Structure and Distribution of Organelles and Cellular Location of Calcium Transporters in *Neurospora crassa*\(^\text{\textcopyright}\)

Barry J. Bowman,¹* Marija Draskovic,¹ Michael Freitag,² and Emma Jean Bowman¹
Department of Molecular, Cell, and Developmental Biology, University of California; Santa Cruz, California 95064,¹ and Department of Biochemistry and Biophysics, Oregon State University, ALS 2011, Corvallis, Oregon 97331-7305²

Received 18 June 2009/Accepted 25 September 2009

We wanted to examine the cellular locations of four *Neurospora crassa* proteins that transport calcium. However, the structure and distribution of organelles in live hyphae of *N. crassa* have not been comprehensively described. Therefore, we made recombinant genes that generate translational fusions of putative organelar marker proteins with green or red fluorescent protein. We observed putative endoplasmic reticulum proteins, encoded by grp-78 and dpm, in the nuclear envelope and associated membranes. Proteins of the vacuolar membrane, encoded by vam-3 and vma-1, were in an interconnected network of small tubules and vesicles near the hyphal tip, while in more distant regions they were in large and small spherical vacuoles. Mitochondria, visualized with tagged ARG-4, were abundant in all regions of the hyphae. Similarly, we tagged the four *N. crassa* proteins that transport calcium with green or red fluorescent protein to examine their cellular locations.

NCA-1 protein, a homolog of the SERCA-type Ca\(^{2+}\)-ATPase of animal cells, colocalized with the endoplasmic reticulum markers. The NCA-2 and NCA-3 proteins are homologs of Ca\(^{2+}\)-ATPases in the vacuolar membrane in yeast or in the plasma membrane in animal cells. They colocalized with markers in the vacuolar membrane, and they also occurred in the plasma membrane in regions of the hyphae more than 1 mm from the tip. The *cax* gene encodes a Ca\(^{2+}\)/H\(^+\) exchange protein found in vacuoles. As expected, the CAX protein localized to the vacuolar compartment. We observed, approximately 50 to 100 μm from the tip, a few spherical organelles that had high amounts of tagged CAX protein and tagged subunits of the vacuolar ATPase (VMA-1 and VMA-5).

We suggest that this organelle, not described previously in *N. crassa*, may have a role in sequestering calcium.

All cells maintain intracellular concentrations of calcium at precise levels, typically about 0.1 μM in the cytosol. Calcium is often present at high levels in the environment, significantly above the level that is tolerated within the cell. Nevertheless, high concentrations are maintained in some organelles because calcium has an essential role in signaling physiological processes (3, 7, 29). In root hairs, pollen tubes, and the hyphae of filamentous fungi calcium has been postulated to have a role in signaling physiological associated processes (20).

In plant and animal cells, three types of calcium-pumping ATPases have been described (3, 7, 9, 58). The PMCA type (most closely related to the Pmc1p ATPase of *S. cerevisiae*) primarily pumps calcium across the plasma membrane, removing excess calcium from the cytosol. The SERCA type, named by its location in the smooth ER, has a major role in transporting calcium in muscle cells but is also present in the ER in many types of cells. *S. cerevisiae* has no homolog to the SERCA ATPase. The SPCA type (secretory pathway Ca\(^{2+}\)-ATPases) is found in the Golgi bodies and is homologous to the Pmr1p ATPase of *S. cerevisiae* (3, 42). The mitochondria also contain a significant share of intracellular calcium. No calcium-pumping ATPase has been identified in this organelle, and transport has been hypothesized to occur through a channel protein, driven by the same electrochemical gradient that drives the synthesis of ATP (18, 35). In addition, calcium is sequestered in small vesicles and in lysosomalike compartments, presumably transported by Ca\(^{2+}\)/H\(^+\) exchange proteins (29).

The availability of the complete genomes for *N. crassa* and other filamentous fungi has allowed us to assess the number and types of calcium transport proteins in these organisms (62). Focusing on *N. crassa*, we found that all three types of calcium-pumping ATPases are present. These genes had been identified earlier in a PCR-based search for P-type ATPases (2). The *N. crassa* gene *nca-1* encodes a SERCA type ATPase.

* Corresponding author. Mailing address: Department of Molecular, Cell, and Developmental Biology, University of California, Santa Cruz, CA 95064. Phone: (831) 459-2245. Fax: (831) 459-3139. E-mail: bowman@biology.ucsc.edu.

¹ Published ahead of print on 2 October 2009.
The nca-2 and nca-3 genes are closely related to each other and appear to encode PMCA type ATPases. The pmr gene is a SPCA type. We also identified the nca gene as a homolog of \textit{VXI}, the gene encoding the Ca\textsuperscript{2+}/H\textsuperscript{+} exchanger that plays a key role in vacuolar transport in \textit{S. cerevisiae}.

Our long-term goal is to use these five genes (nca-1, nca-2, nca-3, pmr, and cax) to find out where calcium is localized in cells and how it gets there. We first wanted to determine the intracellular location of each transporter, using proteins tagged with green and red fluorescent proteins (GFP and RFP, respectively) that these change with distance from the hyphal tip.

Our transformation of \textit{N. crassa} and most other filamentous fungi is a comprehensive analysis of the structure and distribution of organelles in living cells is lacking. GFP and fluorescent dyes have been used successfully to examine nuclei and mitochondria in living cells. In Studies parallel to those reported here, we have fused GFP and RFP to proteins predicted to be localized to nuclei, mitochondria, the ER, the Golgi body, and the vacuole. Similarly, we have made GFP-tdimer2(12) and NCA-2-GFP and NCA-3-GFP also showed some cross-reaction with a band of antibody to GFP (Anti-GFP; Roche Applied Science, catalog no. 11 814 460 001). We attempted to do Western blots with the RFP fusions using two different poly-

### MATERIALS AND METHODS

#### Construction of plasmids.

We used plasmid pMF272 to generate proteins with cGFP fused to the C terminus and plasmid pMF334 to generate proteins with tdimer2(12), a variant of dsRED, fused to the N terminus (25–27). To simplify nomenclature, we use "RFP" here when referring to the \textit{tdimer2}(12) gene (6) or "RFP" when we refer to the protein produced.

Fragments of genomic DNA containing the genes of interest were amplified by PCR from \textit{N. crassa} strain 74A (FGCS987), using the \textit{Pfu} Turbo DNA polymerase from Stratagene (now sold by Agilent Technologies). The primers, shown in Table 1, contained restriction endonuclease sites to facilitate cloning into the pMF272 and pMF334 plasmids. Amplified DNA from the PCR was initially ligated into the vector gATE1 (Pamela Life Sciences) for screening clones containing PCR products with blunt ends. The pGEl1-2 plasmid containing the insert was digested with the two restriction endonucleases that recognize the sites in the primers. In this way, a DNA fragment of the correct size was obtained with a high likelihood that each end had been cut with the correct enzyme. This fragment was ligated with pMF272 or pMF334 digested with the same pair of restriction endonucleases. All new \textit{his} genes were transferred to tubes with Vogel's minimal medium. These isolates were examined by laser scanning confocal microscopy (see below). For some transformations, none of the transformed isolates gave a red or green signal that was clearly above the background fluorescence. However, for most strains 50 to 100% gave a strong signal. Because \textit{N. crassa} conidia used for transformation are typically multinucleate, primary transformants can be heterokaryons. We obtained homokaryons for each strain by isolating microconidia (21). For each of the strains described in this report we obtained two or three independently isolated transformants that were indistinguishable from each other when observed by laser-scanning confocal microscopy. The transformed strains of \textit{N. crassa} are available from the Fungal Genetics Stock Center.

To verify that the GFP signal came from translational fusions of proteins, we performed Western analysis (data not shown), essentially using the procedure of Garcia et al. (28). Cell extracts were prepared by using phenylmethylsulfonyl fluoride (1 mM) and chymostatin (2 \mu g/ml) as protease inhibitors. Using an antibody to GFP (Anti-GFP; Roche Applied Science, catalog no. 11 814 460 001), we observed a band of the predicted size for all of the GFP fusion proteins. NCA-2-GFP and NCA-3-GFP also showed some cross-reaction with a band of approximately 27 kDa, the size of GFP itself. As shown in Results, NCA-2-GFP and NCA-3-GFP localize to the vacuole, and the free GFP may arise by proteolysis in that compartment. The only significant anomaly we saw was two bands for ARG4-GFP, at 17 kDa as predicted and at 50 kDa. Both bands were observed in cell extracts and in purified mitochondria, which indicated that the anomalous 50-kDa band was also targeted to, or generated in, that organelle.
TABLE 2. Calcium transport proteins

| N. crassa gene | Locus       | Protein                        | Most similar protein                                      |
|---------------|-------------|--------------------------------|----------------------------------------------------------|
| cax           | NCU07075    | Ca\(^{2+}\)/H\(^{+}\) transporter | Vcx\(^{1}\)p in vacuolar membrane of S. cerevisiae         |
| nca-1         | NCU03305    | Ca\(^{2+}\)-ATPase              | SERCA ATPase in the endoplasmic reticula of animal cells  |
| nca-2         | NCU04736    | Ca\(^{2+}\)-ATPase              | Pmc1p in vacuolar membrane of S. cerevisiae               |
| nca-3         | NCU05154    | Ca\(^{2+}\)-ATPase              | Pmc1p in vacuolar membrane of S. cerevisiae               |
| pmr           | NCU03292    | Ca\(^{2+}\)-ATPase              | Pmr1p in Golgi membrane of S. cerevisiae                  |

TABLE 3. Proteins used as markers for organelles

| N. crassa gene | Locus         | Protein                                           | Homolog in S. cerevisiae |
|---------------|---------------|---------------------------------------------------|--------------------------|
| gpr-78        | NCU03082      | Endoplasmic reticulum-associated HSP               | Kar2p                    |
| dpm           | NCU07965      | Endoplasmic reticulum dolichol-phosphate mannosyltransferase | Dpm1p                    |
| vps-52        | NCU05273      | Golgi-body-associated retrograde protein          | Vps52p                   |
| vam-3         | NCU06777      | Vacuole-associated SNARE protein                  | Vam3p                    |
| vma-1         | NCU01207      | Subunit A of vacuolar ATPase                       | Vma1p                    |
| vma-5         | NCU09897      | Subunit C of vacuolar ATPase                       | Vma5p                    |
| arg-4         | NCU10468      | Mitochondrial acetylornithine-glutamate transacetylase | Arg4p                    |
| H1            | NCU06863      | Histone H1                                        | Hho1p                    |
| hpo           | NCU04018      | Chromosomal protein HP1                           |                          |

* See reference 25.
mm farther back, which typically contains large spherical vacuoles. With NCA-1 the GFP-labeled membranes were seen in all regions, from the tip to the older segments. The spherical organelles had the same size and abundance as nuclei (compare panels A to C with panels D to F in Fig. 1). To verify that the GFP was in nuclear envelopes, we coinoculated the NCA-1-GFP strain and the RFP-HP1 strain to obtain a heterokaryon that expressed both green and red proteins (Fig. 2A, B, and C). Merging the two images confirmed the location of NCA-1-GFP in the nuclear envelope. Although we observed fairly uniform diameters in nuclei located in regions of the hypha away from the tip, at the tip itself we saw considerable heterogeneity in the apparent size of nuclei as visualized by tagged histone H1 and NCA-1 (compare panels A and B with panels D and E in Fig. 1).

The two presumptive endoplasmic reticulum markers, GRP-78 and DPM, appeared in the same cellular structures as NCA-1 (Fig. 2D to I). The signal from GRP-78 and DPM was not as strong as for NCA-1; however, nuclear envelopes and attached membranes were visible with these constructs (Fig. 2D, E, F, and H). A heterokaryon of NCA-1-GFP plus RFP-GRP-78 showed colocalization of the green and red fluorescent proteins, although RFP-GRP-78 sometimes appeared as a “patch” on one side of the nucleus (Fig. 2G, H, and I). These results suggest that the ER of *N. crassa* is composed of the nuclear envelope and associated membranes. This observation is consistent with investigations of other organisms, where the nuclear envelope is a specialized region of the ER (23, 44, 48, 61).

The localization of GRP-78 and DPM was the same either with RFP fused to the N terminus (Fig. 2D, E, F, and H) or with GFP fused to the C terminus (DPM-GFP and GRP-78-GFP not shown). Likewise, RFP fused to the N terminus of NCA-1 localized to the same structures as NCA-1-GFP (not shown). With NCA-1-GFP we also compared the results obtained when the host cell for transformation carried the normal wild-type copy of *nca-1* or had the endogenous *nca-1* gene replaced by the *hyg* gene (11); NCA-1-GFP appeared in the same intracellular structures, with the same signal intensity (data not shown).

**NCA-2, VAM-3, and the vacuolar network.** Investigators have previously shown that dyes targeted to the vacuole localize in a tubular network near the tips of hyphae (10, 24, 31, 33). The cellular compartments visualized with RFP-VAM-3 protein, a vacuolar SNARE, looked like components of this vacuolar network. The first 50 to 100 μm at the hyphal tip contained little visible RFP-VAM-3. Next came a short region with a mixture of unorganized vesicles and tubular structures (Fig. 3A and C). The dense tubular network typically started 50 to 100 μm from the tip and continued for 300 to 500 μm in our growth conditions. RFP-NCA-2 (Fig. 3B), NCA-2-GFP (Fig. 3E and F), and RFP-CAX (Fig. 3G and H) localized to the same structures as RFP-VAM-3. In hyphal segments further back from the tip RFP-VAM-3, NCA-2-GFP, and RFP-CAX

![FIG. 1. NCA-1-GFP appears in the nuclear envelope and associated membranes. The scale bar in all panels is 10 μm. (A) Tip of a hypha transformed with *nca-1::sgfp*. (B) A region approximately 200 μm from the tip in a hypha transformed with *nca-1::sgfp*. (C) A region approximately 2 mm from the tip in a hypha transformed with *nca-1::sgfp*. (D) Tip of a hypha transformed with *hH1::sgfp*. (E) A region approximately 200 μm from the tip in a hypha transformed with *hH1::sgfp*. (F) A region approximately 2 mm from the tip in a hypha transformed with *hH1::sgfp*.](image-url)
appeared primarily in small vesicles and also in the large spherical vacuoles that are prominent in distal hyphal segments (Fig. 3D, E, F, and H). In these older segments NCA-2-GFP was also visible in the plasma membrane (Fig. 3E and F, discussed further below).

Examination of heterokaryons of NCA-2-GFP plus RFP-VAM-3 or NCA-2-GFP plus RFP-CAX showed that all three of these proteins were in the same vacuolar network. As shown in Fig. 4A to F, the tagged NCA-2 protein colocalized in the same thin tubules and small vesicles as the tagged CAX and VAM-3 proteins.

ARG-4 and mitochondria. Using dyes that localized to the mitochondria other investigators have observed mitochondria in *N. crassa* as abundant long thin tubules (24). We transformed *N. crassa* with *arg-4* /H11001 ::sgfp and observed tubular mitochondria (Fig. 4G and Fig. 5G), which looked similar to those observed with the dye FM4-64 (24). In hyphal segments 1 to 2 mm from the tip the mitochondria appeared to be shorter but were still abundant (Fig. 5H). The mitochondrial network differed from the vacuolar network by having fewer small bright vesicles associated with the tubular structures. Another obvious difference was the abundance of mitochondria throughout the hyphal tip, where few vacuoles were seen. To look at the vacuolar network and mitochondria in the same hyphae, we examined a heterokaryon expressing both RFP-CAX and ARG-4-GFP proteins (Fig. 4G to I). Although the cytoplasm was densely packed with tubular elements, the vacuolar and mitochondrial proteins clearly localized to different compartments.

NCA-2, NCA-3, and the plasma membrane. NCA-2 and NCA-3 ATPases share equal sequence similarity to the Pmc1p protein of *S. cerevisiae*. The PMCA-type ATPases are in the vacuole in *S. cerevisiae* but in the plasma membrane in most animal cells. In *N. crassa* these proteins appear to be in both locations (Fig. 3B, E, and F; Fig. 5A, B, and C). The organelles visualized with RFP-NCA-2, NCA-2-GFP, and NCA-3-GFP were indistinguishable from those seen with RFP-VAM-3 and RFP-CAX. There was a dark area immediately behind the hyphal tip, then a region with a network of tubules and small vesicles, and farther back small vesicles and large spherical vacuoles. Unlike VAM-3 and CAX, both NCA-2 and NCA-3 appeared in the plasma membrane. The signal was weak and variable near the tip; however, in hyphal segments that were 200 to 400 μm distal to the tip fluorescent proteins in the plasma membrane became clearly visible. Although the GFP could possibly be in the cell wall, observations of the septum between hyphal compartments (Fig. 5B) showed a dark space in the middle of the septum, indicating that the fusion proteins were in the plasma membrane, not the wall. Tagged NCA-3 gave a particularly strong signal in the plasma membrane in older regions of hyphae (>2 mm from the tip) and, conversely, was weaker than NCA-2-GFP in the tubular network near the tip. None of the other GFP or RFP fusion proteins tested appeared in the plasma membrane. Examination of a hetero-
karyon of NCA-2-GFP plus RFP-NCA-3 showed that both were in the same intracellular, vacuolar membranes (Fig. 5D, E, and F).

VPS-52 and the Golgi compartment. In *S. cerevisiae* the Ca\(^{2+}\)-ATPase encoded by the *PMR1* gene is localized in the medial Golgi compartment (20). Our preliminary experiments suggest that in *N. crassa*, as in *S. cerevisiae*, the cis-, medial, and trans-Golgi bodies may be distinct vesicular structures. This has necessitated the construction and analysis of a number of putative Golgi markers, work that is not presented here. However, to show a more complete set of organelles, we show the localization of VPS-52-GFP (Fig. 5I), which is predicted to be in a late Golgi compartment (12). In *N. crassa*, VPS-52-GFP does not colocalize with the vacuolar or ER proteins discussed above. PMR-GFP and RFP-PMR appear in vesicles of similar size and abundance to those shown for VPS-52, but PMR and VPS-52 do not colocalize (data not shown).

Colocalization of CAX and the vacuolar ATPase. By transporting protons across the vacuolar membrane the vacuolar ATPase generates an acidic environment within the vacuolar compartment and also generates an electrochemical gradient. The *vma-1* gene encodes the ATP-binding “A” subunit of the enzyme. RFP-VMA-1 gave a strong signal in the vacuolar membranes (Fig. 6A, B, C, and E). A heterokaryon composed of RFP-VMA-1 plus NCA-2-GFP showed these two vacuolar markers to colocalize (Fig. 6E, F, and G). In some hyphal compartments with large spherical vacuoles the RFP-VMA-1 signal was predominantly in the vacuolar membrane (Fig. 6C), not in the interior of the vacuole, as observed with other vacuolar proteins (e.g., Fig. 3E and H). The lack of fluorescence in the vacuolar lumen correlated with the degree of cytoplasmic flow in the hyphal segment. In segments with very active flow the interior of the vacuole was not red. For reasons we do not understand, internalization of the vacuolar ATPase may occur at lower rates in hyphae with active cytoplasmic flow.

A striking difference between RFP-VMA-1 and the tagged VAM-3 (the vacuolar SNARE), NCA-2, and NCA-3 proteins was its strong appearance in the membrane of unidentified organelles near the hyphal tip (Fig. 6A and E). These organelles, 2 to 5 \(\mu\)m in diameter, were seen only in the region near the tip where the tubular vacuolar network starts to appear. A typical hyphal tip contained 1 to 10 of the novel organelles. We made a GFP fusion with another subunit of the vacuolar ATPase, VMA-5-GFP. Although the signal from this construct was not as strong as for RFP-VMA-1, the unidenti-
fied organelle was clearly observable (Fig. 6D). The only other protein that strongly colocalized with the vacuolar ATPase in these intriguing organelles was RFP-CAX (Fig. 7A). A heterokaryon of VMA-5-GFP and RFP-CAX proteins showed that the vacuolar ATPase and the CAX protein were in identical organelles (Fig. 7B, C, and D).

DISCUSSION

We have found that tagging gene products with green and red fluorescent proteins is an effective and efficient means of examining the structure and abundance of organelles in growing *N. crassa* hyphae. Using this approach allowed us to determine the organellar location of five calcium transporters.

In this report we studied 14 genes: 7 were fused to either GFP or RFP, and 7 were fused to both GFP and RFP. Using both green and red tags, we succeeded in obtaining transformants that expressed fusion proteins with good signals. Nonetheless, some strains had weak GFP signals, which were difficult to distinguish from background green fluorescence. In contrast, virtually no background fluorescence was seen when using this particular RFP, tdimer2(12). Fusion of the fluorescent protein to the N terminus versus the C terminus did not affect the localization of the protein for six of the seven genes we examined. Only for the *cax* gene, discussed below, did we observe different behavior in GFP-tagged versus RFP-tagged proteins. The organelles in the endomembrane system were dynamic and variable when observed in actively growing hyphae. Our observations are summarized in Fig. 8. We are not aware of previous reports of systematically examining all of these organelles in living filamentous fungi. We limited our observations to young hyphae vigorously growing on the surface of agar medium, comparing the appearance of organelles at the tip, in the region 5 to 200 μm behind the tip and in the region 1 to 5 mm further back. In each of these regions we observed a difference in the structure and abundance of organelles. Some of the structural variability was surprising.

As shown in previous examinations of GFP- and RFP-tagged nuclei in *N. crassa* (25–27, 50), we found abundant nuclei in all regions except for the small zone 20 to 40 μm immediately behind the tip. The structure of nuclei was relatively uniform, with the exception of a surprising heterogeneity of sizes near the hyphal tip. We located proteins predicted to reside in the ER (products of the *dpm* and *grp-78* genes) both in the nuclear envelope and also in other associated, irregular membranes. Thus, our results with *N. crassa* are consistent with studies done in other fungi showing that the nuclear envelope is a specialized region of the ER (23, 39, 44, 48, 61). The ER was abundant throughout the hyphae from the region immediately behind the tip to the older segments. In older segments of hyphae the fluorescence signals from ER proteins were primarily in the nuclear envelope, with less fluorescence seen in other membranes. Because fungal cells have many nuclei, perhaps the nuclear envelope suffices as the major component of the ER in older hyphal segments.
The product of the nca-1 gene was predicted to be a SERCA-type Ca\(^{2+}\)/H\(^{+}\)-ATPase, based on DNA sequence similarity and the presence of a canonical ER-retention signal (KKDL) at the C terminus. GFP- and RFP-tagged versions of NCA-1 strongly localized to the nuclear envelope and associated membranes, confirming its ER residence. We speculate that SERCA-type ATPases might play a role in filamentous growth because they are present in the filamentous fungi but not in yeasts.

Visualization of the Golgi compartments in N. crassa has proven to be more complicated than we anticipated. A protein predicted to localize to the Golgi, VPS-52, appeared in small vesicles (0.5 to 2.0 \(\mu\)m) that were distinct from other organelles. In our preliminary analysis we saw the PMR Ca\(^{2+}\)-ATPase, which is in the medial Golgi compartment in S. cerevisiae (53), in small vesicles not associated with VPS-52. The data suggest that the Golgi complex in N. crassa may consist of disconnected cis, medial, and trans compartments, as reported for S. cerevisiae (37). We are currently pursuing this hypothesis.

In older textbooks, “the vacuole” was presented as a large spherical organelle, and in N. crassa such vacuoles are often seen in older hyphal segments. However, work pioneered by Anne Ashford and coworkers and since extended by other labs has shown that vacuolar compartments in filamentous fungi can also be in the form of small vesicles or an interconnected network of tubules (10, 24, 31, 33). Using tagged versions of the VAM-3 SNARE protein and the VMA-1 polypeptide of the V-ATPase, we visualized the vacuole in N. crassa. Vacuolar structure varied greatly in different regions of a hypha. Vacuolar markers were not present in the first 20 to 50 \(\mu\)m behind the tip. They appeared as small vesicles and tubules in the zone between about 20 to 50 and 200 \(\mu\)m behind the tip. In the region before the first septum the vacuolar markers were abundant in an interconnected network of thin (<1 \(\mu\)m) tubules, often with small, attached vesicles. These results with N. crassa are generally consistent with observations made using GFP-tagged VAM-3 in Aspergillus oryzae (45, 54). N. crassa grows faster, and the diameter of hyphae can be fivefold larger. The network of vacuolar tubules near the tips is denser near the tip, and fast-growing hyphae have fewer of the large spherical vacuoles. Shoji et al. (54) reported that small punctate structures tagged with GFP-VAM-3 associated with large vacuoles. These authors tentatively identified these as endosomes or prevacuolar compartments. We observed VAM-3 in N. crassa only in compartments that also contained other vacuolar proteins - VMA-1, CAX, NCA-2, or NCA-3.

The tubular network was highly dynamic, moving rapidly and changing shape as the hyphae grew. In older hyphal segments, typically more than 300 \(\mu\)m from the tip, we observed spherical vacuoles of widely ranging sizes, plus numerous small vesicles. This region, of course, represents the majority of the hyphal colony. The dramatic dynamic activity occurs in the growing tip cells.

The tubular network seen with tagged VAM-3 and VMA-1 in some ways resembled the structure of mitochondria, as observed previously with dyes (24). Using ARG-4-GFP we
obtained images of mitochondria that agreed well with the images obtained with dyes. Abundant in all regions examined, the mitochondria were seen as thin tubes with a more uniform thickness, lacking the punctate vesicular structures that appear to be part of the vacuolar network. Mitochondrial structure also varied with position in the hypha, the organelles appearing significantly shorter in older hyphal segments.

The Ca\textsuperscript{2+}-H\textsuperscript{+} transporter encoded by the cax gene is located in the vacuole in \emph{S. cerevisiae} (13, 47). We have found that, consistent with the results obtained from yeast, vacuoles isolated from cax-deficient strains of \emph{N. crassa} have less than 10\% of the calcium found in vacuoles from the wild-type strain (unpublished results). The CAX protein tagged with RFP localized to all components of the vacuole, both the tubular network and the larger spherical structures. Surprisingly, CAX-GFP appeared in the ER. A likely explanation is that fusion of the GFP protein to the C terminus of CAX interfered with a vacuolar targeting signal, causing the protein to be retained in the ER immediately after synthesis. Retention in the ER has been reported for other modified membrane proteins, e.g., the plasma membrane ATPase, PMA1 (40).

As described earlier, nca-2 and nca-3 encode PMCA-type Ca\textsuperscript{2+}-ATPases. These enzymes are located in the plasma membrane in animal cells but in the vacuolar membrane in \emph{S. cerevisiae}. Interestingly, in \emph{N. crassa} these Ca\textsuperscript{2+}-ATPases appeared in both the vacuolar and the plasma membranes. In the region within 200 to 500 \textmu m from the hyphal tip neither NCA2 nor NCA3 was visible in the plasma membrane, but the fluorescent signal was clearly visible in the plasma membrane in older segments of the hyphae. The one difference noted with NCA-2 versus NCA-3 is that tagged NCA-2 was relatively more abundant in the vacuolar compartments, whereas tagged NCA-3 was strongly incorporated into the plasma membrane, especially in regions 1 mm or more away from the tip. These data suggest that in \emph{N. crassa} NCA-2 and NCA-3 may serve to pump calcium out of the cell, as do the homologs of these enzymes in animal cells.
Although the genes we examined—nca-2, nca-3, cax, vam-3, and vma-1—encode membrane proteins, all of the proteins appeared to be internalized to some extent in the large spherical vacuoles. Because of the small size of the tubular components in the vacuolar network we could not determine whether the tagged proteins were inside the tubules. Many types of proteins can be targeted to the vacuole for degradation, but of the 14 different gene products we examined only those predicted to have their primary function in the vacuole localized to the vacuolar interior. The internalization seen in the larger vacuoles could be due to normal turnover of vacuolar membrane proteins. Only for RFP-VMA-1 did we observe hyphae with the tagged protein predominantly in the membrane of larger vacuoles. This is interesting because VMA-1 encodes the A subunit of the vacuolar ATPase and has no membrane-spanning region, unlike the other proteins we examined.

VMA-1, as part of the V1 sector of the enzyme, lies on the cytosolic side of the membrane and can dissociate from the integral membrane sector of the enzyme into the cytosol (5).

From the analysis of these 14 gene products our most surprising finding was an apparently novel organelle. The vacuolar ATPase subunits and CAX allowed visualization of a type of organelle not seen with any of the other tagged marker proteins. RFP-VMA-1, VMA-5-GFP, and RFP-CAX appeared in the membranes of roughly spherical organelles, 2 to 5 µm in diameter. These unidentified structures occurred only in a small region of each hypha, 100 to 200 µm behind the tip. Conceivably, they are compartments of the vacuole specialized for sequestration of calcium. We speculate that they could be related to organelles called acidocalcisomes, described primarily in trypanosomes but also identified in other types of organisms (17). Acidocalcisomes are approximately the same size, have membranes enriched in vacuolar ATPase, and contain high concentrations of calcium bound to polyphosphate.

ACKNOWLEDGMENTS

This study was supported by Public Health Service grant GM058903 from the Institute of General Medicine to B.J.B. and American Cancer Society grant RSG-08-030-01-CCG to M.F.

REFERENCES

1. Antebi, A., and G. R. Fink. 1992. The yeast Ca\(^{2+}\)-ATPase homologue, PMR1, is required for normal Golgi function and localizes in a novel Golgi-like distribution. Mol. Biol. Cell 3:633–645.
2. Benito, B., B. Garcia-Deblacas, and A. Rodriguez-Navarro. 2000. Molecular cloning of the calcium and sodium ATPases in Neurospora crassa. Mol. Microbiol. 35:1079–1089.
3. Berridge, M. J., M. D. Bootman, and H. L. Roderick. 2003. Calcium signaling: dynamics, homeostasis and remodeling. Nat. Rev. Mol. Cell Biol. 4:517–529.
4. Bok, J. W., T. Sone, L. B. Silverman-Gavril, R. R. Lew, F. J. Bowring, D. E. Catcheaside, and A. J. Grillibbs. 2001. Structure and function analysis of the calcium-related gene spray in Neurospora crusta. Fungal Genet. Biol. 32:145–158.
5. Bowman, R. J., W. J. Dochida, T. Harris, and E. J. Bowman. 1989. The vacuolar ATPase of Neurospora crassa contains an F1-like structure. J. Biol. Chem. 264:15606–15612.
6. Campbell, R. E., O. Tour, A. E. Palmer, P. A. Steinbach, G. S. Baird, D. A. Zacharias, and R. Y. Tsien. 2002. A monomeric red fluorescent protein. Proc. Natl. Acad. Sci. USA 99:7877–7882.
7. Carafoli, E. 2002. Calcium signaling: a tale for all seasons. Proc. Natl. Acad. Sci. USA 99:1115–1122.
8. Chavez, C., E. J. Bowman, J. C. Reidling, K. H. Haw, and B. J. Bowman. 2006. Analysis of strains with mutations in six genes encoding subunits of the V-ATPase: eukaryotes differ in the composition of the V0 sector of the enzyme. J. Biol. Chem. 281:27052–27062.
9. Clapham, D. E. 2007. Calcium signaling. Cell 131:1047–1058.
10. Cole, L., D. A. Orlovich, and A. E. Ashford. 1998. Structure, function, and diversity of vacuoles in filamentous fungi. Fungal Genet. Biol. 24:86–100.
11. Colot, H. V., G. Park, G. E. Turner, C. Ringelberg, C. M. Crew, L. Cunningham, K. W., and G. R. Fink. 1994. Calcium deficiencies and apical hyperbranching in wild-type and ‘frost’ and ‘spray’ morphological mutants of Neurospora crassa. J. Gen. Microbiol. 136:1413–1420.
12. Conibear, E., and T. H. Stevens. 2000. Vps52p, Vps53p, and Vps54p form a novel multisubunit complex required for protein sorting at the yeast late Golgi. Mol. Biol. Cell 11:305–323.
13. Cunningham, K. W., and G. R. Fink. 1996. Calcineurin inhibits VCX1-dependent H\(^+\)Ca\(^{2+}\) exchanger and inducible Ca\(^{2+}\) ATPases in Saccharomyces cerevisiae. Mol. Cell. Biol. 16:2222–2377.
14. Cunningham, K. W., and G. R. Fink. 1994. Calcineurin-dependent growth control in Saccharomyces cerevisiae mutants lacking PMU1, a homolog of plasma membrane Ca\(^{2+}\) ATPases. J. Cell Biol. 124:351–363.
15. Cybis, J., and R. R. Davis. 1975. Organization and control in the arginine biosynthetic pathway of Neurospora. J. Bacteriol. 123:196–202.
16. Dicker, J. W., and G. Turian. 1990. Calcium deficiencies and apical hyperbranching in wild-type and ‘frost and ‘spray’ morphological mutants of Neurospora crassa. J. Gen. Microbiol. 136:1413–1420.
17. Docampo, R., W. de Souza, K. Miranda, P. Rohlhoff, and S. N. Moreno. 2005. Acidocalcisomes: conserved from bacteria to man. Nat. Rev. Microbiol. 3:251–261.
18. Duchen, M. R., A. Verkhratsky, and S. Mulalem. 2008. Mitochondria and calcium in health and disease. Cell Calcium 44:1–5.
19. Dunn, T., K. Gable, and T. Beeler. 1994. Regulation of cellular Ca\(^{2+}\) by yeast vacuoles. J. Biol. Chem. J. 269:7273–7278.
20. Dury, G., J. Strayie, R. Pleenier, S. Elbo, S. K. Klee, P. Catty, D. H. Wolf, and H. K. Rudolph. 1998. The medial-Golgi ion pump Pmr1 supplies the yeast secretory pathway with Ca\(^{2+}\) and Mn\(^{2+}\) required for glycosylation, sorting, and endoplasmic reticulum-associated protein degradation. Mol. Biol. Cell 9:1149–1162.
21. Ebbole, D., and M. S. Sach. 1990. A rapid and simple method of isolation of Neurospora crassa homokaryons using microcomidia. Fungal Genet. News 37:17–18.
22. Eilam, Y., H. Lai, and N. Grossowicz. 1985. Cytoplasmic Ca\(^{2+}\) homeostasis maintained by a vacuolar calcium transport system in the yeast Saccharomyces cerevisiae. J. Gen. Microbiol. 131:623–629.
23. Fernandez-Abalos, J. M., H. Fox, C. Pitt, B. Wells, and J. H. Doonan. 1998. Plant-adapted green fluorescent protein is a versatile vital reporter for gene expression, protein localization and mitosis in the filamentous fungus, Aspergillus nidulans. Mol. Microbiol. 27:121–130.
24. Fischer-Parton, R., S. R. Parton, P. C. Hickey, J. Dijkstra, H. A. Atkin-son, and N. D. Read. 2000. Confocal microscopy of FM4-64 as a tool for analyzing endocytosis and vesicle trafficking in living fungal hyphae. J. Microsc. 198:246–259.
25. Freitag, M., P. C. Hickey, T. K. Klahalfall, N. D. Read, and E. U. Selker. 2004. HP1 is essential for DNA methylation in neurospora. Mol. Cell 13:427–434.
26. Freitag, M., P. C. Hickey, N. B. Raju, E. U. Selker, and N. D. Read. 2004. GFP as a tool to analyze the organization, dynamics, and function of nuclei and microtubules in Neurospora crassa. Fungal Genet. Biol. 41:897–910.
27. Freitag, M., and E. U. Selker. 2005. Expression and visualization of red
fluctuation of the yeast plasma membrane [Ca2+] gradient. Annu. Rev. Biochem.
28. Garreau, N. Y., Y. Liu, J. J. Lorus, and J. C. Dunlap, 1997. Alternative
29. Gerasimenko, O., and A. Tepikin. 2005. How to measure Ca2+ in cellular
30. Harold, F. M. 2002. Force and compliance: rethinking morphogenesis in
31. Hickey, P. C., S. R. Swift, and M. G. Roca. 2004. Live-cell imaging of
32. Holdaway-Clarke, T. L., J. A. Feijo, G. R. Hackett, J. G. Kunkel, and P. K.
33. McGilviray, A. M., and N. A. R. Gow.
34. Malho, R., and A. J. Trewavas.
35. Kirchok, Y., G. Krapivinsky, and D. E. Clapham. 2004. The mitochondrial
calcium uniporter is a highly selective ion channel. Nature 427:560–364.
36. Lew, R. R. 1999. Comparative analysis of Ca2+ and H+ flux magnitude and
location along growing hyphae of Saprolegnia ferax and Neurospora crassa.
Eur. J. Cell Biol. 78:982–902.
37. Losev, E., C. A. Reineke, J. Jellen, D. E. Strongin, B. J. Bevis, and B. S. Glick.
38. Malho, R., and A. J. Trewavas.
39. Maruyama, J., S. Kikuchi, and K. Kitamoto. 2006. Differential distribution of
the endoplasmic reticulum network as visualized by the Bpa-A-EGFP fusion protein in
hyphal compartments across the septum of the filamentous fungus, Aspergillus oryzae.
Fungal Genet. Biol. 43:642–654.
40. Mason, A. B., K. E. Allen, and C. W. Slaman. 2006. Effects of C-terminal
truncations on trafficking of the yeast plasma membrane H+–ATPase. J. Biol.
Chem. 281:23887–23898.
41. McGillivray, A. M., and N. A. R. Gow. 1987. The transhyphal electrical
current of Neurospora crassa is carried principally by protons. J. Gen.
Microbiol. 133:2875–2881.
42. Missiaen, L., L. Doce, J. Vanoeveren, L. Raeymaekers, and F. Wuytack.
2007. Calcium in the Golgi apparatus. Cell Calcium 41:405–416.
43. Monnerjahn, C., D. Teuch, U. Meyer, and L. Rensing. 2001. The grp78
promoter of Neurospora crassa: constitutive, stress and differentiation-
dependent protein-binding patterns. Curr. Genet. 39:319–326.
44. Newport, J. W., and D. J. Forbes. 1987. The nucleus: structure, function, and
dynamics. Annu. Rev. Biochem. 56:535–565.
45. Ohneda, M., M. Arioka, H. Nakajima, and K. Kitamoto. 2002. Visualization of
vacuoles in Aspergillus oryzae by expression of CPY-EGFP. Fungal Genet.
Biol. 37:29–39.
46. Orlean, P., C. Albright, and P. W. Robbins. 1988. Cloning and sequencing of
the yeast gene for dolichol phosphate mannose synthase, an essential
protein. J. Biol. Chem. 263:17499–17507.
47. Pozos, T. C., I. Sekler, and M. S. Cyert. 1996. The product of HUM1, a novel
yeast gene, is required for vacuolar Ca2+/H+ exchange and is related to
mammalian Na+/Ca2+ exchangers. Mol. Cell. Biol. 16:3730–3741.
48. Prinz, W. A., L. Gryz, M. Veenhuis, J. A. Kahana, P. A. Silver, and T. A.
Rapoport. 2000. Mutants affecting the structure of the cortical endoplasmic
reticulum in Saccharomyces cerevisiae. J. Cell Biol. 150:461–474.
49. Prokisch, H., O. Yardem, M. Dieminger, M. Tropschug, and I. B. Bartel-
thness. 1997. Impairment of calcineurin function in Neurospora crassa
reveals its essential role in hyphal growth, morphology and maintenance of
the apical Ca2+ gradient. Mol. Gen. Genet. 256:104–114.
50. Ramos-Garcia, S. L., R. W. Roberston, M. Freitag, S. Bartnicki-Garcia, and
R. R. Mourino-Perez. 2009. Cytoplasmic bulk flow propels nuclei in mature
hyphae of Neurospora crassa. Eurykaryot. Cell 8:1880–1890.
51. Rudolph, H. K., A. Antebi, G. R. Fink, C. M. Buckley, T. E. Dorman, J.
LeVitre, L. S. Davidow, J. I. Mao, and D. T. Moir. 1989. The yeast secretory
pathway is perturbed by mutations in PMR1, a member of a Ca2+-ATPase
family. Cell 58:133–145.
52. Schmid, J., and F. M. Harold. 1998. Dual roles for calcium ions in apical
growth of Neurospora crassa. J. Gen. Microbiol. 134:2623–2631.
53. Schroder, S., F. Schimmoller, B. Singer-Kruger, and H. Riezman. 1995. The
Golgolocalization of yeast Emp47p depends on its di-lysine motif but is not
affected by the ret1-1 mutation in alpha-COP. J. Cell Biol. 131:895–912.
54. Shojo, J. Y., M. Arioka, and K. Kitamoto. 2006. Vacular membrane dynam-
ics in the filamentous fungus Aspergillus oryzae. Eurykaryot. Cell 5:411–421.
55. Silverman-Gavila, L., and R. R. Lew. 2000. Calcium and tip growth in
Neurospora crassa. Protoplasma 213:203–217.
56. Silverman-Gavila, L. B., and R. R. Lew. 2001. Regulation of the tip-high
[Ca2+] gradient in growing hyphae of the fungus Neurospora crassa. Eur.
J. Cell Biol. 80:379–390.
57. Sorin, A., G. Rosas, and R. Rao. 1997. PMR1, a Ca2+-ATPase in yeast Golgi,
has properties distinct from sarco/endoplasmic reticulum and plasma mem-
brane calcium pumps. J. Biol. Chem. 272:9895–9901.
58. Sze, H., F. Liang, I. Hwang, A. C. Curran, and J. F. Harper. 2006. Vacuolar membrane dynam-
ics in Magnaporthe grisea: Implications for fungal pathogenesis.
Eur. J. Cell Biol. 85:189–195.
59. Wedlich-Soldner, R., I. Schulz, A. Straube, and G. Steinberg. 2002. Dynin
supports motility of endoplasmic reticulum in the fungus Ustilago maydis.
Mol. Biol. Cell 13:965–979.
60. Zelter, A., M. Bencina, B. J. Bowman, O. Yarden, and N. D. Read. 2004. A
comparative genomic analysis of the calcium signaling machinery in Neuro-
spora crassa, Magnaporthe grisea, and Saccharomyces cerevisiae. Fungal
Genet. Biol. 41:827–841.