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Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*

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Summary

The cell wall of pathogenic fungi such as *Cryptococcus neoformans*, provides a formidable barrier to secrete virulence factors that produce host cell damage. To study secretion of virulence factors to the cell periphery, *sec6* RNAi mutant strains of *C. neoformans* were tested for virulence factor expression. The studies reported here show that *SEC6* RNAi mutant strains were defective in a number of virulence factors including laccase, urease as well as soluble polysaccharide and demonstrated attenuated virulence in mice. Further analysis by transmission electron microscopy detected the production of abundant extracellular exosomes in wild-type strains containing empty plasmid, but a complete absence in the i*SEC6* strain. In addition, a green fluorescent protein–laccase fusion protein demonstrated aberrant localization within cytoplasmic vesicles in i*SEC6* strains. In contrast, i*SEC6* strains retained normal growth at 37°C, as well as substantially normal capsule formation, phospholipase activity and total secreted protein. These results provide the first molecular evidence for the existence of fungal exosomes and associate these vesicles with the virulence of *C. neoformans*.

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Introduction

Cryptococcus neoformans is a basidiomycete fungal pathogen that causes disease in both immunocompetent and immunocompromised individuals including those with AIDS (Chayakulkeeree and Perfect, 2006). However, the spectrum of disease in AIDS has been changing with the advent of highly active antiretroviral therapy and the recognition of an immune reconstitution syndrome associated with cryptococcosis (Jenny-Avital and Abadi, 2002). In the developing world, low rates of antiretroviral use and lack of credible fungal preventative and treatment strategies have led to an explosion in deaths from cryptococcosis in these regions (Bicanic *et al.*, 2005).

An important property of an opportunistic pathogen such as *C. neoformans* is its ability to rapidly adapt to the stressful environment of the host, by expressing and transporting virulence factors that affect host cell damage at the fungal periphery. *C. neoformans* expresses a number of virulence factors including an antiphagocytic polysaccharide capsule and secreted polysaccharide (Chang and Kwon-Chung, 1994; 1998; Chang *et al.*, 1995; 1996; 1997), a membrane-disruptive phospholipase (Cox *et al.*, 2001), and the neurotropic factors, urease (Olszewski *et al.*, 2004) and laccase (Noverr *et al.*, 2004). These factors contribute to virulence by virtue of their location outside of the cell membrane. For example, laccase is predominantly a cell wall-associated protein with additional secretion into the media, both of which facilitate access of the enzyme to macrophage Fenton reactants and host catecholamines (Zhu *et al.*, 2001). However, little is known about molecular mechanisms of how these virulence factors are exported to the fungal periphery. Fungal cells are encased in a rigid and complex cell wall consisting of polysaccharides, proteins and pigments (Nimrichter *et al.*, 2005). While different studies demonstrate that structures with molecular masses higher than 1000 kDa can cross the cell wall and reach the extracellular milieu (McFadden *et al.*, 2006a,b), the mechanism by which such molecules traverse the cell wall barrier remains poorly understood. However, the importance of intact cellular trafficking of these factors to virulence is suggested by the presence of host defence processes that inhibit virulence factor secretion. In one study of *C. neoformans*, lung macrophages were found to

inhibit secretion of the virulence factor laccase to the cell wall, whereas in brain, secretion was intact, facilitating the role of this factor in neurotropism (Waterman *et al*., 2007).

Recently, glucuronoxylomannan (GXM)-containing vesicles were described in supernatants of *C. neoformans* cultures (Rodrigues *et al*., 2008), and were also found to contain a number of virulence factors including laccase, urease and phosphatase as well as potential immunomodulatory proteins such as Hsp70 (Rodrigues *et al*., 2008). These appeared as multiple 100 nm to 200 nm vesicles visualized by electron microscopy that resembled exosome-like compartments (Rodrigues *et al*., 2008). Exosomes in mammalian cells develop from internal vesicles of multivesicular bodies that are then released into the extracellular milieu upon fusion of the multivesicular bodies with the cell surface (Raposo *et al*., 1996; Zitvogel *et al*., 1998; 1999) and have been described as transporting a number of leaderless proteins such as Hsp70 in mammalian cells. Such extracellular exosomes are novel in fungi, and provide a potential mechanism to both transport important virulence factors to the surface through the cell wall and potentially insulate their cargos from host cell proteolytic enzymes.

In the present study, an RNAi knockdown of a *C. neoformans* homologue of *SEC6* was used to assess the role of secretory processes in the expression of cryptococcal virulence factors. Sec6 is essential in yeast and is part of a protein complex involved in polarized fusion of exocytic vesicles with the plasma membrane (Novick *et al*., 1980). We report that *SEC6* suppression resulted in a complete absence of extracellular vesicle production, with absent laccase secretion to the extracellular milieu or the cell wall. Virulence in mice was also reduced. However, capsule production and phospholipase activities remained intact. These results thus provide a molecular dissection of secretory pathways involving virulence and molecular proof of the existence of virulence-associated fungal extracellular exosomes in *C. neoformans*.

Results

Identification of a cryptococcal homologue of the 'exocyst' component, Sec6

SEC6 is an essential gene in yeast and is part of a protein complex known as the 'exocyst' that is involved in the polarized fusion of exocytic vesicles with the plasma membrane (TerBush *et al*., 1996). *SEC6* temperature-sensitive mutants are growth arrested at elevated temperatures and accumulate multiple populations of cytoplasmic vesicles that differ in protein cargo and destination (Harsay and Schekman, 2002). A BLAST search of the cryptococcal genomic database (http://www.broad.mit.edu) yielded a single annotated putative protein sequence of 770 aa (CNAG_01911) having 23% identity to the *Saccharomyces cerevisiae* Sec6 protein sequence (EDN61430). To further characterize cryptococcal Sec6 functionality, a *S. cerevisiae sec6-4* mutant was complemented with a cDNA fragment of the cryptococcal gene expressed under a *Saccharomyces* constitutive promoter. As shown in Fig. 1A, complementation of the Sc *sec6-4* with Cn *SEC6* resulted in partial restoration of growth at a non-permissive temperature (34°C). As the *Saccharomyces* mutant is a temperature-sensitive mutant, partial restoration may have been due to continued competition between the mutant *S. cerevisiae* Sec6 protein and the heterologously expressed *C. neoformans* protein as well as difficulties in the ability of cryptococcal proteins to fully function in ascomycete yeast, as described previously (Blakely *et al*., 2001). Nevertheless, these data suggest a similarity between *SEC6* function in the two yeast species.

Cryptococcal strains expressing SEC6*-interfering RNA grow normally, but exhibit accumulation of vesicles at bud necks and a mild cell wall defect*

Cryptococcus neoformans strains harbouring an episomal vector expressing interfering RNA to *SEC6* in similar copy number to a wild-type strain harbouring an empty vector (iControl) were chosen for analysis (i*SEC6*-1 and i*SEC6*-2). We used RNAi to reduce *SEC6* expression of this essential gene rather than a temperature-sensitive mutation as described in *S. cerevisiae* (Lamping *et al*., 2005) because it allows analysis of *SEC6* suppression at host temperatures, whereas temperature-sensitive mutants (including the *sec6* mutant in yeast) do not grow at these temperatures. In addition, constitutive RNAi suppression allows more facile identification of phenotypes that are retained after suppression because retention of a given phenotype in a temperature-sensitive mutant at the non-permissive temperature could be due to a pre-existing phenotype synthesized prior to the temperature shift. To determine steady-state levels of intact *SEC6* transcript achieved after expression of interfering RNA, Northern blot analysis was performed and quantified by densitometry in reference to rRNA ethidium bromide-stained bands. Residual *SEC6* expression was found to be 47 ± 6% in i*SEC6*-1 and 67 ± 10% in i*SEC6*-2, relative to the iControl strain, demonstrating functionality of the RNAi construct [± standard error of the mean (SEM), $n = 2$]. The strains were compared for their ability to grow in liquid minimal media (YNB + 2% glucose) at 37°C and all were found to grow similarly, suggesting that partial suppression of *SEC6* did not affect growth rate of *C. neoformans* (doubling times: 4.5 ± 0.4 h for iControl, 4.3 ± 0.2 h for i*SEC6*-1 and 4.2 ± 0.3 h for *SEC6*-2, $P = 0.79$).

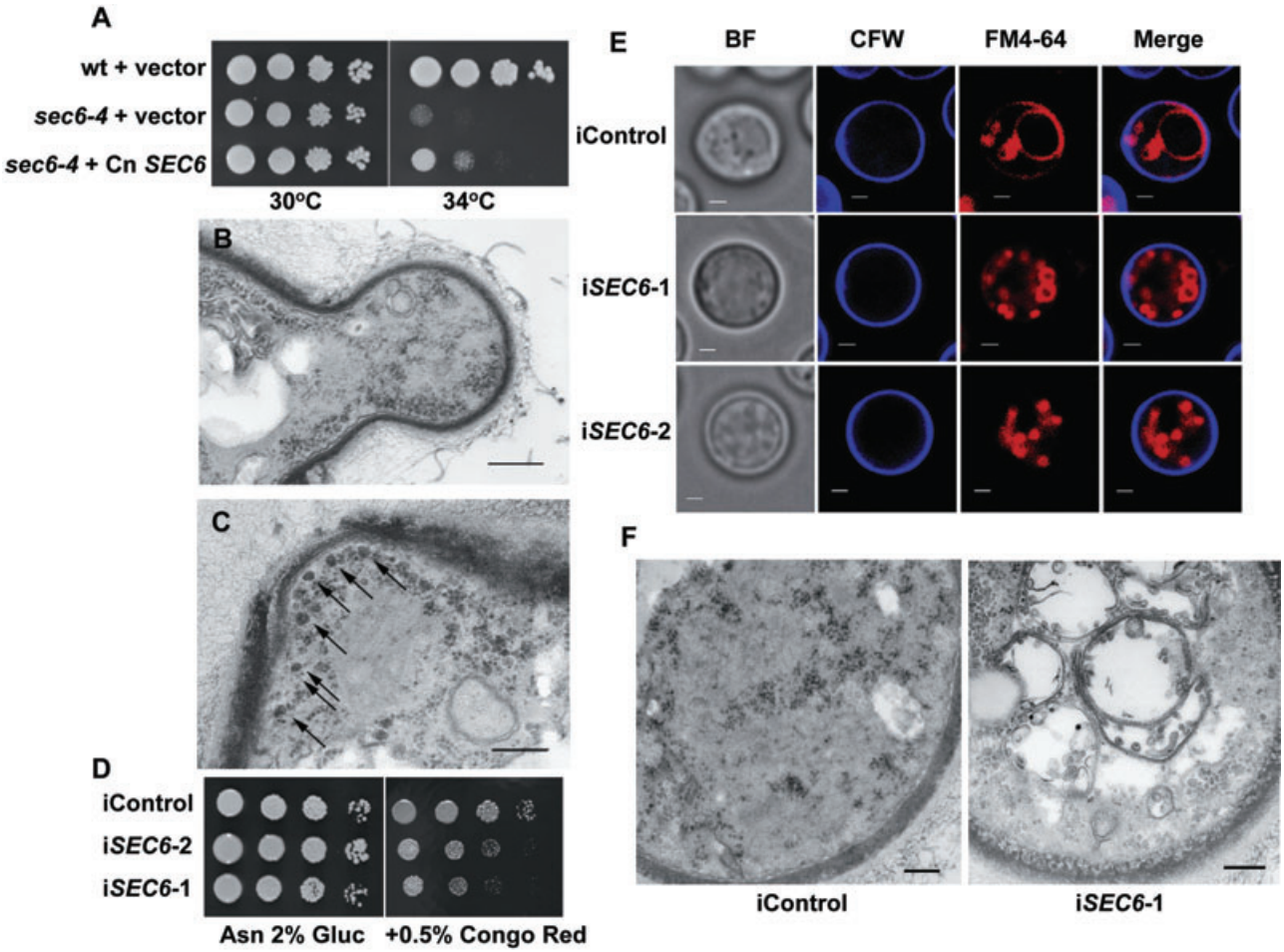

Fig. 1. *Cryptococcus neoformans* contains a Sec6 homologue.
A. Indicated strains of a *S. cerevisiae sec6-4* temperature-sensitive mutant expressing a cryptococcal *SEC6* homologue was assayed for growth at the indicated temperatures. Electron microscopy of wild-type iControl strain (B) or an i*SEC6*-1 strain of *C. neoformans* (C). Arrows point to accumulated vesicles, Scale bar = 500 nm in B and 250 nm in C.
D. Indicated *C. neoformans* cells were assayed for growth on asparagine agar containing 2% glucose with or without addition of 0.5% Congo Red for 3 days at 30°C.
E. Accumulation of intracellular vesicles in i*SEC6* cryptococcal strains: indicated mid-log phase cells were stained with FM4-64 and observed for epifluorescence. Scale bar: 1.0 μm.
F. Low power TEM of indicated strains. Scale bar: 200 nm.

Mutation of genes involved in exocytic functions in *S. cerevisiae* typically results in the accumulation of 100 nm vesicles that contain cell wall synthetic enzymes such as glucanase (Novick and Schekman, 1979; Harsay and Schekman, 2002). The Sec6 component of the exocyst complex in particular has been associated with regions of the bud tip and neck (TerBush and Novick, 1995). To examine this in detail, iControl (Fig. 1B) and the i*SEC6*-1 strains (Fig. 1C) were subjected to transmission electron microscopy (TEM) which demonstrated accumulation of typical 100 nm Sec6-associated vesicles in the bud necks of the RNAi-suppressed cells. To examine effects of *SEC6* suppression on cell wall integrity, the susceptibility of the i*SEC6*-1 and i*SEC6*-2 strains to a panel of cell wall stressors was compared with that of the empty-vector control using a spot-plate assay. A mild susceptibility to the glucan-binding dye Congo Red was observed (Fig. 1D) while no susceptibility was observed to caffeine, the chitin-binding dye calcofluor white or SDS (data not shown). Interestingly, Congo Red susceptibility correlated with the degree of *SEC6* suppression, as the i*SEC6*-1 strain exhibited a greater degree of Congo Red sensitivity than did the i*SEC6*-2 strain, demonstrating a gene dosage effect. This may suggest that synthesis of cryptococcal cell wall glucan, but not chitin, is sensitive to *SEC6* suppression.

The lipophilic dye FM4-64 was used in conjunction with light microscopy to determine if expression of *SEC6*-interfering RNA would result in accumulation of larger cytoplasmic vesicles in addition to the typical 100 nm

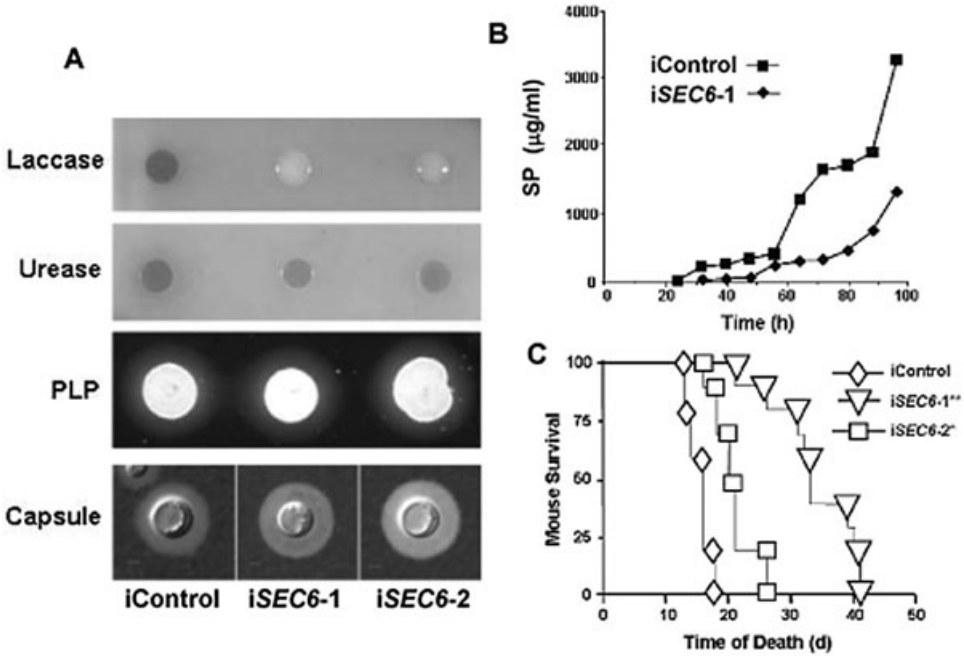

Fig. 2. Virulence-related phenotypes of cryptococcal i*SEC6*-suppressed strains.
A. Indicated strains were assayed for laccase by melanin formation, urease by incubation on Chistenson's media, phospholipase (PLP) by incubation on egg yolk media and capsule by Indian ink microscopy according to *Experimental procedures*.
B. Supernatants of indicated cells grown in yeast nitrogen base with 2% glucose were assayed for soluble polysaccharide (SP) by ELISA as described in *Experimental procedures*.
C. Cumulative mortality in mice injected in the lateral tail vein with 1×10^6 cells of i*SEC6*-1, i*SEC6*-2 or a control strain harbouring an empty vector (iControl). $*P < 0.01$, $**P < 0.001$ versus iControl strain.

vesicles described previously in yeast (Harsay and Bretscher, 1995). As shown in Fig. 1E, microscopic observation revealed accumulation of large FM4-64-stained cytoplasmic vesicles in the i*SEC6* strains compared with the empty-vector control, which was also demonstrated by TEM (Fig. 1F). Statistical analysis of FM4-64 staining from 10 cells of each strain demonstrated that the i*SEC6*-1 and i*SEC6*-2 strains contained an average of 7.2 ± 2.2 vesicles and 4.9 ± 1.9 vesicles respectively, whereas the strain carrying the empty vector (iControl) had an average of 2 ± 1.2 vesicles ($\pm$SEM, $P < 0.01$ iControl versus either i*SEC6*-1 or i*SEC6*-2).

*i*SEC6-*expressing strains demonstrate reduced levels of laccase, urease, and extracellular polysaccharide as well as attenuated virulence in mice*

Assessment of virulence-related phenotypes of *C. neoformans* found a significant reduction in laccase activity in i*SEC*6 strains compared with the iControl strain evidenced by reduced melanin pigment formation after incubation of the fungus in the presence of nor-epinephrine (Fig. 2A). In addition, using a quantitative laccase assay (Zhang *et al.*, 2006), the iControl strain produced 506 ± 24 U l^{-1} of extracellular laccase while both i*SEC6*-1 and i*SEC6*-2 cells displayed no detectable levels of extracellular laccase (< 25 U l^{-1}). Similarly, urease activity demonstrated by a zone of phenol red-detectable pH change in the presence of urea (Fig. 2A) was also reduced in the i*SEC6* strains. Further analysis of the i*SEC6*-1 strain demonstrated reduced soluble extracellular polysaccharide measured by enzyme-linked immunosorbent assay (ELISA) (Fig. 2B). In contrast, extracellular phospholipase activity measured on egg yolk media (Panepinto *et al.*, 2005) and capsule production assayed by Indian ink microscopy was unaffected (Fig. 2A), suggesting differing sensitivities of these two sets of virulence factors to *SEC6* suppression. Quantitative analysis of secreted protein and phospholipase activity using a radiometric assay (Siafakas *et al.*, 2007) showed similar amounts of secreted total protein in the *iSEC6*-1 strain versus the iControl strain (total protein per 4×10^9 cells, iControl: 220 ± 12 μg, i*SEC6*-1: 156 ± 13 μg; i*SEC6*-2: 182 ± 19 μg, where $\pm$SEM) as well as in PLB1 activity (phospholipase activity: iControl: 1.69 ± 0.11 U μg^{-1}, i*SEC6*-1: 1.22 ± 0.11 U μg^{-1}, i*SEC6*-2 2.02 ± 0.03 U μg^{-1}, $\pm$SEM) ($P > 0.05$ for iControl vs i*SEC6*-1 or versus i*SEC6*-2 strains for both secreted protein and PLB1). This suggests differences in secretory pathways between laccase, urease and secreted polysaccharide and that of capsule and secreted phospholipase activity.

As growth rates of the two i*SEC6* strains were equivalent at 37°C to the iControl strain, we assessed for virulence using a mouse model. For these experiments, 1×10^6 cells of i*SEC6*-1, i*SEC6*-2 or iControl strains were injected into the lateral tail vein of NIH Swiss Albino mice. Mouse health was monitored and moribund mice sacrificed in accordance with the UIC IACUC protocol guidelines. Previous experiments have found that the H99*ura5* strain transformed with empty plasmid (iControl) containing the *URA5* gene manifested near equivalent virulence to wild-type H99 strains, with only a 1 day increase in median time to death that was not significant (Hu *et al*., 2008). Experiments injecting the iControl and the i*SEC6* strains into groups of two mice each demonstrated retained prototrophy in all colonies from a sample of 100 colonies of each strain and successful PCR amplification of a 511 bp fragment of the episomal vector backbone in 10 of 10 colonies, each recovered from mouse brains after inoculation on YPD (data not shown). As shown in Fig. 2C, both the i*SEC6*-1 and i*SEC6*-2 strains exhibited a significant decrease in virulence relative to the empty-vector control ($P < 0.01$ and < 0.001 respectively). Interestingly, the reduction in virulence correlated with the amount of *SEC6* suppression, demonstrating a dosage-dependent effect on virulence.

Suppression of SEC6 *mRNA results in the absence of extracellular vesicles*

As laccase, urease and soluble polysaccharide secretion have been associated with extracellular vesicle production (Rodrigues *et al*., 2007; 2008), we next analysed one of the i*SEC6* mutants for production of these vesicles by TEM. As shown in Fig. 3A–C, while the wild-type control strains demonstrated robust production of 100–200 nm extracellular vesicles, the i*SEC6*-1 strain demonstrated no detectable vesicles. Further examination revealed 5425 vesicles in the iControl strain visualized in a total of 100 fields (10 fields examined in each of 10 sectioned grids), whereas examination of the same number of fields from i*SEC6*-1 cells incubated under the same conditions failed to identify any vesicles or any partial debris suggestive of vesicles ($P < 0.01$).

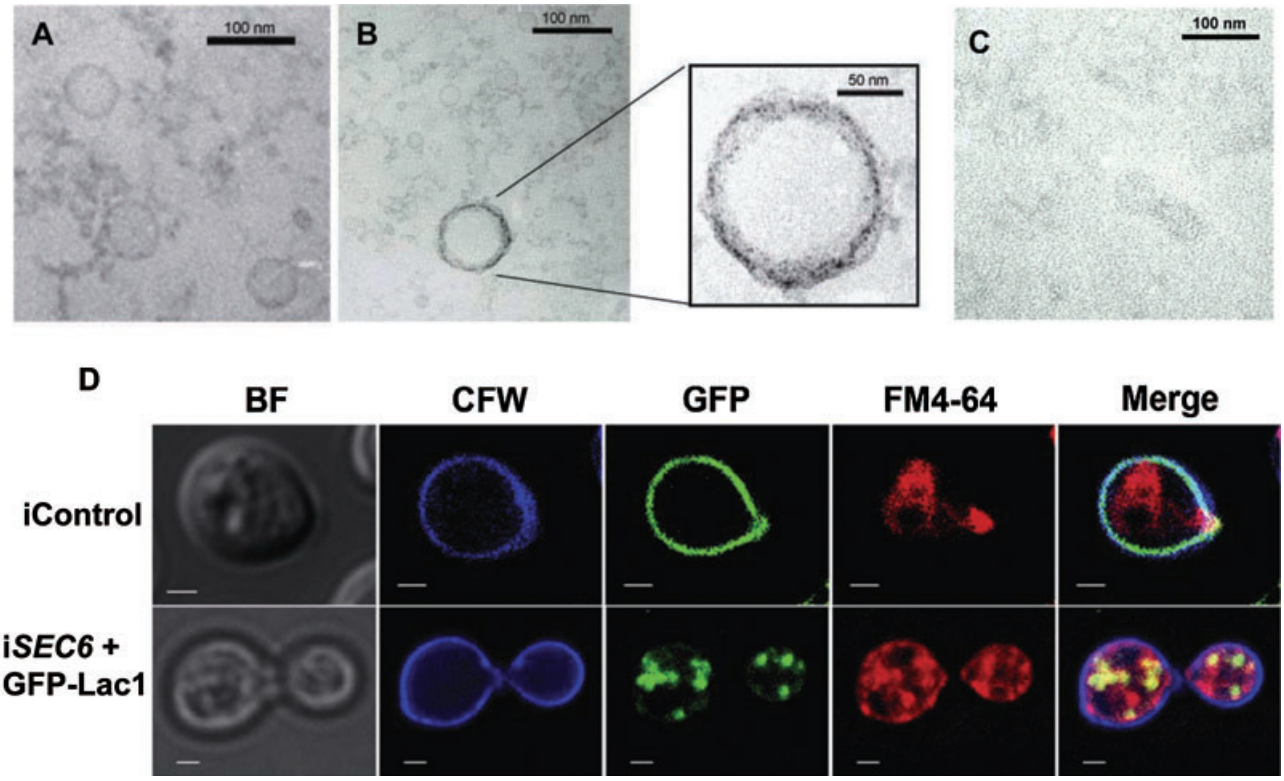

Fig. 3. Extracellular and intracellular vesicles in iControl and i*SEC6* strains of *C. neoformans.* H99FOA strains transformed with either empty plasmid (iControl; A and B) or an RNA interference suppressing SEC6 plasmid (i*SEC6*-1; C) were observed by TEM after 4 days' incubation. Bar = 100 nm.
D. Cryptococcal strains coexpressing either an i*SEC6* plasmid and a GFP-*LAC1* plasmid (i*SEC6* + GFP-Lac1) or GFP-Lac1 with an iControl plasmid (iControl) were induced for 24 h at 30°C for laccase production, stained with calcofluor white (CFW) and the vesicular dye, FM4-64, and epifluorescence observed by microscopy.

*Trafficking of laccase is defective in i*SEC6*-expressing strains*

Because laccase is predominantly a cell wall-associated virulence factor, we hypothesized that reduced laccase activity in the i*SEC6*-expressing strains may be due to aberrant trafficking of the laccase protein. Northern blot analysis of RNA obtained from cells induced for 3 h at 30°C showed identical laccase transcription between both i*SEC6* strains and the iControl (data not shown), suggesting the defect was not due to a *SEC6*-dependent transcriptional defect. Thus, to assess the effect of *SEC6* suppression on laccase trafficking, a Δ*lac1* ura$^-$ strain was cotransformed with the i*SEC6* construct and a plasmid expressing an N-terminal green fluorescent protein (GFP)–laccase fusion (i*SEC6* + GFP-Lac1). Retention of the i*SEC6* construct was verified in transformants by PCR-specific amplification of the i*SEC6* construct. As shown in Fig. 3D, the GFP–laccase fluorescence in the control wild-type strain was localized to the cell wall in all cells after 24 h of induction as previously reported (Zhu *et al.*, 2001). However, the i*SEC6* + GFP-Lac1 strain exhibited increased punctate cytoplasmic fluorescence with very little cell wall targeting in any of the cells over the same time period. Use of the lipophilic vesicle stain, FM4-64 showed that the GFP–laccase fluorescence was found almost exclusively within a subpopulation of the FM4-64-stained vesicles. Quantification of GFP fluorescence in 10 cells using calcofluor fluorescence for cell wall localization demonstrated a cell wall fluorescence of 1100 ± 210 arbitrary units for the iControl strain versus 170 ± 50 units for the i*SEC6* strain (±SEM; $P < 0.01$). Analysis of 16 cells demonstrated that 82.40 ± 4.49% of FM4-64-stained vesicles contained GFP–laccase. However, no GFP-positive vesicles were found in control cells. A small amount of laccase–GFP signal was observed in the cell wall of the i*SEC6* strain, most likely because of residual *SEC6* expression. Further analysis by immunoelectron microscopy confirmed the location of the laccase protein within the intracellular vesicles. As shown in Fig. 4, multiple gold beads labelled the small intracellular vesicular compart-

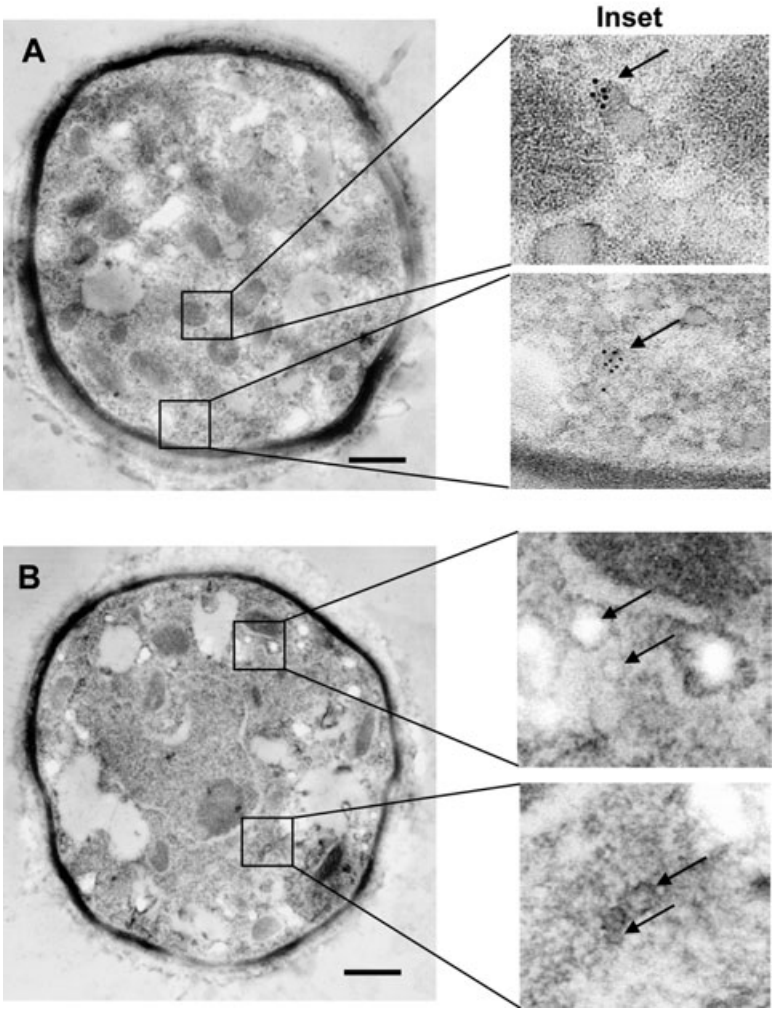

Fig. 4. Vesicular localization of laccase protein in the i*SEC6*-1 strain. The i*SEC6*-1 strain was induced for laccase expression similar to that for Fig. 3D and subjected to immunoelectron microscopy using 2 μg ml^{-1} of an anti-laccase monoclonal antibody (A) or an equivalent concentration of an isotype control antibody (B). Scale bar: 500 nm.

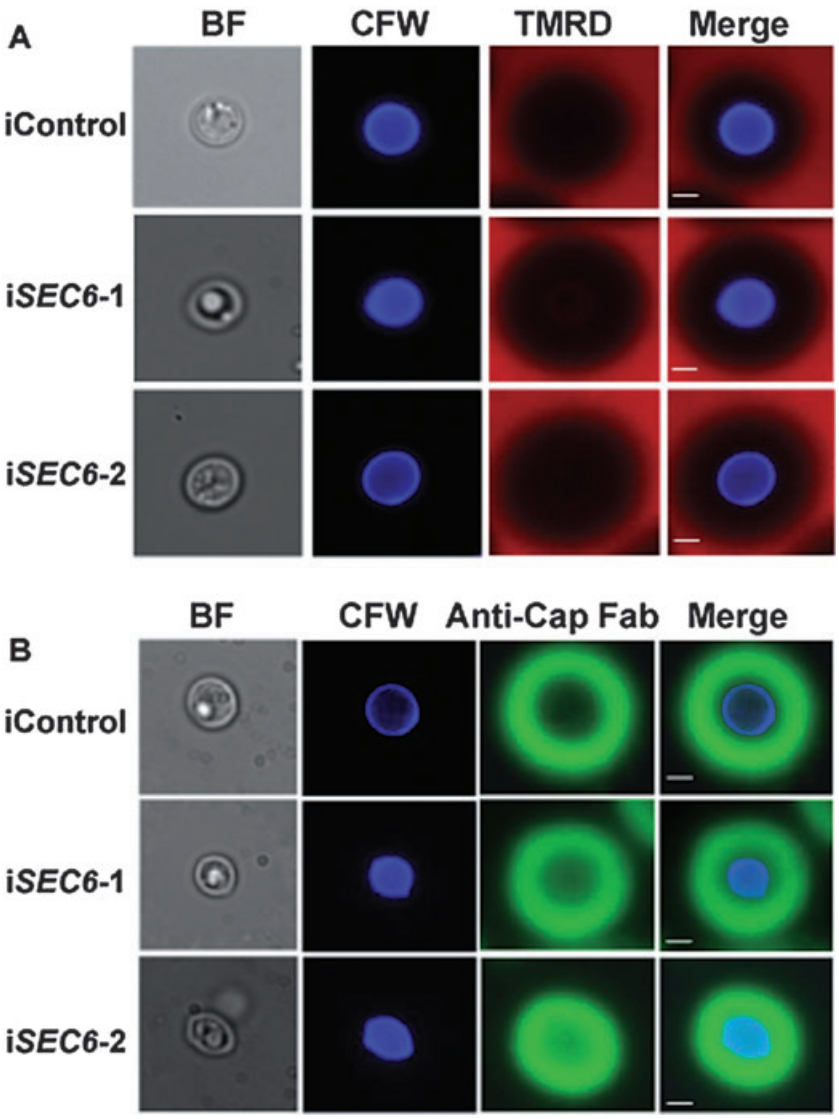

Fig. 5. Assessment of capsule production in the i*SEC6*-suppressed strains. Indicated strains were induced for capsule production and stained with calcofluor white (CFW) tetramethylrhodamine-dextran (TRMD) ~2000 kDa for 1 h and observed for epifluorescence (A) or an anti-capsular FAb (anti-Cap Fab) monoclonal fragment 3C2 (B). Scale bar: 5.0 μm.

ments (Fig. 4A) that was not evident using an isotype control antibody (Fig. 4B). Observation of 200 beads in multiple sections identified 193 within vesicular compartments, and seven in other regions of the cell, whereas only 10 beads were found in the isotype control-labelled cells in the same number of sections, with none in vesicular compartments. We did not assay for localization of intracellular polysaccharide because of the unavailability of reagents that would distinguish secreted polysaccharide from capsular polysaccharide. However, these data demonstrate that secretion of laccase-containing vesicles occurs within a *SEC6*-sensitive pathway.

Cryptococcus neoformans *i*SEC6 *strains produce extracellular capsule similar to wild type*

While Indian ink staining of the i*SEC6* strains in Fig. 2 showed a similar capsule radius, additional studies were performed to compare the size and porosity of the polysaccharide structure, using size exclusion of fluorescent-labelled dextran particles (Gates *et al*., 2004). Multiple cells were observed and statistics performed, as the H99 *C. neoformans* parent strain exhibits heterogeneity in size (Feldmesser *et al*., 2001). Using this method, the radius of dextran exclusion was found to be slightly increased in cells examined 15 min after staining (i*SEC6*-1*:* 7.08 ± 0.35 μm, i*SEC6*-2: 6.38 ± 0.39 μm and iControl: 5.01 ± 0.29 μm; ±SEM, $P < 0.001$ for i*SEC6*-1 and $P < 0.05$ for i*SEC6*-2 versus iControl diameter, Fig. 5A). An increased dextran exclusion diameter in the i*SEC6* mutants suggests that this high-molecular-weight dye did not penetrate as well in these mutants, consistent with a reduction in capsule porosity (Gates *et al*., 2004). However, the distribution of Fab fragments of monoclonal antibody against GXM (3C2) was not different among the strains (i*SEC6*-1*:* 0.51 ± 0.13 μm, i*SEC6*-2:

0.42 ± 0.09 μm and control: 0.47 ± 0.11 μm, $P > 0.05$), consistent with normal molecular architecture of GXM within the matrix of the capsule (Fig. 5B). In summary, capsule in the i*SEC6* mutants was grossly intact, with subtle reductions in porosity.

Discussion

Secretion of virulence factors to the cell periphery is required to allow these host-damaging agents to interact with their intended targets. In the present study, sensitivity to *SEC6* suppression was used to molecularly dissect possible differences in virulence factor secretory pathways. Previously, temperature-sensitive mutations in the *SEC6* locus of *S. cerevisiae* have been used to differentiate the sorting pathways of cargo proteins in yeast (Harsay and Schekman, 2002). Using this approach, secretion of virulence factors in *C. neoformans* was observed to exhibit divergent sensitivity to *SEC6* suppression. Laccase, urease and secreted soluble GXM were highly dependent on the *SEC6* locus, whereas the GXM-containing capsule as well as secretion of phospholipase activity showed less dependency. However, there is little similarity between the structures of the *SEC6*-dependent cargos that would suggest similar transport mechanisms. For example, laccase, which is targeted to the cell wall and secreted to the extracellular space (Zhu *et al*., 2001), contains a typical N-terminal leader sequence which is cleaved in the mature protein (Williamson, 1994) and a hydrophilic C-terminal fragment associated with cell wall targeting (Waterman *et al*., 2007). Urease, which is also detectable as a secreted protein (Rodrigues *et al*., 2008), has no consensus leader sequence (Cox *et al*., 2000), and secreted polysaccharide contains negligible protein (Eisenman *et al*., 2007). Despite these structural differences, all three virulence factors were recently detected in isolated extracellular vesicles in *C. neoformans* believed to represent fungal exosome structures (Rodrigues *et al*., 2008). Therefore, TEM was used in the present study to evaluate whether the production of fungal exosome was also dependent on *SEC6*. The lack of extracellular vesicles in the mutant suggests that fungal exosomes are a physiological phenomenon and not an artefact of lipid self-assembly, or cell lysis, because a mutation can eliminate them. It is possible that a defect in vesicle stability in the i*SEC6* strain could have resulted in the observed defect, although such a scenario would have resulted in a release of the exosomal cargo such as laccase, which was not detected in extracellular assays. Furthermore, as would be expected for a secretory pathway mutation, the abrogation of extracellular vesicles was associated with an increased accumulation of intracellular vesicles after a mutation with the secretory pathway, further strengthening the case for the existence of fungal exosomes.

Interestingly, extracellular capsule production as well as phospholipase secretion occurred in the absence of exosome production and was less sensitive to *SEC6* suppression. Cell wall integrity was essentially intact, but showed a mild defect that may be due to a minor population of synthetic enzymes of this structure that are aberrantly secreted. This differing sensitivity to *SEC6* suppression may be due to different pathways of transit through the plasma membrane. For example, extracellular phospholipase B, the principal component of phospholipase activity in this fungus (Chen *et al*., 2000; Cox *et al*., 2001), is anchored to the plasma membrane by a glycosylphosphatidylinositol anchor and released to the extracellular space after cleavage of the mannose linkage (Siafakas *et al*., 2007). In contrast, extracellular exosomes from other eukaryotes such as vertebrates have been shown to originate via multivesicular bodies from alternative structures such as the *trans*-Golgi network (Fevrier and Raposo, 2004). Indeed, lipid analysis of cryptococcal exosomes also suggests their origin as non-plasma-membrane-derived vesicles (Rodrigues *et al*., 2008). Recently, GXM polysaccharide has also been found to reside within intracellular vesicles of a mutant of a cryptococcal Sec4 homologue (Yoneda and Doering, 2006), suggesting an interrelationship between these trafficking pathways. Interestingly, these previous studies found exclusively 100 nm vesicles in Sec4 mutants, rather than the large vesicles demonstrated in the i*SEC6* strains. This may be due to the role of Sec6 in more proximal events of secretion (Harsay and Bretscher, 1995) versus the role of Sec4 in distal events such as plasma membrane fusion (Salminen and Novick, 1987) or to the differing ways in which the two mutants were generated, i.e. temperature sensitive versus RNAi. It is important to note that we did not directly address the origin of the Sec6-associated vesicles including whether they are a product of recycling endosomes as previously described (Harsay and Schekman, 2002).

Sorting of virulence factors by both exosome-dependent and exosome-independent pathways may allow specific spatial or temporal regulation of exocytosis. Such regulation of secretion has been demonstrated in polarized epithelial cells in which distinct vesicles are targeted to the apical and basolateral surfaces (Mostov *et al*., 2000), allowing both constitutive and regulated pathways of secretion (Burgess and Kelly, 1987). In the present case, while expression of soluble GXM and urease is mostly a constitutive phenomenon (Rodrigues *et al*., 2008), formation of capsule is highly regulated, induced by glucose as well as low iron and high carbon dioxide concentrations (Granger *et al*., 1985; Zaragoza *et al*., 2006). The exosome-dependent laccase is also highly regulated, but divergently from capsule in that it is highly repressed by glucose (Williamson, 1994).

Given its effect on the secretion of several virulence factors it is perhaps not surprising that *SEC6* suppression resulted in a dose-dependent reduction in virulence in mice even after a large intravenous inoculum. This was despite robust growth of the i*SEC6* mutants in minimal media at 37°C and the presence of virtually normal capsule. This implies that *SEC6*-dependent pathways are an important component of virulence and the absence of exosome secretion could have contributed to this reduced virulence. It is interesting to speculate that immune responses to mammalian exosome proteins (Admyre *et al*., 2008) may have originated from a primordial response to pathogen exosome-transported virulence factors such as laccase. The results presented here provide the initial framework for further study of the molecular pathways of virulence factor secretion within fungal exosomes that may provide a critical component of host–pathogen interactions.

Experimental procedures

Fungal strains, plasmids and media

Cryptococcus neoformans ATCC 208821 (H99) was a generous gift of J. Perfect and strain H99 *ura5* was described previously (Zhu and Williamson, 2004). The asparagine minimum selective media used for transformant selection and for detection of laccase production have been previously described (Zhu and Williamson, 2003). Plasmid pCIP containing the *URA5* gene was a kind gift of K.J. Kwon-Chung. Construction of the pKUTAP plasmid used for RNAi expression was described previously (Liu *et al*., 2006).

Complementation of a S. cerevisiae sec6-4 *mutant with Cn* SEC6

First-strand cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad) as directed by the manufacturer using total RNA isolated from log-phase cells of wild-type *C. neoformans* strain H99 as template. The *C. neoformans SEC6* cDNA fragment was amplified from first-strand cDNA using sense primer 5′-GGAGGACCTAGGATGTCATCAG CAAACCCATCGTC and antisense primer 5′-TTATTAG AGCTCCACGGGTAAGCCTATGACTGC. After digestion with SacI and AvrII, the cDNA fragment was cloned into the *S. cerevisiae* expression vector pYX112 (R&D Systems) in frame with the *TPI* promoter to generate p*CnSEC6*. Yeast cells of *S. cerevisiae* strain NY-13 (*MATa, ura3–52*) and NY-19 (*MATa, ura3–52, sec6-4*), generous gifts of Peter Novick, Yale University, were transformed with either p*CnSEC6* or the empty vector by lithium acetate/polyethylene glycol and selected on uracil dropout medium as described (Hu *et al*., 2008). Temperature-dependent growth was assessed on synthetic dextrose medium (2% dextrose, 6.7 mg ml^{-1} YNB without amino acids) using a spot-plate assay. Plates were incubated for 3 days at the indicated temperatures.

*Construction of an i*SEC6 C. neoformans *mutant strain*

The cryptococcal shuttle vector, pORA-KUTAP, containing the *URA5* transformation marker, was used to effect RNAi suppression of *SEC6* after the method of Liu *et al*. (2002), modified by replacement by a 500 bp fragment of Intron I of *LAC1* for the intervening region between sense and antisense strands. First, pORA-KUTAP containing sequence of the EF-1α terminator was digested with EcoRI and ligated simultaneously to a mixture of a XhoI-digested PCR-amplified *LAC1* intron fragment from H99 (using primers: IntronS-Xho: 5′-GCCGCTCGAGATCCTAATCGGTAAATATT TCTTTC and IntronA-Xho: 5′-GCCGCTCGAGCGTCGGT ATAGCTAAATTG) and a second XhoI and EcoRI-digested PCR-amplifed fragment of the *SEC6* ORF (using primers iSEC6-S-RI: 5′-GAATTCGAATTCCCTGCCGACTATGAGAT TACCGC and iSEC6-A-XI: 5′-CTCGAGCTCGAGCCCTG GTAACACCTCTTCCGG), to produce pORA-iSec6. The plasmid was recovered, sequence verified, linearized with SceI and transformed into *C. neoformans* H99 Matα, *ura5* cells using electroporation by standard methods (Erickson *et al*., 2001). An H99 Matα, *ura5* strain transformed with a pORA-KUTAP plasmid without the RNAi construct served as a control for *URA5* expression for *in vivo* studies (iControl strain). Transformants were selected by equivalent copy number demonstrated on uncut Southern analysis as described previously (Zhang *et al*., 1999). For *in vitro* expression, cells were incubated in asparagine media for the indicated times and examined by deconvolution microscopy using an IX-70 Olympus microscope and the soft agar embedding technique described previously (Liu *et al*., 2006).

Laccase activity assay

To assess laccase activity by the production of melanin, age-matched strains were patched onto ASN agar without glucose with 50 μg ml^{-1} norepinephrine. Plates were incubated at 30°C for 24 h and photographed. For quantitative analysis of laccase activity, a colorimetric assay was used as described previously (Zhang *et al*., 2006), using the laccase substrate, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sufonic acid) (ABTS, Sigma, St Louis, MO, USA). The assay was performed in triplicate. 1 U is defined as 0.001A_{415} in 1 h.

Capsule size measurements

iControl and i*SEC6* cells were visualized in an Indian ink suspension using an Olympus AX70 microscope. Pictures were taken using a QImaging Retiga 1300 Digital camera using the QCapture Suite V2.46 software (QImaging, Burnaby BC, Canada). Capsule size of 100 cells of each strain was measured in these images using Adobe Photoshop 7.0 for Windows (San Jose, CA, USA). Average and standard deviation was calculated.

Assessment of capsular permeability

To induce capsule, yeast cells were grown in 3 ml of RPMI in a 12 well plate incubated in a CO_2-enriched environment

(GasPak EZ CO_2, Becton Dickinson) in a 37°C water jacketed incubator for 4 days. TMR-Dextran 2000 kDa (TMRD, Invitrogen) staining of *C. neoformans* capsule on cells grown under capsule-inducing conditions was performed as described previously (Gates *et al*., 2004). Cells were also stained with calcofluor white and visualized by fluorescence microscopy 15 min after staining with TMRD. Staining with AlexaFluor 488-conjugated anti-capsular Fab fragments (generous gift of Tom Kozel, University of Nevada, Reno) was performed as described previously, except with the inclusion of calcofluor white stain in the reaction (Gates *et al*., 2004). The distance between the outside of the cell wall and the staining front was measured using Slidebook software.

Virulence factor expression and virulence studies

Capsule was measured by Indian ink microscopy (Erickson *et al*., 2001) and urease production by incubation on Christensen's agar (Cox *et al*., 2000), and the method of Liu *et al*. (1999) was used to measure laccase activity. Phospholipase activity was measured using an egg yolk medium (Panepinto *et al*., 2005) as well as by a radiometric quantitative assay (Siafakas *et al*., 2007). Virulence studies were conducted according to a previously described intravenous mouse meningoencephalitis model (Salas *et al*., 1996) using 10 Swiss Albino mice. Animal studies were approved by the University of Illinois at Chicago Animal Care Committee. Studies involving human tissue were approved by the University of Illinois at Chicago Office for the Protection of Research Subjects and the Institutional Review Board (protocol # 2004–0600). *C. neoformans* strains were recovered from brains of mice by inoculation on YPD agar and retention of plasmids verified by growth on yeast nitrogen base + 2% glucose as well as amplification from DNA of a 511 bp fragment of the pKUTAP shuttle vector using primers, STAB-446S: 5′-CGGCA TATCCACGGTGAAAACCAG and STAB-957A: 5′-GGTGA GCATAATGAGCATTCGAGGG.

Extracellular vesicle purification

Cryptococcus neoformans strains were grown in minimal media for 3 days at 30°C. Vesicle purification was carried out as described by Rodrigues *et al*. (2008). Briefly, fungal cells were separated from culture supernatants by centrifugation at 4000 *g* for 15 min at 4°C. The supernatants were collected and centrifuged at 15 000 *g* (4°C) to remove smaller debris. Supernatant was concentrated approximately 20-fold using an Amicon ultrafiltration system (cut-off, 100 kDa). Concentrated culture fluid was again centrifuged as described above and the resulting supernatant was then centrifuged at 100 000 *g* for 1 h at 4°C. The supernatants were then discarded, and the pellets were washed by several resuspension and centrifugation steps, each consisting of 100 000 *g* for 1 h at 4°C with 0.1 M Tris-buffered saline.

Electron microscopy

Transmission electron microscopy was used to visualize vesicles isolated from supernatants. The pellets obtained after washing and centrifugation at 100 000 *g* were fixed in 2% glutaraldehyde in 0.1 M cacodylate at room temperature for 2 h and were then incubated overnight in 4% formaldehyde, 1% glutaraldehyde and 0.1% phosphate-buffered saline. The samples were incubated for 90 min in 2% osmium, serially dehydrated in ethanol, and embedded in Spurr's epoxy resin. Thin sections were obtained on a Reichert Ultracut and stained with 0.5% uranyl acetate and 0.5% lead citrate. Samples for immunoelectron microscopy were prepared as described (Zhu *et al*., 2001) using 2 μg ml^{-1} of either laccase monoclonal antibody clone G3P4D3 or an equivalent concentration of a *c-myc* monoclonal IgG1 as a negative isotype control (Invitrogen). Samples were observed in a JEOL 1200EX transmission electron microscope operating at 80 kV.

Measurement of exopolysaccharides

Five thousand *C. neoformans* cells were grown in YNB minimal media for 96 h at 30°C. A 50 μl aliquot of each culture was analysed every 8 h for exopolysaccharide quantification. Quantification of exopolysaccharide was done by capture ELISA (Casadevall *et al*., 1992), using monoclonal antibody 2D10, an IgM monoclonal antibody with specificity for secreted cryptococcal polysaccharide. Purified polysaccharide was used as a positive control.

Statistics

Intracellular vesicle numbers of fungal cells were counted for 10 cells in the largest diameter plane for each cell and numbers compared between observed cells using a two-tailed Student's *t*-test as described (Mendenhall, 1975). Northern blot signals of i*SEC6* strains were quantified by densitometry, normalizing for ethidium bromide staining of rRNA bands and signals averaged and expressed as mean intensity. The capsule radius was measured in 10 cells for each strain and means compared using ANOVA with Tukey's test post hoc. Errors were expressed as SEM. Phospholipase activities were compared using a Mann–Whitney test. Statistical significance of mouse survival times was assessed by Kruskall–Wallis analysis (ANOVA on Ranks).

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