

Cell wall α -1,3-glucan is required to anchor the *Cryptococcus neoformans* capsule

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Summary

***Cryptococcus neoformans* is an opportunistic pathogen responsible for serious disease in humans. Critical for virulence of this fungus is an elaborate polysaccharide capsule, which impedes the host immune response. We found that association of the capsule with the cell requires a specific component of the cell wall, α -1,3-glucan. Post-transcriptional inhibition of α -1,3-glucan synthase expression, using double-stranded RNA interference, yields cells that are unable to assemble a capsule although they generate its polysaccharide components. The resulting cryptococci are slow-growing and acapsular. This finding demonstrates a novel mode of polysaccharide attachment and an important application of RNA interference in fungi. The elimination of the capsule by reducing the expression of a single gene suggests a potential avenue for antifungal chemotherapy.**

Introduction

With the advent of AIDS, opportunistic pathogens that were previously biological curiosities have become significant sources of disease. One of these is the fungus *Cryptococcus neoformans*, which causes cryptococcosis and a potentially fatal meningitis (Casadevall and Perfect, 1998). This organism is characterized by an extensive polysaccharide capsule that is absolutely required for its virulence (Chang and Kwon-Chung, 1994; Kozel, 1995). Capsular material, both associated with cells and shed copiously from them, impedes the host immune response by altering defensive processes including phagocytosis, proinflammatory cytokine production and leucocyte migration (Buchanan and Murphy, 1998). The bulk of the capsule is composed of a partially acetylated linear mannan decorated with monosaccharide side-chains of xylose and

glucuronic acid; the remainder is a galactan-based polysaccharide with side-chains containing mannose, galactose and xylose (Cherniak *et al.*, 1998; Vaishnav *et al.*, 1998). Although the structures of the capsule components have been determined, and many capsule functions have been well studied, elucidation of the synthesis and assembly of this complex structure is just beginning (reviewed by Bose *et al.*, 2003). For example, although a number of acapsular (*cap*) mutants have been identified, all of which are completely avirulent in animal models, no function has been ascribed to the affected genes (Chang and Kwon-Chung, 1994; 1998; 1999; Chang *et al.*, 1997). A few enzymes required for capsule synthesis have been identified (Bar-Peled *et al.*, 2001; Wills *et al.*, 2001; Moyrand *et al.*, 2002; Sommer *et al.*, 2003; M. Bar-Peled, unpubl.), but nothing is known about how the overall structure is retained at the cell surface.

In both prokaryotic and eukaryotic microbes, surface glycoconjugates play critical roles in pathogenesis. Association of these molecules with the cell surface may be mediated by lipid, glycan, glycolipid moieties or peptide components. Determining how surface polysaccharides are linked to cells and, in some cases, how they are shed is required to understand their biosynthesis and function. Accordingly, the major question in the study of the cryptococcal capsule is how it associates with the fungal surface. High-resolution electron micrographs indicate a close association of capsule polysaccharide with the cell (Sakaguchi *et al.*, 1993; J. E. Heuser and T. L. Doering, unpublished data), and studies of actively growing capsule show that new material is incorporated directly adjacent to the cell wall (Pierini and Doering, 2001), but the fundamental interaction remains obscure. We have focused on determining the nature of the interface between the capsule and the cryptococcal cell surface.

Results and discussion

Previous studies in *Cryptococcus* demonstrated that capsular material that was chemically precipitated from conditioned medium could bind to acapsular cells (Kozel, 1977; Kozel and Hermerath, 1984; Small and Mitchell, 1986). Studies of this association suggested that the interaction between capsule components and the cell surface was mediated by a specific receptor, but its molecular character was not determined (Bulmer and Sans, 1968;

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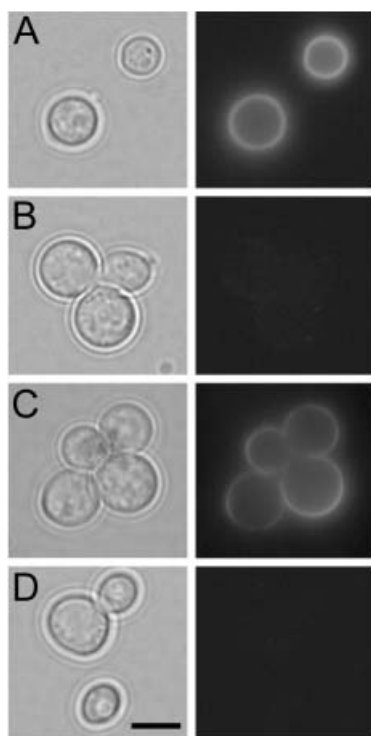


Fig. 1. Capsule material shed from wild-type *C. neoformans* into conditioned medium (CM) can be transferred to acapsular cells. Cells were labelled with Cy3-conjugated anti-capsule monoclonal antibody with (+) or without (-) previous treatment with wild-type CM. Phase-contrast (left) and immunofluorescence (right) images are shown of *C. neoformans* wild-type strain JEC43 -CM (A); acapsular strain, *cap59* -CM (B); *cap59* +CM (C); and *S. cerevisiae* cells +CM (D). Scale bar, 5 μ m.

Tacker *et al.*, 1972; Kozel, 1977; Kozel and Hermerath, 1984; Small and Mitchell, 1986). To understand the mechanism of capsule binding, we developed a physiological capsule transfer assay in which acapsular mutant cells (strain *cap59*) serve as acceptors for the capsular material that is shed constitutively by wild-type *C. neoformans*. In this assay, acceptor cells are first exposed to medium in which wild-type cells have grown, then washed, and their surfaces probed with monoclonal antibodies against capsular polysaccharide (Fig. 1). This assay showed that shed capsule material bound to acapsular strains of *Cryptococcus* (Fig. 1C), but not to *Saccharomyces cerevisiae* (Fig. 1D) or to the pathogenic yeast *Candida albicans* (data not shown). The binding of capsule components was not altered by previous or simultaneous treatment of cells with chelating agents or high salt (Table 1), suggesting that it does not involve non-specific charge-mediated association.

Our earlier studies suggested that new capsule material is incorporated immediately adjacent to the distal boundary of the fungal cell wall (Pierini and Doering, 2001), a complex interwoven matrix of glucose polymers, chitin and

cell wall mannoproteins (Cabib *et al.*, 1988; 2001; Lipke and Ovalle, 1998; Smits *et al.*, 1999; Kapteyn *et al.*, 2000; Bernard and Latgé, 2001; Klis *et al.*, 2001). We addressed the potential role in capsule binding of each of these cell wall constituents. First, to test the involvement of a protein receptor, we treated cells with a mixture of proteases to degrade exposed polypeptide moieties. This did not alter the ability of cells to bind capsule (compare Fig. 2B and A), suggesting that, contrary to previous speculation (Bulmer and Sans, 1968), a protein receptor is not required for capsule binding. We next examined whether the N-glycans of cell wall proteins might play a role in binding, by treating cells with either peptide-N-glycosidase F (PNGase F) to remove surface N-glycans or concanavalin A to bind mannose side-chains and hinder putative capsule-binding interactions. Neither of these treatments altered capsule binding (Table 1), although control experiments indicated that the glycanase was active and that the lectin bound to the cell surface (not shown).

Chitin in fungal cell walls is the binding site for an important surface virulence factor in the fungal pathogen *Blasotryces dermatitidis* (Brandhorst and Klein, 2000). To investigate a potential role for this polymer in capsule binding, we treated cells with either chitinase or an excess of the N-acetylglucosamine-binding lectin, wheatgerm agglutinin. Neither of these altered capsule binding (Table 1), suggesting that the chitin component of cryptococcal cell wall is not responsible for capsule binding. Phospholipases C and D also had no impact on binding (Table 1).

Table 1. Effect on capsule binding after treatment of acceptor cells with various compounds.

| Treatment | Conditions | Staining |
|------------------------|-----------------------------|----------|
| - | - | + |
| β -1-6-glucanase | 2 mg ml ⁻¹ | + |
| Calcium chloride | 10 mM | + |
| Chitinase | 0.8 mg ml ⁻¹ | + |
| Concanavalin A | 0.1–0.5 mg ml ⁻¹ | + |
| EGTA | 10 mM | + |
| HCl | 1 M | - |
| NaCl | 2 M | + |
| NaOH | 25 mM–1 M | - |
| Novozyme | 4 mg ml ⁻¹ | ± |
| Phospholipase C | 0.9 mg ml ⁻¹ | + |
| Phospholipase D | 1.8 mg ml ⁻¹ | + |
| PNGase F | 20 U ml ⁻¹ | + |
| Protease | 4 mg ml ⁻¹ | + |
| Quantazyme | 75 U ml ⁻¹ | + |
| SDS | 10% | + |
| Sodium azide | 10% | + |
| Sodium periodate | 0.02 M, pH 4.5 | + |
| Urea | 6 M | + |
| Wheatgerm agglutinin | 0.1–0.4 mg ml ⁻¹ | + |
| Zymolyase | 2 mg ml ⁻¹ | + |

Capsule binding was judged by the fluorescence pattern of Cy3-conjugated anti-capsule antibody. Cells were scored as to the fraction with uniformly labelled surfaces, which was one of three patterns: +, >95%; ±, 40–60%; -, <10%.

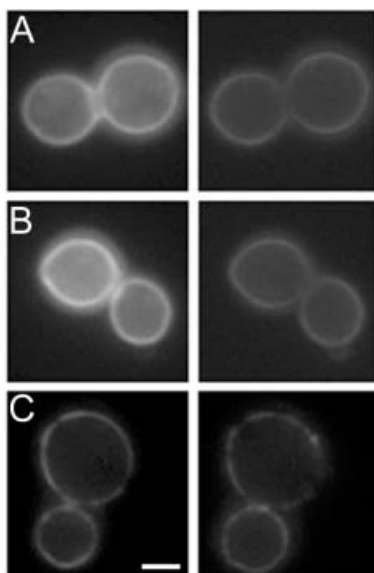


Fig. 2. Treatment with glucanase, but not protease, perturbs capsule binding. *Cap59* cell walls were fluorescein labelled, and the cells were treated with protease or glucanase (see *Experimental procedures*) before exposure to CM and labelling with Cy3-conjugated anti-capsule antibody. Fluorescence of tagged cell walls (left) and indirect immunofluorescence of capsule material (right) are shown for cells treated with buffer alone (A), protease (B) or glucanase (C). Scale bar, 2.5 μ m. The image in (C) was chosen to show residual cell wall (left) and consequent capsule binding (right).

The final candidate for capsule binding was cell wall glucan. We used various glycanases (both crude mixtures and pure enzymes) to treat cryptococcal cell walls before performing the capsule transfer assay. When the enzyme treatment included α -1,3-glucanase, we noted that the binding of capsular polysaccharide was altered in parallel with degradation of the cell wall, suggesting that α -1,3-glucan mediates an interaction between the two structures (compare Fig. 2C and A). α -1,3-glucan is found in the cell walls of several fungi besides *C. neoformans*, including *Histoplasma capsulatum*, *Blastomyces dermatitidis* and *Paracoccidioides brasiliensis* (Klimpel and Goldman, 1988; James *et al.*, 1990; Hogan and Klein, 1994; Borges-Walmsley *et al.*, 2002), but is not a component of *S. cerevisiae* (Lipke and Ovalle, 1998) or *C. albicans* cell walls (Klis *et al.*, 2001). (For a review and comparison of the cell wall components of these yeasts, see Bose *et al.*, 2003.)

To examine the surface accessibility of cryptococcal α -1,3-glucan, we labelled cells with monoclonal antibodies specific for this moiety. Antibodies bound well to acapsular cryptococcal cells (not shown), but not to encapsulated *C. neoformans* (not shown), presumably because the capsule blocks access to this epitope. The α -1,3-glucan antibody also did not bind to *S. cerevisiae* (not shown), consistent with the absence of this linkage in cell walls of this yeast (Lipke and Ovalle, 1998).

We used the dimorphic fungal pathogen *H. capsulatum* to examine the role of α -1,3-glucan in capsule binding. This organism contains surface-accessible α -1,3-glucan in its cell wall, but can spontaneously mutate to a form lacking this polymer (Klimpel and Goldman, 1988). Cryptococcal polysaccharide bound extensively to wild-type *H. capsulatum*, but not at all to the isogenic strain lacking α -1,3-glucan (Fig. 3). This assay demonstrated that this specific glucan moiety can mediate capsule association.

We next wished specifically to test the role of α -1,3-glucan in cryptococcal capsule construction. We first identified a putative *C. neoformans* α -1,3-glucan synthase gene (*AGS1*) by homology with sequences encoding these enzymes in *Aspergillus fumigatus* (one of two α -1,3-glucan synthase genes; Bernard and Latgé, 2001) and *Schizosaccharomyces pombe* (one of a family of α -1,3-glucan synthase genes; Katayama *et al.*, 1999). We hypothesized that reducing expression of this gene would deplete the α -1,3-glucan from *C. neoformans*, and thus abolish the ability of cells to bind capsule. To accomplish this, we turned to double-stranded RNA interference (Hammond *et al.*, 2001; Hannon, 2002), which we recently developed as a tool in *C. neoformans* (Liu *et al.*, 2002; Cottrell and Doering, 2003). We chose this method because it is rapid, it allowed us to study the role of this extremely large gene without knowing the entire coding sequence, it is readily reversible, and it would allow us to investigate a potentially essential gene.

We performed RNA interference as described in *Experimental procedures*, using a hairpin construct to target a 438-nucleotide portion of the *AGS1* sequence (Fig. 4).

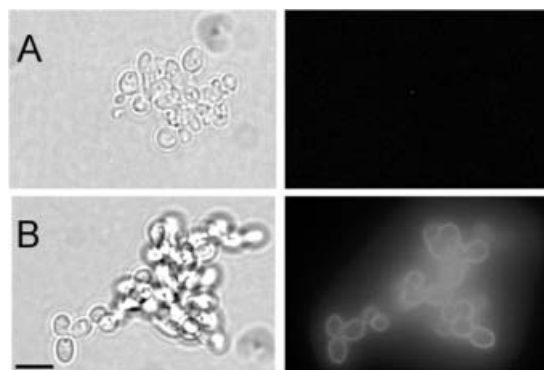


Fig. 3. Capsule material from *C. neoformans* binds only to *H. capsulatum* strains with α -1,3-glucan. Fixed *H. capsulatum* cells were exposed to CM and labelled with Cy3-conjugated anti-capsule monoclonal antibody. Phase-contrast (left) and immunofluorescence (right) images are shown of G186AS, which lacks cell wall α -1,3-glucan (A), and wild-type strain G186AR, which contains α -1,3-glucan and grows in larger clumps (B). Scale bar, 5 μ m. Note that the blurred appearance of the fluorescent image in (B) results from the intense staining; the image was not deconvoluted.

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Cn KIGGLGVMSLLMGKAMTDVLDLIWVVKVQD
Af KIGGLGVMAQLMGKTLGHQDLIWVVPVGG
Sp KIGGLGVMAQLMAQHLKHEDLVWVVPVGD

Cn LEYPOGEYRAEPIEVIIFGEPYLLIEVETHKI
Af VDYPVDRPAEPMIVTILGKPYEQVQYHII
Sp VVYPEAEEASPEIEVKIIDQTYTINVYHYL

Cn DNITYVILDSPVFRQTKADPYPPORMDDL
Af QNITYVILDAPVFRQSKSEPPPRMDDL
Sp DNIKYVILDAPVFRQTSKEPPPARMDL

Cn SAIFYSTWNOATAETIRRNPIVDIYHINDY
Af SAIFYSAWNOCIAQTIKREPI-DIYHINDY
Sp SAIFYSAWNOCIAEVIRRNPI-DIYHINDY

Cn HGALAPLYILPKVVEVCLSLHNAEFQGL
Af HGSLAPLYILPQTIPACLTLHNAEFQGL
Sp HGALAPCYLLPDIIPCALSLHNAEFQGL

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Fig. 4. A putative α -1,3-glucan synthase gene from *C. neoformans* strain H99 (Cn) is homologous to genes in other fungi. A portion of translated sequence from the predicted *C. neoformans* enzyme synthase domain (Hochstenbach *et al.*, 1998; Bernard and Latgé, 2001) is compared with the homologous portions of Ags1p from *Aspergillus fumigatus* (Af) and Mok1p from *Schizosaccharomyces pombe* (Sp). The region shown corresponds to amino acids 1186 to 1332 of Mok1p, which is the portion of *C. neoformans* Ags1p used for RNAi.

RNA blotting demonstrated the efficacy of interference (Fig. 5). AGS1-i cells showed a 93% reduction in AGS1 mRNA [after correction for the expression of a control gene, actin (*ACT*)]. Immunoelectron microscopy using gold-labelled anti- α -1,3-glucan antibody (Eissenberg *et al.*, 1997) showed that transformants in which RNAi was effective (AGS1-i cells) had dramatically reduced α -1,3-glucan in their cell walls [0.006 gold particles per cell ($n = 156$)] compared with wild-type cells [0.74 gold particles per cell ($n = 77$)].

We noted that growth of the AGS1-i cells was greatly slowed at 30°C, with a doubling time of 7 h compared with 3.5 h for wild-type cells or cells expressing a control plasmid. AGS1-i cells did not grow visibly on solid media at 37°C, and growth was severely depressed in culture at 37°C; cell numbers decreased throughout 4 days at 37°C, although at 10 days, colony-forming units were still present. Slowed growth is characteristic of α -glucan synthase mutants in *A. fumigatus* (Bernard and Latgé, 2001), and *S. pombe* α -glucan synthase mutants are temperature sensitive (Hochstenbach *et al.*, 1998; Katayama *et al.*, 1999). Although the latter phenotype is reversed in *S. pombe* by growth under conditions of high osmolarity (Katayama *et al.*, 1999), this was not the case for the AGS1-i cells (not shown). AGS1-i cells also demonstrated increased susceptibility to stressful growth conditions, as they did not grow in the presence of 0.005% sodium dodecyl sulphate (SDS) at 30°C, whereas the parental strain grew well in that medium (not shown). This phenotype suggests a problem with cell integrity (de Groot *et al.*, 2001).

We next examined the capsule of AGS1-i cells. In comparison with wild type, AGS1-i cells had no capsule visible by electron microscopy (compare Fig. 6B and A). This characteristic, along with all other phenotypic changes in AGS1-i cells, reverted to the parental wild-type upon growth in the presence of 5-fluoroorotic acid (5-FOA) to select against maintenance of the *URA5*-marked interference plasmid (Fig. 6C). AGS1-i cells also did not react with anticapsular antibody, confirming the lack of capsule (Fig. 7E), and appeared to be clumpy in liquid culture, typical of acapsular cryptococci (Chang and Kwon-Chung, 1994) (compare Fig. 7C and E).

The acapsular phenotype of AGS1-i cells confirmed the importance of α -1,3-glucan, but lack of capsule could indicate inability to generate capsule components, inability to bind them or both. We first tested whether the AGS1-i cells could bind capsule in our transfer assay. When AGS1-i cells (seven independent transformants) were incubated with fluorophore-tagged anti-capsule antibodies, none exhibited uniform fluorescent rings. Furthermore, when the AGS1-i cells were incubated with conditioned medium (CM) from wild-type cells, they were unable to acquire capsule, in contrast to *cap59* cells (compare Fig. 7F and D). Growth in 5-FOA reversed this inability to bind capsule (Fig. 7H).

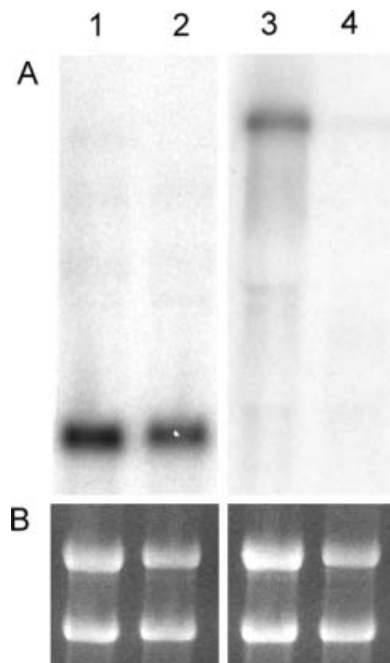


Fig. 5. Total RNA from wild-type JEC43 cells (lanes 1 and 3) or AGS1-i cells was probed with a radiolabelled segment of either *ACT*, which encodes actin (lanes 1 and 2), or *AGS1* (lanes 3 and 4). Phosphorimager analysis of the RNA blot (A) and an ethidium bromide image of the original gel (B) are shown. Results identical to those shown in lanes 3 and 4 were obtained with two additional non-overlapping *AGS1* probes (not shown).

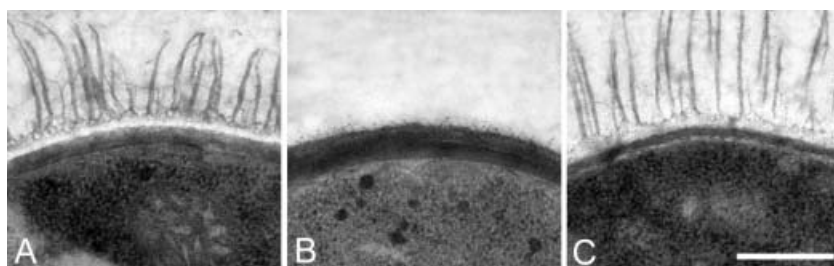


Fig. 6. AGS1-i cells do not have capsule. Thin-section electron micrographs show portions of the edge of a wild-type cell (A), an AGS1-i cell (B) and an AGS1-i cell grown on 5-FOA (C). Scale bar, 0.5 μ m.

Finally, we investigated the ability of AGS1-i cells to generate capsule polysaccharide. Notably, CM prepared from AGS1-i cells did confer the ability to bind anticapsular antibody on *cap59* cells, similar to CM from wild-type cells or from AGS1-i cells cured of the interference plasmid with 5-FOA (compare Fig. 7J with I and L). This is in contrast to CM from *cap59* cells, which does not confer the ability to bind anti-capsule antibody (Fig. 7K). These results demonstrate that cryptococci lacking α -1,3-glucan in their cell walls do generate and shed functional capsule components, but no longer express the determinants required for binding them and thereby assembling a capsule.

We have used an *in vivo* assay to model the interactions between cryptococcal cells and their major virulence factor, the polysaccharide capsule. We found that, contrary to previous speculation, the critical interaction is mediated by α -1,3-glucan in the cell wall of the fungus. The presence of α -1,3-glucan has previously been implicated in fungal virulence for filamentous and dimorphic fungi (Klimpel and Goldman, 1988; Hogan and Klein, 1994; Borges-Walmsley *et al.*, 2002), but not for any organisms that grow only as yeast, such as *C. neoformans*. This moiety is also important in pathology caused by prokaryotes. For example, karyogenic strains of *Streptococcus mutans* form dental plaque of α -1,3-glucan (Tsumori *et al.*, 1985; Wiater *et al.*, 1999).

We confirmed the importance of α -1,3-glucan in capsule binding using RNA interference specifically to target the corresponding glucan synthase. This approach yielded cells that lack α -1,3-glucan in their cell walls and are acapsular. The cells barely grow at 37°C in culture, as described above, and are therefore likely to be avirulent in a mammalian host. This work demonstrates a new function for fungal cell walls, and also shows the applicability of double-stranded RNAi (dsRNAi) to investigations of fungal virulence. The complete disruption of capsule association with the cell by targeting a single enzyme, α -1,3-glucan synthase, also suggests a potential candidate for greatly needed anticryptococcal chemotherapy. β -Glucan synthase inhibitors are effective antifungals for treatments of candidiasis and aspergillosis, but do not work against cryptococcosis (Kurtz and Rex, 2001). α -1,3-Glucan synthesis may provide a new antifungal target to fill this gap in efficacy.

Experimental procedures

Reagents

Peptide-N-glycosidase F was obtained from Prozyme; β -1,2-xylosidase, β -1,4-xylosidase and α -1,3-glucan antibody (mouse IgM, myeloma) were from Calbiochem; fluorescein-conjugated rabbit anti-mouse IgM (μ -chain specific) was

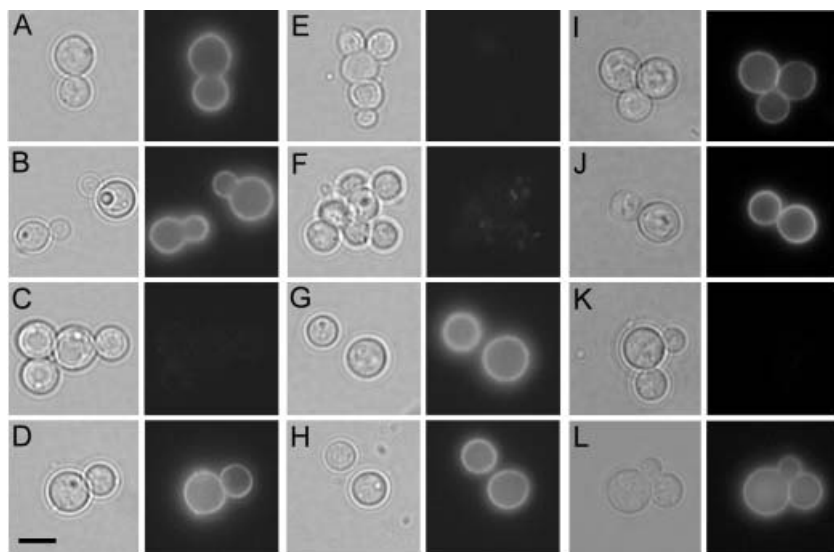


Fig. 7. AGS1-i cells are acapsular and do not bind exogenous capsule material, but do shed capsule polysaccharide. Phase-contrast (left) and immunofluorescence (right, as in Fig. 1) image pairs are shown of samples without (A, C, E and G) and with (B, D, F and H–L) CM treatment. Cells tested were JEC43 (A and B); *cap59* (C, D and I–L); AGS1-i (E and F); or AGS1-i grown in media containing 5-FOA (G and H). CM used was from wild-type cells (B, D, F, H and I), AGS1-i cells (J), *cap59* cells (K) or AGS1-i cells grown in media containing 5-FOA (L). Scale bar, 5 μ m.

from Pierce; Jack bean α -mannosidase was from Oxford GlycoSciences; zymolyase was from Zymo Research; Quantazyme was from InterSpex Products; fluorescein-conjugated concanavalin A, fluorescein-conjugated wheat-germ agglutinin, 4'-(aminomethyl)fluorescein hydrochloride and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride were from Molecular Probes; Cy3 monofunctional dye and the Megaprime™ DNA labelling system were from Amersham Pharmacia Biotech; TRIzol® reagent was from Gibco BRL; RNA loading mix was from GenHunter; ULTRAhyb® ultrasensitive hybridization buffer was from Ambion; [α -³²P]-dATP 3000 mCi mol⁻¹ was from Perkin-Elmer Life Sciences; and oligonucleotide primers were from Integrated DNA Technologies. Protease type XIV from *Streptomyces griseus*, lysing enzyme, chitinase, phospholipases C and D, laminarinase, SigmaSpin™ Post-Reaction purification columns and any unspecified reagents were obtained from Sigma. β -1,6-Glucanase was a generous gift from Johan C. Kapteyn and Franz Klis (University of Amsterdam, The Netherlands). Anti-capsular monoclonal antibodies 3C2 (MacGill *et al.*, 2000) and 2H1 (Casadevall *et al.*, 1992) were generous gifts from Thomas R. Kozel (University of Nevada) and Arturo Casadevall (Albert Einstein College of Medicine) respectively.

Strains and culture conditions

Cryptococcus neoformans strains included H99 (serotype A MAT α) from Dr Gary Cox (Duke University Medical Center), acapsular *cap59* [TYCC33 (Chang and Kwon-Chung, 1994); serotype D MAT α *cap59*] from Dr June Kwon-Chung (National Institutes of Health) and JEC43 [(Wickes *et al.*, 1997); serotype D MAT α *ura5*] from Dr Joseph Heitman (Duke University Medical Center). *Saccharomyces cerevisiae* strain RSY255 was from Dr Randy Schekman (University of California, Berkeley). All cells were grown at 30°C with continuous shaking in YPD medium [1% (w/v) yeast extract; 2% (w/v) peptone, 2% dextrose], minimal medium lacking uracil (Chanda, 2001) or minimal medium supplemented with 1 mg ml⁻¹ 5-FOA. *Histoplasma capsulatum* G186AR and G186AS strains were obtained from Dr Linda Eissenberg and Dr William Goldman (Washington University School of Medicine), grown as described by Eissenberg and Goldman (1987). These strains are isogenic, except that G186AS is α -1,3-glucan deficient.

Capsule transfer assay

Conditioned medium (CM) was prepared as a source of capsule polysaccharide by growing H99 wild-type cells for 1 week in YPD or minimal medium (CM from JEC43 also works in our assay; data not shown). Culture supernatant was sterile filtered, dialysed against phosphate-buffered saline (PBS) using a 3500 molecular weight cut-off dialysis membrane and stored at 4°C. CM from other strains was prepared in a similar way, but without the dialysis step. For capsule transfer assays, acapsular cells were used as acceptors of polysaccharide. Approximately 2.5×10^6 cells were washed twice in PBS, resuspended in 0.5 ml of PBS, rotated for 1 h at 23°C with 1 μ l of CM and then washed twice with

PBS. In some experiments, the cell walls of the acapsular acceptor strain were tagged with fluorescein before treatment with CM, using the method described by Pierini and Doering (2001). When acceptor cells were treated with enzymes before exposure to CM, they were incubated for 90–120 min at 37°C with 100 μ l of either 20 mg ml⁻¹ protease or 20 mg ml⁻¹ lysing enzyme, as a source of glucanase, and then washed twice with PBS.

Indirect immunofluorescence

For indirect immunofluorescence, cells were incubated for 60 min with 0.8 μ g ml⁻¹ Cy3-tagged anticapsular antibody (Pierini and Doering, 2001). Both anticapsular antibodies used, 3C2 or 2H1, gave equivalent results for all experiments shown. Cells were then washed twice with PBS and visualized on an Olympus BX60 microscope under phase-contrast and fluorescence-filtered conditions. Images were captured with IPLab SPECTRUM software and prepared for publication using Adobe PHOTOSHOP.

Electron microscopy

Ultrastructural analysis was performed as described by Eisenberg *et al.* (1997) with minor modifications. Yeast cells were fixed for 2 h at 4°C in 2% glutaraldehyde (Polysciences) in 100 mM phosphate buffer, pH 7.2. Cells were then washed in phosphate buffer, post-fixed in 1% osmium tetroxide (Polysciences) for 1 h at 4°C, rinsed in buffer and dehydrated in a graded series of ethanol and propylene oxide before embedding in Eponate 12 resin (Ted Pella). Samples were sectioned with a Leica Ultracut UCT ultramicrotome. Sections (70–90 nm) were stained with uranyl acetate and lead citrate and viewed with a Jeol 1200EX transmission electron microscope.

For immunolabelling studies, thin sections were blocked for 30 min with 5% fetal bovine serum/5% normal goat serum before a 1 h incubation with a 1:1 500 000 dilution of mouse monoclonal anti- α -1,3 glucan antibody in PBS. After washing, samples were probed for 1 h with a 1:30 dilution of 18 nm colloidal gold-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch). Parallel controls omitting the primary antibody were consistently negative at this concentration of colloidal gold-conjugated secondary antibodies. Sections were washed in phosphate buffer, rinsed with water and stained as above for imaging.

RNA interference

A single putative *C. neoformans* α -1,3-glucan synthase (AGS1) gene was identified in strain H99 genome sequence (<http://cneo.genetics.duke.edu/>) by homology with sequences encoding AGS1 in *Aspergillus fumigatus* (GenBank AAL28129) and *mok1+* in *Schizosaccharomyces pombe* (GenBank BAA3405). A 438-nucleotide portion of the α -1,3-glucan synthase sequence was polymerase chain reaction (PCR) amplified from H99 genomic DNA prepared as described previously (Casadevall and Perfect, 1998), using primers AGS1_RNAi1 (5'-GGAATTCATATGGAATTCGAA GATCGGTGGTTTGG-3') and AGS1_RNAi2 (5'-CGGTTC

CTAGGGAGACCTTGGAAGCTCGG-3') to add restriction sites *Nde*I and *Avr*II. The same gene fragment was amplified using primers AGS1_RNAi3 (5'-GAAGATCTTCAAGATCG GTGGTTTGG-3') and AGS1_RNAi4 (5'-CCGCTCGAGCGG GAGACCTTGGGAAGCTCGG-3') to add restriction sites *Bgl*II and *Xho*I. This region is 96% identical at the DNA level between H99 and JEC43 (<http://www.tigr.org/tdb/e2k1/cna1/>). These fragments were cloned into the appropriate sites of the *URA5*-marked plasmid pADE2i frame adjacent to 500-nucleotide portions of the cryptococcal *ADE2* gene detailed by Liu *et al.* (2002). Transcription of the resulting pAGS1-i/ADE2i-URA5 construct should yield a dsRNA hairpin product in which the stem contains both *AGS1* and *ADE2* sequences. JEC43 cryptococcal cells were transformed with pAGS1-i/ADE2i-URA5 by electroporation (Wickes and Edman, 1994), and transformants were selected on minimal medium lacking uracil. Pink colonies, indicating active RNAi targeting *ADE2* (Liu *et al.*, 2002), constituted 23% of all transformants and were chosen for further study. The resulting pink colonies could be reproduced upon repeated electroporation. All pink colonies tested exhibited reduced capsule binding in the transfer assay.

RNA blotting

RNA was isolated from cryptococcal cells using TRIzol® reagent and a modification of the method described by Liu *et al.* (2002), and RNA blotting was performed using standard methods (Brown and Mackey, 2001). A 348-nucleotide region of *AGS1* (downstream of the *AGS1* RNAi site) was chosen based on its probable protein coding nature (Flicek *et al.*, 2003). This was amplified using primers AGS1-T1 5'-CCT TCCAAGGTGTATTCCTCGC-3' and AGS1-T2 5'-GGCGG GATCTGACGGTAGTACT-3'. A 151-nucleotide region of *ACT* was amplified for a control probe template using primers actin-C3 5'-GGACCTCTATGGTAACATTGTCA-3' and actin-B3 5'-GATCCAGACACTGTACTTTCGC-3'. Probes were labelled with [α -³²P]-dATP using the Megaprime™ DNA labelling system according to the manufacturer's specifications. Images were obtained with a Fujifilm FLA-5000 PhosphorImager using IMAGE READER software and quantified using IMAGE GAUGE software.

Acknowledgements

We thank Hong Liu and Cara Griffith for technical assistance, and Wandy Beatty and Lori LaRose in the Molecular Microbiology Imaging Facility at Washington University School of Medicine for electron microscopy. We are grateful to William Goldman, Scott Hultgren and members of the Doering laboratory for helpful discussion during this work and comments on the manuscript. We thank William Goldman for the use of his fluorescence microscope, and Linda Eissenberg, Arturo Casadevall and Thomas Kozel for reagents. We appreciate helpful suggestions by Natalia Akopyants, Randall Brown, Mark Drew, Kathryn Luker, Chad Rappleye, Elizabeth Slawson and Stephanie Strand. Work on this project was supported by National Institutes of Health Award AI07172 (A.J.R.) and a Burroughs Wellcome Fund New Investigator Award in Pathogenic Molecular Mycology (T.L.D.). Develop-

ment of RNA interference techniques was supported by National Institutes of Health award AI49173.

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