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CELLULAR ROLE OF THE V-ATPase IN *NEUROSPORA CRASSA*: ANALYSIS OF MUTANTS RESISTANT TO CONCANAMYCIN OR LACKING THE CATALYTIC SUBUNIT A

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Summary

Vacuolar ATPases (V-ATPases) are large complex enzymes that are structural and mechanistic relatives of F_1F_o-ATPases. They hydrolyze ATP and pump protons across membranes to hyperpolarize membranes and, often, to acidify cellular compartments. The proton gradients generated are used to drive the movement of various compounds across membranes. V-ATPases are found in membranes of archaebacteria and some eubacteria, in various components of the endomembrane system of all eukaryotes and in the plasma membranes of many specialized eukaryotic cells. They have been implicated in a wide variety of cellular processes and are associated with several diseases. Bafilomycin and concanamycin, specific inhibitors of V-ATPases, have been instrumental in implicating the V-ATPase in many of these roles. To understand further the mechanism of inhibition by these antibiotics and the physiological role of the enzyme in the cell, we have isolated mutants of the filamentous fungus *Neurospora crassa* that are resistant to concanamycin. Concanamycin has a dramatic effect on hyphal morphology at acid pH and is lethal at basic pH. In the resistant mutants, the cells can germinate and grow, although abnormally, in basic medium. Thus far, none of the mutants we have characterized is mutated in a gene encoding a subunit of the V-ATPase. Instead, the largest class of mutants is mutated in the gene encoding the plasma-membrane H^+-ATPase. Mutations in at least four uncharacterized genes can also confer resistance. Inactivation of the V-ATPase by disruption of *vma-1*, which encodes the catalytic subunit (A) of the enzyme, causes a much more severe phenotype than inhibition by concanamycin. A strain lacking *vma-1* is seriously impaired in rate of growth, differentiation and capacity to produce viable spores. It is also completely resistant to concanamycin, indicating that the inhibitory effects of concanamycin *in vivo* are due to inhibition of the V-ATPase. How the multiplicity of ATPases within a cell is regulated and how their activity is integrated with other metabolic reactions is poorly understood. Mutant analysis should help unravel this puzzle.

Key words: V-ATPase, vacuolar ATPase, P-ATPase, concanamycin, subunit A, *Neurospora crassa*, vacuole, fungus.

Introduction

Vacuolar ATPases (V-ATPases) are large complex enzymes that are structural and mechanistic relatives of F_1F_o-ATPases (for reviews, see Bowman and Bowman, 1996; Dow, 1999; Finbow and Harrison, 1997; Forgac, 1999; Graham and Stevens, 1999; Kakinuma et al., 1999; Kane, 1999; Margolles-Clark et al., 1999; Müller et al., 1999; Nelson and Harvey, 1999; Stevens and Forgac, 1997; Wieczorek et al., 1999). They hydrolyze ATP and pump protons across membranes to hyperpolarize membranes and, often, to acidify cellular compartments. The transport of various compounds across membranes is often coupled to the electrochemical gradients generated by the V-ATPases. V-ATPases have been described in components of the endomembrane system of eukaryotes, in the plasma membranes of many specialized eukaryotic cells and in the cell membranes of archaebacteria and some eubacteria. Experiments with bafilomycin and concanamycin, specific inhibitors of V-ATPases, have shown that V-ATPases are involved in a wide range of cellular processes and are associated with several diseases (for a review, see Dröse and Altendorf, 1997).

Our laboratory has investigated the structure and function of the V-ATPase found on vacuolar membranes of the filamentous fungus *Neurospora crassa*. The subunit composition and electron microscopic image of this enzyme are essentially identical to those described for other eukaryotic V-ATPases. The enzyme behaves like other V-ATPases in its response to inhibitors and appears to be regulated by similar mechanisms. We believe that *N. crassa* has proved to be especially suitable for certain investigations. Because the vacuolar membranes are thickly studded with V-ATPases and

Table 1. *Subunits and genes of the* Neurospora crassa *V-ATPase*

Subunit name		Gene name	Chromosome location	Number of introns	Molecular mass (kDa)	F-ATPase homolog	Reference
V_1	A	*vma-1*	VR	6	67	β	Bowman et al. (1988c)
	B	*vma-2*	IIR	5	57	α	Bowman et al. (1988a)
	H	*vma-13*	?	?	50		J. C. Reidling and B. J. Bowman (unpublished results)
	C	*vma-5*	?	?	(47)		J. C. Reidling and B. J. Bowman (unpublished results)
	D	*vma-8*	IVR	3	28	γ?	Margolles-Clark et al. (1999)
	E	*vma-4*	IL	2	26	γ?	Bowman et al. (1995)
	F	*vma-7*	IVR	3	13	δ?	Margolles-Clark et al. (1999)
	G	*vma-10*	IIR	5	13	b	Hunt and Bowman (1997)
V_o	a	*vph-1*	IIL	4	98	a	GenBank U36396
	d	*vma-6*	IIR	3	41		Melnik and Bowman (1996)
	c	*vma-3*	VR	4	16	c	Sista et al. (1994)
	c′	*vma-11*	IR	?	16	c	J. I. Pounder and B. J. Bowman (unpublished results)
	c″	*vma-16*	not identified	?	?	c	–

Subunit and gene names are based on the nomenclature of Stevens and Forgac (1997) for the V-ATPase of *Saccharomyces cerevisiae*.
R and L refer to the right and left arms of the chromosomes.
Molecular masses are typically derived from sequences of genes and cDNAs.
For subunits C, H and c′, only cDNA sequences are available.
The molecular mass of subunit C was estimated by gel electrophoresis.

are amenable to examination by electron microscopy, we could directly compare the size and shape of the V-ATPase with the F_1F_o-ATPase on mitochondrial membranes (Dschida and Bowman, 1992). We have regularly isolated and sequenced both cDNAs and genomic DNAs for genes encoding V-ATPase subunits, allowing us to look for possible regulatory elements at this level (Hunt and Bowman, 1997; Wechser and Bowman, 1995). Fortuitously, the bafilomycins and concanamycins were first identified as specific inhibitors of V-ATPases using *N. crassa* (Bowman et al., 1988b; Dröse et al., 1993). We have isolated mutant strains of *N. crassa* that are resistant to concanamycin (Bowman et al., 1997), as well as mutant strains that lack a subunit of the ATPase (E. J. Bowman and B. J. Bowman, unpublished observations). These mutants have the potential both to answer questions concerning the reaction mechanism of the V-ATPase and to elucidate the function of the enzyme in the cell acting in concert with other metabolic processes.

Subunit composition and electron microscopic structure

Comparison of data from many laboratories suggests that the subunit composition of V-ATPases is remarkably conserved. The highest level of complexity, and probably the most complete set of data, has been reported for *Saccharomyces cerevisiae*. Thirteen different subunits are found in the functional enzyme, eight in the V_1 sector and five in the V_o sector (Nelson and Harvey, 1999; Stevens and Forgac, 1997). In *S. cerevisiae*, the largest subunit of the V-ATPase, subunit a, is encoded by two genes (Manolson et al., 1992, 1994), which may specify organelle-specific isoforms of the enzyme. Isoforms are relatively common for subunits of V-ATPases from more complex organisms, both animals and plants, where they can define tissue-specific or organelle-specific V-ATPases (e.g. Crider et al., 1997; Gogarten et al., 1992; Hernando et al., 1995; Peng et al., 1999; Puopolo et al., 1992). Their functional roles are just beginning to be understood.

The V-ATPase from *N. crassa* also appears to have 13 subunits (Table 1). In our best preparations of purified vacuolar ATPase, we can see gel bands corresponding to all 13 subunits, assuming that the proteolipid subunits (c, c′ and c″) co-migrate. However, the strongest evidence that all 13 subunits are present is based on gene isolation and analogy to the *S. cerevisiae* system. Our report of sequences for genes encoding subunits A (Bowman et al., 1988c) and B (Bowman et al., 1988a) of the V-ATPase from *N. crassa* together with the sequence of subunit A from *Daucus carota* (Zimniak et al., 1988) provided the first evidence that V-ATPases are related to F-ATPases. Since then, we have cloned genes or cDNAs encoding V_1 subunits C, D, E, F, G and H, and V_o subunits a, c, c′ and d (Margolles-Clark et al., 1999). Although we have not yet succeeded in isolating the gene for subunit c″, such a gene has been identified in the genome of a close relative of *N. crassa*, *Magnaporthe grisea*, and is therefore probably present in *N. crassa*. In contrast to the higher eukaryotes, *N. crassa* does not appear to have isoforms for any subunit, including subunit a, which is encoded by two genes in *S. cerevisiae* (Manolson et al., 1992, 1994). Table 1 summarizes the subunit composition of the V-ATPase of *N. crassa*; where possible, the homologous subunit from F-type ATPases is also indicated.

Viewed in the electron microscope, the V-ATPase has a ball-and-stalk structure much like that of the F-ATPase. However, a direct comparison of negatively stained preparations of V-ATPase from vacuolar membranes and F-ATPase from mitochondrial membranes of *N. crassa* showed significant differences in size and shape between the two enzymes (Dschida and Bowman, 1992). The V-ATPase is larger; the V_1 sector is approximately 2 nm wider, and the stalk is apparently 2 nm longer and 0.5 nm wider than in the F_1 sector. The larger mass in V_1 could be accounted for by the larger size of subunit A (67 kDa) compared with its homolog β (56 kDa) and to the inclusion of more subunits in V_1 (eight) than in F_1 (five). The actual composition of the ball-and-stalk regions, however, has not been determined. In our electron microscopic images, we consistently observed extra protrusions originating from the base of the V_1 stalk. Whether these structures are related to the second stalk recently seen by others in both F-ATPases (Wilkens and Capaldi, 1998) and V-ATPases (Boekema et al., 1997) or perhaps represent a unique feature of V-ATPases is not clear at this time.

Genes, introns and expression

We have isolated and characterized genomic DNA encoding nine subunits of the V-ATPase, V_1 subunits A, B, D, E, F and G and V_o subunits a, c and d (Table 1). We have sequenced cDNAs only for subunits C, H and c′. All the genes have relatively short, multiple introns (2–6), which tend to be clustered near the ends of the coding region and may be important for high levels of expression of these genes (Bowman et al., 1992). The chromosomal locations of *vma* genes are scattered throughout the genome (Table 1), raising the interesting question of whether transcription of the genes is regulated in a coordinated manner. Consistent with this possibility, the relative amount of mRNA present for *vma-1*, *vma-2* and *vma-3* transcripts was found to be roughly proportional to the copy number of each subunit in the enzyme complex. *vma* mRNA was approximately 10- to 12-fold less abundant than mRNA for genes encoding the α and β subunits of the mitochondrial F-ATPase, which is an extremely abundant enzyme in *N. crassa*. Thus, the ATPase genes seem to be transcribed at levels that approximately reflect the relative amount of gene product found in the cell (Wechser and Bowman, 1995). Examination of the 5′ upstream regions of four of the genes, *vma-1*, *vma-2*, *vma-3* and *vma-10*, suggests that they share features common to constitutively expressed housekeeping genes in higher eukaryotes in this region (Hunt and Bowman, 1997; Wechser and Bowman, 1995). They have multiple initiation sites 100–200 base pairs (bp) upstream of the translation start site, a high GC content just upstream of the transcription initiation site and a GC box, but lack a TATAAAT sequence element. Moreover, these four genes all have three similar sequence elements in similar positions in their upstream sequences, a GGGCG sequence similar to an Sp1 binding site farther upstream, a GGGCCG sequence 200–300 bp from the translation start site and a CCAACCTC element 100–200 bp from the ATG start codon. These features are not found in all *vma* genes; they are absent in *vma-4*, *vma-6* and *vph-1*. Because the first four genes encode subunits that are present in multiple copies in the V-ATPase, and the latter three encode single-copy subunits, we have speculated that the conserved elements may be involved in expression of genes that encode multiple copies in the enzyme (Hunt and Bowman, 1997). When we have all 13 genes ‘in hand’, we would like to pursue further the question of how gene expression might be regulated in a coordinated manner for such a complex enzyme.

With one exception, the cDNAs derived from the V-ATPase genes of *N. crassa* all have rather short 5′ leaders (100–200 bp) and 3′ tails (150–350 bp) and appear to produce one major transcript. The exception is *vma-3*, which had cDNAs terminating at 361, 701 and 794 bp after the stop codon. Northern blot analysis revealed two prominent classes of transcript, differing in size by approximately 200 bp. We demonstrated that this difference was due to heterogeneity at the 3′ end of the transcript (Sista et al., 1994). The function of the heterogeneity is not known.

Regulation of V-ATPase activity

There is convincing experimental evidence that V-ATPases are regulated *in vivo* by the novel mechanism of dissociation and reassociation of the V_1V_o complex. This mechanism occurs as part of the life cycle of the tobacco hornworm *Manduca sexta*: V_1 sectors are released from the membrane in the early molting stages and become reattached after ecdysis (Sumner et al., 1995; Wieczorek et al., 1999). Similarly, in *S. cerevisiae*, starvation for glucose induces a rapid, reversible release of V_1 complexes (Kane, 1995, 1999).

Moriyama and Nelson (1989) first demonstrated the release of the V_1 sector *in vitro* by cold treatment of chromaffin granule membranes. Subsequently, we and others reported that treatment with high concentrations of nitrate and other chaotropic agents inhibited V-ATPase activity and induced dissociation of V_1 from the membrane *in vitro* (Arai et al., 1989; Bowman et al., 1989; Kane et al., 1989; Rea et al., 1987). Further analysis of this phenomenon led us to propose that the mechanism of release involved an oxidation–reduction reaction. In our model, nitrate and other oxidizing agents inhibit the activity of V-ATPases by promoting the formation of a disulfide bond, which is quickly followed by dissociation of the V_1 sector; agents such as sulfite block inhibition by keeping essential cysteine residues reduced (Dschida and Bowman, 1995). These results support proposals from other laboratories that, within the cell, the redox state of the immediate environment may play a role in regulating V-ATPase activity (Feng and Forgac, 1992a,b, 1994; Kibak et al., 1993).

In *N. crassa*, we have found that resuspension of cells in a medium that lacks sugar causes the rapid release of the V_1 sector (J. C. Reidling and B. J. Bowman, unpublished results). Our results are similar to those obtained with *S. cerevisiae* (Kane, 1995). A significant fraction of the V-ATPase

dissociates within 2 min, but another fraction fails to dissociate even after 1–2 h in sugar-free medium. After dissociation, the peripheral subunits of the enzyme appear to stay together in a complex. The cytosolic fraction obtained from these cells may be a rich source of the V_1 sector of the ATPase, suitable for purification and possibly for crystallization. This experimental approach is currently being pursued with the V_1 sector of the *M. sexta* V-ATPase (Svergun et al., 1998; Wieczorek et al., 1999).

Concanamycin-resistant mutants

In 1988, we and our collaborators discovered that the macrolide antibiotic bafilomycin A_1 was a highly potent and specific inhibitor of the V-type ATPase (Bowman et al., 1988b). Later, the concanamycins, a related family of molecules, were found to be even more potent inhibitors (Dröse et al., 1993). These antibiotics have proved to be enormously useful for investigating the role of V-type ATPases in many systems both *in vitro* and *in vivo* (for an excellent review, see Dröse and Altendorf, 1997).

In *N. crassa*, concanamycin completely inhibits the activity of the V-ATPase *in vitro* at a concentration of 10 nmol l^{-1} and has no effect on either the mitochondrial F-ATPase or the plasma membrane H^+-ATPase at concentrations up to 10 μmol l^{-1} (Bowman et al., 1997). In normal growth medium (pH 5.8), concanamycin does not kill *N. crassa* cells, but it does slow growth and cause significant changes in cell morphology (see Figs 1, 2). The cells cannot grow in alkaline medium (pH 7.2) containing 0.2 μmol l^{-1} concanamycin. With these characteristics in mind, we set out to select mutant strains of *N. crassa* that were resistant to concanamycin. We hoped the mutants would be altered in genes encoding subunits of the V-ATPase and would prove valuable in uncovering both the role of the V-ATPase in the cell and the mechanism by which concanamycin inhibits V-ATPase.

We isolated 94 mutant strains, designated ccr for concanamycin-resistant, which could grow in alkaline medium with 0.2 μmol l^{-1} (or 1.0 μmol l^{-1}) concanamycin A (Bowman et al., 1997). To our surprise, the largest class of strains (77 isolates) had mutations in the gene encoding the plasma membrane H^+-ATPase, *pma-1*, which is a 'P-type' ATPase. Our analysis supported the conclusion that the mutations did not affect a direct interaction between concanamycin and the plasma membrane H^+-ATPase. The V-ATPase was indeed inhibited by concanamycin in the mutant cells. The vacuoles were not acidified, as shown by the lack of fluorescence in the presence of chloroquine, and they were unable to accumulate large concentrations of arginine, both processes dependent on the activity of the V-ATPase. However, the mutations caused a specific type of change in the plasma membrane H^+-ATPase, which suppressed the toxic effects of concanamycin in the cell. We analyzed seven mutated *pma-1* alleles. All seven exhibited an intriguing change in the kinetic behavior of the enzyme. The wild-type *N. crassa* plasma membrane H^+-ATPase (like the *S. cerevisiae* enzyme; Koland and Hammes, 1986) shows a strongly sigmoidal dependence of ATPase activity on substrate concentration, with a K_m for ATP of 1.2 mmol l^{-1} MgATP. The mutated strains exhibited simple Michaelis–Menten kinetic behavior, and the K_m was lower, 0.18–0.60 mmol l^{-1} MgATP. We have suggested that the mutants are resistant to concanamycin because the mutant plasma membrane H^+-ATPase can use low levels of ATP more efficiently than the wild-type enzyme. Thus, substances such as Ca^{2+} or lysine, that are released from the vacuole into the cytosol when the vacuolar ATPase is inhibited by concanamycin, can be pumped out of the cell and prevented from attaining toxic concentrations in the cytosol.

We thought the *pma-1* mutants might help resolve an old controversy regarding P-type ATPases. Considerable biochemical and genetic data suggest that these enzymes function as dimers. Indeed, the kinetic behavior of the *N. crassa* enzyme is best fitted by a model with two interacting active sites, both of which must be filled for catalysis to occur (Bowman, 1983). However, some experiments in *N. crassa* and other organisms have indicated that the isolated enzyme can function as a monomer (Craig, 1982; Goormaghtigh et al., 1986; Moller et al., 1980). We reasoned that the changes in the kinetic behavior of the mutant H^+-ATPase were consistent with its functioning as a monomer. As a test of this hypothesis, we subjected wild-type and mutant enzymes to radiation inactivation with a ^{60}Co source to determine their functional sizes. Both enzymes responded identically, yielding an estimated functional size of approximately 200 kDa (E. J. Bowman and B. J. Bowman, unpublished results), a dimer by this test and thus not supporting our hypothesis.

We were surprised, and disappointed, that none of the ccr mutations mapped to a known structural gene for the V-ATPase (E. J. Bowman, unpublished results). (We have mapped ten of the expected 13 genes; Table 1.) Among the 17 strains that are not *pma-1* mutants, six mapped to linkage group I-left, one to linkage group I-far left, two to linkage group V-right, and eight to other regions in the genome. Several of the mutants demonstrated properties that might be indicative of a V-ATPase malfunction, such as slow growth, sensitivity to histidine in the medium or sensitivity to high pH. Vacuolar ATPase isolated from these mutants had the same K_i for concanamycin as the wild-type enzyme, with one exception. In the exception, *ccr66-9*, the K_i for concanamycin was reproducibly two- to threefold higher. This mutation mapped close to, but not at, *vma-3*.

We suspect that the mutant strains obtained thus far all act by partially suppressing the effects of concanamycin. Other laboratories have suggested that concanamycin binds to the 98 kDa subunit a (Zhang et al., 1994) or to the proteolipid subunit c (Rautiala et al., 1993). The binding site might also be complex, making it difficult to confer resistance by changing a single residue. Such a conclusion is premature for the V-ATPase, however, and we plan to select and characterize more concanamycin-resistant mutants in the future. Having shown that *vma* deletion strains of *N. crassa* are completely resistant to concanamycin (see below), we believe that further

mutant analysis has a high probability of producing strains with altered V-ATPase genes. We also look forward to surprises, such as the *pma-1* mutants, which will lead us to investigations of the complex interactions between the V-ATPase and other proteins in the cell.

Inactivation of the gene encoding subunit A

The effect of deleting a gene encoding a V-ATPase subunit has been extensively studied in only one organism, *S. cerevisiae*. Such deletion strains grow more slowly than wild-type strains in rich medium and cannot grow in medium buffered to pH 7.5. They are particularly sensitive to high concentrations of Ca^{2+}, as well as other ions (e.g. Zn^{2+}, Mn^{2+}, Cu^{2+} and Na^{+}), and require a non-fermentable carbon source (Eide et al., 1993; Nelson and Nelson, 1990; Ohya et al., 1991). (For more complete descriptions, see reviews by Jones et al., 1997; Stevens and Forgac, 1997.) A *VMA1* deletion strain of *Ashbya gossypii* was also recently described (Förster et al., 1999). Although impaired in growth, the deletion strain could grow even at pH 7.5 and appeared to be efficient at excreting vacuolar metabolites into the growth medium. Attempts to make V-ATPase deletion mutants in *Dictyostelium discoideum* were not successful, presumably because the genes are essential for cell viability (Xie et al., 1996). Null mutants of a V-ATPase gene in *Drosophila melanogaster* have been generated and show an early larval lethal phenotype (Davies et al., 1996; Dow, 1999).

We have also reported that the gene encoding *vma-1* (subunit A) is essential in *N. crassa* (Ferea and Bowman, 1996). In that study, we generated a RIP/Rescue strain, which contained an inactivated *vma-1* gene at the endogenous site and a functional second copy at an ectopic site (*N. crassa* is a haploid organism). The endogenous gene was inactivated by RIP (repeat induced point mutations), a novel process in *N. crassa* that scans the genome and introduces multiple point mutations into genes present in duplicate copies (Selker and Garrett, 1988). When the RIP/Rescue strain was crossed with the wild type, the mutated *vma-1* gene behaved like a simple recessive lethal, consistent with the interpretation that the gene was indispensable. Subsequently, in another cross involving the RIP/Rescue strain, we serendipitously isolated a rare, very slowly growing progeny strain, which has proved to be a true *vma-1* null strain (E. J. Bowman, unpublished results). Thus, the gene is not essential in *N. crassa*; however, *vma-1* null ascospores have unusually low viability. Of interest, haploid spores of *S. cerevisiae* with a *VMA* delete gene also exhibit lowered viability (P. Kane, personal communication), and sporulation was never observed in *VMA1* disruptant strains of *A. gossypii* (Förster et al., 1999).

Like *vma* null strains in *S. cerevisiae*, our mutant grows slowly in acidic medium, cannot grow in basic medium and does not grow in medium with high salt concentrations (1 mol l^{-1} NaCl) or in the presence of some metals (Zn^{2+}). Unlike *S. cerevisiae*, the *N. crassa* mutant can grow on a non-fermentable substrate (glycerol), does not require inositol and is not affected by Mn^{2+} or Fe^{2+} in the medium. To our surprise, the mutant is not particularly sensitive to Ca^{2+} in the medium. Pleasingly, the null mutant is completely resistant to concanamycin added to the growth medium (Fig. 1), supporting the hypothesis that the V-ATPase is the only, or at least the primary, target of the inhibitor in the cell. Of particular interest, the null strain shows striking abnormalities in morphology and development (Figs 1, 2). Wild-type *N. crassa* forms a mat of long thread-like cells called hyphae, and the growth of individual hyphae shows strong apical dominance. Some cells grow up into the air (aerial hyphae) and differentiate by pinching off small, round, asexual spores called conidia. In the *vma-1* null strain, the branching pattern of the hyphae is altered, colony morphology on agar plates is altered and differentiation into conidia does not occur. Starvation for carbon or nitrogen induces a sexual cycle in *N. crassa*. The *vma-1* null strain functions normally as a nuclear donor in this process, but only 0.5 % of the haploid spores carrying the *vma-1* null gene germinate and grow. Although not recognized in the *vma* null mutants of *S. cerevisiae*, a temperature-sensitive *VMA4* mutant strain displayed aberrant cellular morphologies (Kane, 1999; Zhang et al., 1998), which might be the functional equivalents of the abnormalities seen in the *N. crassa vma-1* null strain.

Cellular function of the V-ATPase in *Neurospora crassa*

The fungal vacuole is the lysosomal counterpart in these organisms. It contains a variety of hydrolytic enzymes and harbors high concentrations of basic amino acids such as arginine, ornithine and lysine, of the counterion polyphosphate and of critical ions such as Ca^{2+} (Jones et al., 1997). At first viewed as a static storage compartment, the vacuole is now recognized as playing a dynamic role in regulating some metabolic pathways and in contributing to cell homeostasis. The vacuole of *N. crassa* has been best studied for its role in arginine metabolism (Davis, 1986) and its contribution to maintaining low levels of cytosolic Ca^{2+} (Miller et al., 1990). These functions depend on the activity of the V-ATPase, which generates the electrochemical gradient that drives transport of basic amino acids and Ca^{2+} into the vacuole.

Our experiments with inhibition by concanamycin (Bowman et al., 1997) and inactivation of *vma-1* indicate that the V-ATPase also plays a significant role in cell morphogenesis and development. In both cases, the growth of cells is pH-sensitive, and the vacuoles are unable to accumulate arginine. The wild-type pattern of apical dominance in hyphal branching, the defining feature of growth in a filamentous fungus, is disrupted, albeit not in exactly the same way, and growth in shaking liquid culture results in the formation of pellet-like structures. Germination of spores, conidia or ascospores is seriously impaired. Gene inactivation and growth on concanamycin do not produce identical phenotypes (Figs 1, 2). Gene inactivation is the more severe; growth is very slow, disruptions in hyphal morphology are uniform throughout the culture and differentiation into aerial hyphae with conidia cannot occur.

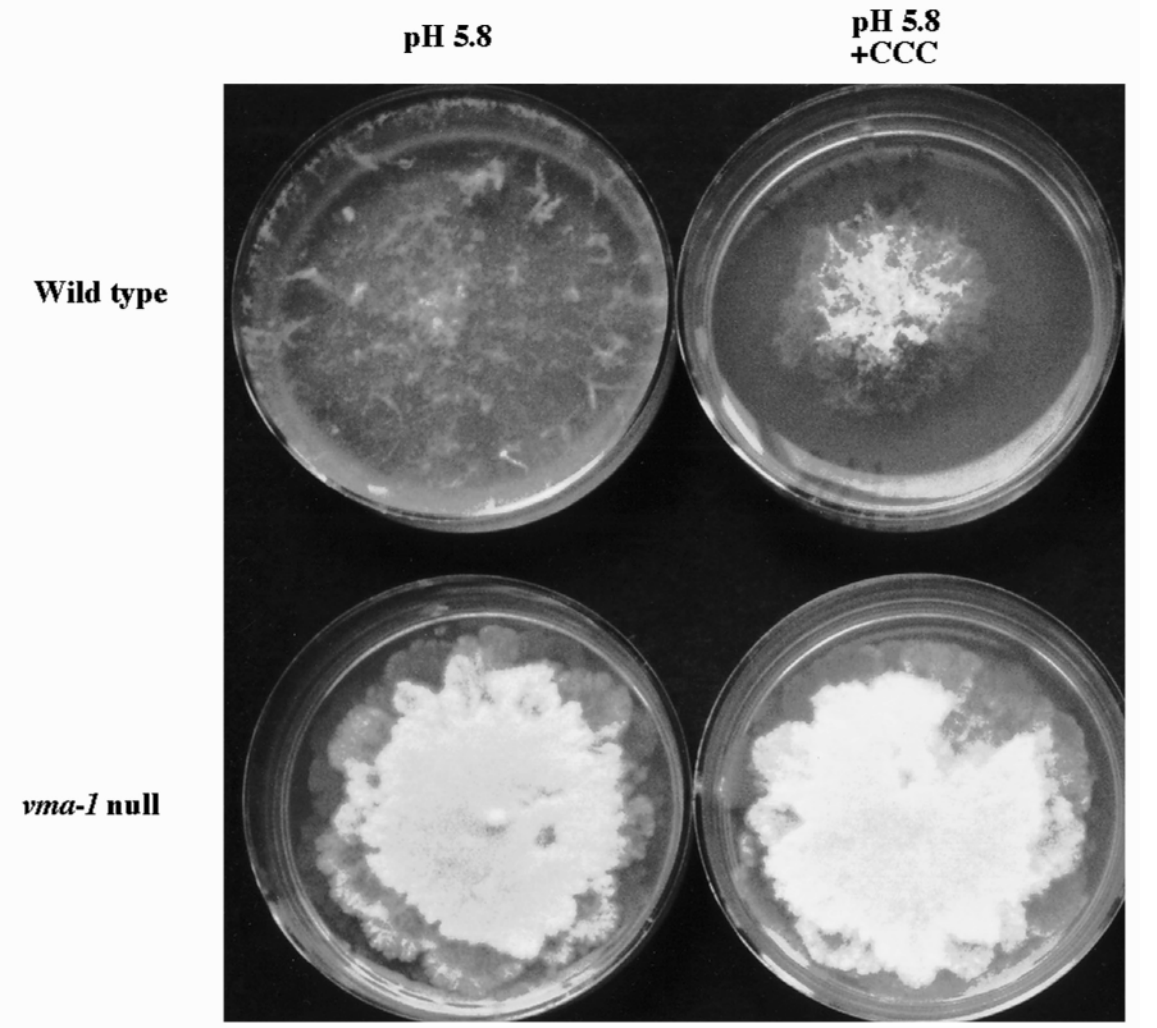

Fig. 1. Comparison of the effects of inhibition with concanamycin *versus* inactivation of *vma-1* on growth of *Neurospora crassa*. Wild-type (74A) and *vma-1* null strains were grown on agar plates containing standard minimal medium at pH 5.8 in the absence or presence of 0.2 μmol l^{-1} concanamycin C (CCC). The wild type was grown for 1 day and the *vma-1* mutant for 4 days at 30 °C.

When the V-ATPase is inhibited by concanamycin on pH 5.8 medium, hyphal morphology is disrupted in an irregular manner, aerial hyphae with conidia can form and the rate of linear growth is not quite so slow. It may be that in the presence of concanamycin the cells maintain a low level of V-ATPase activity or that the cell can adapt in some unknown manner, as reported for *Dictyostelium discoideum* (Temesvari et al., 1996). Alternatively, the complete absence of V-ATPase, one of the most abundant proteins in the vacuolar membrane, might disrupt organellar function more severely than inhibition of activity alone.

A possible explanation for the morphological changes observed is that the V-ATPase is essential for maintaining or generating Ca^{2+} and pH gradients in the cytosol. Although not understood in detail, there is a body of evidence implicating both cytosolic free Ca^{2+} (or Ca^{2+} gradients) and localized pH differences as necessary for normal apical extension or branching in fungi (for a review, see Jackson and Heath, 1993). Sequestration of Ca^{2+} in the vacuole (Miller et al., 1990) and perhaps control of cellular pH depend on the activity of the V-ATPase. Thus, we speculate that inactivation of the V-ATPase by mutation or by inhibition with concanamycin exerts its primary effect on morphology as a result of disruption of cytosolic Ca^{2+} concentrations or pH.

Conclusions and prospects

The V-ATPase of *N. crassa* was one of the first V-type ATPases to be discovered. The first sequences of genes encoding V-ATPase subunits, A from *D. carota* (Zimniak et al., 1988) and A and B from *N. crassa* (Bowman et al., 1988a,c), established the relationship between F- and V-type ATPases, and electron microscopy comparing the two *N. crassa* enzymes was instrumental in suggesting some of their differences (Dschida and Bowman, 1992). The inhibitory effect of bafilomycin on a V-ATPase was discovered with the *N. crassa* enzyme (Bowman et al., 1988b). Most recently, our studies on inhibition and inactivation of the V-ATPase in *N.*

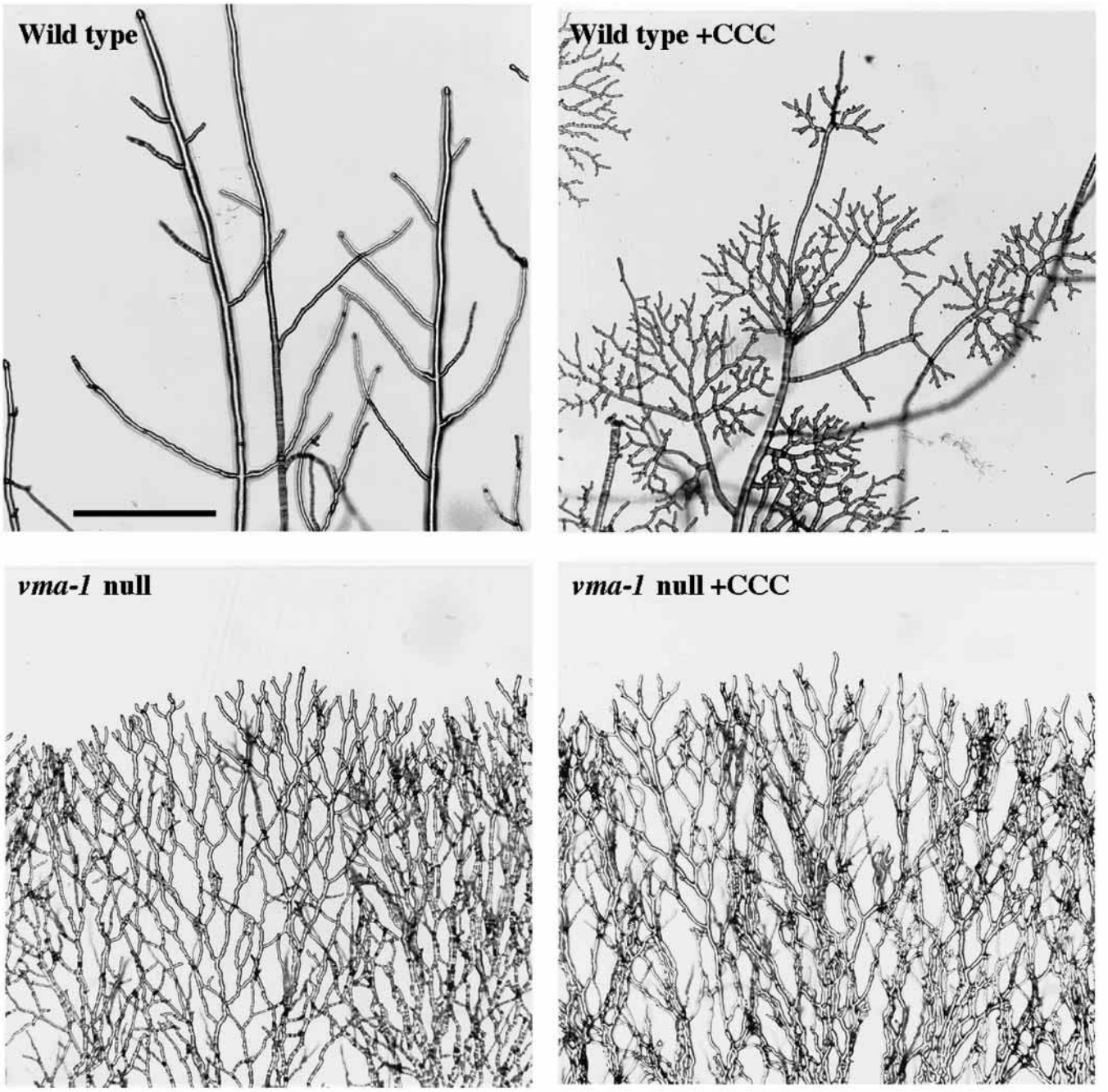

Fig. 2. Comparison of the effects of inhibition with concanamycin *versus* inactivation of *vma-1* on hyphal morphology of *Neurospora crassa*. Wild-type (74A) and *vma-1* null strains were grown on dialysis membrane lying on an agar plate with minimal medium (pH 5.8) in the absence or presence of 0.3 μmol l^{-1} concanamycin C (CCC). The dialysis membranes were moved to a Leitz Aristoplan microscope and camera, where growing fronts of hyphae were photographed with Kodak print film, ASA 100. Scale bar, 100 μm.

crassa have demonstrated an important functional role for this enzyme in determining how cells grow and differentiate (Bowman et al., 1997).

Because cell growth in *N. crassa* exhibits a high degree of morphological differentiation without the multicellular complexity of higher organisms, we believe *N. crassa* will prove valuable for unveiling the role of the V-ATPase in these processes. Mutant strains may provide an approach to probing the role of the enzyme in other cellular processes also. The concanamycin-resistant mutants that are not mutated in a V-ATPase gene partially compensate for the deleterious effects of inhibiting the V-ATPase. For example, mutant forms of the *pma-1* gene can partially suppress the pH-conditional phenotype of *vma* deletion strains, but do not affect the altered morphology (E. J. Bowman, unpublished results). Mutations in other genes may suppress specific aspects of the *vma* deletion

phenotype, allowing us to dissect the role of the V-ATPase in different cellular events.

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