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

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ABSTRACT

Cryptococcus 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 *SAV1* gene, which encodes a homolog of the Sec4/Rab8 subfamily GTPases. Under restrictive conditions there are two notable morphological changes in the *sav1* mutant: accumulation of post-Golgi vesicles and the appearance of an unusual organelle, which we term the *sav1* body (SB). The SB is an electron-transparent structure 0.2–1 μm 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 A_1. Bafilomycin A_1 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.

1. Introduction

Cryptococcus 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 *sav1* (Yoneda and Doering, 2006). Sav1p is a cryptococcal homolog of the Sec4/Rab8 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 *sav1* body (SB), which appears in parallel with the accumulation of secretory vesicles in the *sav1* 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 *sav1* mutant generated in H99, the serotype D wild type strain JEC21, and a *sav1* 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-

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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% CO_2 for the indicated time.

2.2. Capsule induction with drugs

Bafilomycin A_1 (Axxora, ALX-380-030-C100), brefeldin A (Sigma, B7651), and rapamycin (VWR, 101416-492) were dissolved in DMSO as 1000× stock solutions and stored at −20 °C. NaCl and NH_4Cl were prepared as 10× stocks in deionized water and filter sterilized. Final working concentrations were: bafilomycin A_1, 10 μM; brefeldin A, 500 μM; rapamycin, 1 μg/ml; NaCl and NH_4Cl, 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 h 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 (Fig. 1). We have named this structure the *sav1* body (SB). The boundary of the SB appeared discontinuous (Fig. 1, arrows) and a spiral double-membrane structure was often observed near its membrane (Fig. 1A, closed arrowheads and inset on the left panel). Moreover, vesicles and small vacuoles were often observed in contact with the SB (Fig. 1, open arrowheads). SBs progressively increased in size as cells were maintained at the semi-permissive temperature (compare Fig. 1A (3 h) and B (10 h)) although there was no consistent change in cell size. At the 10 h time point, the boundary of the SB appeared granular (Fig. 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).

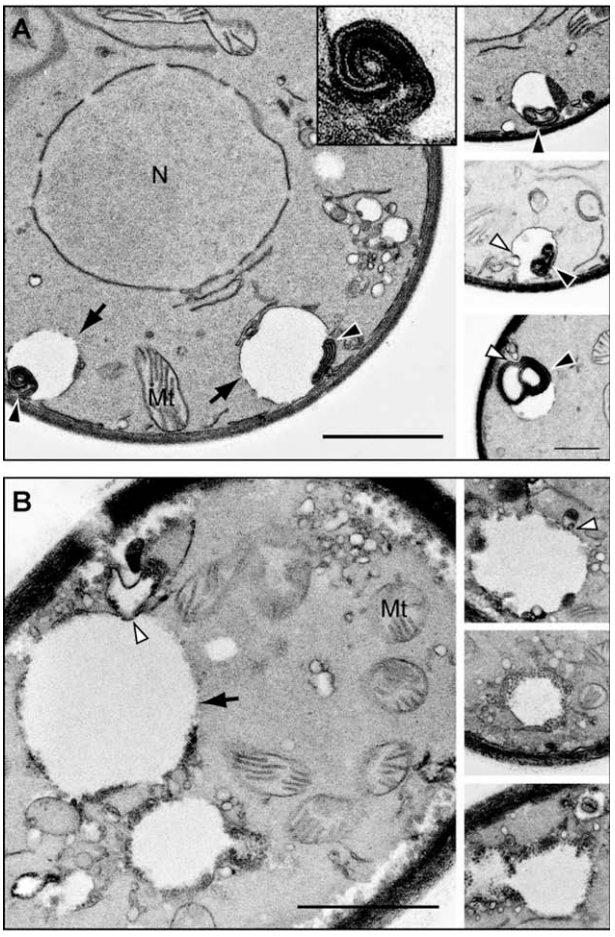

Fig. 1. An unusual, electron-transparent structure (the *sav1* body; SB) induced in the serotype A *sav1* mutant. (A) The *sav1* mutant shifted to 30 °C for 3 h. Inset, a spiral structure at higher magnification. (B) The same strain shifted to 30 °C for 10 h. N, nucleus; Mt, mitochondrion. Closed arrowheads, 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.

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 (Fig. 2A); in serotype A *sav1* cells, this structure typically only appeared after 3 h (Fig. 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 (Fig. 2B). The processing method required for these immunolabeling studies confers a different appearance on the SBs compared to methods that optimize membrane visualization (compare Figs. 1 and 2A to Fig. 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.