Molecular Microbiology (2011) 81(1), 206–218 ■
doi:10.1111/j.1365-2958.2011.07686.x
First published online 17 May 2011

Role for Golgi reassembly and stacking protein (GRASP) in polysaccharide secretion and fungal virulence

Lívia Kmetzsch,[1‡] Luna S. Joffe,[3,4‡] Charley C. Staats,[1,2] Débora L. de Oliveira,[3] Fernanda L. Fonseca,[3] Radames J.B. Cordero,[5] Arturo Casadevall,[5,6] Leonardo Nimrichter,[3] Augusto Schrank,[1,2] Marilene H. Vainstein[1,2†] and Marcio L. Rodrigues[3*†]

[1]*Centro de Biotecnologia and* [2]*Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves 9500, 43421, Caixa Postal 15005, Porto Alegre, RS 91501-970, Brazil.*

[3]*Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-902, Brazil.*

[4]*Instituto de Bioquímica Médica, Programa de Glicobiologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, 21941-902, Brazil.*

[5]*Department of Microbiology and Immunology and* [6]*Division of Infectious Diseases of the Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave, Bronx, NY 10461, USA.*

Summary

Secretion of virulence factors is a critical mechanism for the establishment of cryptococcosis, a disease caused by the yeast pathogen *Cryptococcus neoformans*. One key virulence strategy of *C. neoformans* is the release of glucuronoxylomannan (GXM), a capsule-associated immune-modulatory polysaccharide that reaches the extracellular space through secretory vesicles. Golgi reassembly and stacking protein (GRASP) is required for unconventional protein secretion mechanisms in different eukaryotic cells, but its role in polysaccharide secretion is unknown. This study demonstrates that a *C. neoformans* functional mutant of a GRASP orthologue had attenuated virulence in an animal model of cryptococcosis, in comparison with wild-type (WT) and reconstituted cells. Mutant cells manifested altered Golgi morphology, failed to produce typical polysaccharide capsules and showed a reduced ability to secrete GXM both *in vitro* and during animal infection. Isolation of GXM from cultures of WT, reconstituted or mutant strains revealed that the GRASP orthologue mutant produced polysaccharides with reduced dimensions. The mutant was also more efficiently associated to and killed by macrophages than WT and reconstituted cells. These results demonstrate that GRASP, a protein involved in unconventional protein secretion, is also required for polysaccharide secretion and virulence in *C. neoformans*.

Accepted 25 April, 2011. *For correspondence. E-mail marcio@micro.ufrj.br; Tel. (+55) 21 2598 3035; Fax (+55) 21 2560 8344. †M.H.V. and M.L.R. share senior authorship on this article. ‡L.K. and L.S.J. contributed equally to this work.

Introduction

Cryptococcus neoformans is a yeast-like pathogen associated with high mortality rates in immunosuppressed individuals (Park *et al*., 2009; Prado *et al*., 2009). *C. neoformans* virulence is dependent on the expression of a number of virulence factors, including enzymes, pigments, polysaccharides and lipids (Li and Mody, 2010). Like many bacterial pathogens, *C. neoformans* is surrounded by a polysaccharide capsule that is an important virulence factor (Zaragoza *et al*., 2009). Capsule formation in this fungal pathogen requires intracellular polysaccharide synthesis (Feldmesser *et al*., 2001; Yoneda and Doering, 2006), followed by secretion of capsular components to the extracellular space and their incorporation into the cell surface (Rodrigues *et al*., 2008a; Zaragoza *et al*., 2009). Capsule expression is purportedly the most important constraint for cryptococcal virulence (McClelland *et al*., 2005).

Glucuronoxylomannan (GXM), the major capsular component of *C. neoformans*, is presumably synthesized in the Golgi and targeted to the cell surface (Yoneda and Doering, 2006). The polysaccharide then traverses the cell wall in vesicles that reach the extracellular space (Rodrigues *et al*., 2007), where GXM is used for enlargement of the cryptococcal capsule (Zaragoza *et al*., 2006). Exposure of *C. neoformans* to brefeldin A, which affects the formation of Golgi-related transport vesicles, results in a strong inhibition of capsule assembly (Hu *et al*., 2007). Consequently, the Golgi apparatus is suggested to be required for GXM synthesis and secretion, based on

results of two independent studies. Yoneda and Doering demonstrated that a *C. neoformans* mutant lacking expression of Sav1p, a putative secretory vesicle-associated Rab GTPase essential for exocytosis, accumulates intracellular, post-Golgi vesicles containing GXM (Yoneda and Doering, 2006). This is in agreement with results described by Panepinto and colleagues, who showed that *C. neoformans* cells with deficient expression of Sec6p, which mediates polarized targeting of secretory vesicles to active sites of exocytosis, had a decreased rate of GXM secretion (Panepinto *et al*., 2009). Although both studies clearly indicated an association of Golgi-derived pathways with GXM secretion, the fact that capsular expression was apparently normal in both *sav1* and *sec6* mutants suggested that other components of Golgi-associated secretory pathways could have a role in GXM traffic in *C. neoformans*.

Golgi reassembly and stacking proteins (GRASPs) have been implicated in the stacking of Golgi cisternae, vesicle tethering and mitotic progression (Nickel and Seedorf, 2008; Nickel and Rabouille, 2009). GRASP is primarily attached peripherally to the cytoplasmic surface of Golgi membranes, but its distribution into other cellular compartments is also expected (Nickel and Rabouille, 2009). In *Dictyostelium discoideum*, the single GRASP orthologue (GrpA) is required for unconventional secretion of acyl-coenzyme A-binding protein (AcbA) during spore differentiation (Kinseth *et al*., 2007), in a process that requires secretory vesicles (Cabral *et al*., 2010). GRASP is also required for the delivery of integrin α subunits to the plasma membrane of *Drosophila melanogaster* in a Golgi-independent manner (Schotman *et al*., 2008). More recently, it has been demonstrated GRASP is also required for starvation-induced secretion of AcbA in *Saccharomyces cerevisiae* and *Pichia pastoris* (Duran *et al*., 2010; Manjithaya *et al*., 2010).

The fact that a number of the cryptococcal virulence factors are exocellular components (Li and Mody, 2010) implies that secretory activity is directly linked to virulence in *C. neoformans*. Most of the *C. neoformans* virulence factors are released to the extracellular space apparently through unconventional mechanisms of secretion (Rodrigues *et al*., 2007; 2008a,b; Nosanchuk *et al*., 2008; Casadevall *et al*., 2009). The possible link between cryptococcal virulence and unconventional secretion led us to investigate the role of GRASP in an animal model of *C. neoformans* infection. Our results suggest that virulence is attenuated in a GRASP orthologue mutant of *C. neoformans*, which was associated with a defect in the ability of yeast cells to secrete GXM. To our knowledge, this is the first report showing a role for a GRASP orthologue in microbial virulence and in polysaccharide secretion in eukaryotic cells.

Results

The construction of the GRASP orthologue mutant strain used in this study is summarized in Fig. 1. GRASP 5′ and

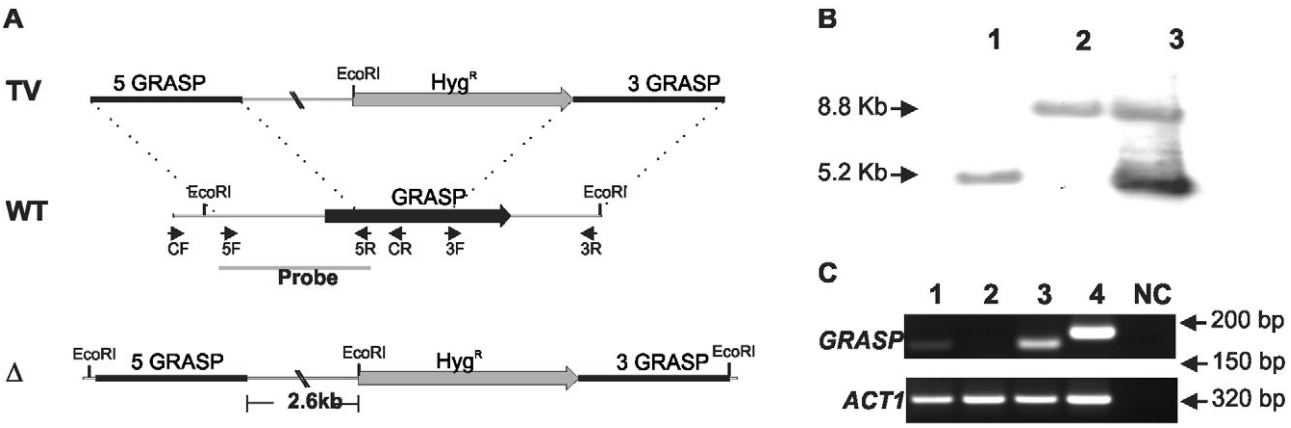

Fig. 1. Deletion and complementation of the *C. neoformans GRASP* orthologue.
A. Scheme for the construction of the mutant strain. *GRASP* gene was replaced with the hygromycin-resistant marker (Hyg^R) cassette (grey box). GRASP 5′ and 3′ flanks (5 GRASP and 3 GRASP respectively) were fused with Hyg^R cassette by Delsgate methodology (Garcia-Pedrajas *et al*., 2008). The resulting targeting vector (TV) was used for *C. neoformans* transformation. The wild-type locus of *GRASP* (WT) and the position of primers used for *GRASP* gene disruption are also indicated. The black bar scale corresponds to 500 base pairs (bp). The cleavage sites of EcoRI restriction enzyme are indicated in the deletion scheme.
B. Southern blot analysis. Genomic DNA (10 μg) from WT (lane 1), *grasp* mutant (lane 2) and *grasp::GRASP* reconstituted (lane 3) strains were digested with EcoRI restriction enzyme. The 5′ gene flank was used as probe in Southern hybridization. Numbers at left indicate the hybridization signal sizes based upon the position of molecular size marker.
C. Semi-quantitative RT-PCR with cDNA from WT (lane 1), *grasp* mutant (lane 2) and *grasp::GRASP* reconstituted (lane 3) strains as template. Numbers at right indicate the length of the transcript amplification for *GRASP* (upper panel) and *ACT1* (lower panel) genes. Lane 4: positive control with genomic DNA as template. NC: negative control of the PCR reaction.

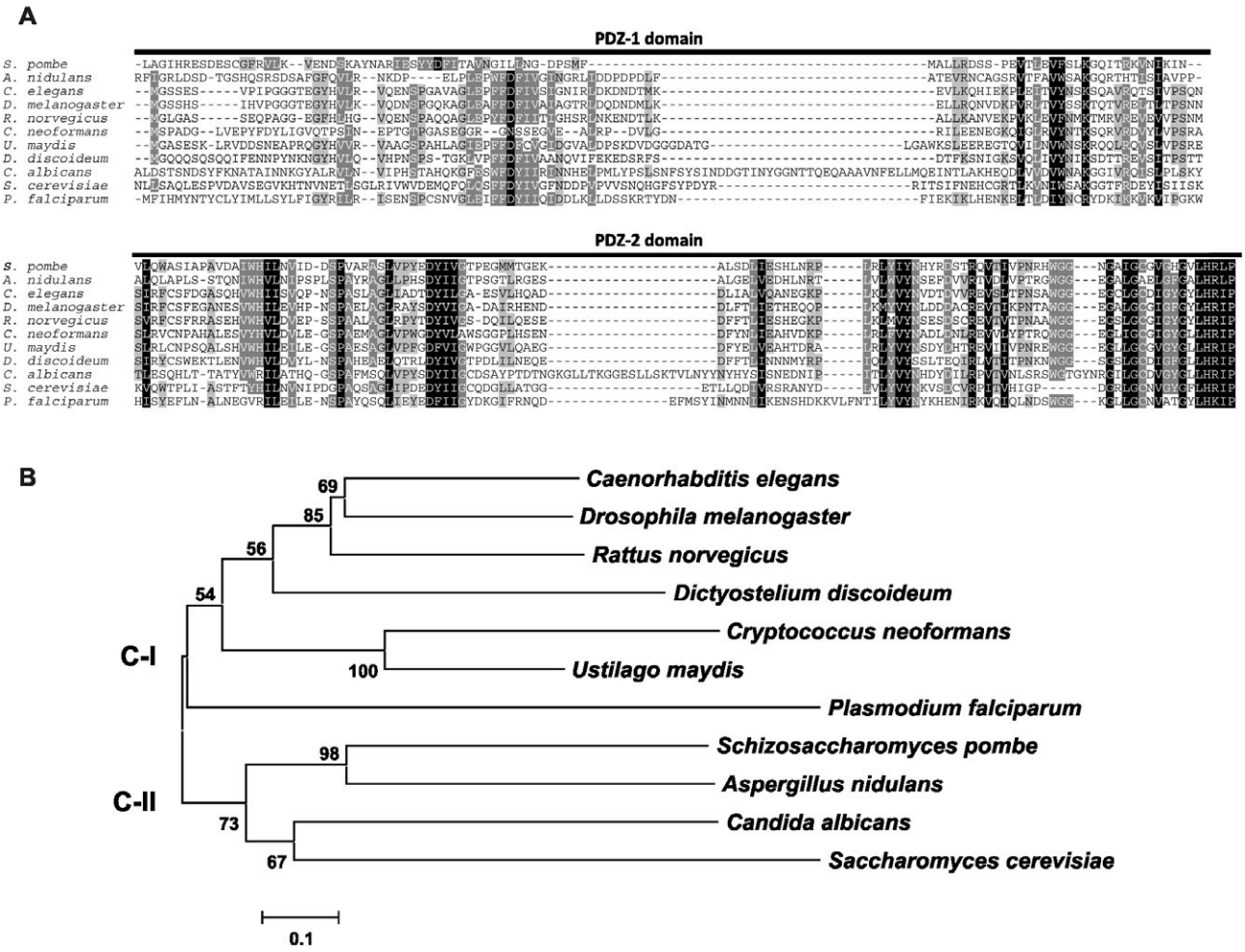

Fig. 2. Phylogenetic analysis of the *C. neoformans GRASP* orthologue.
A. Alignment of the *GRASP* PDZ domains (PDZ-1 and PDZ-2) from *R. norvegicus* (AAB81355.2), *C. elegans* (NP_501354), *D. melanogaster* (AAF49092), *S. cerevisiae* (NP_010805), *D. discoideum* (EAL60823), *S. pombe* (NP_593015.1), *P. falciparum* (AAN35366), *A. nidulans* (Broad Institute Accession No. ANID_11248), *U. maydis* (Broad Institute Accession No. UM01076), *C. albicans* (Broad Institute Accession No. CAWG_01021) and *C. neoformans* (Broad Institute Accession No. CNAG_03291) using CLUSTALX2.
B. Phylogenetic analysis applying the Neighbour-Joining method including *GRASP* sequences from distinct eukaryotic organisms listed above. The phylogeny tree splits into two major clades. C-I and C-II represents clades I and II respectively. The bar marker indicates the genetic distance, which is proportional to the number of amino acid substitutions.

3′ flanks were fused with the hygromycin-resistant marker cassette by Delsgate methodology (Garcia-Pedrajas *et al*., 2008; Kmetzsch *et al*., 2010; 2011). The resulting targeting vector was used for *C. neoformans* transformation, which was monitored by Southern hybridization and semi-quantitative RT-PCR. We identified a putative *GRASP* orthologue in the *C. neoformans* var. *grubii* (serotype A) genomic database (Broad Institute, Accession No. CNAG_03291.2). The 1051 bp putative *GRASP* has four introns and predicts a 256-amino-acid protein. GRASPs are characterized by the presence of two PDZ-like domains in the N-terminal region (Kinseth *et al*., 2007). A BLAST search using the PDZ domains sequences from *Rattus norvegicus* GRASP revealed that these regions are also present in the *C. neoformans* GRASP orthologue. Sequence comparisons revealed that the identity range for the PDZ-1 and PDZ-2 domains were 40% and 48% respectively (Fig. 2A).

A broad phylogenetic analysis including GRASP sequences from distinct eukaryotic organisms rendered a phylogeny tree split into two major clades (Fig. 2B). Clade C-II encompasses the GRASPs from the majority of the fungi analysed, all belonging to the ascomycetes. Clade C-I harboured the GRASPs from the basidiomycetous fungi *C. neoformans* and *Ustilago maydis*, as well as those from mammalian (*R. norvegicus*), fly (*D. melanogaster*), worm (*Caenorhabditis elegans*), protist (*Plasmodium falciparum*) and amoeba (*D. discoideum*).

GRASP is primarily localized to the cytoplasmic surface of Golgi membranes, where it is apparently involved in the

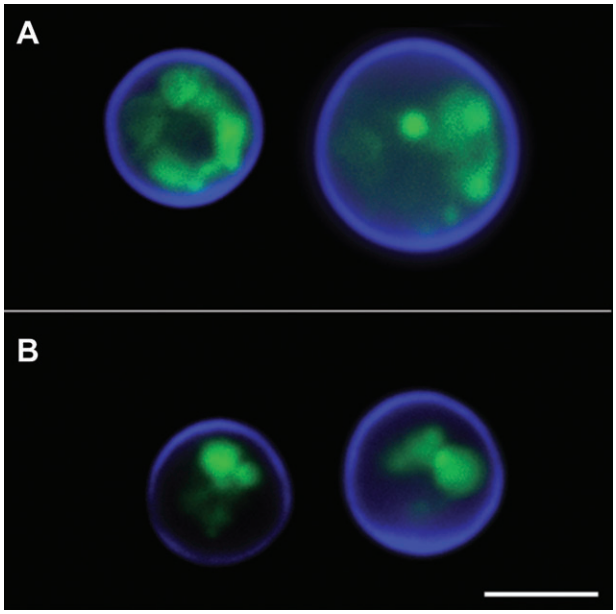

Fig. 3. Modified morphology of the Golgi apparatus in *C. neoformans* GRASP orthologue mutant cells. WT cells (A) and the GRASP orthologue mutant (B) were sequentially incubated with C6-NBD-ceramide for Golgi visualization (green) and Uvitex 2B for staining of the cell wall (blue). Scale bar, 3 μm.

stacking of Golgi cisternae in eukaryotic cells (Vinke *et al.*, 2011). Based on this observation, we evaluated whether lack of GRASP would affect Golgi morphology in *C. neoformans*. The Golgi marker *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazole)]-6-aminocaproyl-D-erythro-sphingosine (C6-NBD-ceramide) stained wild-type (WT) cells producing a pattern that was very similar to that described for *S. cerevisiae* (Levine *et al.*, 2000) (Fig. 3). Mutant cells, however, showed altered Golgi morphology. In these cells, the Golgi apparatus appeared to be compacted and limited to a smaller area of the cytoplasm.

Extracellular molecules are crucial to the cryptococcal pathogenesis (Li and Mody, 2010). Post-Golgi secretion is also involved in the release of *C. neoformans* virulence factors (Yoneda and Doering, 2006; Panepinto *et al.*, 2009; Chayakulkeeree *et al.*, 2011). These observations and the recently described roles of GRASP in unconventional secretion (Kinseth *et al.*, 2007; Levi and Glick, 2007; Schotman *et al.*, 2008; Duran *et al.*, 2010; Manjithaya *et al.*, 2010) led us to hypothesize that Golgi integrity would be required for the pathogenic mechanisms of *C. neoformans*. Therefore, we evaluated some virulence determinants in the GRASP orthologue mutant in comparison with both WT and complemented cells.

The GRASP orthologue mutant showed normal growth rates at 37°C (Fig. 4A). In an intranasal model of animal infection, this mutant caused death of all animals in 18

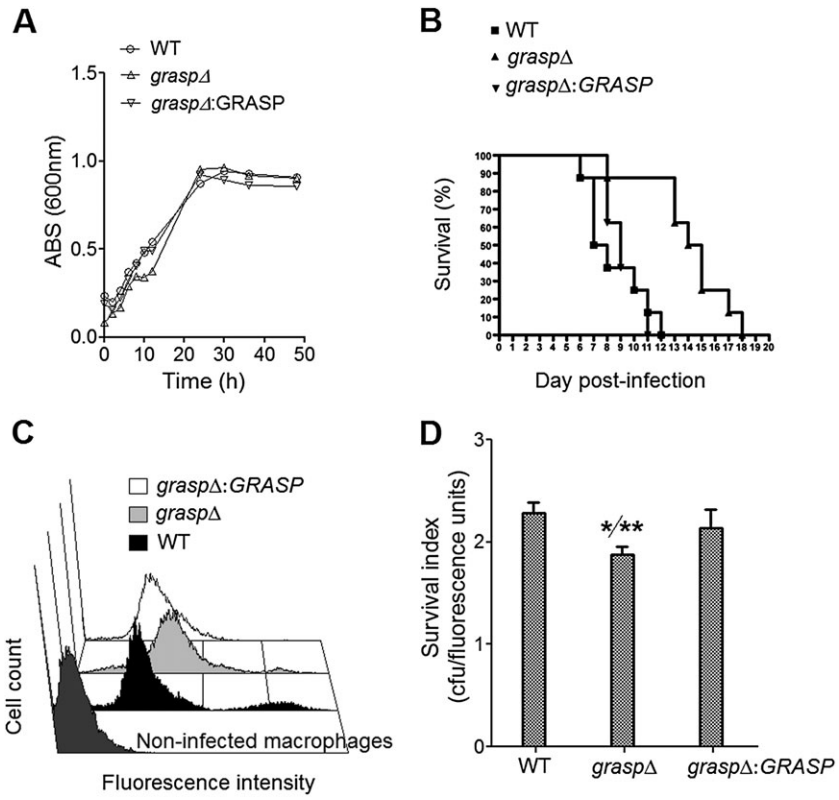

Fig. 4. Virulence phenotype of the GRASP orthologue mutant.
A. Growth rates of WT, mutant and complemented cells.
B. The GRASP orthologue mutant exhibited attenuated virulence in an animal model of cryptococcosis. Mice were lethally infected with *C. neoformans* for daily monitoring of survival. Animals infected with the mutant strain survived significantly longer ($P < 0.01$).
C. Association of FITC-labelled *C. neoformans* cells with murine phagocytes. The similarity in the fluorescence levels of macrophages after infection with non-opsonized WT and reconstituted cells is indicative of similar indices of association between fungal and host cells. Higher fluorescence levels were observed for the mutant, suggesting increased phagocytosis.
D. Survival of cryptococci after interaction with the phagocytes. The GRASP orthologue mutant was significantly more susceptible to killing by macrophages than WT (*) and complemented (**) cells ($P < 0.01$).

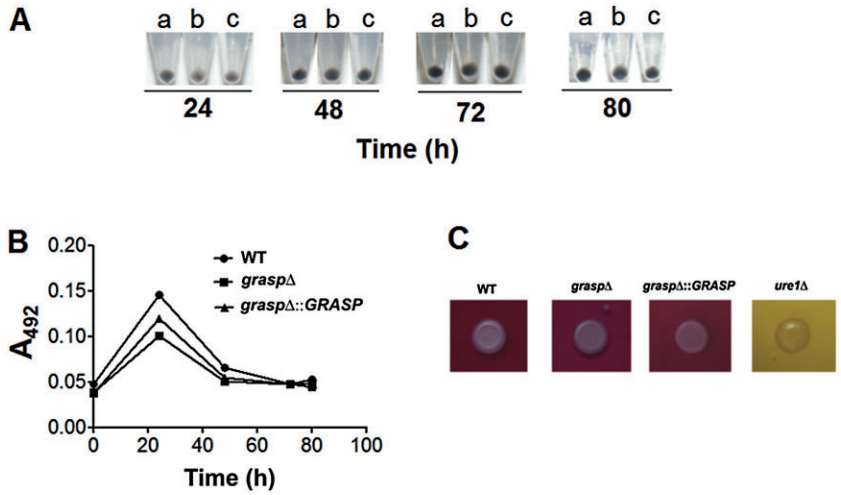

Fig. 5. Absence of GRASP does not affect pigmentation (A and B) or urease activity (C) in cryptococcal cultures.
A. Pigmentation of *C. neoformans* cells after growth in the presence of L-DOPA. Pigmented pellets of WT (a), mutant (b) and reconstituted cells (c) are shown.
B. Release of pigment-like molecules into *C. neoformans* cultures.
C. Urease activity was detected (pink colour) in cultures of WT, mutant and reconstituted cells, but not in cultures of a urease deletion mutant of *C. neoformans* (*ure1*Δ, yellow).

days (Fig. 4B). In contrast, when animals were infected with either WT or complemented cells, 100% killing was observed within 11 and 12 days post infection respectively ($P < 0.01$, in comparison with mutant cells). These results revealed that the GRASP orthologue mutant had attenuated virulence in an animal model of cryptococcosis.

Since the interaction of *C. neoformans* with macrophages is considered to be determinant in a number fungal infections (Seider *et al.*, 2010), we have also evaluated whether the attenuated virulence of the GRASP orthologue mutant was related to decreased levels of association of *C. neoformans* with macrophages. For this purpose, we incubated phagocytes with FITC-labelled yeast cells, and measured phagocytosis in an assay where the index of fluorescence of each macrophage in flow cytometry analysis was proportional to the efficacy of the fungi–host cell interaction. Our results indicate that WT and complemented strains showed similar levels of association with murine phagocytes (Fig. 4C). The mutant, however, was more efficiently associated to macrophages, suggesting increased phagocytic indices. Treatment of the macrophage–fungi complexes with trypan blue, which quenches the fluorescence of extracellularly associated yeast cells, resulted in a decreased of fluorescence levels corresponding to 8%, 12% and 7% for WT, mutant and reconstituted cells respectively (data not shown). The relatively high resistance of infected macrophages to lose fluorescence after exposure to trypan blue indicates that, in all systems, *C. neoformans* cells were internalized by the macrophages in high rates. WT and complemented cells were significantly more resistant than the GRASP orthologue mutant against the microbicidal activity of macrophages ($P < 0.01$). Similar experiments were performed after opsonization of yeast cells with monoclonal antibody (mAb) 18B7, which recognizes GXM (Casadevall *et al.*, 1998) (data not shown). Antibody treatment did not influence phagocytosis of both WT and mutant cells (not shown), which can be linked to the high efficacy of fungal ingestion by the phagocytes after the 18 h incubation.

Pigmentation, urease activity and synthesis and release of capsular components are associated with the survival of *C. neoformans* during infection of host cells (reviewed in Zaragoza *et al.*, 2009). We therefore analysed the ability of the GRASP orthologue mutant to pigment, to produce extracellular urease activity, to secrete GXM and to form a polysaccharide capsule. Levels of pigmentation and urease activity in cultures of WT, mutant and reconstituted cells were similar (Fig. 5). After cultivation under regular (non-inducing) conditions of growth and capsule synthesis, the GRASP orthologue mutant manifested a hypocapsular phenotype, in comparison with WT and reconstituted cells (Fig. 6A–C). Fluorescence microscopy revealed that GXM was detected at the cell surface of both WT and reconstituted cells, as well as in the mutant. In the latter, some of the cells appeared to lack a detectable capsule, while other cells showed the hypocapsular phenotype as observed by India ink counterstaining.

The hypocapsular phenotype of the GRASP mutant was apparently related to a defective ability to secrete GXM, since the concentration of this major capsular component in culture supernatants of the GRASP orthologue mutant was significantly lower than in supernatants of WT and reconstituted cultures (Fig. 6D). Analysis of infected organs revealed that the concentration of lung GXM was not significantly affected by lack of GRASP (data not shown). In the brain, however, deletion of GRASP resulted in a marked decrease in the ability of cryptococci to release GXM (Fig. 6E).

Although secretion of capsular components is required for capsule assembly, it was recently demonstrated that capsule enlargement also requires polysaccharide molecules with higher effective diameters (Frases *et al.*,

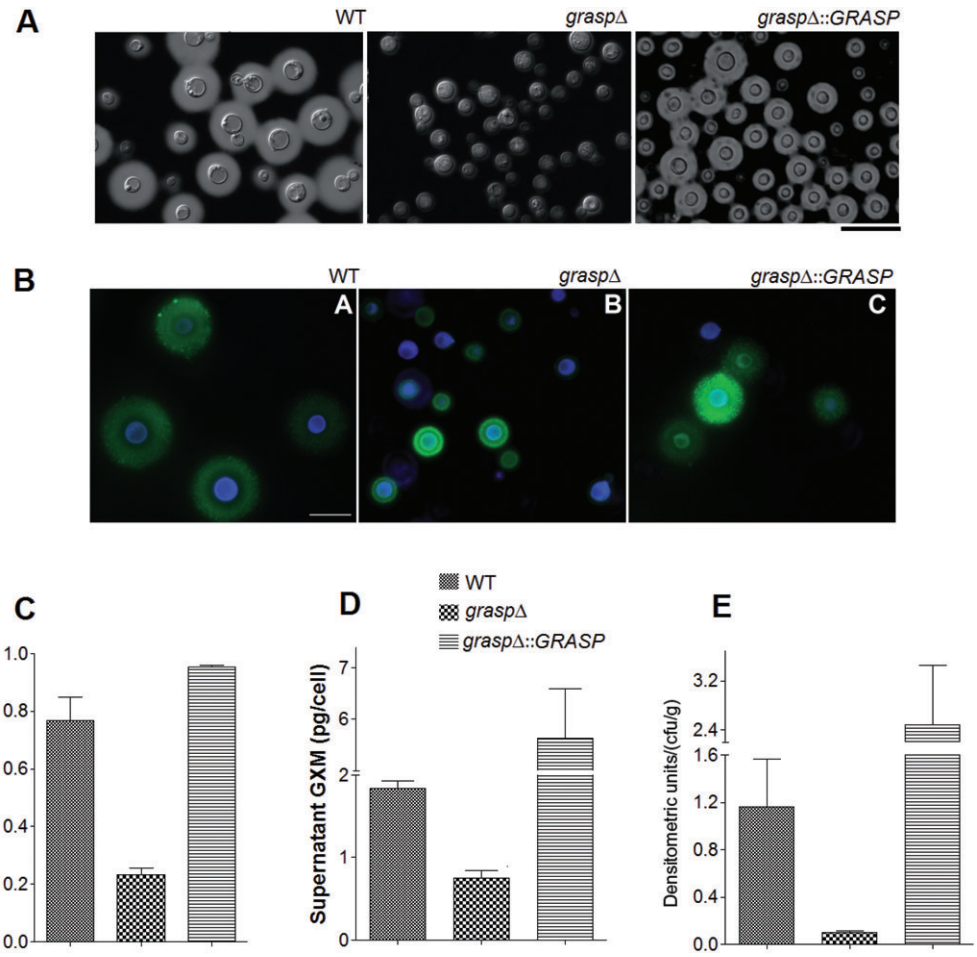

Fig. 6. GRASP is required for normal GXM secretion and capsule assembly.
A. India ink counterstaining of *C. neoformans* cells. Yeast strains are indicated on the top of each panel.
B. Reactivity of *C. neoformans* cells with calcofluor white (blue fluorescence) and a monoclonal antibody raised against GXM (green fluorescence).
Scale bars in (A) and (B) represent 20 and 10 μm respectively.
C. Determination of capsule size of the *C. neoformans* cells illustrated in (A) and (B).
D and E. GXM determination in culture supernatants (D) and infected brains (E) are shown, indicating that *C. neoformans* GRASP orthologue mutant shows a reduced content of extracellular GXM.
Statistical analysis of the results shown in (C), (D) and (E) indicates that values obtained for the GRASP orthologue mutant are significantly smaller than those found for WT and complemented systems ($P < 0.05$ in all cases).

2009). We then analysed this parameter in GXM fractions from WT and mutant cells (Fig. 7). Profiles of diameter distribution of GXM isolated from culture supernatants of WT and complemented cells were very similar. However, values for the effective diameter for polysaccharide fractions obtained from cultures of the mutant were below the detection limit under the conditions used in this study. Since interaction with divalent cations is known to promote GXM aggregation and to increase the dimensions of polysaccharide molecules (Nimrichter *et al.*, 2007; Frases *et al.*, 2008) we incubated the extracellular GXM samples with 1 mM $CaCl_2$ for 1 h to favour detection of smaller molecules. Under these conditions, extracellular GXM fractions from the GRASP orthologue mutant reached dimensions that were within the limit of detection of the method.

In the presence of $CaCl_2$, the complemented strain and the GRASP orthologue mutant exhibited a similar pattern of GXM diameter, in contrast to the apparently ordered structures formed by WT cells. The explanation for these findings is still unclear. GXM aggregation is dependent on the availability of glucuronic acid residues, which is favoured when larger polysaccharide fractions are tested (Nimrichter *et al.*, 2007; Frases *et al.*, 2008; 2009). Therefore, the pattern in size observed for each sample after addition of Ca^{2+} is likely influenced by the availability of glucuronic acid residues. However, this remains to be determined. In fact, properties of capsule/GXM were

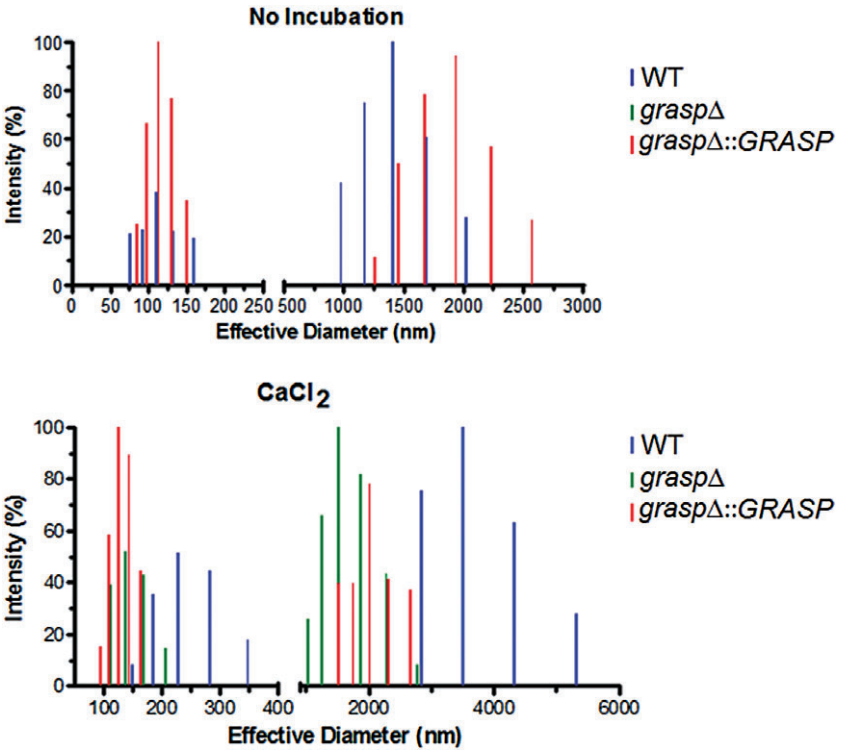

Fig. 7. Effective diameter determination of GXM obtained from cultures of WT, mutant and reconstituted cells. The upper panel shows diameter determination under regular conditions of GXM analysis. The lower panel illustrates diameter determination after incubation of polysaccharide fractions with 1 mM $CaCl_2$.

modified in some of the experiments in which the complemented strain was used. These modified characteristics included exacerbated GXM secretion (Fig. 6) and aggregation in the presence of Ca^{2+} (Fig. 7). The complemented *grasp*Δ::GRASP strain used in this study was generated by ectopic integration of the WT gene in the GRASP orthologue mutant, which is likely responsible for overexpression of the GRASP-coding gene.

Discussion

Fungal cells evolved a number of unconventional secretion mechanisms to release proteins to the extracellular milieu (Albuquerque *et al*., 2008; Rodrigues *et al*., 2008b; Panepinto *et al*., 2009; Duran *et al*., 2010; Manjithaya *et al*., 2010; Oliveira *et al*., 2010a,b). In addition to the well-characterized unconventional secretory pathways, fungi have been proposed to exploit a vesicular pathway for the *trans*-cell wall passage of molecules to the extracellular milieu (Albuquerque *et al*., 2008; Rodrigues *et al*., 2007; 2008a,b; Nosanchuk *et al*., 2008; Casadevall *et al*., 2009; Eisenman *et al*., 2009; Oliveira *et al*., 2009; 2010b; Vallejo *et al*., 2010). The genes and cognate proteins involved in such mechanisms, however, are only partially characterized. Polysaccharides, lipids and pigments can also be extracellularly secreted by fungi (Rodrigues *et al*., 2007; Eisenman *et al*., 2009), but the pathways required for these processes remain largely unknown. GXM biosynthesis in *C. neoformans*, in fact, illustrates this scenario. Many of the enzymes required for synthesis of this polysaccharide have been described (reviewed in Doering, 2009), but several aspects related to its cellular distribution and traffic remain unknown. Intracellular GXM is associated to the membranes of still uncharacterized organelles (Oliveira *et al*., 2009). Extracellular GXM is found either free as a soluble polysaccharide (reviewed in Zaragoza *et al*., 2009), or associated to exosome-like structures (Rodrigues *et al*., 2007). Defects in GXM production and capsule assembly led to avirulent phenotypes in *C. neoformans* (reviewed in Zaragoza *et al*., 2009). Since GXM production and extracellular release are crucial for cryptococcal pathogenesis, this is clearly an area of active study.

A few examples of cryptococcal genes implicated in GXM traffic and secretion are available in the literature, including *CAP59, SAV1* and *SEC6*. *CAP59* gene produces a 458-amino-acid protein of unknown function that has identity to *CMT1*, whose product has α-1,3-mannosyltransferase activity (Chang *et al*., 1995; Sommer *et al*., 2003). Deletion of *CAP59* resulted in acapsular cells with increased cell body diameters (Garcia-Rivera *et al*., 2004), which was attributed to intracellular polysaccharide accumulation. In fact, a missense mutation of *CAP59* partially hampered the trafficking of GXM, but not of proteins (Garcia-Rivera *et al*., 2004). The existence of cellular pathways required for GXM export was further confirmed in studies focused on the role of post-Golgi secretion events in *C. neoformans*. The

products of *SAV1* and *SEC6* genes, which correspond to a putative vesicle-associated Rab GTPase and to a member of the post-Golgi exocytic complex, respectively, were demonstrated to be involved in vesicle-mediated export of GXM to the surface of *C. neoformans* (Yoneda and Doering, 2006; Panepinto *et al*., 2009). GXM is then released to the extracellular space in vesicles that traverse the cell wall (Rodrigues *et al*., 2007). Therefore, extracellular GXM release is linked to elements of the conventional, post-Golgi secretory pathway (Hu *et al*., 2007; Panepinto *et al*., 2009; Yoneda and Doering, 2006) and to exosome-like structures (Rodrigues *et al*., 2007). Nevertheless, the involvement of other cellular pathways in the traffic of the polysaccharide is largely unknown.

In *S. cerevisiae* and *P. pastoris*, GRASP is involved in unconventional secretory mechanisms that require the participation of genes related to autophagy, early endosomal compartments and MVBs (Duran *et al*., 2010; Manjithaya *et al*., 2010). The possibility raised by many authors that GXM secretion could involve such organelles (Takeo *et al*., 1973a,b; Yoneda and Doering, 2006; Albuquerque *et al*., 2008; Nosanchuk *et al*., 2008; Rodrigues *et al*., 2008a; Casadevall *et al*., 2009; Oliveira *et al*., 2009) combined to the emerging roles of GRASP in unconventional secretory pathways (Kinseth *et al*., 2007; Levi and Glick, 2007; Schotman *et al*., 2008; Nickel and Rabouille, 2009; Cabral *et al*., 2010; Duran *et al*., 2010) prompted us to ask whether GRASP would also regulate GXM secretion and virulence in *C. neoformans*.

In comparison with WT and reconstituted cells, the GRASP orthologue mutant of *C. neoformans* was less efficient in killing lethally infected mice and more effectively phagocytized by macrophages. A possible explanation for this finding would be its reduced ability to secrete GXM and/or to form regular capsules. Since the polysaccharide is believed to cause many deleterious effects to the host (reviewed in Zaragoza *et al*., 2009), the reduction of the extracellular concentration of GXM would favour host defence resulting in infection control. Although the absence of GRASP was correlated with attenuated virulence, the *C. neoformans* mutant lacking this protein was still able to kill all the mice. Hence, it would be reasonable to infer that GRASP deletion generated a partial phenotype in that capsule assembly, GXM secretion and virulence were affected. This scenario, therefore, would reflect quantitative and not qualitative alterations, suggesting that other molecules in addition to GRASP may play redundant roles in polysaccharide secretion and capsule assembly. Remarkably, GXM obtained from the GRASP orthologue mutant had altered physical chemical properties (reduced dimensions), although still recognized by an antibody raised to the polysaccharide. The fact that the mutant produced smaller GXM molecules is consistent with the observation of a reduced capsule. Capsule enlargement was demonstrated to be linked to the availability of polysaccharide molecules of increased dimensions (Frases *et al*., 2009).

Based on studies with mAbs, it is generally accepted that the first steps of GXM synthesis occur intracellularly (Feldmesser *et al*., 2001; Garcia-Rivera *et al*., 2004; Yoneda and Doering, 2006; Oliveira *et al*., 2009). However, it is not known whether polysaccharide molecules distributed to intracellular organelles show the same structural features observed in extracellular fractions. For instance, antibody-binding GXM small precursors could be synthesized in intracellular compartments to be then transferred to the extracellular space, where polymerization would occur by aggregative mechanisms (Nimrichter *et al*., 2007; Frases *et al*., 2009). In this case, the reduced secretion of GXM by the GRASP orthologue mutant would be simply related to impaired cellular traffic, as described for other systems (Kinseth *et al*., 2007; Duran *et al*., 2010; Manjithaya *et al*., 2010). GXM polymerization would be favoured in conditions where the polysaccharide is more abundant (Nimrichter *et al*., 2007), and this might explain why the effective diameter of GXM is reduced in supernatants of the mutant.

GRASP is required for unconventional secretion in *D. discoideum*, *S. cerevisiae*, *P. pastoris* and *D. melanogaster* (Kinseth *et al*., 2007; Duran *et al*., 2010; Manjithaya *et al*., 2010). It is well accepted that GRASP is a Golgi-associated protein, which is in agreement with our current results. However, it has also been proposed that this protein could mediate vesicle fusion events at the plasma membrane (Nickel and Rabouille, 2009). This proposal is in agreement with the fact that GRASP is localized at the plasma membrane during epithelial cell remodelling in *D. melanogaster* (Schotman *et al*., 2008). Remarkably, *D. discoideum* cells lacking GRASP show defects in the final stage of fusion of vesicles required for unconventional secretion with the plasma membrane (Cabral *et al*., 2010). In contrast to what has been demonstrated for a *S. cerevisiae grasp* mutant (Oliveira *et al*., 2010b), *C. neoformans* cells lacking GRASP showed normal extracellular release of vesicles (L. Sobrino, unpubl. data). This could suggest that, in *C. neoformans*, GRASP is required for GXM loading into secretory vesicles rather than in the release of these structures to the extracellular space, although we still do not have any evidence that GXM and GRASP interact. Alternatively, the role played by GRASP in polysaccharide traffic could be related to its presence in Golgi cisternae, which also requires experimental confirmation.

Protein secretion has been extensively explored in bacterial and eukaryotic cells. Although polysaccharide secretion in fungi was described many decades ago, the regulatory mechanisms are largely unexplored. To our knowledge, this is the first study to demonstrate a role for GRASP in polysaccharide secretion, as well as in micro-

bial virulence. The observation that GRASP regulates a process required for the pathogenesis of *C. neoformans* adds a new function to the list of the important roles played by this protein in the biology of eukaryotic organisms.

Experimental procedures

Fungal strains, plasmids and media

Cryptococcus neoformans H99 strain was employed as a recipient for creating target gene deletion. Plasmid pJAF15, which contains the hygromycin marker cassette, was a generous gift of Joseph Heitman (Duke University, Durham, NC USA). Plasmid pAI4, which contains the nourseothricin marker cassette, was kindly provided by Alexander Idnurm (University of Missouri-Kansas City Kansas City, MO USA). The strains were maintained on YPD medium (1% yeast extract, 2% peptone, 2% dextrose and 1.5% agar). YPD plates containing hygromycin (200 μg ml^{-1}) were used to select *C. neoformans grasp* deletion transformants (*grasp*Δ strain). YPD plates containing nourseothricin (100 μg ml^{-1}) were used to select *C. neoformans grasp* reconstituted transformants (*grasp*Δ*::GRASP* strain).

In silico *analysis of the* C. neoformans *GRASP orthologue*

The putative *C. neoformans GRASP* gene sequence was identified by a BLAST search of the *C. neoformans* var. *grubii* strain H99 genomic database at the Broad Institute using *GRASP* sequences of *S. cerevisiae* (GenBank Accession No. NP_593015) and *D. discoideum* (GenBank Accession No. EAL60823). The amino acid sequences of *GRASP* orthologues from *R. norvegicus*, *C. elegans*, *D. melanogaster*, *S. cerevisiae*, *D. discoideum*, *Schizosaccharomyces pombe*, *P. falciparum*, *Aspergillus nidulans*, *U. maydis*, *Candida albicans* and *C. neoformans* were aligned using CLUSTALX2 (Larkin *et al.*, 2007). Mega4 were used for phylogenetic analysis applying the Neighbour-Joining method and the tree architecture was inferred from 10 000 bootstraps (Tamura *et al.*, 2007). The identification of the PDZ domains in the sequences was performed by BLAST search of the previous sequences with the *R. norvegicus* PDZ domains as previously described (Kinseth *et al.*, 2007).

Disruption and complementation of GRASP

Disruption of *GRASP* was achieved employing the Delsgate methodology (Garcia-Pedrajas *et al.*, 2008; Kmetzsch *et al.*, 2010; 2011). A Gateway cloning system donor vector (Invitrogen, Carlsbad, CA) containing the hygromycin selectable marker for *C. neoformans* transformation was constructed. A 2.2 kb PCR product spanning the hygromycin marker cassette fragment was amplified from pJAF15 and cloned into the EcoRV site of pDONR201 (Invitrogen, Carlsbad, CA). The resulting vector was named pDONRHYG. The 5′ and 3′ GRASP flanks (702 bp and 700 bp respectively) were PCR-amplified and purified from agarose gels (Illustra GFX PCR DNA and Gel Band Purification kit, GE Healthcare, Buckinghamshire, UK). Approximately 300 ng of pDONRHYG and 30 ng of each PCR product were submitted to BP clonase reaction, according to manufacturer's instructions (Invitrogen, Carlsbad, CA). The product of this reaction was transformed into *Escherichia coli* OmniMAX 2-T1. After confirmation of the correct deletion construct, the plasmid was linearized with I-SceI prior to *C. neoformans* biolistic transformation (Toffaletti *et al.*, 1993). The mutants were screened by colony PCR, and the deletion was confirmed by Southern blot and semi-quantitative RT-PCR analyses. For complementation, a 3 kb genomic PCR fragment containing the WT *GRASP* gene was cloned into the SmaI site of pAI4. The resulting plasmid was used for transformation of the GRASP orthologue mutant strain. Genomic insertion of the complemented gene was confirmed by Southern blot and semi-quantitative RT-PCR analyses. The primers used in these plasmid constructions are listed in Table 1. The strategy used for generation of *C. neoformans grasp* mutant strain is summarized in Fig. 1.

Staining of the Golgi apparatus

Staining of the Golgi apparatus was based on the protocols described by Pagano and colleagues (Pagano, 1989; Pagano *et al.*, 1989). The Golgi staining reagent was C6-NBD-ceramide, which accumulates at the Golgi apparatus of either living or fixed cells (Pagano, 1989). C6-NBD-ceramide has been successfully used to stain *S. cerevisiae* Golgi-related structures (Levine *et al.*, 2000). Yeast cells were fixed with paraformaldehyde 4% in PBS, followed by washing with PBS and incubation with C6-NBD-ceramide (5 μM) for 16 h at 4°C. The cells were then incubated with

Table 1. List of primers used in this study.

Primer name	Sequence (5′–3′)	Purpose
CnGRASPF	AAAATAGGGATAACAGGGTAATGAGATACCAGATGGACTGAA	Disruption construct for *GRASP1*, 5′ flank
CnGRASP5R	GGGGACAAGTTTGTACAAAAAAGCAGGCTATATATTCTGCCCAGCACATCT	Disruption construct for *GRASP1*, 5′ flank
CnGRASP3F	GGGGACCACTTTGTACAAGAAAGCTGGGTAATGCTAATGTGAAACGCAAT	Disruption construct for *GRASP1*, 3′ flank
CnGRASP3R	AAAAATTACCCTGTTATCCCTAGTAACGAGAAGTGCTGTCTC	Disruption construct for *GRASP1*, 3′ flank
GRASPcompF	ATAATGCAATGCGATGGCTGC	Amplification of *GRASP* for complementation
GRASPcompR	AATCCCCTCAAGAGCTCACGG	Amplification of *GRASP* for complementation
RTGRASPF	AGTTCTTTACCCTACTAGACAG	Amplification of *GRASP* for RT-PCR
RTGRASPR	TCTCCTCACATTGTCAGATTC	Amplification of *GRASP* for RT-PCR
RTACTF	CCTTCTACGTCTCTATCCAG	Amplification of *ACT1* for RT-PCR
RTACTR	TTTCAAGCTGAGAAGACTGG	Amplification of *ACT1* for RT-PCR

fetal calf serum (10%) at 4°C for 1 h to remove the excess of C6-NBD-ceramide (Pagano, 1989). For staining of the cell wall, the cells were then incubated for 15 min with Uvitex 2B (0.1 mg ml^{-1}; Polysciences, Warrington, PA), followed by washing with PBS and analysis by fluorescence microscopy. Images were generated in an ApoTome 2 epifluorescence microscope (Carl Zeiss, Germany) and processed with the AxioVision software (Carl Zeiss, Germany).

Virulence assay

Virulence studies were conducted according to a previously described intranasal inhalation infection model (Cox *et al.*, 2000) using eight female BALB/c mice (approximately 6 weeks old) for each strain. Mice were infected with 10^7 yeast cells suspended in 50 µl of saline and monitored daily. Animal studies were approved by the Federal University of Rio Grande do Sul Ethics Committee. Kaplan–Meier analysis of survival was performed using GraphPad Software.

Phagocytosis of C. neoformans *cells by murine macrophages*

Murine macrophage-like cells (RAW 264.7 lineage) were obtained from the American Type Culture Collection (ATCC). Cultures were maintained and grown to confluence in 25 cm^2 culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C and 5% CO_2. The culture medium was replaced with fresh media for further incubation with fluorescein isothiocyanate (FITC, Sigma Aldrich Corp, St Louis, MO)-labelled *C. neoformans* yeast cells (Barbosa *et al.*, 2006). Fluorescent yeast cells were prepared by staining with 0.5 mg ml^{-1} FITC in PBS (25°C) for 10 min. FITC-labelled *C. neoformans* were then suspended in DMEM to generate a ratio of 10 fungal cells per macrophage for further incubation at 37°C and 5% CO_2 for 18 h. In some systems, yeast cells were opsonized by treatment with mAb 18B7 (10 µg ml^{-1}) for 1 h at 37°C. For negative control we used an isotype-matched irrelevant IgG at the same concentrations used for mAb 18B7. Non-adherent yeast cells were removed by washing with PBS. Fungi–host cell complexes were then treated for 10 min at 25°C with trypan blue (200 µg ml^{-1}) to discriminate between surface-associated and intracellular yeast cells. After removal from the plastic surface with a cell scrapper, the cells were analysed by flow cytometry as described previously (Barbosa *et al.*, 2006). Control preparations were developed as described above using uninfected cells and non-stained yeast. Alternatively, infected macrophages were lysed with cold water and the remaining suspension was plated onto Sabouraud solid agar for counting of colony-forming units (cfu). These values were used for calculation of the survival indices of *C. neoformans* after interaction with the phagocytes. The survival index was defined as the number of cfu divided by the fluorescence index of macrophages in each experimental system.

GXM and capsule determination

Cryptococcus neoformans cells (WT, mutant and reconstituted strains) were placed onto glass slides and mixed with similar volumes of India ink. The suspensions were covered with glass coverslips and analysed with an Axioplan 2 (Zeiss, Germany) microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). Capsule sizes, defined as the distances between the cell wall and the outer border of the capsule in India ink stained yeast cells, were determined by using the ImageJ Software (version 1.33), elaborated and provided by National Institutes of Health (NIH, http://rsb.info.nih.gov/ij/). Cell diameters were determined using the same software. Final measurements were presented as ratio of capsule size/cell diameter. Cellular suspensions were analysed by fluorescence microscopy. The staining reagents used in fluorescence microscopy included calcofluor white, which has been extensively used to stain chitin in fungal cell walls, and the mAb 18B7, a mouse IgG1 with high affinity for GXM of different cryptococcal serotypes (Casadevall *et al.*, 1998). Yeast cells (10^6) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS (PBS-BSA) for 1 h. The cells were then suspended in 100 µl of a 25 µM calcofluor white solution (Invitrogen, Carlsbad, CA) for 30 min at 37°C. After washing in PBS, the cells were incubated for 1 h in the presence of mAb 18B7 (1 µg ml^{-1}). The cells were finally incubated with a fluorescein isothiocyanate (FITC)-labelled goat anti-mouse IgG (Fc-specific) antibody (Sigma Aldrich Corp, St Louis, MO). For negative control we used an isotype-matched irrelevant IgG at the same concentrations used for mAb 18B7. Cell suspensions were mounted over glass slides as described above and analysed under an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired and processed as described above.

Determination of GXM concentration in fungal supernatants and infected tissues

Culture supernatants were obtained as recently described (Fonseca *et al.*, 2010). GXM concentration in fungal supernatants was determined by ELISA with mAb 18B7, using modifications of a previously described protocol for GXM detection (Casadevall *et al.*, 1992; Fonseca *et al.*, 2009). Briefly, 96-well polystyrene plates were coated with standard GXM, supernatant samples for further blocking with bovine serum albumin. The plates were sequentially incubated with mAb 18B7 and an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate, followed by measuring absorbance at 405 nm with a microplate reader (TP-reader, Thermo Plate). Antibody concentration in this assay corresponded to 1 µg ml^{-1}. For *in vivo* determinations, tissue samples were obtained after intranasal infection female BALB/c ($n = 10$) with 10^6 yeast cells of WT, mutant or reconstituted *C. neoformans* cells (Cox *et al.*, 2000). At day 7 post infection, animals were euthanized and their lungs and brains were aseptically excised. Tissues were then homogenized by maceration in PBS. These suspensions were plated on Sabouraud agar for cfu counting or were processed for GXM determination. For this purpose, suspen-

sions were supplemented with proteinase K (0.2 mg ml^{-1}, final concentration) and incubated overnight at 37°C. Samples were then heated for 20 min at 100°C, placed on ice and centrifuged at 10 000 *g*. Supernatants were then used in for GXM determination. For the *in vivo* systems, high backgrounds were observed when samples were analysed by ELISA (data not shown). We therefore analysed *in vivo* samples by dot blotting. Samples were loaded onto nitrocellulose membranes, which were allowed to dry for 1 h at 37°C and then were blocked with PBS containing 5% bovine serum albumin. Blocked membranes were incubated with mAb 18B7 at a starting dilution of 1 μg ml^{-1}. After being washed extensively, membranes were sequentially incubated with peroxidase-conjugated goat anti-mouse IgG1 and orthophenylene diamine solutions. Reactions were quantified by densitometry with the Scion Image software (version 4.03) and normalized to cfu values and weight of infected tissues.

Analysis of virulence factors

Urease activity and melanin formation, two well-defined *C. neoformans* virulence factors that are related to vesicular secretion (Rodrigues *et al*., 2008b; Eisenman *et al*., 2009), were used as prototypes in assays aiming at the evaluation of virulence determinants that are not associated to capsule expression. Urease activity was assayed after growth of WT, mutant and reconstituted strains in Christensen's agar medium at 30°C for 48 h (Cox *et al*., 2000). This medium contains 300 mM urea and phenol red as a pH indicator. The urease activity is expected to convert urea into ammonia, resulting in an increase in the pH of the medium. This feature is reflected by a colour change from yellow to bright pink. A *ure1* mutant was used as a negative control. Analysis of melanin production followed the methodology described by Baker *et al*. (2007). WT, mutant and complemented cells were inoculated in a chemically defined medium containing L-asparagine (1 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), KH_2PO_4 (3 g l^{-1}) and thiamine (1 mg l^{-1}), supplemented with 10 mM L-3,4-dihydroxyphenylalanine (L-DOPA). *C. neoformans* cells were then cultivated at 30°C. After 24 h intervals, the cultures were centrifuged 1000 *g* for 10 min; pellets were photographed for visual analysis of pigmentation and the supernatants were spectrophotometrically analysed (absorbance 492 nm). Shorter times were also analysed, but pigmentation was only observed after 24 h in the culture medium (data not shown).

GXM effective diameter

For diameter determination, extracellular GXM was isolated from culture supernatants as previously described by our group (Nimrichter *et al*., 2007). Yeast cells were cultivated in a minimal medium composed of glucose (15 mM), $MgSO_4$ (10 mM), KH_2PO_4 (29.4 mM), glycine (13 mM) and thiamine-HCl (3 μM), pH 5.5, for 2 days at room temperature with shaking and separated from culture supernatants by centrifugation at 4000 *g* (15 min, 4°C). The supernatant fluids were collected and again centrifuged at 15 000 *g* (15 min, 4°C), to remove smaller debris. The pellets were discarded and the resulting supernatant was concentrated approximately 20-fold using an Amicon (Millipore, Danvers, MA) ultrafiltration cell (Nimrichter *et al*., 2007). After supernatant concentration, the viscous layer formed was collected with a cell scraper and transferred to graduated plastic tubes for GXM determinations. The effective diameter of GXM in these samples was determined by Quasi elastic light scattering in a 90Plus/BI-MAS Multi Angle Particle Sizing analyser (Brookhaven Instruments Corp., Holtsville, NY), using minor modifications of the method described by Frases *et al*. (2009). Polysaccharide diameter was modulated by incubation of dialysed fractions with 1 mM $CaCl_2$ for 1 h at room temperature.

Acknowledgements

M.L.R., M.H.V., L.N. and A.S. are supported by grants from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Brazil), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq, Brazil), Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP, Brazil), Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ, Brazil) and Financiadora de Estudos e Projetos (FINEP, Brazil). A.C. is supported by NIH Grants AI033142, AI033774, AI052733 and HL059842. We thank Priscila C. Albuquerque and Ana Claudia Zimbres for help with handling cultures of fungal mutants. We are thankful to the reviewers for their insightful comments that helped strengthen this article. We are also thankful to Prof. Vivek Malhotra for initial suggestions on the role of GRASP in polysaccharide secretion.

References

Albuquerque, P.C., Nakayasu, E.S., Rodrigues, M.L., Frases, S., Casadevall, A., Zancope-Oliveira, R.M., *et al*. (2008) Vesicular transport in *Histoplasma capsulatum*: an effective mechanism for trans-cell wall transfer of proteins and lipids in ascomycetes. *Cell Microbiol* **10:** 1695–1710.

Baker, L.G., Specht, C.A., Donlin, M.J., and Lodge, J.K. (2007) Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity in *Cryptococcus neoformans*. *Eukaryot Cell* **6:** 855–867.

Barbosa, F.M., Fonseca, F.L., Holandino, C., Alviano, C.S., Nimrichter, L., and Rodrigues, M.L. (2006) Glucuronoxylomannan-mediated interaction of *Cryptococcus neoformans* with human alveolar cells results in fungal internalization and host cell damage. *Microbes Infect* **8:** 493–502.

Cabral, M., Anjard, C., Malhotra, V., Loomis, W.F., and Kuspa, A. (2010) Unconventional secretion of AcbA in *Dictyostelium discoideum* through a vesicular intermediate. *Eukaryot Cell* **9:** 1009–1017.

Casadevall, A., Mukherjee, J., and Scharff, M.D. (1992) Monoclonal antibody based ELISAs for cryptococcal polysaccharide. *J Immunol Methods* **154:** 27–35.

Casadevall, A., Cleare, W., Feldmesser, M., Glatman-Freedman, A., Goldman, D.L., Kozel, T.R., *et al*. (1998) Characterization of a murine monoclonal antibody to *Cryptococcus neoformans* polysaccharide that is a candidate for human therapeutic studies. *Antimicrob Agents Chemother* **42:** 1437–1446.

Casadevall, A., Nosanchuk, J.D., Williamson, P., and Rodrigues, M.L. (2009) Vesicular transport across the fungal cell wall. *Trends Microbiol* **17:** 158–162.

Chang, Y.C., Wickes, B.L., and Kwon-Chung, K.J. (1995) Further analysis of the CAP59 locus of *Cryptococcus neoformans*: structure defined by forced expression and description of a new ribosomal protein-encoding gene. *Gene* **167:** 179–183.

Chayakulkeeree, M., Johnston, S.A., Oei, J.B., Lev, S., Williamson, P.R., Wilson, C.F., *et al*. (2011) SEC14 is a specific requirement for secretion of phospholipase B1 and pathogenicity of *Cryptococcus neoformans*. *Mol Microbiol* **80:** 1088–1101.

Cox, G.M., Mukherjee, J., Cole, G.T., Casadevall, A., and Perfect, J.R. (2000) Urease as a virulence factor in experimental cryptococcosis. *Infect Immun* **68:** 443–448.

Doering, T.L. (2009) How sweet it is! Cell wall biogenesis and polysaccharide capsule formation in *Cryptococcus neoformans*. *Annu Rev Microbiol* **63:** 223–247.

Duran, J.M., Anjard, C., Stefan, C., Loomis, W.F., and Malhotra, V. (2010) Unconventional secretion of Acb1 is mediated by autophagosomes. *J Cell Biol* **188:** 527–536.

Eisenman, H.C., Frases, S., Nicola, A.M., Rodrigues, M.L., and Casadevall, A. (2009) Vesicle-associated melanization in *Cryptococcus neoformans*. *Microbiology* **155:** 3860–3867.

Feldmesser, M., Kress, Y., and Casadevall, A. (2001) Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection. *Microbiology* **147:** 2355–2365.

Fonseca, F.L., Frases, S., Casadevall, A., Fischman-Gompertz, O., Nimrichter, L., and Rodrigues, M.L. (2009) Structural and functional properties of the *Trichosporon asahii* glucuronoxylomannan. *Fungal Genet Biol* **46:** 496–505.

Fonseca, F.L., Nohara, L.L., Cordero, R.J., Frases, S., Casadevall, A., Almeida, I.C., *et al*. (2010) Immunomodulatory effects of serotype B glucuronoxylomannan from *Cryptococcus gattii* correlate with polysaccharide diameter. *Infect Immun* **78:** 3861–3870.

Frases, S., Nimrichter, L., Viana, N.B., Nakouzi, A., and Casadevall, A. (2008) *Cryptococcus neoformans* capsular polysaccharide and exopolysaccharide fractions manifest physical, chemical, and antigenic differences. *Eukaryot Cell* **7:** 319–327.

Frases, S., Pontes, B., Nimrichter, L., Viana, N.B., Rodrigues, M.L., and Casadevall, A. (2009) Capsule of *Cryptococcus neoformans* grows by enlargement of polysaccharide molecules. *Proc Natl Acad Sci USA* **106:** 1228–1233.

Garcia-Pedrajas, M.D., Nadal, M., Kapa, L.B., Perlin, M.H., Andrews, D.L., and Gold, S.E. (2008) DelsGate, a robust and rapid gene deletion construction method. *Fungal Genet Biol* **45:** 379–388.

Garcia-Rivera, J., Chang, Y.C., Kwon-Chung, K.J., and Casadevall, A. (2004) *Cryptococcus neoformans* CAP59 (or Cap59p) is involved in the extracellular trafficking of capsular glucuronoxylomannan. *Eukaryot Cell* **3:** 385–392.

Hu, G., Steen, B.R., Lian, T., Sham, A.P., Tam, N., Tangen, K.L., and Kronstad, J.W. (2007) Transcriptional regulation by protein kinase A in *Cryptococcus neoformans*. *PLoS Pathog* **3:** e42.

Kinseth, M.A., Anjard, C., Fuller, D., Guizzunti, G., Loomis, W.F., and Malhotra, V. (2007) The Golgi-associated protein GRASP is required for unconventional protein secretion during development. *Cell* **130:** 524–534.

Kmetzsch, L., Staats, C.C., Simon, E., Fonseca, F.L., de Oliveira, D.L., Sobrino, L., *et al*. (2010) The vacuolar Ca(2)(+) exchanger Vcx1 is involved in calcineurin-dependent Ca(2)(+) tolerance and virulence in *Cryptococcus neoformans*. *Eukaryot Cell* **9:** 1798–1805.

Kmetzsch, L., Staats, C.C., Simon, E., Fonseca, F.L., Oliveira, D.L., Joffe, L.S., *et al*. (2011) The GATA-type transcriptional activator Gat1 regulates nitrogen uptake and metabolism in the human pathogen *Cryptococcus neoformans*. *Fungal Genet Biol* **48:** 192–199.

Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., McWilliam, H., *et al*. (2007) Clustal W and Clustal X version 2.0. *Bioinformatics* **23:** 2947–2948.

Levi, S.K., and Glick, B.S. (2007) GRASPing unconventional secretion. *Cell* **130:** 407–409.

Levine, T.P., Wiggins, C.A., and Munro, S. (2000) Inositol phosphorylceramide synthase is located in the Golgi apparatus of *Saccharomyces cerevisiae*. *Mol Biol Cell* **11:** 2267–2281.

Li, S.S., and Mody, C.H. (2010) Cryptococcus. *Proc Am Thorac Soc* **7:** 186–196.

McClelland, E.E., Bernhardt, P., and Casadevall, A. (2005) Coping with multiple virulence factors: which is most important? *PLoS Pathog* **1:** e40.

Manjithaya, R., Anjard, C., Loomis, W.F., and Subramani, S. (2010) Unconventional secretion of *Pichia pastoris* Acb1 is dependent on GRASP protein, peroxisomal functions, and autophagosome formation. *J Cell Biol* **188:** 537–546.

Nickel, W., and Rabouille, C. (2009) Mechanisms of regulated unconventional protein secretion. *Nat Rev Mol Cell Biol* **10:** 148–155.

Nickel, W., and Seedorf, M. (2008) Unconventional mechanisms of protein transport to the cell surface of eukaryotic cells. *Annu Rev Cell Dev Biol* **24:** 287–308.

Nimrichter, L., Frases, S., Cinelli, L.P., Viana, N.B., Nakouzi, A., Travassos, L.R., *et al*. (2007) Self-aggregation of *Cryptococcus neoformans* capsular glucuronoxylomannan is dependent on divalent cations. *Eukaryot Cell* **6:** 1400–1410.

Nosanchuk, J.D., Nimrichter, L., Casadevall, A., and Rodrigues, M.L. (2008) A role for vesicular transport of macromolecules across cell walls in fungal pathogenesis. *Commun Integr Biol* **1:** 37–39.

Oliveira, D.L., Nimrichter, L., Miranda, K., Frases, S., Faull, K.F., Casadevall, A., and Rodrigues, M.L. (2009) *Cryptococcus neoformans* cryoultramicrotomy and vesicle fractionation reveals an intimate association between membrane lipids and glucuronoxylomannan. *Fungal Genet Biol* **46:** 956–963.

Oliveira, D.L., Nakayasu, E.S., Joffe, L.S., Guimaraes, A.J., Sobreira, T.J., Nosanchuk, J.D., *et al*. (2010a) Biogenesis of extracellular vesicles in yeast: many questions with few answers. *Commun Integr Biol* **3:** 533–535.

Oliveira, D.L., Nakayasu, E.S., Joffe, L.S., Guimaraes, A.J., Sobreira, T.J., Nosanchuk, J.D., *et al*. (2010b) Character-

ization of yeast extracellular vesicles: evidence for the participation of different pathways of cellular traffic in vesicle biogenesis. *PLoS ONE* **5:** e11113.

Pagano, R.E. (1989) A fluorescent derivative of ceramide: physical properties and use in studying the Golgi apparatus of animal cells. *Methods Cell Biol* **29:** 75–85.

Pagano, R.E., Sepanski, M.A., and Martin, O.C. (1989) Molecular trapping of a fluorescent ceramide analogue at the Golgi apparatus of fixed cells: interaction with endogenous lipids provides a trans-Golgi marker for both light and electron microscopy. *J Cell Biol* **109:** 2067–2079.

Panepinto, J., Komperda, K., Frases, S., Park, Y.D., Djordjevic, J.T., Casadevall, A., and Williamson, P.R. (2009) Sec6-dependent sorting of fungal extracellular exosomes and laccase of *Cryptococcus neoformans*. *Mol Microbiol* **71:** 1165–1176.

Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., and Chiller, T.M. (2009) Estimation of the current global burden of cryptococcal meningitis among persons living with HIV/AIDS. *AIDS* **23:** 525–530.

Prado, M., Silva, M.B., Laurenti, R., Travassos, L.R., and Taborda, C.P. (2009) Mortality due to systemic mycoses as a primary cause of death or in association with AIDS in Brazil: a review from 1996 to 2006. *Mem Inst Oswaldo Cruz* **104:** 513–521.

Rodrigues, M.L., Nimrichter, L., Oliveira, D.L., Frases, S., Miranda, K., Zaragoza, O., *et al*. (2007) Vesicular polysaccharide export in *Cryptococcus neoformans* is a eukaryotic solution to the problem of fungal trans-cell wall transport. *Eukaryot Cell* **6:** 48–59.

Rodrigues, M.L., Nimrichter, L., Oliveira, D.L., Nosanchuk, J.D., and Casadevall, A. (2008a) Vesicular trans-cell wall transport in fungi: a mechanism for the delivery of virulence-associated macromolecules? *Lipid Insights* **2:** 27–40.

Rodrigues, M.L., Nakayasu, E.S., Oliveira, D.L., Nimrichter, L., Nosanchuk, J.D., Almeida, I.C., and Casadevall, A. (2008b) Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence. *Eukaryot Cell* **7:** 58–67.

Schotman, H., Karhinen, L., and Rabouille, C. (2008) dGRASP-mediated noncanonical integrin secretion is required for *Drosophila* epithelial remodeling. *Dev Cell* **14:** 171–182.

Seider, K., Heyken, A., Luttich, A., Miramon, P., and Hube, B. (2010) Interaction of pathogenic yeasts with phagocytes: survival, persistence and escape. *Curr Opin Microbiol* **13:** 392–400.

Sommer, U., Liu, H., and Doering, T.L. (2003) An alpha-1,3-mannosyltransferase of *Cryptococcus neoformans*. *J Biol Chem* **278:** 47724–47730.

Takeo, K., Uesaka, I., Uehira, K., and Nishiura, M. (1973a) Fine structure of *Cryptococcus neoformans* grown *in vitro* as observed by freeze-etching. *J Bacteriol* **113:** 1442–1448.

Takeo, K., Uesaka, I., Uehira, K., and Nishiura, M. (1973b) Fine structure of *Cryptococcus neoformans* grown *in vivo* as observed by freeze-etching. *J Bacteriol* **113:** 1449–1454.

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol Biol Evol* **24:** 1596–1599.

Toffaletti, D.L., Rude, T.H., Johnston, S.A., Durack, D.T., and Perfect, J.R. (1993) Gene transfer in *Cryptococcus neoformans* by use of biolistic delivery of DNA. *J Bacteriol* **175:** 1405–1411.

Vallejo, M.C., Matsuo, A.L., Ganiko, L., Medeiros, L.C., Miranda, K., Silva, L.S., *et al*. (2010) The pathogenic fungus *Paracoccidioides brasiliensis* exports extracellular vesicles containing highly immunogenic {alpha}-galactosyl epitopes. *Eukaryot Cell* **10:** 343–351.

Vinke, F.P., Grieve, A.G., and Rabouille, C. (2011) The multiple facets of the Golgi reassembly stacking proteins. *Biochem J* **433:** 423–433.

Yoneda, A., and Doering, T.L. (2006) A eukaryotic capsular polysaccharide is synthesized intracellularly and secreted via exocytosis. *Mol Biol Cell* **17:** 5131–5140.

Zaragoza, O., Telzak, A., Bryan, R.A., Dadachova, E., and Casadevall, A. (2006) The polysaccharide capsule of the pathogenic fungus *Cryptococcus neoformans* enlarges by distal growth and is rearranged during budding. *Mol Microbiol* **59:** 67–83.

Zaragoza, O., Rodrigues, M.L., De Jesus, M., Frases, S., Dadachova, E., and Casadevall, A. (2009) The capsule of the fungal pathogen *Cryptococcus neoformans*. *Adv Appl Microbiol* **68:** 133–216.