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The Long Physiological Reach of the Yeast Vacuolar H⁺-ATPase

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Abstract

V-ATPases are structurally conserved and functionally versatile proton pumps found in all eukaryotes. The yeast V-ATPase has emerged as a major model system, in part because yeast mutants lacking V-ATPase subunits (*vma* mutants) are viable and exhibit a distinctive Vma-phenotype. Yeast *vma* mutants are present in ordered collections of all non-essential yeast deletion mutants, and a number of additional phenotypes of these mutants have emerged in recent years from genomic screens. This review summarizes the many phenotypes that have been associated with *vma* mutants through genomic screening. The results suggest that V-ATPase activity is important for an unexpectedly wide range of cellular processes. For example, *vma* mutants are hypersensitive to multiple forms of oxidative stress, suggesting an antioxidant role for the V-ATPase. Consistent with such a role, *vma* mutants display oxidative protein damage and elevated levels of reactive oxygen species, even in the absence of an exogenous oxidant. This endogenous oxidative stress does not originate at the electron transport chain, and may be extra-mitochondrial, perhaps linked to defective metal ion homeostasis in the absence of a functional V-ATPase. Taken together, genomic data indicate that the physiological reach of the V-ATPase is much longer than anticipated. Further biochemical and genetic dissection is necessary to distinguish those physiological effects arising directly from the enzyme's core functions in proton pumping and organelle acidification from those that reflect broader requirements for cellular pH homeostasis or alternative functions of V-ATPase subunits.

Keywords

V-ATPase; yeast; *vma* mutant; acidification; genomic; oxidative stress

Introduction

Vacuolar proton-translocating ATPases (V-ATPases) are functionally diverse proton pumps with a highly conserved structure (Kane, 2006; Nishi & Forgac, 2002). In all eukaryotic cells, V-ATPases are responsible for acidification of a variety of organelles, including lysosomes/vacuoles, endosomes, and the late Golgi apparatus. In certain cells, they have also been adapted to export protons from the cytosol across the plasma membrane (Breton & Brown, 2007) Wieczorek et al., 1999). V-ATPases contribute to cellular pH control in all of these different cellular contexts.

All eukaryotic V-ATPases have a very similar structure, consisting of approximately 14 subunits arranged into two subcomplexes: a peripheral membrane subcomplex called V_1 and an integral membrane subcomplex designated V_o (Nishi & Forgac, 2002). In mammalian cells, many of these subunits exist as several tissue- or organelle-specific isoforms, but in yeast, all subunits except the V_o "a" subunit are encoded by a single gene (Figure 1). Deletion of any one of these genes, any of four assembly factors dedicated to the V-ATPase (Davis-Kaplan et al., 2006; Graham, Hill & Stevens, 1998), or both of the a subunit isoforms, results in a very similar phenotype. This Vma^- phenotype, is characterized by a distinct pattern of pH and

calcium sensitive growth, metal ion sensitivity, and inability to grow on non-fermentable carbon sources (Kane, 2006). In contrast, complete loss of V-ATPase activity in eukaryotes other than fungi is lethal, often at very early stages of development (Allan et al., 2005; Sun-Wada et al., 2000). Null mutations of subunit isoforms permit viability in some cases in metazoans, and result in specific defects characteristic of the sphere of influence of V-ATPases containing that specific isoform (Borthwick & Karet, 2002).

Because yeast *vma* deletion mutants are viable, it is possible to assess the downstream consequences of abrogating V-ATPase function from the properties of the *vma* mutants. Both direct analysis of the *vma* mutants and phenotypic screens of ordered yeast deletion mutant arrays have revealed that loss of V-ATPase activity has unexpectedly diverse consequences. These results indicate that tight-control of pH and/or "moonlighting" functions of V-ATPase subunits are intertwined with a large number of cellular processes. In this review, we will describe the diverse functional connections to the V-ATPase revealed by yeast genomic screens in recent years, and then focus on recent data suggesting that V-ATPases may help to provide resistance to oxidative stress.

Genomic screens highlight widespread defects in *vma* mutants

The development of ordered deletion mutant arrays lacking individual, marked, non-essential yeast genes has permitted many non-biased genomic screens for specific growth phenotypes and inhibitor sensitivities (Giaever et al., 2002). In these screens, the collection of almost 5000 non-essential deletion mutants is tested for growth under varied conditions, and mutants with selectively compromised growth are identified. The resulting collection of mutants is further analyzed to look for enrichment of certain classes of genes. Statistically enriched classes can identify a complex or pathway that enables wild-type cells to grow under the conditions tested. Although it is certainly true that mutations can rather indirectly impact the ability of cells to grow under any given set of conditions, it is a significant advantage that genomic screens can potentially highlight the full scope of gene products required for growth, and provide evidence of important functional connections that were not appreciated previously. For example, this approach was used to screen the haploid non-essential deletion collection for all mutants exhibiting the classical Vma^{-} phenotype, specifically, compromised growth at high pH and/or high calcium concentrations (Sambade et al., 2005; Serrano et al., 2004). These genomic screens identified almost all of the previously characterized V-ATPase subunits (in our screen, the strains lacking the other subunits were not represented in our library), as well as a previously unidentified subunit of the enzyme (Sambade & Kane, 2004) and a number of potential regulators (Sambade et al., 2005). A parallel screen also identified a novel assembly factor for the V-ATPase (Davis-Kaplan et al., 2004). All of these screens identified other strains that may have compromised vacuolar acidification.

In addition to their expected prominence in the total set of deletion mutants sensitive to elevated pH and high calcium, *vma* mutants emerged as a major class of mutants that display metabolic and morphological aberrations and sensitivity to a number of other treatments. As summarized in Table 1, *vma* mutants were overrepresented in genomic screens for: 1) sensitivity to multiple drugs, 2) sensitivity to elevated metal ion concentration, 3) sensitivity to limited iron availability, 4) sensitivity to both low and high extracellular calcium, 5) sensitivity to alcohol stress, 6) poor growth on high salt, 7) aberrant vacuolar morphology, 8) excess glycogen accumulation, 9) sensitivity to DNA damaging reagents, and 10) senstivity to multiple forms of oxidative stress (see Table 1 for references to specific screens). Some of these results may be readily explained by established roles of the vacuole in metal ion and calcium homeostasis, in nutrient storage and in sequestration of multiple metabolites and toxins (Klionsky, Herman & Emr, 1990). Additionally, V-ATPases that reside outside the vacuole may also have critical functions, as suggested by a recent study comparing the phenotypic consequences of V-ATPase

activity loss to effects of losing Pmr1p, a calcium pump localized in the Golgi (Yadav et al., 2007). Other results, however, such as multidrug sensitivity and sensitivity to DNA damaging agents, are harder to incorporate into our current understanding of V-ATPase function, and suggest that the influence of the V-ATPase in overall cell physiology is far more extensive than anticipated.

An antioxidant role for V-ATPases

One unexpected phenotype of the *vma* mutants revealed by genomic screens is hypersensitivity to oxidative stress (Outten, Falk & Culotta, 2005; Thorpe et al., 2004). Oxidative stress is an occupational hazard for cells growing in an oxygen environment (Imlay, 2003), and uncontrolled stress is implicated as an important factor in aging and multiple human disease states ranging from Parkinson's disease and amyotrophic lateral schlerosis to diabetes (Davies, 1995; Valko et al., 2007). Many mechanisms for oxidative stress resistance, including superoxide dismutase, the glutathione buffer system, thioredoxins, and redox-sensitive transcription factors, are ancient and conserved, tuned to responding to stresses from different sources and/or in different intracellular locations (Carmel-Harel & Storz, 2000; Temple, Perrone & Dawes, 2005). Screening yeast deletion libraries for mutants with enhanced sensitivity to oxidative stress confirmed that deletions in these well-established systems compromised resistance to multiple oxidants (Outten et al., 2005; Thorpe et al., 2004). Surprisingly, these screens also indicated that loss of V-ATPase activity rendered cells sensitive to multiple oxidants, suggesting that V-ATPase activity also played a critical role in defending against oxidative stress.

We confirmed this result by testing two *vma* mutants, *vma*2Δ and *vma*3Δ, for sensitivity to hydrogen peroxide, the superoxide-generating agent menadione, and diamide (Milgrom et al., 2007). We found that the *vma* mutants were particularly sensitive to hydrogen peroxide, but also showed increased sensitivity to menadione and diamide. It was particularly striking that the sensitivity of *vma* mutants to hydrogen peroxide approached that of mutations lacking the cytosolic or mitochondrial superoxide dismutases (SODs), which are well-established as important antioxidant systems. This supports a critical antioxidant role for the V-ATPase. Many mutations that disrupt antioxidant mechanisms result in elevation of endogenous reactive oxygen species (ROS) and evidence of oxidative damage to protein, even in the absence of an exogenous oxidant (O'Brien et al., 2004). Consistent with this, we found significantly increased ROS and oxidative protein damage in the *vma*2Δ mutant, suggesting that these cells have an endogenous source of ROS that may be taxing the redox buffering system(Milgrom et al., 2007).

In recent years it has become clear that, in addition to promoting multiple forms of cell damage, ROS can play complex roles in signalling and adaptation and can originate from multiple sources (Temple et al., 2005; Valko et al., 2007). We explored possible sources of the excess endogenous ROS in the *vma* mutants. The respiratory chain can be a major source of reactive oxygen species (Dirmeier et al., 2002), and the failure of *vma* mutants to grow on non-fermentable carbon sources suggested the possibility that defective oxidative phosphorylation might give rise to enhanced oxidative stress. We tested this by examining the sensitivity of *vma* mutant cells lacking mitochondrial DNA (*rho*^{0} cell s) to oxidative stress. Yeast *rho*^{0} cells contain mitochondria, but lack an intact electron transport chain; in some cases, loss of mitochondrial DNA suppresses ROS production (Haynes, Titus & Cooper, 2004). The *vma*2Δ *rho*^{0} mutant did not show a significant decrease in ROS levels, however, and was at least as sensitive as the *vma*2Δ *rho*$^{+}$ mutant to peroxide addition (Milgrom et al., 2007), suggesting that the source of oxidative stress in the mutants is non-mitochondrial. Furthermore, consistent with a cytosolic source of endogenous ROS, the sensitivity of the *vma*2Δ mutant to oxidative stress was exacerbated much more severely when the *vma*2 deletion was combined with a

deletion in the cytosolic antioxidant systems (the *trx2Δ*, *glr1Δ* and *sod1Δ* mutations, which affect the cytosolic thioredoxin, glutathione, and superoxide dismutase defenses, respectively) than when combined with deletions in the mitochondrial antioxidant systems (*trx3*Δ, and *sod2Δ*, affecting the mitochondrial thioredoxin and superoxide dismutase, respectively) (Milgrom et al., 2007). In fact, combination of *vma*2Δ and *sod1Δ* mutations appeared to be lethal unless the *vma*2Δ mutation was complemented by a *VMA2*-containing plasmid. Taken together, these results suggest an extra-mitochondrial source of oxidative stress, but the exact nature of that stress and the primary source of ROS in the *vma* mutants remain to be determined.

Transcriptional profiling can also provide unique insights into the physiological state of a cell. Through microarray analysis, we compared mRNA levels from *vma*2Δ and wild-type cells grown under optimal conditions for the mutant (Milgrom et al., 2007). The set of genes upregulated in the *vma*2Δ mutant was most highly enriched in genes associated with two functional categories: 1) metal ion, and particularly iron, transport and homeostasis, and 2) arginine, glutamine, and ornithine biosynthesis and metabolism. In yeast, many of the iron transport and homeostasis genes (collectively called the “iron regulon”) are co-regulated under the control of two homologous but non-redundant transcription factors, Aft1p and Aft2p (Rutherford & Bird, 2004). The upregulation of the iron regulon is potentially significant in the context of oxidative stress, both because perturbed metal ion homeostasis may promote ROS formation, and because upregulation of these genes in response to oxidative stress had been reported previously (Belli et al., 2004; Pujol-Carrion et al., 2006). We tested one mechanism by which a cycle of iron deprivation and ROS production might be operating in the *vma* mutants. Yeast cells require an acidic compartment to support maturation of the high affinity iron transporter Fet3p/Ftr1p, so Fet3p does not mature in *vma* mutants and high-affinity iron transport is lost (Davis-Kaplan et al., 2004). It was possible that this in itself might potentiate oxidative stress by introducing a requirement for low affinity/low specificity transporters that less efficiently control influx of redox active metals (Li & Kaplan, 1998). We found, however, that a *fet3*Δ mutant, which completely lacks the high affinity iron transporter, is not much more sensitive to peroxide stress than the wild-type cells. Therefore, defective metal ion homeostasis is still a potential source of oxidative stress in the vma mutants, but iron deprivation does not fully explain this stress.

Significantly, most of the genes encoding typical antioxidant enzymes were not upregulated in the *vma*2Δ mutant. Acute oxidative stress generates a potent transcriptional response via the Yap1p and Skn7p transcription factors, which results in upregulation of much of the antioxidant machinery (Coleman et al., 1999; Lee et al., 1999). There was little evidence of activation of Yap1 or Skn7-regulated genes in the *vma*2Δ mutant, but one potential antioxidant gene, the cytosolic, stress-induced, thioredoxin peroxidase Tsa2p was highly upregulated. Thioredoxin peroxidases are capable of reducing reactive oxygen species such as H_2O_2 or organic hydroperoxides (Munhoz & Netto, 2004; Park et al., 2000), so it is possible that upregulation of *TSA2* helps provde relief from oxidative stress in the *vma*2Δ mutant.

Why does loss of V-ATPase activity have such far-reaching effects?

As discussed above, the surprising antioxidant effects of the V-ATPase have a number of potential physiological explanations that can be readily explored. However, these results also highlight again the general question raised by the wide-ranging phenotypes associated with the *vma* mutants outlined in Table I; specifically, why does loss of V-ATPase activity have such far-reaching effects on cell physiology? Furthermore, it is entirely possible that these far-reaching effects are present not only in yeast, but in mammalian cells as well. The full range of these effects may not have been fully appreciated in animals because the current tools for abolishing V-ATPase activity are much more limited. Notably, not only is genetic loss of V-ATPase activity lethal at very early stages of mouse development (Sun-Wada et al., 2000), but

treatment of a number of cell types with the specific V-ATPase inhibitors concanamycin A or bafilomycin A1 results in wide-ranging consequences, often culminating in cell death (De Milito et al., 2007;Manabe et al., 1993;Nishihara et al., 1995;Okahashi et al., 1997). These results indicate that the V-ATPase is centrally important in mammalian cells as well as yeast cells.

There is no question that the primary function of V-ATPases is proton pumping. In most eukaryotic cells, this pumping is directed toward organelle acidification, and organelle acidification in turn is important for a variety of other processes ranging from protein sorting and degradation to overall ion homeostasis (reviewed in (Kane, 2006; Paroutis, Touret & Grinstein, 2004)). Proton pumping into organelles is accompanied by removal of protons from the cytosol, but the extent to which V-ATPase activity makes a significant contribution to cytosolic pH homeostasis under normal growth conditions is somewhat unclear. There is clear evidence that their activity may become important for cytosolic pH control under certain conditions at least (Swallow et al., 1991; Swallow et al., 1993). We have recently found that the yeast *vma* mutants have dramatically perturbed cytosolic pH homeostasis (Martinez-Munoz and Kane, manuscript in preparation); therefore, cytosolic pH changes could help account for some of the unexpected defects of the yeast *vma* mutants. This overall perturbation in pH homeostasis in yeast may help to account for the central position of the V-ATPase in homeostasis of multiple other ions (Eide et al., 2005), and could even be contributing to the lifetime or distribution of ROS(Imlay, 2003), helping to explain the antioxidant function of V-ATPases.

It should also be noted, however, that certain V-ATPase subunits have been implicated in a number of "moonlighting functions" that appear to be separate from the enzyme's proton pump function. The most prominent recent example of this is the proposed role of the V_o sector subunits in membrane-membrane fusion (Hiesinger et al., 2005; Peters et al., 2001). Disruption of such additional functions of the V-ATPase could also give rise to diverse phenotypes, but it should be noted that in these cases, only mutations in the subunits involved, i.e. the V_o subunits in the case of membrane fusion, should give rise to phenotypes arising from loss of fusion. Significantly, virtually all of the screens summarized in Table 1 identified *vma* genes encoding both V_1 and V_o sector subunits.

Taken together, these data suggest that the long reach of V-ATPase function is likely to be rooted in one or more aspects of pH homeostasis. Sorting out how pH ultimately influences different aspects of cell physiology and determining how far removed the different phenotypes are from loss of V-ATPase activity in yeast will help to define the physiological reach of V-ATPase activity in mammalian cells and clarify the many facets of V-ATPase function.

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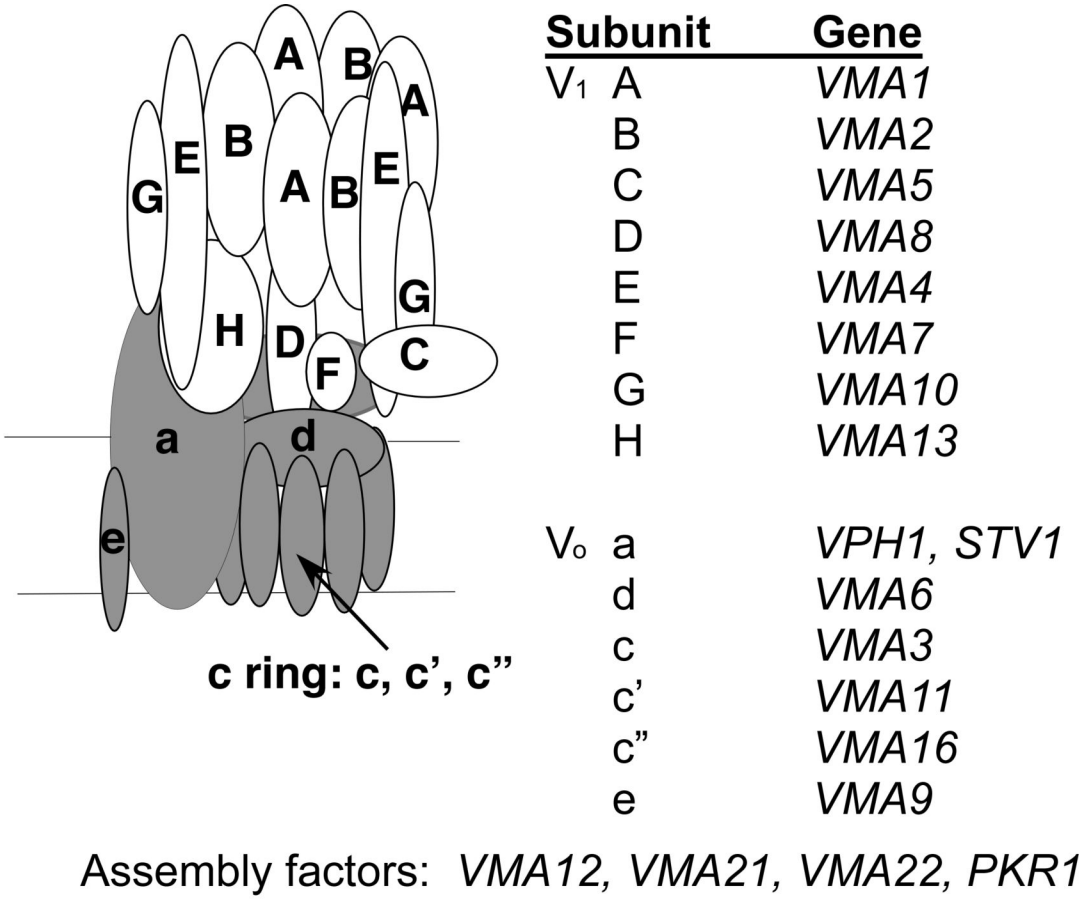

Figure 1.
Model of yeast V-ATPase structure and subunit gene designations. V_1 subunits are shown in white, and V_o subunits are shown in grey in the model. *VPH1* and *STV1* encode two isoforms of the V_o a subunit.

Table 1

Multiple phenotypes of yeast *vma* deletion mutants as revealed by genomic screening

Phenotype targeted in genomic screen	Reference
Poor growth at elevated pH and extracellular calcium concentrations	{Sambade, 2005 #646}
Alkaline pH sensitivity	{Serrano, 2004 #539}
Sensitivity to organic acids	{Rand, 2006 #1283}
Poor growth under conditions of limited iron availability	{Davis-Kaplan, 2004 #547}
Poor growth at elevated metal or metalloid concentrations	{Eide, 1993 #1166; Hamilton, 2002 #1288; Gharieb, 1998 #1300}
Altered uptake and distribution of cellular iron	{Lesuisse, 2005 #580}
Poor NaCl tolerance	{Hamilton, 2002 #1290}
Multidrug hypersensitivity; vma mutants were hypersensitive to: cyclosporin A, FK506, fluconazole, sulfometuron methyl wortmannin, tunicamycin, caffiene, rapamycin, hydroxyurea, and cycloheximide	{Parsons, 2004 #521}
Hypersensitivity to BAPTA (low Ca^{2+}), amiodarone, and $MnCl_2$	{Yadav, 2007 #1280}
Hypersensitivity to DNA damaging agents (cisplatin)	{Liao, 2007 #1284}
Sensitivity to multiple oxidants	{Thorpe, 2004 #579}
Poor growth at high oxygen pressure	{Outten, 2005 #578}
Sensitivity to dithiothreitol (reductive stress)	{Rand, 2006 #1283}
Poor tolerance of ethanol and other alcohols	{Fujita, 2006 #1299}
Altered glycogen accumulation	{Wilson, 2002 #1287}
Perturbed vacuolar morphology; defective vacuolar protein sorting	{Seeley, 2002 #573; Bonangelino, 2002 #762}