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Vacuoles in Filamentous Fungi

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Major contributions of filamentous fungi to the ecosystem include degradation of detritus by saprotrophic fungi, support of plant growth by mycorrhizal fungi, and invasion of humans and major crop plants by pathogenic fungi. Although the fungal vacuole plays a critical role in all of these functions, we are only just beginning to understand what specific genes and proteins are involved, how they are regulated, and the molecular mechanism of their action in formation of this organelle.

AQ1

Investigators are in good agreement that the vacuole is a dynamic organelle, enclosed by a single membrane, with a variety of structural forms: small vesicles, tubules, and large vesicles. It is an acidic compartment as a consequence of the activity of the vacuolar H^+-translocating ATPase (V-ATPase) in the membrane. It contains high concentrations of basic amino acids, polyphosphate, hydrolytic enzymes, and divalent cations. It has a presumed role in nitrogen and phosphate storage, solute transport, regulation of cellular amino acid metabolism, recycling of macromolecules, osmoregulation, and cytosolic ion and pH homeostasis. The vacuole is an important organelle.

STRUCTURE OF THE VACUOLE

The Vacuolar System as a Motile Tubular Complex

The old textbook view of vacuoles as large round organelles changed to a dynamic picture of connected tubules, vesicles, and some large vesicles as the result of work by Anne Ashford and her colleagues (Shepherd et al., 1993; Cole et al., 1998). Using the ectomycorrhizal fungus *Pisolithus tinctorius*, they observed four zones of vacuolar morphology from the growing hyphal tip: (i) the apical zone, which has few or no vacuoles; (ii) the subapical zone with small ovoid-spherical vacuoles; (iii) the nuclear zone, where tubular vacuoles predominate; and (iv) the basal zone, where large spherical vacuoles are most common. (Fig. 1 through 3 in Hyde et al., 2002, show fine examples of these zones.) The parts appear to form a continuous vacuolar system with numerous small tubes connecting small vesicles, large vesicles, and highly tubulated regions. Most striking is the active nature of the vacuolar system in living hyphae. Tubules extend, retract, fuse with each other and with spherical vacuoles, and can deform (reversibly) into small vesicles. Vesicles may sprout small tubules and appear to slide along larger tubules.

Such a dynamic pleiomorphic tubular vacuolar structure has been documented in many other filamentous fungi, including *Paxillus involutus* (Tuszynska et al., 2006), *Phanerochaete velutina* (Darrah et al., 2006), *Phialocephala fortinii* (Saito et al., 2006), *Gigaspora margarita* (Uetake et al., 2002), *Aspergillus oryzae* (Shoji et al., 2006a), *Neurospora crassa* (Hickey et al., 2004), and several members of the oomycete genus *Saprolegnia* (Allaway et al., 1997).

Visualizing the Vacuolar Complex

The vacuolar structure described above is the product of live-cell imaging in microscopes utilizing fluorescent dyes that accumulate in vacuoles and green fluorescent protein (GFP)- or red fluorescent protein (RFP)-tagged proteins targeted to vacuoles or vacuolar membranes. Both fluorescent microscopes and confocal laser scanning microscopes are widely used. Delights of the live-imaging technology are movies showing the dynamic vacuolar system in action. Examples are included on a CD-ROM produced for educational purposes, which can be obtained from www.fungalcell.org (Hickey and Read, 2003).

The dye preferred by at least two research groups is Oregon green 488 carboxylic acid diacetate (carboxy-DFFDA) 6 isomer (catalog no. 06151; Invitrogen-Molecular Probes, Eugene, OR), which accumulates specifically in the lumen of both tubular and spherical vacuoles (Cole et al., 1998; Hickey et al., 2004). The dye diffuses into cells, where it is colorless and nonfluorescent until esterases cleave off the acetate groups to yield the fluorescent, amine-reactive carboxy-DFFDA. Cole and colleagues (Cole et al., 1997) proposed that in *P. tinctorius* the dye crosses the vacuolar membrane via a nonspecific anion transporter and becomes trapped there. Supporting this hypothesis, probenecid, an anion transport inhibitor, prevented uptake of the carboxy-DFFDA from the cytosol to the vacuolar system.

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Other fluorescent dyes from Molecular Probes are also suitable for staining fungal vacuoles. Earlier studies used 6-carboxyfluorescein diacetate (6-CFDA); it works well but is being replaced by its more stable derivative, carboxy-DFFDA. Kitamoto and coworkers (Shoji et al., 2006a) have used 7-amino-chloromethyl coumarin (CMAC), which is converted to a cell-impermeant conjugate with glutathione and accumulates in fungal vacuoles via glutathione pumps. Another useful dye is FM4-64 because it labels plasma membranes, endosomal membranes, and vacuolar membranes in a progressive manner. Commonly used as an indicator dye for endocytosis, FM4-64 beautifully illuminated the endosomal/vacuolar systems of *A. oryzae* (Shoji et al., 2006a) and *N. crassa* (Hickey et al., 2004) but labeled only plasma membranes and vacuoles in dead or damaged cells in *P. tinctorius* (Cole et al., 1998).

More recently, investigators have been visualizing the vacuolar system with probes containing marker proteins tagged with GFP and RFP. The availability of complete genome sequences for *A. oryzae* and *N. crassa* greatly facilitated these studies. Genes for proteins targeted to the vacuole have been obtained by PCR and cloned into GFP and RFP vectors for transformation to *A. oryzae* and *N. crassa*.

Kitamoto and his colleagues initiated the studies with GFP and RFP in vacuoles of filamentous fungi. Based on studies with *Saccharomyces cerevisiae*, they constructed a strain of *A. oryzae* expressing the fusion protein of carboxypeptidase Y, a protease in the vacuolar lumen, and enhanced GFP (CPY-EGFP) (Ohneda et al., 2002). Unexpectedly, the fluorescence of CPY-EGFP was weak and unclear in cells grown on acidic medium (pH 5.5, which is optimal for growth) but appeared in the lumen of vacuole-

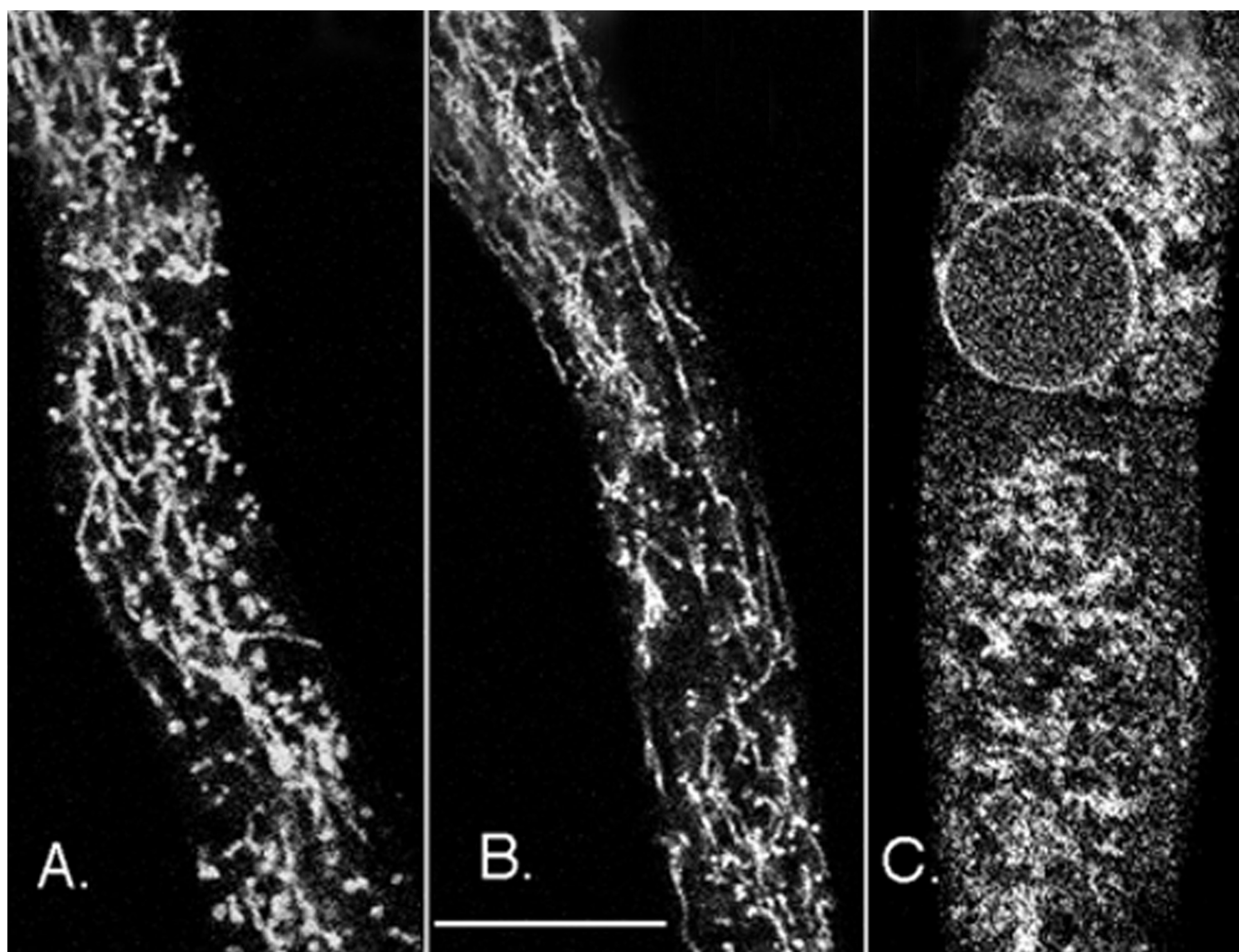

FIGURE 1 Visualizing the vacuole with RFP and GFP. RFP (dsRED) or GFP was fused to proteins predicted to be in the membrane of the vacuole, using the plasmids pMF272 or pMF334 constructed by M. Freitag (Oregon State University, Corvallis). By transformation into *N. crassa* the recombinant genes were targeted to the *his-3* locus. Images were obtained by confocal microscopy (B. Bowman and E. J. Bowman, unpublished results). (A) RFP dsRED was fused to the N terminus of the CAX protein. (B) RFP dsRED was fused to the N terminus of the VAM-3 protein. In panels A and B the region shown is approximately 100 μm behind the hyphal tip. The vacuolar system consists of tubules and small vesicles. (C) GFP was fused to the C terminus of the A subunit of the V-ATPase (encoded by *vma-1*). The region shown is approximately 2 mm behind the hyphal tip. In this older part of the hypha the fluorescence is localized to the membrane of a large vacuole and to many small vesicles. In this strain the vacuole appears as a network of tubules and small vesicles nearer the hyphal tip (not shown), as in panels A and B. The bar represents 10 μm, and the scale is the same for all panels.

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like structures grown on medium of alkaline pH (8.0). Apparently, EGFP fluorescence is sensitive to low pH. A subsequent paper was more satisfying. Vam3p is a tSNARE protein located on the vacuolar membrane in *S. cerevisiae*. Shoji et al. (2006a) examined vacuolar membranes in strains of *A. oryzae* expressing the fusion protein EGFP-AoVam3p and saw the dynamic, pleiomorphic structure that has become a hallmark of the vacuolar system in filamentous fungi as described above. Thus, AoVam3p became the first protein to be localized on membranes of tubular vacuoles. Beautiful confocal images of the vacuolar membrane system in *N. crassa* are obtained by using fusion proteins between GFP or RFP and VAM-3, proteins involved in calcium transport, such as CAX, and subunits of the V-ATPase (Fig. 1).

A Cautionary Note

Because it is dynamic and flexible, the fungal vacuolar system is subject to variation and change induced by the experimenter. The cells are surprisingly fragile and must be handled with extreme care to avoid damage and artifactual findings. Damage can result from high concentrations of dyes, excessive light intensity, too-long exposure to light, oxygen deprivation, and rough handling of samples. Signs that cells have been perturbed include slowing of hyphal growth, narrowing of the hyphal tip, retraction of the Spitzenkörper from the tip, and changes in organelle morphology (Hickey et al., 2004; Cole et al., 1998). The vacuolar system in particular responds to insult by forming many spherical vesicles with a range of sizes.

Complications in assessing the condition of a sample come from natural variation in the material. Vacuolar form and motility vary with the age of the culture, growth conditions, and the location within the culture (Cole et al., 1998; Ohneda et al., 2002; Shoji et al., 2006a). Furthermore, experimental treatments such as addition of inhibitors may affect growth and morphology of the vacuolar system in the same way as damage does (Hyde et al., 1999, 2002; Tuszynska et al., 2006). When feasible, showing that a response is reversible is desirable.

Another View of the Vacuole

For biochemical studies in vitro, vacuoles can be isolated from filamentous fungi as a pure organellar fraction of round vesicles, approximately 0.2 to 2 μ in diameter. Cramer et al. (1983) developed the original procedure for *N. crassa* in the early 1980s. Cells are grown for 14 to 15 h in aerated liquid culture and lysed in 1 M sorbitol by the action of glass beads in a bead beater, and vacuoles are then separated from other cell fractions by differential centrifugation. The vacuoles, behaving like osmometers, become very dense and pellet with mitochondria. Centrifugation on a sucrose gradient separates mitochondria from vacuoles, which pellet under a 60% sucrose solution. To isolate vacuolar membranes, the pelleted dense vacuoles are lysed by suspension in a low-osmotic solution (1 mM Tris-HCl, pH 7.5) and then the light vacuolar membranes are pelleted at high speed (Bowman and Bowman, 1997).

AQ2

After viewing the tubular vacuolar system by microscopy, one might anticipate a large yield of vacuoles and vacuolar membranes from such a preparation. Instead, from a typical cell fractionation procedure we obtain ~200 mg of mitochondrial protein, 20 mg of plasma membrane protein, and 2 mg of vacuolar membrane protein. This is a reminder that the dense tubular vacuolar system occurs only near the growing tips of hyphae, while large spherical vacuoles predominate behind them. The structure of vacuoles in a mass of mycelium grown in the lab, in liquid or on plates, remains to be investigated. Similarly, results from in vitro and in vivo studies should be compared with caution.

MAINTENANCE AND BIOGENESIS OF THE VACUOLAR SYSTEM

Microtubules

For filamentous fungi, the study of formation and biogenesis of the vacuole is a nascent research area. In 1993, Steinberg and Schliwa proposed that all organellar formation and movement in *N. crassa* were microtubule-dependent processes (Steinberg and Schliwa, 1993). Hyde et al. (1999) observed the effects of microtubule inhibitors on the structure of the vacuolar system of *P. tinctorius* visualized with carboxy-DFFDA. The antimicrotubule drug oryzalin largely eliminated the tubular vacuolar system, replacing it with spherical vacuoles. Upon removal of the microtubule inhibitor, the tubular vacuolar system re-formed. Antiactin drugs had little effect on the morphology and motility of the majority of the vacuolar system, but they did cause "massed tubules" at and just behind the tip, possibly because growth was inhibited and tubules piled up as mass flow continued. Electron microscope pictures of freeze-substituted material consistently showed a close alignment of microtubules and vacuoles. Taken together, these results pointed to microtubules, but not microfilaments, as important for determining vacuolar morphology and motility (Hyde et al., 1999).

GTPases

Dynamin-like proteins, which are GTPases, have been implicated in the formation of the interconnected vacuolar system of filamentous fungi. In *P. tinctorius* GTPγS induced a dramatic increase in tubular vacuoles in the first five cells. (Typically, tubular vacuoles are primarily in the first cell in this fungus.) The change was reversible and prevented by preincubation with GDPγS. This behavior was consistent with the action of a dynamin-like protein (Hyde et al., 2002).

A molecular approach was taken in the study of *Aspergillus nidulans*. Kitamoto and his group began choosing homologs of *S. cerevisiae* genes known to be involved in vacuolar biogenesis to evaluate their functions in filamentous fungi. Deletion of the *vpsA* gene (*S. cerevisiae* homolog *VPS1*), which encodes a protein related to dynamin, led to severe alterations in the structure of the vacuolar system of *A. nidulans*. Vacuole-like structures, identified by staining with the vacuolar dye 6-CFDA in the disruptant strain, were highly fragmented rather than large and spherical as in the wild-type strain (Tarutani et al., 2001). Deletion of the *avaA* gene (*S. cerevisiae* homolog *VAM4/YPT7*), which encodes a small GTPase in the Rab family, elicited a similar phenotype, i.e., highly fragmented vacuoles (Ohsumi et al., 2002). If Vam4p is fixed in the GDP-bound state by mutation in *S. cerevisiae*, it loses its function in homotypic vacuolar fusion. A strain with the comparable mutation in the *avaA* gene produced highly fragmented vacuoles, consistent with a role for the GDP-bound form of the enzyme in preventing activity. For both of the *A. nidulans* genes the authors suggested that the small vacuole-like compartments might be intermediates in vacuolar biogenesis (Tarutani et al., 2001; Ohsumi et al., 2002). Interestingly, although both *A. nidulans* genes had functions similar to those of their yeast homologs, neither one was able to rescue its respective *S. cerevisiae* deletion strain.

Late Endosomal Markers

Data from other deletion strains led to more complex interpretations (Tatsumi et al., 2007). An *S. cerevisiae* strain lacking Vps24p, a marker protein for late endosomes, grows normally and has intact vacuoles because an alternative route for vacuolar biogenesis is available. In contrast, the homologous deletion in *A. oryzae* resulted in a strain (Aovps24) that grew poorly and contained fragmented and aggregated vacuoles, much like the two *A. nidulans* strains described above. Late endosomal and vacuolar functions appeared to be functionally fused in the *A. oryzae* mutant. Strains with three other late endosomal markers (again by homology to *S. cerevisiae*) fused to EGFP were constructed and examined by confocal microscopy. The EGFP signal appeared as static dot-like structures near vacuoles that were stained by FM4-64 (an endomembrane system dye) but not by CMAC (a vacuolar dye), suggesting an endosomal location only. Expression of the dsRED- and EGFP-tagged late endosomal markers in the Aovps24 strain did not label dispersed dot-like structures but aggregated bodies previously identified as fragmented vacuoles. Thus, the fragmented vacuoles behaved like hybrid organelles with features of both late endosomes and vacuoles. Maybe AoVps 24 is required for the functional separation of these two compartments. The data can be interpreted as evidence for a late endosomal function in vacuolar biogenesis (Tatsumi et al., 2007).

Vacuolar Membrane Marker

AoVam3p was the first protein shown to locate on the membrane of tubular vacuoles in filamentous fungi (Shoji et al., 2006a). The yeast homolog, Vam3p, is a vacuolar membrane syntaxin that regulates vesicular traffic to vacuoles and the homotypic fusion between vacuoles. As anticipated, the fusion protein of EGFP-AoVam3p localized to small punctate structures in the apex, tubular vacuoles behind the apex, and the membrane of large vacuoles further back, like a typical vacuolar network in *A. oryzae*. Surprisingly, it also colocalized with FM4-64. Sequence analysis revealed equal similarity of AoVam3p to two *S. cerevisiae* proteins, Vam3p and Pep12p. (Pep12p is a tSNARE in the late endosome/prevacuolar compartment.) In the absence of a second homolog in *A. oryzae*, it is possible that AoVam3p has the functions and cellular locations of both proteins. Time-lapse imaging of EGFP-AoVam3p for a short period showed multiple changes in vacuolar structure, consistent with a role for the protein in mediating fusion of small vesicular and tubular structures with large spherical vacuoles (Shoji et al., 2006a).

How filamentous fungi form their unique, variable, and vibrant vacuolar system is just beginning to be studied and understood. One lesson from the experiments above is that understanding the biogenesis of the vacuolar system in *S. cerevisiae* cannot be transferred directly to filamentous fungi and that additional studies are essential.

FUNCTIONS OF THE VACUOLAR SYSTEM

Storage of Nitrogen and Phosphorus Compounds

Fungal vacuoles serve as storage reservoirs for high levels of phosphorus and nitrogen in the form of basic amino acids and polyphosphate (polyPi), respectively. R. H. Davis used *N. crassa* in a classic analysis of basic amino acid metabolism and its regulatory role in fungi. The amino acid story and its partner polyamine story are covered in chapter 27 of this volume, and we do not discuss it further here. AQ3

The history of polyPi in fungal vacuoles has elements of mystery. What does it do in the vacuole, what form does it take, and how does it get there? Early studies suggested that polyPi served as a counterbalance to the positive charges in the vacuole. Extracts of isolated vacuoles of *N. crassa* were found to contain millimolar concentrations of ornithine (18.1 mM), arginine (13.6 mM), histidine (1.9 mM), and lysine (1.8 mM), as well as spermidine (2.5 mM), Mg^{2+} (2.7 mM), Na^{+} (1.5 mM), and K^{+} (0.7 mM). The polyphosphate-P level (36.7 mM) was sufficient to counterbalance about one-half of the total positive charges (Cramer and Davis, 1984). As a counterion for neutralization of basic amino acids (and other positive ions), the polyPi allows cells to store both P and N compounds in a small volume.

PolyPi was once thought to occur in the form of granules in living cells, but the picture changed with improvements in sample preparation for electron microscopy. Electron microscopic analysis of growing cells of *P. tinctorius*, prepared by anhydrous freeze substitution, showed dispersed phosphorus, largely balanced by potassium ions in large spherical and tubular vacuoles (Cole et al., 1998). X-ray microanalysis of the same samples showed colocalization of phosphorus with potassium. Similar results were obtained with *P. tinctorius* associated with *Eucalyptus pilularis* as an ectomycorrhiza: the polyPi was concentrated in vacuoles of the fungus in dispersed form, not in granules (Ashford et al., 1999). AQ4

More recently Saito et al. (2006) used an innovative enzymatic method to localize polyPi in the dark root fungal endophyte, *P. fortinii*. The probe consisted of the recombinant polyPi binding domain of *Escherichia coli* exopolyphosphatase (PPBD) linked to Xpress tag, which could be visualized in confocal microscope images as a fluorescent signal by using anti-mouse Xpress conjugated with Alexa 488 or in transmission electron micrographs as gold particles conjugated with goat anti-mouse-immunoglobulin G antibody interacting with the PPBD mouse-anti-Xpress antibody complex. Again, the polyPi occurred in dispersed form in the fungal vacuoles, with tubular vacuoles appearing to contain less polyPi than spherical or elongated vacuoles.

A new insight into the importance of polyPi storage comes from a study of the corn smut fungus, *Ustilago maydis* (Boyce et al., 2006). Infection of corn by the fungus depends on a morphological transition from budding to filamentous growth. Mutants that limited polyPi accumulation in the vacuole favored the transition to the filamentous form, yet the resultant hyphae had reduced virulence in maize seedlings. One interpretation of these results is that vacuolar polyPi levels play an important part in mediating both morphogenesis and infectivity in this pathogenic fungus.

Vacuolar Calcium

Fungal vacuoles also act as reservoirs for divalent cations, including Ca^{2+}, Zn^{2+}, and Fe^{2+}, to prevent them from reaching toxic levels in the cytosol. In *S. cerevisiae* over 95% of the cellular Ca^{2+} has been reported to be in the vacuole (Eilam et al., 1985). Purified vacuoles of *N. crassa* were found to contain only 0.3 mM Ca^{2+} ions (Cramer and Davis, 1984), and calcium did not colocalize with phosphorus in X-ray microanalysis of *P. tinctorius* (Cole et al., 1998). Electrophysiological experiments demonstrated that the vacuole of *N. crassa* accumulates high concentrations of Ca^{2+} (300 μM) and that this reservoir is largely responsible for maintenance of low concentrations of Ca^{2+} in the cytosol (0.1 μM) (Miller et al., 1990). No calcium was detected in vacuoles of a mutant strain with an inactivated *cax* gene that encodes a putative Ca^{2+}/proton exchanger in the vacuolar membrane (Zelter et al., 2004). Yet the *cax* mutant is not sensitive to high concentrations of Ca^{2+} in the medium. A second *N. crassa* mutant, *nca-2*, is defective

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in the homolog of a CaATPase (Pmcp) in the vacuolar membrane of yeast. The *nca-2* mutant retains normal, possibly elevated, levels of vacuolar Ca^{2+} but is sensitive to high amounts (200 mM) of Ca^{2+} in the medium (Abreu, 2003; B. J. Bowman, unpublished results). The phenotypes of the *cax* and *nca-2* mutants suggest that additional components are involved in mediating proper cytosolic Ca^{2+} levels. Because of the universal importance of Ca^{2+} as a signaling molecule, it is important to pursue the question of how the vacuole regulates cellular distribution of Ca^{2+} in a filamentous fungus.

Transport of Solutes

Filamentous fungi that are associated with plants as mycorrhizae or as endophytes are believed to supply nutrients to the plant. That transport of nutrients such as polyPi along the hypha to the plant occurs via the dynamic vacuolar system is a logical and attractive hypothesis. Retrograde transport of carbon compounds from the plant to the fungus could also utilize the vacuolar system. The nature of filamentous growth itself requires the constant translocation of new material to the growing tip. Cytosolic streaming is vigorous and must contribute to growth. Because of its orientation and constant flux, the vacuolar system is a likely conduit as well.

From the Kitamoto lab comes other evidence suggesting that the structure of the vacuolar system points to a role in transport (Shoji et al., 2006a). Using the strain of *A. oryzae* that expressed EGFP-AoVam3p to label the vacuolar system (see above), the researchers observed an increase in the amount of tubular vacuolar structure in hyphae that extended onto a glass surface compared to the amount seen in hyphae growing on medium. Perhaps the increased tubular vacuoles served to transport nutrients to the hyphae not in contact with the medium. In mature hyphae, the EGFP fluorescence filled the lumen of the large vacuoles, as though the membrane protein had been internalized. An attractive hypothesis was that the vacuoles degrade cytosolic material by microautophagy (see below) in old vacuoles and recycle it via transport through the tubular connections in the vacuolar system to growing regions of the mycelium.

A detailed study by Darrah et al. (2006) generated the first direct evidence that the tubular vacuolar system functions in transport by following the movement of carboxy-DFFDA through the vacuolar system of *P. velutina*. The researchers monitored the dye's progress by using the technique of fluorescence recovery after photobleaching. They concluded that longitudinal transport through the dynamic, interconnected vacuolar system did occur, over distances of millimeters or even centimeters, and that solute movement could be described by a diffusion model. Moreover, these workers noted that diffusion through the vacuolar system could permit movement of solutes in both directions, thus counteracting the force of mass flow in the cytosol.

Degradative Enzymes

In the wild, filamentous fungi are important agents of recycling, and degrading litter is a major activity. Fungal vacuoles are well known for their high contents of hydrolytic enzymes (Klionsky et al., 1990). Although many of these enzymes have been extensively studied in *S. cerevisiae*, they have to date received much less attention in filamentous fungi. During our efforts to identify subunits of the V-ATPase in *N. crassa*, we isolated an abundant vacuolar protein that proved to be the homolog of proteinase A, the product of the *PEP4* gene in *S. cerevisiae*. Because proteinase A is at the top of an activation cascade, yeast cells containing Pep4p null mutations are deficient in at least three proteases. Inactivation of the *pep-4* gene in *N. crassa* resulted in phenotypes different from those of the mutants in yeast. The *N. crassa* mutant strains were not sporulation deficient, and they were not deficient in protease B and carboxypeptidase Y. Instead, the activities of the other proteases in the *pep-4* mutants were higher than in the wild type, as if the loss of proteinase A was compensated by higher levels of other proteases (Vázquez-Laslop et al., 1996). However, in *Aspergillus niger*, inactivation of the *pepE* gene, which encodes the homolog of *S. cerevisiae* proteinase A, gave a different result. The mutant strain was not deficient in sporulation, but it did exhibit reduced levels of activity for three proteases, similar to the *S. cerevisiae* proteinase A mutant strain (van den Hombergh et al., 1997).

While investigating the incompatibility reaction (described below) in *Podospora anserina*, Paoletti et al. (2001) characterized the homolog of proteinase B, a vacuolar protein involved in autophagy in *S. cerevisiae*. The proteinase B homolog was also purified in *A. niger* (Frederick et al., 1993).

Autophagy

One of the most fascinating and far-reaching roles of the vacuolar system in filamentous fungi is the involvement in autophagy. The process of autophagy has an essential role during starvation, cell differentiation, cell death, and aging in all eukaryotic organisms (Reggiori and Klionsky, 2002; Levine and Klionsky, 2004; Klionsky et al., 2007). One of two cellular pathways for the turnover of proteins and organelles, autophagy takes place in lysosomes of mammals and vacuoles of fungi, both of which have a range of hydrolases capable of degrading all cellular constituents. (The other pathway, proteasome-mediated degradation, is not discussed here.) Microautophagy involves uptake of cytosolic material directly at the vacuolar membrane and is poorly understood. In macroautophagy, bulk cytoplasm or cytosolic organelles are first sequestered in double-membrane vesicles called autophagosomes. The autophagosomes dock with vacuoles and fuse with them. In both sorts of autophagy the membrane vesicles and their contents are released into the vacuole and broken down by vacuolar hydrolases.

Thoroughly studied in *S. cerevisiae*, autophagy has received less attention in the filamentous fungi. However, the basic phenomenon has been demonstrated by using orthologues of marker proteins for autophagy in *S. cerevisiae*. In *S. cerevisiae*, Atg1p is required for the formation of preautophagosomes, and Atg8p moves from the cytoplasm to autophagosomes and to autophagic bodies in the vacuole upon induction of autophagy (Reggiori and Klionsky, 2002). When autophagy was induced by nitrogen starvation in *P. anserina*, the fluorescent GFP-PaAtg8 fusion protein localized to perivacuolar bodies and to the vacuolar lumen (Pinan-Lucarré et al., 2003, 2005). Starvation also induced transcription of two genes involved in autophagy (Pinan-Lucarré et al., 2003). Similar results were obtained with *A. oryzae*. Fusion proteins of EGFP-AoAtg8 and DsRed2-AoAtg8 localized in dot structures similar to preautophagosomes under normal growth conditions and transferred into vacuoles under starvation conditions or following addition of the inhibitor rapamycin, which is convincing evidence that autophagy was induced (Kikuma et al., 2006).

As proposed for other organisms (Levine and Klionsky, 2004), autophagy plays an important role in the differentiation and development of filamentous fungi. Strains of *A. oryzae* with AoAtg8 deleted, which did not accumulate

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DsRed-2 from the cytosol in their vacuoles (an indication that autophagy did not occur), were deficient in the differentiation of aerial hyphae and in conidial germination. Furthermore, EGFP-AoAtg8 localized to vacuoles in swollen conidia, germlings, and germ tubes even under nutrient-rich conditions. These (and other) results point to a role for autophagy in differentiation and development (Kikuma et al., 2006). Autophagy also affected differentiation in *P. anserina*. Inactivation of two genes involved in autophagy led to deficiencies in pigmentation, differentiation of aerial hyphae, and development of perithecia (Pinan-Lucarré et al., 2003).

The phenomenon of nonself recognition in filamentous fungi is often associated with autophagy. Also referred to as cell death by incompatibility, vegetative incompatibility, heterokaryon incompatibility, or programmed cell death, it is the subject of chapter 23 in this volume. Cells of growing filamentous fungi fuse with each other to form a mycelial network. However, when two cells that differ genetically at specific loci called *het* or *vic* loci fuse, they undergo cell death by incompatibility. We mention it here because the vacuolar system is intimately involved. As cells of *P. anserina* or *N. crassa* begin to die, the vacuolar system undergoes a morphological change from the tubular network to round vesicles (Pinan-Lucarré et al., 2005; Glass and Kaneko, 2003). The vacuoles fuse into larger vacuoles and then collapse or burst. Because vacuolization is a common feature of cell death by incompatibility, it is likely that vacuolar membrane permeability or rupture releases lytic enzymes and acidifies the cytoplasm, causing cell death in filamentous fungi (Pinan-Lucarré et al., 2007). Destruction of the heterokaryotic cell can be complete within 30 min after hyphal fusion (Glass and Koneko, 2003).

AQ5

The cause-and-effect relationship between autophagy and cell death by incompatibility is not yet clear. A 2003 study (Pinan-Lucarré et al., 2003) involving *P. anserina* reported that autophagy was induced during cell death by incompatibility. Two genes involved in the autophagic process in *S. cerevisiae* were upregulated during incompatibility, and a GFP-PaATG8 fusion protein accumulated in vacuoles in response to either nitrogen starvation or induction of the incompatible reaction in a self-incompatible strain. A later paper (Pinan-Lucarré et al., 2005) showed that cell death by incompatibility could be experimentally separated from autophagy in *P. anserina*. Deletion of either the PaATG1 gene, involved in early autophagy, or the PaATG8 gene blocked the formation of autophagosomes but failed to prevent cell death or the accompanying vacuolization that is characteristic of both autophagy and cell death by incompatibility. In fact, cell death by incompatibility was accelerated in the deletion mutants, as if autophagy played a protective role.

As suggested by Pinan-Lucarré et al. (2007), the vacuole probably functions both in cell death by incompatibility and in autophagy. The inside-out destruction of the vacuole could be necessary for the induction of cell death by incompatibility, and intact vacuoles could be needed for autophagy to function in turning over cytoplasmic constituents and shielding neighboring cells from destruction.

THE FUNGAL VACUOLE AS A MODEL ORGANELLE FOR STUDIES OF THE V-ATPASE

V-ATPases

Discovered in the early 1980s, V-ATPases are large multisubunit enzymes found in all eukaryotic cells. They are present on many components of the endomembrane system and on plasma membranes of specialized cells (Forgac, 2007; Kane, 2006). They have cellular roles in multiple physiological processes, and a number of diseases are associated with their malfunction. V-ATPases were first identified and characterized on vacuolar membranes of fungi and plants. Due to the availability of extensive molecular biology tools and a large number of investigators, *S. cerevisiae* became the premier model organism for investigating the V-ATPase. Later, animal systems became preeminent because of the involvement of V-ATPases in diseases. The filamentous fungus *N. crassa* also served as one of the major model systems for the identification and analysis of the enzyme.

Vacuolar Function and the V-ATPase

The fungal vacuole is an acidic compartment, providing an environment favorable for maturation and activity of hydrolytic enzymes. An electrochemical gradient of protons drives the transport of molecules across the membrane. The V-ATPase in the vacuolar membrane is the driving force for both of these functions. Large enough to be readily seen in an electron micrograph, V-ATPase molecules thickly decorate the vacuolar membrane of *N. crassa* (Fig. 2). *N. crassa* is a favorable model system for study of the V-ATPase because of the ease and economy of growing large numbers of cells, the availability of a procedure for isolating clean vacuolar membranes, and the density of the enzyme on vacuolar membranes.

Structure and Mechanism of the V-ATPase

V-ATPases are composed of two domains that form a ball-and-stalk structure, similar to, but larger than, the related F_OF_1 ATP synthase in membranes of mitochondria, chloroplasts, and bacteria (Forgac, 2007; Kane, 2006; Venzke et al., 2005). The proton-translocating domain of the enzyme, named V_0, contains six types of subunits embedded in the membrane, with a reported stoichiometry of $a_1,c_{4\text{–}5},c'_1,c''_1,d_1,e_1$. ATP is hydrolyzed within the V_1 domain of the enzyme, a roughly globular structure connected to V_0 by at least two stalks. V_1 contains eight subunits with a stoichiometry of $A_3B_3CDE_2FG_2H_{1\text{–}2}$. Like the F-type ATPase, the V-ATPase functions as a rotary motor. In current models, hydrolysis of ATP by the "motor" (primarily the A and B subunits) drives the rotation in the membrane of the "rotor" (subunits D,F,c,c′,c″, and d). The remaining subunits connect and stabilize the complex structure (Fig. 3). One difference between the subunit content of V-ATPases of fungi and higher eukaryotes has been found. Subunit c′ is present in all fungi but not in other organisms (Chavez et al., 2006; Forgac, 2007).

Genes Encoding V-ATPase Subunits

The first published sequences for subunits of the V-ATPase were for *Daucus carota* and *N. crassa* (Bowman et al., 1988a, 1988c; Zimniak et al., 1988). Today it is known that 14 genes encode the 14 subunits of the V-ATPase in *N. crassa*, making it likely that all the V-ATPases in the cell have the same structure. By contrast, higher eukaryotes express multiple isoforms of some subunits: four for a, two for B, E, H, and d, and three for C and G, making the total number of possible isoform combinations in the enzyme enormous (Forgac, 2007). Most of the isoforms in mammals are expressed in different tissues or in some cases on different membranes within a cell. Even in *S. cerevisiae*, two genes encode one of the subunits, the 100-kDa subunit a of the V_0 sector of the enzyme. The two isoforms localize

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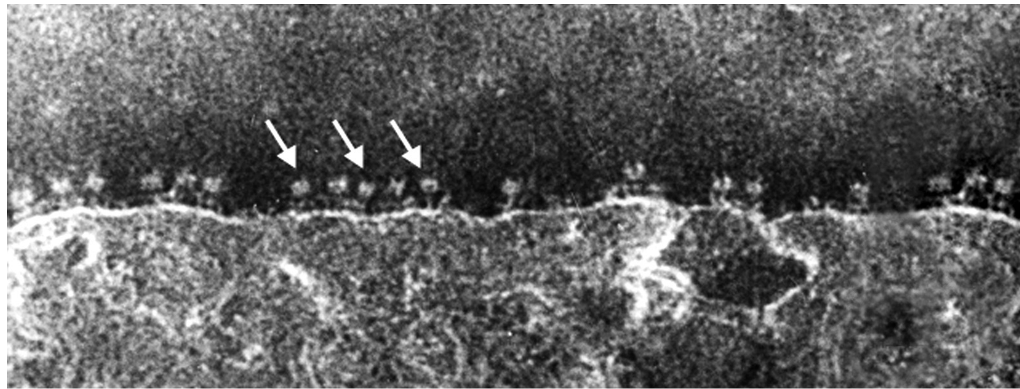

FIGURE 2 Electron micrograph of the V-ATPase in vacuolar membranes. Vacuolar membranes were isolated from *N. crassa*, negatively stained, and examined by transmission electron microscopy as described previously (Dschida and Bowman, 1992). A few of the V-ATPases are indicated by arrows. The globular head is 12 nm wide and is attached to the membrane by a 3-nm-wide stalk.

either to the vacuolar membrane or to the Golgi membrane, and both forms can partially substitute for each other (Manolson et al., 1994).

We have named the genes in *N. crassa* as follows. *vma-1*, *vma-2*, *vma-5*, *vma-8*, *vma-4*, *vma-7*, *vma-10*, and *vma-13* encode the V_1 subunits A, B, C, D, E, F, G, and H, respectively. *Vph-1*, *vma-3*, *vma-11*, *vma-16*, *vma-6*, and *vma-9* encode V_0 subunits a, c, c′, c″, d, and e. The *vma/vph-1* genes have relatively short, multiple introns (two to six introns), which tend to cluster near the ends of the coding region and may be important for high levels of expression (Bowman and Bowman, 2000; Chavez et al., 2006). The genes in *N. crassa* are scattered throughout the genome, raising the question of whether their transcription is regulated in a coordinated manner. This is an interesting point, because variability in numbers of V-ATPase subunits can have severe effects on vacuolar acidification in yeast (Rizzo et al., 2007). Expression levels of all the genes have not been determined, but at least three *vma* genes seem to be transcribed at levels that reflect the relative amount of gene product found in the cell (Wechser and Bowman, 1995). Furthermore, the upstream sequences of four *vma* genes share features with constitutively expressed housekeeping genes (Hunt and Bowman, 1997; Wechser and Bowman, 1995).

Bafilomycin and Concanamycin, Specific Inhibitors of V-ATPases

The most significant contributions from filamentous fungi to the study of V-ATPases resulted from the fortuitous discovery that bafilomycin specifically inhibits V-ATPases of fungi, plants, and animals (Bowman et al., 1988b). The bafilomycins and the related concanamycins are macrolide antibiotics with 16- or 18-membered lactone rings, isolated from *Streptomyces* species (Bowman et al., 1988b; Dröse et al., 1993). Their contribution to the study of V-ATPases is threefold. (i) Sensitivity to these inhibitors was the initial criterion used to identify V-ATPases in multiple organelles and organisms. Currently they are used to investigate numerous cellular processes. (ii) Bafilomycin and concanamycin spawned a new area of drug discovery,

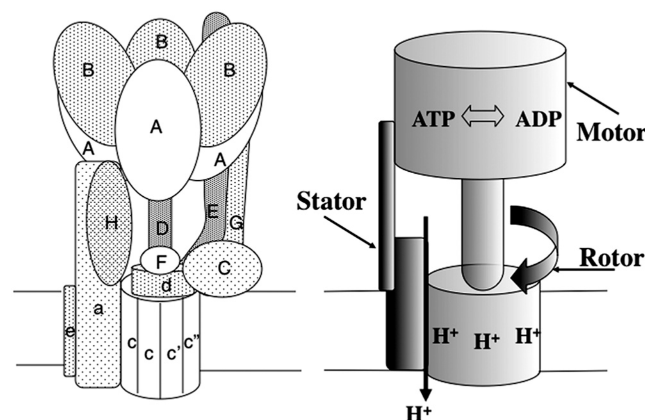

FIGURE 3 Two models of the V-ATPase. As shown on the left, the V-ATPase is composed of 14 different types of subunits, some of which are present in multiple copies. The diagram is modified from the model of the *N. crassa* V-ATPase in Venzke et al. (2005). The model on the right shows the major functional domains. The rotor portion of the enzyme is composed of subunits D, F, d, c, c′, and c″. The A and B subunits form the ATP binding sites and constitute the motor domain. It is not known for certain which subunits form the stator domain.

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in which the major targets were V-ATPases involved in osteoporosis and cancer (Bowman and Bowman, 2005; Boyd et al., 2001). (iii) Analysis of mutants resistant to bafilomycin and concanamycin led to a better understanding of the structure and mechanism of the V-ATPase (Bowman et al., 2006).

For reasons that are not clear, mutant strains with an altered V-ATPase that were resistant to bafilomycin or concanamycin in vivo could only be isolated in filamentous fungi. Attempts to isolate resistant mutants in *S. cerevisiae* failed, as did our first attempts with concanamycin as the selective agent in *N. crassa* (Bowman et al., 1997). Selection with bafilomycin was successful. A hallmark of fungal strains with an inactivated V-ATPase gene is the inability to grow at alkaline pH (Nelson and Nelson, 1990). Similarly, wild-type strains cannot grow on medium buffered to pH 7.5 and containing bafilomycin or concanamycin (0.2 to 1.0 μM). We mutagenized *N. crassa* conidia with UV light and selected for strains that were able to grow on medium buffered to pH 7.5 and containing bafilomycin. While a number of conidia germinated, only the strong growers showing wild-type hyphal morphology proved to contain an altered V-ATPase gene (Bowman and Bowman, 2002). In vitro analyses of V-ATPase activity and isolation of the mutated gene confirmed these were V-ATPase mutants: the K_i for inhibition of the enzyme by bafilomycin was increased, and *vma-3*, the gene encoding the proteolipid subunit c in the V_0 domain, was mutated. We named the strains bfr (bafilomycin resistant) strains.

Because bafilomycin and concanamycin have similar structures, we expected the V-ATPases in the bfr strains to be resistant to concanamycin. They were not. However, by subjecting bfr strains to UV mutagenesis and selecting for strains growing at pH 7.5 in the presence of concanamycin, we isolated ccr strains (concanamycin resistant) that were resistant to both inhibitors in vivo and in vitro. The ccr strains all had additional mutations in the *vma-3* gene (Bowman et al., 2004). Altogether we have isolated 55 independent bafilomycin/concanamycin-resistant strains that have amino acid changes at 12 different sites, all in the c subunit of the V-ATPase. Site-directed mutagenesis targeted another 12 residues (Bowman et al., 2006). The increase in resistance to bafilomycin among all the mutants ranged from 2-fold to 450-fold; the increase in resistance to concanamycin ranged from no effect to 97-fold. We hypothesize that both inhibitors bind the same region of subunit c but that concanamycin, which is slightly larger, may have more contact points and bind more tightly.

One goal of the mutagenesis studies was to identify the binding site(s) of bafilomycin and concanamycin on the V-ATPase to determine how the inhibitors act. Subunit c is a highly conserved hydrophobic protein with four predicted membrane helices, an evolutionary duplicate of the two-helix subunit c in F-type ATPases (Mandel et al., 1988). We constructed a model of the arrangement of the V-ATPase helices based on the crystal structure of the c-subunit ring of the F-type ATPase (Stock et al., 1999). Most of the residues implicated in binding bafilomycin/concanamycin clustered together in this model. Positions of the residues supported a model in which the drug-binding site is a pocket formed by helices 1, 2, and 4. We hypothesized that the drugs inhibit by preventing the rotation of the c subunits in the V-ATPase rotor mechanism (Bowman et al., 2004).

We also hoped to generate a good model for the structure of c subunits of the V-ATPase. Our modeling efforts were greatly facilitated by the publication of a crystal structure for the homologous subunits of the V-ATPase in the bacterium *Enterococcus hirae* (Murata et al., 2005). The new model shows the positions of the residues that confer resistance to bafilomycin and concanamycin in a 10-membered subunit c ring. Ten of the 11 mutation sites that confer the highest degree of resistance are closely clustered. They form a putative drug-binding pocket at the interface between helices 1 and 2 on one c subunit and helix 4 of the adjacent c subunit. The excellent fit of the *N. crassa* sequence to the *E. hirae* structure and the degree to which the structural model predicted the clustering of these residues suggest that the folding of the eukaryotic polypeptides is very similar to that of the bacterial protein (Bowman et al., 2006).

Phenotype of V-ATPase Null Mutants

Inactivation of *vma* genes has a myriad of effects on vacuolar function, growth rate, and development. After publishing our conclusion that *vma-1* was an essential gene (Ferea and Bowman, 1996), we stumbled upon a null strain by letting cultures growing from spores in liquid medium sit on the benchtop for 2 weeks. One tube showed weak mycelial growth in the lower half of the medium. (*N. crassa* is a fast-growing fungus. Wild-type strains reach the surface overnight and produce abundant conidia within 4 days.) The rescued mycelium proved to be an authentic *vma* null strain (Bowman et al., 2000). generated by the process of repeat-induced point mutation (see chapter 11 in this volume). AQ6

Disruption or replacement of *vma* genes encoding different V-ATPase subunits gives almost identical phenotypes (Bowman et al., 2000; Chavez et al., 2006). Many functions attributed to vacuoles are lost. Vacuoles contribute to pH homeostasis, and *vma* null strains grow poorly in acidic medium (the optimum pH is 5.5 to 5.8), weakly in more acidic medium, and not at all in basic medium (pH 7.2 and above). Vacuoles store large amounts of basic amino acids; *vma* mutant cells have one-fifth the amount of acid-soluble arginine found in wild-type cells. Vacuoles store potentially toxic divalent cations; the presence of 4 mM Zn^{2+} in the medium inhibits growth of *vma* null strains by 85% but does not affect the wild type. As mentioned above, the finding that Ca^{2+} in the medium is less toxic to V-ATPase mutants in *N. crassa* (50% inhibition by 100 mM Ca^{2+} versus 13% inhibition of wild-type growth) than to mutants in *S. cerevisiae* needs to be explained. Vacuoles have a role in osmotic homeostasis, and vacuoles do not pellet as dense organelles in *vma* mutants, apparently having lost the capacity to behave like osmometers. The phenotypes of *vma* null strains provide strong evidence for the involvement of vacuoles in these various processes.

The most severe consequences of inactivating the V-ATPase are on growth and development. The primary effect appears to be on the control of tip elongation and morphology. We measured growth as the increase in colony diameter on an agar surface. The *vma-1* (encodes catalytic subunit A) null strain grew at 0.5 mm/h, eight times slower than the wild type (4.2 mm/h). In the slowly advancing mutant strain, branching is primarily dichotomous and frequent, resulting in a crowded growth front that often looks layered. Aerial hyphae are short and rare. By comparison, the wild type grows mainly by apical extension, producing side branches at less frequent intervals, and forms aerial hyphae that result in a cottony appearance. *vma* null strains cannot differentiate conidia (asexual spores) or form perithecia (female fruiting bodies). However, they can act as a male and contribute a nucleus in a fertile sexual cross.

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Fewer than 1% of the spores carrying a *vma* null mutation germinate. We constructed heterokaryons between *vma-1* null mutants of opposite mating types and a helper strain. The heterokaryons grew normally, indicating that the *vma* mutation is recessive. The helper strain contributes to perithecial development but not to ascus and ascospore development. Crossing two *vma* null strains to each other resulted in arrest of ascus development in meiotic prophase (Bowman et al., 2000). The V-ATPase clearly has a prominent and essential role in morphogenesis and development of *N. crassa*. However, little is known about the mechanisms that the vacuole employs to mediate these functions in filamentous fungi.

We have found some interesting differences in the phenotypes of different *vma* mutants. For example, two mutant alleles of *vma-1* (which encodes the A subunit) were generated by repeat-induced point mutation. One of these, named *pvn1*, has a stop codon at amino acid residue 130 (of a total of 607) and is unlikely to make a polypeptide, while the second, *pvn2*, has only four amino acid changes and produces a membrane-bound protein detectable on a Western blot (E. J. Bowman, unpublished results). No V-ATPase activity was detected in membrane fractions from either strain, and both had the phenotype of the *vma* null mutants described above for almost all characteristics (Bowman et al., 2000). However, it was surprising that growth on 1 M sorbitol induced conidiation in the *pvn2* mutant. Although not detectable in vitro, perhaps a very low level of V-ATPase activity was present and accounted for the ability to conidiate. We speculated that sorbitol could desiccate the culture and induce a stress response, which might include upregulating expression of V-ATPase genes. More unexpected was the difference in ascospore viability; *pvn-2* spores germinated as well as the wild type. This could result from a low level of V-ATPase activity, or because the presence of the protein product, even though inactive, is required for spore germination.

vma-11 mutant strains, which are either defective in, or completely lacking, the c′ subunit, have a less severe *vma* null phenotype (Chavez et al., 2006). These strains grow slightly better than the other *vma* null strains. The "extra" growth is inhibited by concanamycin, suggesting that some V-ATPase activity is present. Subunit c′ occurs in V-ATPases of fungi but not other organisms. Perhaps even in fungi it is not completely essential or can be replaced by an extra copy of the closely related c subunit. Like *pvn-2*, the *vma-11* mutants produce conidia when grown on 1.5 M sorbitol. However, *vma-11* strains with nonsense mutations or with complete deletion of the gene exhibit very low levels of ascospore germination (Chavez et al., 2006). A functional V_0 rotor domain (an assembled cluster of c, c′, and c″ subunits) may be critical for ascospore germination. *vma-11* and *vma-16* mutant strains produce few viable ascospores, and despite extensive efforts, a viable spore with an inactivated *vma-3* gene has never been recovered.

Role of the V-ATPase in Vacuolar Morphogenesis

Inactivation of a V-ATPase gene changes the structure of the vacuolar system in *N. crassa*. It was reported that vacuoles in a *VMA* null strain of *S. cerevisiae* are normal (Yamashiro et al., 1990). This was not the case in the *vma-1* null strain of *N. crassa*. Compared with wild-type vacuoles, which are spherical, the vacuoles in the mutant were irregular, often misshapen, and frequently multilamellar (Bowman et al., 2000). This is interesting in view of fascinating recent studies involving *S. cerevisiae*, examining the role of the V-ATPase in regulating vacuolar fusion and fission (Baars et al., 2007). The results from Andreas Mayer's lab show that homotypic fusion of vacuoles requires the physical presence of the V-ATPase (at least the V_0 part) but not H^+-translocating activity. Fission, however, does require V-ATPase pump activity. The investigators propose that vacuolar morphology results from an equilibrium of competing fission and fusion reactions, with the ratio of the rates determining the outcome. Vacuolar morphology was visualized with FM4-64 in deletion strains lacking Vma1p, Vma3p, and Vma6p (Baars et al., 2007). They contained one enlarged vacuole/cell, suggesting that fusion was dominant. By analogy, we suggest that similar processes determine the structure of vacuoles in the *vma-1* null strain of *N. crassa*.

V-ATPase and Autophagy

In *S. cerevisiae*, the V-ATPase plays an essential role in autophagy (Reggiori and Klionsky, 2002). The breakdown of engulfed material by vacuoles requires normal acidification of the vacuole, both to provide the acid pH that is optimal for degradative enzymes and to facilitate the autocatalytic cleavage and activation of proteinases A and B. These proteases initiate a proteolytic cascade that leads to the activation of most vacuolar hydrolases. The V-ATPase achieves the acidification. The contribution of the V-ATPase to autophagy in filamentous fungi has not yet been investigated. Such studies will indicate whether the vacuole plays a conserved function in regulating autophagy in yeasts and filamentous fungal species.

SUMMARY

The former image of the vacuole as the junkyard of the cell in filamentous fungi has been replaced by a picture of a constantly changing, multifunctional meshwork of vesicles and tubules. The vacuolar system is highly variable in appearance at different locations within the mycelium and in response to different growth conditions. We know almost nothing about what controls and regulates the structure of the vacuole. Is it as functionally compartmented as it is structurally diverse? Although we have a good idea of what the vacuolar contents are, we are comparatively ignorant as to how they get there, how they are stored, how they are used, and how they are moved about. The nature of tip growth necessitates the transport of materials over long distances in the mycelium; however, the role of vacuoles in polarized growth has been largely ignored. Our understanding of autophagy in filamentous fungi is primitive, yet we suspect that it plays a critical role in recycling resources to support tip growth.

Several labs are introducing new approaches to study the dynamic behavior of the vacuolar system of filamentous fungi. One example is the work by Darrah et al. (2006), in which fluorescence recovery after photobleaching and mathematical modeling are combined to determine the connectivity of membranous compartments in vivo. Other methods are being developed by Watkinson et al. (2005). Using a combination of photon counting scintillation imaging and stable-isotope nuclear magnetic resonance, this group's goals are to observe how a transport system develops and is altered and to follow nitrogen in a mycelial network growing in a "realistic soil."

Molecular approaches similar to that pioneered by the Kitamoto group to take advantage of genome sequence data from filamentous fungi should facilitate rapid progress in understanding vacuolar structure and function. Homologs of *S. cerevisiae* genes with known functions in vacuolar biogenesis, autophagy, and ion transport are excellent candidates for

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identifying the genes involved in these processes in filamentous fungi. Genetic studies of strains with selected genes deleted either singly or in combination with others will allow us to evaluate their roles in the cell. Confocal laser scanning microscopy of fusion proteins with GFP and RFP or other tags will reveal the cellular locations of these proteins. We can anticipate finding novel adaptations in how filamentous fungi use these genes and their products to support their unique lifestyle.

One of the most fascinating questions about the vacuolar system is whether it is structurally and functionally compartmentalized. Are endocytic and lysosomal functions in separate vesicles or membrane segments? Are calcium and arginine enclosed together or in specialized vacuoles? Do forward transport and retrograde transport occur simultaneously in the same tubule? Are the three vacuolar calcium transporters in the same or different regions of the vacuolar complex? Can autophagosomes fuse with any region of the vacuolar system? Methods used by Valkonen et al. (2007) might be applied to such questions. Using fluorescence lifetime imaging microscopy in combination with Foerster resonance energy transfer, these authors were able to demonstrate for the first time the spatial and functional separation of two SNARE proteins on the plasma membrane in a filamentous fungus.

In addition to being intriguing for their contribution to unique growth habits, the vacuoles of filamentous fungi have been valuable as model systems, especially in two areas. First is their role in arginine metabolism: the compartmentalized storage, the regulation of cytosolic concentrations, and the mobilization in times of need (chapter 27). Second is their contribution to the discovery of V-ATPases and to studies of the structure and function of this major class of enzymes. The investigations performed in recent years demonstrate that the vacuole is a dynamic organelle with many important roles in metabolism, growth, and development. Although it is the most visible organelle in fungal hyphae, the vacuole has not yet received the attention it deserves.

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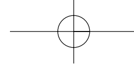

Author Queries

QU1: Please indicate where the following references should be cited: Kugo et al., 2008; Schadeck et al., 2003; Shoji et al., 2006b.
QU2: Is "bead beater" okay (as a generic term), or do you mean "BeadBeater" (trade name, BioSpec Products)?
QU3: Some chapter numbers have changed. Chapter 27 is now Kubicek et al., "Plant Cell Wall and Chitin Degradation." If not as meant, please consult Dr. Borkovich.
QU4: Sentence "PolyPi was once thought to occur in the form of granules in living cells, but the picture changed with improvements..." okay as edited? (To clarify what was "once thought to occur.")
QU5: Chapter 23 is now Davis and Wong, "Nitrogen Metabolism in Filamentous Fungi." See Query 3.
QU6: Chapter 11 is now Aramayo and Pratt, "Meiotic trans-Sensing and Silencing in Neurospora." See Query 3.
QU7: See Query 3 for chapter 27.