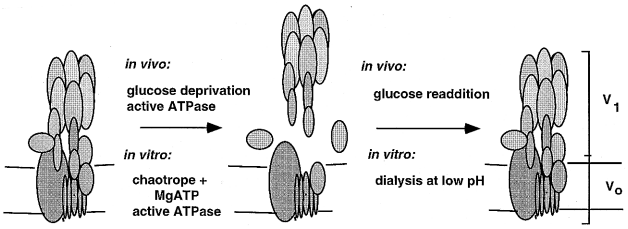

Fig. 3. Disassembly and reassembly of the yeast V-ATPase. Conditions required for disassembly and reassembly of the yeast V-ATPase *in vivo* are indicated above the arrows. Optimal conditions for disassembly and reassembly *in vitro* are indicated below the arrows.

MgATP, and a catalytically active enzyme; mutations that allow V-ATPase assembly but compromise ATPase activity prevented disassembly (Kane *et al.*, 1989; Liu and Kane, 1996). Reassembly, either with the original membranes or with membranes from a mutant strain in which only V_0 sectors are assembled, was achieved by dialyzing to remove the chaotrope. Reassembly and recovery of ATPase activity were tightly coupled. Interestingly, both processes were optimal at pH 5.5, with very little reassembly or activation occurring at pH 7.5 (Parra and Kane, 1996). These results suggested that functional association of the yeast V_1 and V_0 sectors is pH-dependent and have important physiological implications for *in vivo* reassembly. The experiments described above suggest that a drop in cytosolic ATP concentrations may be part of the signal that triggers disassembly of the V-ATPase, which could serve a means of preserving cytosolic ATP concentrations. In the opposite direction, a drop in cytosolic pH, which might occur with resumption of rapid metabolism when glucose is restored, may well be a factor in regenerating V_1–V_0 interactions and ATPase activity *in vivo*. Future studies will focus on both how V_1–V_0 dissociation and reassociation are signaled and how the free V_1 and V_0 sectors are "silenced" to prevent unproductive hydrolysis of cytosolic ATP and loss of proton gradients.

Regulation by Oxidation and Reduction *In Vivo*

A second new *VMA* complementation group identified in the random mutagenesis of yeast cells described above, *VMA41*, proved to be allelic to the *CYS4* gene (Oluwatosin and Kane, 1998). *CYS4* encodes cystathionine β-synthase, an enzyme required for cysteine biosynthesis. *cys4* mutants: (1) display the pH-dependent, Ca^{2+}-sensitive growth phenotype characteristic of *vma* mutants, (2) fail to accumulate quinacrine into the vacuole *in vivo*, indicating a vacuolar acidification defect, and (3) contain structurally unstable V-ATPase complexes with reduced levels of ATPase activity in isolated vacuoles. Interestingly, all of these phenotypes could be reversed by supplementation of the cells' growth medium with reduced glutathione, suggesting that the cysteine biosynthesis defect and the Vma^- phenotype were tightly connected. Previous results had suggested that *cys4* mutant cells have lowered levels of cytoplasmic glutathione, so we hypothesized that regulation of the V-ATPase might be altered in a less-reducing cytosol. Feng and Forgac (1994) have presented strong evidence that the bovine clathrin-coated vesicle V-ATPase can be reversibly inactivated *in vitro* by oxidation of a conserved cysteine in the nucleotide binding site (P-loop) of the catalytic subunit. Mutation of this cysteine to valine in the yeast catalytic subunit (*vma1* C261V) resulted in a fully active enzyme that was insensitive to oxidizing agents (Liu *et al.*, 1997). In order to test whether oxidative inactivation of the V-ATPase was occurring *in vivo* in the *cys4* mutants, we constructed a strain that combined the *cys4* mutation with the *vma1* C261V mutation. The V-ATPase activity in isolated vacuoles from this double mutant strain was similar to that obtained from wild-type cells and was no longer activated by extracellular glutathione. These results indicate strongly that the Vma^- phenotype of *cys4* mutant cells arises from oxidative inactivation of the V-ATPase *in vivo* and that this inactivation specifically involves cys-261 of the catalytic subunit. The *cys4* mutation imposes a very severe oxidative stress on yeast cells and we still do not know whether wild-type yeast cells ever experience changes in cytoplasmic redox potential severe enough to cause oxidative inactivation *in vivo*. However, other eukaryotic cells, such as macrophages, encounter severe oxidative stress regularly and it is certainly possible that oxidative inactivation regulates V-ATPases in these systems.

CELLULAR TARGETS OF V-ATPase ACTIVITY *In Vivo*

V-ATPases appear to play both constitutive roles in internal organelles of all eukaryotic cells and specialized roles distinct to specific cell types (Stevens and Forgac, 1997). Definition of the constitutive roles of V-ATPase has focused on organelle acidification, but in fact, V-ATPases may play a general role in other physiological processes, including cytosolic pH regulation and ion homeostasis. As described above, the yeast V-ATPase is an excellent model for constitutive V-ATPases found in all eukaryotic cells. Although the yeast Vma^-phenotype has been extremely useful in identification of mutations that affect V-ATPase activity and has provided important physiological clues to the constitutive roles of V-ATPases, certain aspects of this phenotype remain rather mysterious. It was initially surprising that several processes believed to be absolutely dependent on organelle acidification, including zymogen activation and vacuolar protein sorting, still occurred in *vma* mutants (Yamashiro *et*

al., 1990). This result suggested that vacuolar acidification is not absolutely essential for these processes and the conditional lethality resulting from inactivation of the V-ATPase must arise from disruption of other essential processes. The calcium sensitivity of *vma* mutants may be explained by loss of the vacuolar pH gradient and a concomitant loss of the driving force for calcium uptake into the vacuole; consistent with this explanation, *vma* mutants have been shown to exhibit elevated cytoplasmic calcium levels (Halachmi and Eilam, 1993). The molecular basis of the pH-dependent growth phenotype is less clear, however. It has been proposed that cells compensate for loss of V-ATPase activity by fluid phase uptake of the acidic medium when they are grown at pH 5 (Munn and Riezman, 1994), but in *Neurospora*, loss of V-ATPase activity during growth in concanamycin A is compensated by mutations in the plasma membrane proton pump (Bowman *et al.*, 1997), suggesting a complex interaction between these two proton pumps. A potential complication with the yeast *vma* deletion mutants is that they may have altered activity of other pumps that compensates for long-term loss of V-ATPase activity. In an effort to trace the immediate cellular consequences of loss of V-ATPase activity, we isolated a temperature conditional *vma* mutant in which the Vma^- phenotype could be induced by a temperature shift.

The *VMA4* gene, which encodes the 27-kDa E subunit, was randomly mutagenized and a temperature conditional allele (*vma4-1^{ts}*) was identified (Zhang *et al.*, 1998). The *vma4-1^{ts}* mutant was able to grow at either pH 5 or 7.5 at 25°C, but failed to grow at pH 7.5 at 37°C. The *vma4-1^{ts}* allele contains a single-point mutation, D145G, at a position perfectly conserved among V-ATPase E subunits. The primary molecular effect of the mutation was destabilization of the unassembled subunit. At elevated temperatures, most of the newly synthesized subunit appeared to be degraded very rapidly, before assembly could occur. We used this mutant to examine the "acute" effects of loss of V-ATPase activity. After shifting cells from 25 to 37°C, quinacrine accumulation into the vacuole was decreased within 1 and absent after 3 h. Mutant cells incubated at pH 5 after the temperature shift retained normal morphology. If the cells were incubated at pH 7.3, however, 25–30% of the cells showed aberrant morphology, including multiple or elongated buds, within 4 h of the shift. This loss of normal morphology was accompanied by delocalization of actin. In contrast, *vma4*Δ mutants, which lack the *VMA4* gene and thus suffer a "chronic" loss of V-ATPase activity, did not show as severe morphological defects at pH 7.3, although they did lose actin localization.

We hypothesize that the V-ATPase plays an essential role in maintaining cytoplasmic pH, cytoplasmic-free ionized calcium concentrations, or both, in the presence of elevated extracellular pH. Loss of control of pH and calcium concentrations under these conditions results in a loss of actin cytoskeletal organization that may be directly responsible for the mutants' inviability. It may be that other proton pumps, particularly the plasma membrane H^+-ATPase, are able to compensate for loss of V-ATPase activity at low pH, but cannot maintain this compensation at high pH. Consistent with this hypothesis, mutants with deficient or defective plasma membrane H^+-ATPase activity also show morphological defects similar to those seen in the *vma4-1^{ts}* mutant, including multiple or elongated buds, and, in some cases, their phenotypes are aggravated at low extracellular pH (McCusker *et al.*, 1987; Vallejo and Serrano, 1989). Higher eukaryotes often use Na^+/H^+ exchangers at the plasma membrane in preference to the fungal P-type proton pumps, but this does not exclude the possibility that the V-ATPase cooperates with the exchanger or other transporters to help regulate cytosolic pH in these cells. Future studies will be directed toward: (1) isolation of other temperature-conditional *vma* mutants with which to test the hypothesis that the *vma4-1^{ts}* mutant is revealing the immediate consequences of loss of V-ATPase activity and (2) direct measurement of cytosolic and vacuolar pH at varied extracellular pH under the permissive and non-permissive conditions for the mutants.

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