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An unusual organelle in *C. neoformans* links luminal pH and capsule biosynthesis

Aki Yoneda and Tamara L. Doering *

Department of Molecular Microbiology, Washington University School of Medicine St. Louis, MO 63110

Abstract

C. neoformans is a basidiomycete that causes deadly infections in the immunocompromised. We previously generated a secretion mutant in this fungus by introducing a mutation in the *SAVI* gene, which encodes a homolog of the Sec4/Rab8 subfamily GTPases. Under restrictive conditions there are two notable morphological changes in the *savI* mutant: accumulation of post-Golgi vesicles and the appearance of an unusual organelle, which we term the *savI* body (SB). The SB is an electron-transparent structure 0.2–1 μ m in diameter, with vesicles or other membranous structures associated with the perimeter. Surprisingly, the SB was heavily labeled with anti-glucuronoxylomannan (GXM) antibodies, suggesting that it contains a secreted capsule component, GXM. A structure similar to the SB, also labeled by anti-GXM antibodies, was induced in wild type cells treated with the vacuolar-ATPase inhibitor, bafilomycin A₁. Bafilomycin A₁ and other agents that increase intraluminal pH also inhibited capsule polysaccharide shedding and capsule growth. These studies highlight an unusual organelle observed in *C. neoformans* with a potential role in polysaccharide synthesis, and a link between luminal pH and GXM biosynthesis.

Keywords

Cryptococcus neoformans; Sec4/Rab8; secretion; bafilomycin A; ultrastructure; GXM; organelle; electron microscopy; yeast; fungi

1. INTRODUCTION

C. neoformans is an encapsulated yeast that belongs to the phylum Basidiomycota. This fungus is widely distributed in the environment and can cause deadly infection in immunocompromised individuals such as AIDS patients (Chayakulkeeree and Perfect, 2006; Lin and Heitman, 2006). The most distinctive virulence determinant of *C. neoformans* is the polysaccharide capsule; the major component of this capsule is a highly acidic, linear polysaccharide termed glucuronoxylomannan (GXM) (Doering, 2009; Janbon, 2004).

In a previous study of capsule synthesis, we generated a conditional exocytosis mutant named *savI* (Yoneda and Doering, 2006). *Sav1p* is a cryptococcal homolog of the Sec4/Rab8

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*Corresponding author Mailing address: Department of Molecular Microbiology, Campus Box 8230, Washington University School of Medicine, St. Louis, MO 63110 Phone: (314) 747-5597 Email: doering@borcim.wustl.edu.

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subfamily of small GTPases, which regulate tethering of post-Golgi vesicles to the site of secretion (Segev, 2001). Under restrictive conditions, this temperature-sensitive mutant exhibits reduced protein secretion and accumulates secretory vesicles (Yoneda and Doering, 2006). These post-Golgi exocytic vesicles can be immunolabeled with anti-GXM monoclonal antibodies (mAbs), suggesting that they contain GXM or a related glycan that is likely synthesized in the Golgi (Yoneda and Doering, 2006). Earlier studies in *Saccharomyces cerevisiae* showed that exocytosis mutants in this model yeast similarly accumulate vesicles, with no dramatic changes in the ultrastructure of other organelles (Aalto et al., 1993; Couve et al., 1995; Finger and Novick, 1997; Roth et al., 1998; Salminen and Novick, 1987).

In this study, we report an unusual organelle termed the *savI* body (SB), which appears in parallel with the accumulation of secretory vesicles in the *savI* secretion mutant. A similar structure is observed in wild type cells when luminal pH is raised, and correlates with impairment of capsule polysaccharide shedding and capsule growth. Formation of these aberrant organelles may result from disturbed membrane trafficking, which ultimately leads to a block in capsule enlargement and shedding; these results suggest possible mechanistic links between luminal pH, GXM synthesis, and capsule enlargement.

2. MATERIALS AND METHODS

2.1. Strains and growth conditions

The serotype A wild type strain H99, a *savI* mutant generated in H99, the serotype D wild type strain JEC21, and a *savI* mutant generated in JEC21 were as previously described (Yoneda and Doering, 2006). Typically, a 50 ml 'starter culture' of Yeast extract Peptone Dextrose medium (YPD) was inoculated with a small portion of a colony from a YPD plate, grown at room temperature (RT) overnight, and then used to inoculate fresh YPD so as to achieve log-phase growth at the time of harvest. For capsule induction, cells from a starter culture were washed twice in Dulbecco's Modified Eagle's Medium (DMEM, Sigma D5796) before resuspension at 10^7 cells/ml in 10 ml DMEM in a small tissue culture flask; these cultures were incubated at 37 °C with 5% CO₂ for the indicated time.

2.2. Capsule induction with drugs

Bafilomycin A₁ (Axxora, ALX-380-030-C100), brefeldin A (Sigma, B7651), and rapamycin (VWR, 101416-492) were dissolved in DMSO as 1000x stock solutions and stored at -20 °C. NaCl and NH₄Cl were prepared as 10x stocks in deionized water and filter sterilized. Final working concentrations were: bafilomycin A₁, 10 µM; brefeldin A, 500 µM; rapamycin, 1 µg/ml; NaCl and NH₄Cl, 400 mM.

For capsule induction in the presence of drugs, washed cells from the starter culture were resuspended in 10 ml DMEM containing drugs and grown as above. At 14 h after capsule induction, 0.5 ml was removed from each culture and the remaining cells were washed to remove the drug, resuspended in 10 ml of fresh DMEM without drug, and returned to culture for the time indicated. Culture samples were centrifuged to separate supernatants and cell pellets, and the supernatants were subjected to mild heating to inactivate proteins (65 °C for 15 min).

2.3. Analyses of surface capsule and shed GXM

Electrophoresis and immunoblotting of shed GXM and India ink staining of capsule were performed as previously described (Yoneda and Doering, 2008). For light microscopy, cells were either immediately stained and observed or fixed in 4% paraformaldehyde for later observation.

2.4. Electron microscopy

Electron microscopy (EM) using KMnO_4 (for morphology) and OsO_4 (for immuno-EM) was performed as previously described (Yoneda and Doering, 2006). Anti-GXM mAb 3C2 (a generous gift from Dr. Kozel, University of Nevada, Reno) was used for immuno-EM. For electron microscopy of drug-treated cryptococci, cells were treated with the drug concentrations noted above (section 2.2.) for 2 hours at 30 °C in YPD (without capsule induction) before primary fixation.

3. RESULTS

3.1. An unusual structure is induced in the *sav1* mutant

A large (0.2–1 μm in diameter) electron-transparent structure appeared in parallel with secretory vesicle accumulation when *sav1* cells were grown at a semi-permissive temperature of 30 °C (Figure 1). We have named this structure the *sav1* body (SB). The boundary of the SB appeared discontinuous (Figure 1, arrows) and a spiral double-membrane structure was often observed near its membrane (Figure 1A, closed arrowheads and inset on the left panel). Moreover, vesicles and small vacuoles were often observed in contact with the SB (Figure 1, open arrowheads). SBs progressively increased in size as cells were maintained at the semi-permissive temperature (compare Figure 1A (3 h) and 1B (10 h)) although there was no consistent change in cell size. At the 10 h time point, the boundary of the SB appeared granular (Figure 1B, all panels). At a lethal temperature (37 °C), secretory vesicles were rarely observed, and the SBs were more prominent than in cells at semi-permissive temperature (not shown).

The serotype D wild type strain, JEC21, has higher constitutive secretion activity than the serotype A wild type H99 (Yoneda and Doering, 2006). Consistent with this observation, the serotype D *sav1* mutant displayed more severe temperature sensitivity and more rapid vesicle accumulation than the serotype A *sav1* strain (Yoneda and Doering, 2006). SBs were observed in a serotype D *sav1* mutant as soon as 90 min after temperature shift to 30 °C (Figure 2A); in serotype A *sav1* cells, this structure typically only appeared after 3 h (Figure 1A).

3.2. The SB is heavily labeled with anti-GXM monoclonal antibodies

In the course of capsule secretion studies using *sav1* cells, we made the unexpected observation that SBs were heavily labeled with an anti-GXM monoclonal antibody (mAb), 3C2 (Figure 2B). The processing method required for these immunolabeling studies confers a different appearance on the SBs compared to methods that optimize membrane visualization (compare Figure 1 and Figures 2A to 2B), but extensive electron microscopic observations indicated that they correspond to the same electron-lucent structure (not shown). The immuno-EM results thus suggest that the SBs contain GXM or a related glycan.

3.3. A GXM-positive structure in wild type cells resembles the SB and is not of autophagic origin

We observed membrane-bounded areas of cytosol, identified by the presence of ribosomes (Figure 2B, arrows), in association with the SB. This finding prompted us to examine the role of autophagy in SB formation. We first tested the effect of bafilomycin A_1 , a compound that has been used to inhibit fusion of autophagosomes with lysosomes (Yamamoto et al., 1998) although its role in this process is controversial (Klionsky et al., 2008). When *C. neoformans sav1* cells were treated with bafilomycin A_1 , formation of the SB was not altered (not shown). This suggested that the SB is not formed via an autophagic process.

Surprisingly, bafilomycin A_1 treatment of the wild type strain JEC21 induced a structure very similar to the SB that was also labeled with 3C2 (Figure 3, closed arrowheads). To test the potential connection of this structure in wild type cells to autophagy, we used rapamycin, an

autophagy-inducing agent. Although some large membrane-bounded structures appeared in wild type cells treated with rapamycin, they were not labeled with 3C2 (Figure 4, arrowheads). We know that the labeling of these cells was effective because we did observe occasional labeled 100 nm-vesicles, probably of secretory origin (Figure 4, arrows); this is similar to what is seen in untreated wild type cells. Beyond the lack of anti-GXM labeling, the dominant rapamycin-induced structures differed from SBs and bafilomycin A₁-induced structures in being extremely electron-dense (Figure 4, open arrowheads); electron-transparent structures that appeared following rapamycin treatment were clearly demarcated with continuous membranes (Figure 4, closed arrowhead). These observations further suggested that neither the SB in *sav1* cells nor the structurally similar bafilomycin A₁-induced bodies in wild type cells are related to autophagy.

3.4. Bafilomycin A₁ inhibits shedding and enlargement of the capsule

The anti-GXM mAb labeling of SBs, which form when secretion is disrupted, suggests interplay between these structures and the trafficking of capsule materials. Bafilomycin A₁, which induces structures similar to SBs in wild type cells, has previously been reported to inhibit capsule enlargement (Erickson et al., 2001). We assessed bound and shed capsule in the presence of this compound, using cells grown in DMEM with CO₂ for capsule induction. We observed that bafilomycin A₁ completely inhibited the formation of large capsules (Figure 5A, panel 5), as in a previous report using different induction conditions (Erickson et al., 2001). We also noted that the treated cells did not shed detectable levels of GXM, as assessed by immunoblotting of the culture medium (Figure 5B, 14h, lane 5). To make sure that the lack of capsule shedding was not simply due to cell death, we washed away the drug, returned the cells to fresh DMEM, and again incubated under inducing conditions. After 24 h, bafilomycin A₁-treated cells recovered the ability to induce capsules (data not shown) and to shed capsule polysaccharide into the medium (Figure 5B, 24 h p.w. lane 5).

Our results support the model that the bafilomycin A₁-induced GXM-positive structure reflects interruption of intracellular polysaccharide trafficking, which also results in inhibition of capsule enlargement and shedding. Parallel studies of rapamycin-treated cells showed no effect on capsule enlargement (Figure 5A, panel 6) or polysaccharide shedding (Figure 5B, lane 6 of each time point), consistent with the lack of immunolabeling of intracellular structures induced by rapamycin.

3.5. Luminal pH-perturbing agents inhibit capsule shedding and enlargement

We had originally utilized bafilomycin A₁ to test autophagy, but this compound is also known as an inhibitor of vacuolar H⁺-ATPases (V-ATPases), causing increased luminal pH in treated cells (Bowman et al., 1988). In *S. cerevisiae*, there are two isoforms of the V₀ subunit of V-ATPase, Vph1p and Stv1p (Manolson et al., 1994). Vph1p functions mainly in vacuoles, while Stv1p localizes to the Golgi and endosomes (Kawasaki-Nishi et al., 2001). The *C. neoformans* *VPH1* was initially identified as a gene that is required for normal laccase activity and capsule enlargement (Erickson et al., 2001). There is no second isoform of the V-ATPase V₀ subunit in *C. neoformans*, suggesting that Vph1p functions in both vacuoles and secretory organelles in this organism (Erickson et al., 2001). The phenotype of *C. neoformans* treated with bafilomycin A₁, which inhibits both vacuolar and Golgi V-ATPases (Bowman et al., 1988; Moriyama and Nelson, 1989), is similar to that of the *vph1* mutant (Erickson et al., 2001), supporting this hypothesis.

Our bafilomycin A₁ studies suggested that increased luminal pH affects capsule enlargement and shedding. To test this further we used ammonium chloride (NH₄Cl), a weak base that has been used to increase Golgi and vacuolar pH in other systems (Axelsson et al., 2001; Thorens and Vassalli, 1986). Treatment of cells with 400 mM NH₄Cl completely inhibited capsule

induction (Figure 5A, panel 3). This treatment also reversibly blocked GXM shedding (Figure 5B, 14h lane 5); washing out the salt allowed recovery of this process over time (Figure 5B, p.w. lanes 5). Sodium chloride (NaCl) was used as a control for NH₄Cl, although it has been reported that 1 M NaCl inhibits capsule enlargement (Jacobson et al., 1989). Consistent with the earlier report, the presence of 400 mM NaCl partially inhibited capsule enlargement (Figure 5A, panel 2) and GXM shedding (Figure 5B, 14h, lane 2). Chloroquine, another weak base that increases pH in secretory organelles and vacuoles (Thorens and Vassalli, 1986), also inhibited capsule induction and shedding (not shown).

We also tested brefeldin A, a general secretion inhibitor that interferes with COPI-coat formation (Donaldson et al., 1992; Helms and Rothman, 1992). Use of this compound induced formation of typical tubular structures (Klausner et al, 1992) but did not induce formation of SB (not shown). This compound strongly inhibited capsule enlargement (Figure 5A, panel 4) and shedding (Figure 5B, 14 h lane 4), and cells were unable to recover from this treatment (Figure 5, p.w. lanes 4). It is likely that inhibition of general secretion at 37 °C for 14 h by brefeldin A was lethal to the cells, consistent with the severe temperature sensitivity of *savI* secretion mutants (Yoneda and Doering, 2006). In contrast, when a microtubule inhibitor, nocodazole, was used to arrest cell growth without interfering with secretory processes, capsule enlargement and shedding were normal (not shown).

4. DISCUSSION

A pH gradient is maintained throughout the secretory pathway in eukaryotic cells, from the ER (slightly basic) through the Golgi to post-Golgi vesicles (slightly acidic) (Paroutis et al., 2004). Maintenance of luminal pH is important for the localization and activity of resident enzymes. When luminal pH is disrupted, glycosyltransferases can be mislocalized, which may result in synthesis of aberrant glycans (Axelsson et al., 2001; Rivinoja et al., 2006; Thorens and Vassalli, 1986).

We have observed an unusual structure in *C. neoformans* that is characterized by electron-transparency, discontinuous membranes, associated vesicular structures, spiral membrane formations, and heavy labeling with anti-GXM mAbs. We have termed this structure the *savI* body (SB) due to its appearance in *savI* mutant cells under semi-permissive and restrictive conditions. Interestingly, a similar structure appears when the maintenance of luminal pH of wild type cells is disturbed by bafilomycin A₁ treatment. Treatment with either bafilomycin A₁ or the weak bases NH₄Cl and chloroquine, which increase pH in secretory organelles (Thorens and Vassalli, 1986), also completely inhibits capsule induction and shedding (Figure 5 and not shown). The effects of these compounds were dose-dependent (not shown). Capsule inhibition is not simply due to disturbance of cell growth, as nocodazole, which completely inhibits fungal growth, does not have an effect on capsule (not shown). Together, these data suggest that elevated luminal pH inhibits capsule enlargement and shedding.

The appearance of the SB in *C. neoformans* cells raises the question of its origin. Involvement of an autophagy-like pathway was suggested by ultrastructural studies showing enclosed cytoplasm (Figure 2B, arrows) near this body. However, it is unlikely that autophagy is involved in generating this structure, which we believe is related to GXM synthesis and capsule enlargement, because a *vps34* mutant that is defective in autophagy is able to induce large capsule normally (Hu et al., 2008). Consistent with this, induction of autophagy by rapamycin treatment did not affect capsule induction and shedding (Figure 5).

The nature of the GXM present in the SB is unclear. The GXM that accumulates in *savI* cells under conditions that induce SB formation migrates like both shed and cell wall-bound GXM by agarose gel electrophoresis (Yoneda and Doering, 2008 and data not shown), suggesting

that these populations are similar. However, purification of intracellular forms of GXM will be required to directly analyze these polysaccharides and define their relationship to extracellular forms.

We can speculate on multiple possibilities for the origin of the SB in our *sav1* exocytosis mutant. It may derive from a secretory sub-compartment that functions in glycan polymer synthesis and is ultrastructurally indistinguishable from other “normal” organelles in wild type cells, but becomes expanded when secretion is impaired. Alternatively, it may occur when vesicles that accumulate in the mutant due to blocked exocytosis fuse with recycling endosomes or vacuoles. Another possibility is that it corresponds to a swollen form of the Golgi apparatus caused by lack of normal protein recycling from the plasma membrane; future work will be needed to resolve these models.

It is noteworthy that no aberrant organelle like the SB has been reported in *S. cerevisiae* mutants blocked in exocytosis (Aalto et al., 1993; Couve et al., 1995; Finger and Novick, 1997; Roth et al., 1998; Salminen and Novick, 1987), although vesicle accumulation in these strains is similar to that seen in *C. neoformans sav1*. When vesicle budding from the Golgi is blocked in *S. cerevisiae*, distended Golgi cisternae or Berkeley bodies are observed (Novick et al., 1980). These structures are morphologically distinct from the SB. These observations highlight a possible fundamental difference in secretory organelles between these two evolutionarily distant fungal species, potentially related to the extensive glycan synthetic requirements of an encapsulated pathogen compared to a model yeast.

We hypothesize that the GXM-positive structure induced in wild type cells by the V-ATPase inhibitor bafilomycin A₁ corresponds to the SB observed in *sav1* cells. We further speculate that these structures correspond to a secretory sub-compartment specialized for glycan synthesis that becomes expanded in conditions where traffic of polysaccharides is perturbed. Support for this model includes our observation that double mutants of *sav1* and *acapsular* strains do not form SBs (not shown), but further work will be needed to explore the formation and function of this putative compartment.

The possibility that the SB may derive from the Golgi apparatus links it to altered pH in that organelle, because the exocytosis block in *sav1* and consequent loss of normal protein recycling may cause depletion of proteins needed to maintain organellar pH. Our drug treatment results (Figure 5) in wild type cells also suggest a relationship between GXM synthesis and luminal pH. Future studies will be required to mechanistically define the relationships between elevated Golgi pH, formation of the unusual structures and glycan synthesis.

Compounds that disrupt luminal pH are promising antifungal drugs for *C. neoformans* (Harrison et al., 2000; Levitz et al., 1999) and focusing on luminal pH maintenance may result in development of new therapies. Although additional experiments are required to investigate the nature of the drug-induced GXM-positive structure and its relationship with capsule biosynthesis, the results presented here suggest that maintenance of the luminal pH gradient is critical for both GXM biosynthesis and capsule enlargement. Future studies involving the *vph1* mutant (Erickson et al., 2001) and additional mutants that cannot normally regulate pH in vacuolar compartments or the Golgi will be needed to assess how pH maintenance affects the fitness of *C. neoformans* during infection.

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Figure 1.

An unusual, electron-transparent structure (the *savI* body; SB) induced in the serotype A *savI* mutant. **A**, The *savI* mutant shifted to 30 °C for 3 h. Inset, a spiral structure at higher magnification. **B**, The same strain shifted to 30 °C for 10 h. N, nucleus; Mt, mitochondrion. Closed arrowheads, spiral structure; open arrowheads, apparent membrane docking; arrows, discontinuous membrane perimeter of the SB. Scale bars in large panels, 1 μm; all small panels on the right are at the same magnification and the scale bar indicates 500 nm.

Figure 2.

SBs in the serotype D *savI* mutant shifted to 30 °C for 90 minutes. **A**, Samples optimized for visualizing membrane structures. Closed arrowhead, spiral structure; open arrowheads, apparent membrane docking. **B**, Samples optimized for immuno-labeling of GXM. (This changes the appearance of membranes compared to the method used in Figure 1A and Figure 2A.) Arrows, ribosomes enclosed in membranes. Scale bars, 500 nm.

Figure 3. Anti-GXM mAb-labeled structures are induced in JEC21 grown in YPD plus bafilomycin A₁ at 30 °C for 2 h. Arrowheads, bafilomycin A₁-induced structures; N, nucleus; Mt, mitochondrion. Scale bar at left, 1 μm; four panels on the right are at the same magnification and the scale bar indicates 500 nm.

Figure 4.

Rapamycin-induced structures in JEC21 grown in YPD at 30° C for 2 h. Arrows, 100-nm vesicles labeled with 3C2; closed arrowhead, boundary of an electron-transparent region; open arrowheads, electron-dense regions; N, nucleus; Mt, mitochondrion. All panels are to the same scale; scale bar, 500 μ m. Note that the multiply membrane-bounded structure at the upper left of the cell in the large panel is consistent in appearance with an autophagic body, and is distinct from the spiral structures associated with the SB (Figure 1B, closed arrows and inset). Neither this nor other rapamycin-induced structures were labeled with 3C2.

Figure 5.

Drug treatment affects capsule enlargement and GXM shedding. **A**, Visualization of capsule by India ink staining. JEC21 cells were incubated in DMEM with 5% CO_2 at 37° C for 14 h in the presence of DMSO as a control or the indicated compounds. **B**, Culture supernatants containing shed GXM were resolved by electrophoresis and immunoblotted with the anti-GXM mAb 3C2. Culture supernatant samples were collected after 14 h of drug treatment (lane numbers refer to the compounds in panel A) or at 4, 8, or 24 h after washing away the drugs (post wash, p.w.). All cells recover after compounds are washed away and can shed capsule materials (and display large capsules, not shown) except for brefeldin A-treated cells. Note that the 4 h and 8 h p.w. samples contain less GXM than the initial induction samples (14 h), as more capsule materials accumulate in the culture supernatant with longer incubation. GXM shedding occurred only in conjunction with capsule induction (not shown).