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Loss of cell wall $\alpha(1-3)$ glucan affects *Cryptococcus neoformans* from ultrastructure to virulence

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SUMMARY

Yeast cell walls are critical for maintaining cell integrity, particularly in the face of challenges such as growth in mammalian hosts. The pathogenic fungus *Cryptococcus neoformans* additionally anchors its polysaccharide capsule to the cell surface via $\alpha(1-3)$ glucan in the wall. Cryptococcal cells disrupted in their alpha glucan synthase gene were sensitive to stresses, including temperature, and showed difficulty dividing. These cells lacked surface capsule, although they continued to shed capsule material into the environment. Electron microscopy showed that the alpha glucan that is usually localized to the outer portion of the cell wall was absent, the outer region of the wall was highly disorganized, and the inner region was hypertrophic. Analysis of cell wall composition demonstrated complete loss of alpha glucan accompanied by a compensatory increase in chitin/chitosan and a redistribution of beta glucan between cell wall fractions. The mutants were unable to grow in a mouse model of infection, but caused death in nematodes. These studies integrate morphological and biochemical investigations of the role of alpha glucan in the cryptococcal cell wall.

Keywords

Cryptococcus neoformans; alpha glucan; cell wall; ultrastructure; capsule

INTRODUCTION

Almost forty years ago, researchers in Scotland detected $\alpha(1-3)$ glucan in the cell walls of several non-pathogenic species of *Cryptococcus* (Bacon *et al.*, 1968). Although this polymer had been tentatively identified in other fungi in 1952 (Duff, 1952), and was isolated from *Aspergillus niger* in 1965 (Johnson, 1965), several decades elapsed before a protein was implicated in its synthesis. Studies of the non-pathogenic fission yeast *Schizosaccharomyces pombe* then identified a protein, termed Ags1p (for alpha glucan synthase; Hochstenbach *et al.*, 1998) or Mok1p (Katayama *et al.*, 1999), whose reduced expression correlated with loss of $\alpha(1-3)$ glucan and aberrant cell morphology. Recent work further suggests that this 272 kDa

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protein may function to both synthesize alpha glucan and to join $\alpha(1-3)$ glucan segments via linkers composed of $\alpha(1-4)$ glucan (Grün *et al.*, 2005). *S. pombe* encodes three additional proteins related to alpha glucan synthase, which are required for spore wall maturation. Two of these (Mok12p and Mok13p) are involved in the synthesis of glucans containing mainly $\alpha(1-3)$ linkages, while a third (Mok14p) acts in formation of glucan polymers which include $\alpha(1-4)$ linkages (Garcia *et al.*, 2006).

Among the pathogenic fungi, alpha glucan synthases have now been studied in *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Cryptococcus neoformans*. *A. fumigatus* has three such enzymes (Beauvais *et al.*, 2005; Maubon *et al.*, 2006). Ags1p is localized to the cell wall and is involved in formation of ~50% of the cell wall $\alpha(1-3)$ glucan, while Ags2p is intracellular and its loss has no detectable effect on glucan levels. Deletion of the genes encoding either Ags1p or Ags2p yields cells with altered hyphal morphology and reduced conidiation, but with normal virulence in a mouse model (Beauvais *et al.*, 2005). Deletion of the third gene, encoding Ags3p, results in cells that overexpress Ags1p, presumably to compensate for the lost enzyme activity and maintain normal wall composition. These cells demonstrate enhanced virulence in a mouse model, although that is probably an indirect effect (Maubon *et al.*, 2006). In contrast to the protein families of *Aspergillus* and *Schizosaccharomyces*, the *H. capsulatum* genome encodes only one alpha glucan synthase. Deletion of the gene results in cells that have reduced ability to kill macrophages in culture and to colonize murine lungs, establishing $\alpha(1-3)$ glucan as a major virulence factor of that pathogen (Rappleye *et al.*, 2004).

Our interest in alpha glucan stems from its role in the biology of the important opportunistic pathogen *Cryptococcus neoformans*. This fungus causes fatal meningoencephalitis in immunocompromised patients, with the majority of infections caused by serotypes A and D. Central to the virulence of *C. neoformans* is an extensive polysaccharide capsule, which surrounds and is linked to the cryptococcal cell wall. The wall is composed of alpha and beta glucans, chitin, chitosan, and mannoproteins. We previously used RNA interference (RNAi) to target the single cryptococcal alpha glucan synthase gene (*AGS1*), and showed that this eliminated proper association of capsule material with the cell surface. These cells also grew slowly and were temperature-sensitive (Reese and Doering, 2003).

The temperature-sensitivity we observed in cells subjected to RNAi targeting *C. neoformans* *AGS1* interested us for two reasons. First, we wondered whether this indicated that the gene was essential, but that the cells survived because of the inherently incomplete nature of RNAi. Second, even if strains deleted for *AGS1* were viable, the observed temperature sensitivity suggested that such cells would not survive in animals infected with *C. neoformans*. If this were true, Ags1p would be a candidate target for antifungal agents, especially as the mammals affected by cryptococcosis do not produce compounds similar to alpha glucans. In support of this idea, a relatively new class of antifungal drugs targets beta glucan synthase, an enzyme required for cell wall synthesis (Kartsonis *et al.*, 2003).

To explore the questions outlined above, and to understand the role of $\alpha(1-3)$ glucan in *C. neoformans* biology, we generated alpha glucan synthase disruption and appropriate complemented strains in serotypes A and D. Below we present our findings on these strains and the relationship between morphology, cell wall composition, virulence, and drug sensitivity.

RESULTS

We used protein sequence homology to *S. pombe* to identify the single cryptococcal *AGS1* gene from serotype D strain JEC21 (see Experimental Procedures). The gene, which is 8,480 nucleotides, includes 13 introns ranging from 46 to 80 nucleotides, and encodes a protein with

high homology to the corresponding enzymes of other fungi studied. Well-conserved regions occur throughout the protein (Fig. 1), covering each putative functional domain (Hochstenbach *et al.*, 1998). This is particularly notable in the putative glycogen synthase domain (Fig. 1), which has been proposed to be involved in binding UDP-glucose or ADP-glucose and in polymer synthesis, based on comparisons with glycogen synthases from *Escherichia coli* (Furukawa *et al.*, 1993). A mutation in the *S. pombe* Ags1p that conferred temperature sensitivity (asterisk in Fig. 1) was located in the putative hydrolase domain (Hochstenbach *et al.*, 1998), and we targeted this region in our gene disruption in *C. neoformans*.

We performed biolistic transformation of serotype D strain JEC21 to replace part of the putative glycosyl hydrolase domain of *AGS1* (Fig. 1) with a *C. neoformans* *URA5* gene. If the inserted sequence were read through from the intact upstream portion of the hydrolase domain, it would encode 20 unrelated amino acids followed by a stop codon. We also generated a version of the resulting *ags1* Δ strain that was complemented by exogenous integration of the intact wild type *AGS1* gene (*ags1* Δ *AGS1*). We confirmed the sequence replacement and subsequent complementation by PCR and DNA blotting (see Experimental Procedures).

To assess the loss of alpha glucan, we performed immunoelectron microscopy using a specific antibody developed against *S. pombe* α (1–3) glucan (Konomi *et al.*, 2003; Sugawara *et al.*, 2003). As shown in Fig. 2, we detected no antibody-reactive material in the cell walls of *ags1* Δ cells, indicating a complete lack of α (1–3) glucan. In contrast, the parental wild type strain showed abundant labeling in the cell wall, primarily in the outer portion of that structure. The results from complemented mutant cells were like those from wild type, indicating that the loss of alpha glucan labeling was indeed due to the engineered *AGS1* disruption. As expected from our previous studies using RNAi, the mutant also lacked capsule fibers extending from the cell wall, in sharp contrast to the other two strains.

To examine larger cell populations for the potential presence of capsule we stained cells with antibody to the major polysaccharide of the capsule (MacGill *et al.*, 2000). Wild type cells and the complemented mutant strain (*ags1* Δ *AGS1*) bound anticapsular antibody to their surfaces (Fig. 3, top panel). In contrast, *ags1* Δ cells did not react at all with this antibody, similar to a known acapsular strain, *cap59* (Fig. 3, top panel), and to the RNAi strain (Reese and Doering, 2003).

Wild type cryptococci constitutively shed capsule material into their surroundings. When mutant strains that lack capsule are incubated in conditioned medium from wild type cells, which contains shed capsule material, they recover the ability to bind anticapsular antibody, even after extensive washing (Reese and Doering, 2003). This is shown for the acapsular strain *cap59* in the lower panel of Fig. 3 (*cap59*/wt). Conditioned medium from the *ags1* Δ mutant similarly transferred capsule material to acapsular acceptor cells (Fig. 3, *cap59*/*ags1* Δ), indicating that this mutant, like wild type cells, sheds capsule components. However, *ags1* Δ cells were not able to bind capsule material to their own surfaces, even when it was provided by wild type conditioned medium (Fig. 3, *ags1* Δ /wt). The mutant cells were present in clumps, a characteristic typical of acapsular strains, although the clumps were larger than those seen for *cap59*. The *ags1* Δ cells were also less regular in size and shape than either acapsular or wild type cells. These characteristics were seen in *ags1* Δ cells of both serotypes A and D (Fig. 3), and in cells in which RNAi targeted *AGS1* (Reese and Doering, 2003).

Typical of cryptococcal strains lacking capsule (Bulmer *et al.*, 1967), *ags1* Δ colonies of both serotypes had a dry, dull appearance, and were not as bright a white color as the wild type (Fig. 4). Like the corresponding RNAi strains, the mutants did not grow at 37 °C and grew slowly at 30 °C: the doubling time for serotype A *ags1* Δ cells at 30 °C was 4.8 hours, compared to parental and complemented strain doubling times of 2.5 and 2.6 hours, respectively. Poor

growth of the mutant cells was exacerbated by the presence of 0.05% SDS (Fig. 4), or by the presence of 30 μ g/ml Congo Red or 1 mg/ml Calcofluor White (not shown), all phenotypes consistent with poor cell integrity (de Groot *et al.*, 2001). These growth defects were common to both serotypes. We detected no difference in the efficiency of mating when serotype D *ags1* Δ cells were used as either the *MATa* or *MAT α* partner in mating reactions (not shown).

We wished to pursue the apparent defect in cell integrity of cells lacking Ags1p by examining the cell wall ultrastructure. Using fixation conditions for electron microscopy that we optimized to preserve even abnormally thickened cell walls (see Experimental Procedures for rationale and details), we observed dramatic alterations in the cell structure (Fig. 5). Compared to wild type or complemented strains, the mutants were misshapen and exhibited abnormally broad junctions at the bud neck (panel B) and the division septum (panel E). Wild type cell walls are uniform, with a more striated inner region that blends smoothly into a more particulate outer region. In the mutants, the wall is thickened and ragged, and appears to be less dense overall. The two regions of the wall no longer form a smooth continuum, but show a hypertrophic striated region with only patchy material clinging to its surface (see panel E, expanded image). This phenotype was enhanced in serotype A cells (panel E), and by growth in minimal medium (not shown).

The apparent overgrowth of the inner layer of the cell wall, and overall thickening of the cell wall structure, suggested that the *ags1* Δ strains were undergoing compensatory changes to maintain cell integrity in the absence of α (1–3) glucan. To address this we wished to examine cell wall composition directly. Such studies in *C. neoformans* have previously been performed in acapsular mutants, to avoid contamination with the abundant capsule polysaccharides (James *et al.*, 1990). We therefore generated an acapsular version of our serotype D *ags1* Δ mutant by crossing it to the acapsular strain B4131, which has a point mutation in the *CAP59* gene (Chang and Kwon-Chung, 1994). As described in the Experimental Procedures, we isolated cell wall material from the resulting double mutant and prepared alkali-insoluble (AI) and alkali-soluble (AS) fractions. The AI fractions in wild type cells are composed predominantly of beta glucans, along with chitin, chitosan and small amounts of mannan (James *et al.*, 1990; Banks *et al.*, 2005). AS fractions typically contain alpha and beta glucans. Cells bearing both the *ags1* Δ and *cap59* mutations showed profound alterations in the composition and distribution of cell wall components compared to those bearing the *cap59* mutation alone (Fig. 6). As expected, α (1–3) glucan itself was completely absent, indicating that no other pathway in *C. neoformans* significantly contributes alpha glucan to the cell wall under these conditions. Interestingly, beta glucan was redistributed from the AS layer of the wall to the AI fraction, although it remained roughly 80% of the total cell wall mass. Concomitant with the loss of α -glucan from the mutant strain, the content of chitin and chitosan was increased to about twice its normal fraction of the cell wall (Fig. 6).

In recent years, the echinocandins, which inhibit β (1–3) glucan synthase, have been developed as a new class of antifungal agents (Kartsonis *et al.*, 2003). Interestingly, *C. neoformans* is not susceptible to these compounds, although the cryptococcal beta(1–3) glucan synthase is essential (Thompson *et al.*, 1999) and is sensitive to them *in vitro* (Maligie and Selitrennikoff, 2005). The altered distribution of beta glucan in the *ags1* Δ cell wall made us wonder whether the mutant cryptococci might have altered susceptibility to antifungal compounds that either target beta glucan specifically, or that might benefit by a disorganized and weakened cell wall. To investigate this we examined the effect of established antifungal agents on the growth of wild type, *ags1* Δ , and complemented strains. These experiments were performed in serotype A because it is more virulent than serotype D, and is most commonly isolated from immunocompromised patients with cryptococcal infections (Mitchell and Perfect, 1995). We found that the susceptibility of the *ags1* Δ strain to the echinocandin tested was not significantly

different from that of parental cells, although the mutant was slightly more susceptible to several azole antifungal drugs (Table 1).

The central role of capsule in cryptococcal pathogenesis is undisputed. Less clear, however, are the relative contributions of capsule material that is present on the cell surface and capsule material that has been shed from cells. The *ags1* Δ strains are unique among cryptococci without capsule, in that they still shed capsule components although they are unable to bind these molecules to their surfaces (Fig. 3). This makes virulence studies with the *ags1* Δ strain important. We used an intranasal inoculation model (Lim *et al.*, 1980) to examine the growth of these cells in mice. Although recovery of serotype A mutant cells from the lungs of infected mice was similar to wild type at 1 hour after infection, by one week later we were unable to recover any cells from the lungs of infected animals while recovery of wild type had increased over 100-fold (data not shown). This was not surprising, given the temperature sensitivity and compromised integrity of this mutant.

Because of the temperature sensitivity of the mutant strain, we turned to a less conventional disease model that can be tested at lower temperatures. The soil nematode, *Caenorhabditis elegans*, has been used to evaluate cryptococcal virulence (London *et al.*, 2006; Mylonakis *et al.*, 2002; Mylonakis *et al.*, 2003; Mylonakis *et al.*, 2004; Nielson *et al.*, 2005; Tang *et al.*, 2005). These investigations have demonstrated that several mutations in cryptococcal cells, which are known to reduce virulence in mammals, also abrogate the shortened lifespan and reduced progeny formation that are caused in worms by *C. neoformans* with wild type virulence (Tang *et al.*, 2005). We observed several differences when we compared worms that were exposed to *C. neoformans* serotype A *ags1* Δ to those exposed to the parental strain (Fig. 7). First, unlike the parental wild type cells, no mutant cryptococci were visible as intact spheres in the gut beyond the grinder (Fig. 7), suggesting that most of them were crushed during passage through this structure. Mutant cells also did not induce the digestive organ distension seen when worms were exposed to the parental strain (Fig. 7, white arrows), and were not recovered to the same extent (3.80 $\pm$ 2.0 colony forming units per worm for *ags1* Δ versus 33.20 $\pm$ 7.79 for the parent; *p* value < 0.05). Despite these differences, exposure to the mutant strain still resulted in death of the worms, killing them with a half time of 5 days compared to 3 days for the wild type (*p* = 0.04). For comparison, worms grown on a nonpathogenic bacterial lawn survive well over 10 days, while those grown with no nutrient source survive no more than two days. Both the mutant strain and its parent dramatically inhibited the formation of progeny, with similar counts of 4.2 $\pm$ 0.77 progeny for the wild type and 3.3 $\pm$ 0.62 for the mutant (*p* value = 0.51), compared to over 100 progeny for worms grown on bacterial lawns.

DISCUSSION

Alpha glucan is critical to the normal function of yeast cell walls and plays an important role in the virulence of multiple fungal pathogens. Beyond *Aspergillus*, *Histoplasma*, and *Cryptococcus*, which have been mentioned above, the cell wall α (1–3) glucan of *Blastomyces dermatitidis* has been suggested to play a role in yeast-phagocyte interactions (Hogan and Klein, 1994). In *Paracoccidioides brasiliensis* a shift in glucan polymer linkage from beta to alpha occurs as cells convert from the environmental mycelium form to pathogenic yeast (reviewed in Borges-Walmsley *et al.*, 2002).

In this study, we investigated *C. neoformans* cells disrupted in their only copy of the alpha glucan synthase gene, *AGS1*. These cells are viable, although they grow slowly and are temperature sensitive. One notable result of their lack of cell wall α (1–3) glucan is that they no longer display capsule polysaccharide on their surfaces, although they do shed this material into their environment, as we observed earlier for a strain in which *AGS1* was targeted by RNA interference (Reese and Doering). Examination of the *ags1* Δ strain showed that the mutant

cells have poorly formed cell walls and have trouble dividing. Direct analysis of cells walls showed that a the loss of $\alpha(1-3)$ glucan is accompanied by an increase in chitin/chitosan content and the redistribution of beta glucan from the AS fraction to the AI fraction. The latter changes may be linked, as there is precedent for beta glucan bound to chitin being alkali insoluble (Hartland *et al.*, 1994), although we have not tested this directly. However, even the compensatory changes we observe are insufficient to restore the integrity of mutant cells, so they remain sensitive to cell wall stress. Our electron microscopy studies highlight the corresponding morphological changes in *ags1* Δ cells, demonstrating that a reduction of the outer region of the cell wall (Fig. 5) accompanies loss of $\alpha(1-3)$ glucan. The small amount of this region that remains is tremendously disorganized, and is adjacent to a hypertrophic variant of the usual striated inner zone (Fig. 5, panel E). The perturbed wall, lacking $\alpha(1-3)$ glucan, can no longer serve as an attachment site for the capsule fibers that are essential for cryptococcal cells to cause disease.

Studies in other fungal pathogens have demonstrated a range of alpha glucan expression within individual species, depending on the strain examined (Hogan and Klein, 1994; Rappleye *et al.*, 2004). To test whether our results could be generalized within *C. neoformans*, we generated *ags1* Δ mutant and appropriate complemented control strains in both serotypes A and D. For all phenotypes tested, these strains were the same, with the exception of more extensive changes in the appearance of serotype A *ags1* Δ cell walls (Fig. 5). We have noted that the cell walls of serotype A are generally thicker and denser in appearance, and are more resistant to both enzymatic and physical disruption, than those of serotype D (A. Yoneda and T. L. Doering, unpublished results). The more dramatic mutant phenotype probably reflects this difference, rather than any fundamental difference in the role of alpha glucan.

Cell wall synthesis is an attractive target for antifungal agents, analogous to the many successful antibiotics that target the corresponding process in bacteria. Echinocandin compounds inhibit fungal beta(1-3) glucan synthesis, and are approved for clinical use for certain manifestations of candidiasis and aspergillosis, as well as for prophylactic or empiric treatment in selected contexts (reviewed in (Morrison, 2006)). Interestingly, these compounds are not effective against *C. neoformans* infections, although *in vitro* studies indicate they do inhibit cryptococcal beta(1-3) glucan synthase (Maligie and Selitrennikoff, 2005), which is essential for cell viability (Thompson *et al.*, 1999). Although all of the cell wall glucan in our mutant cells is present in beta linkage, this did not significantly alter susceptibility to the echinocandin we tested. This supports the conclusion that the resistance to echinocandins displayed by cryptococci is not due to any lack of importance of beta glucan to cell wall structure in this organism, but rather to one or more other mechanisms. These might include degradation or elimination of the drug, modulation of pathways involved in maintaining cell integrity (Kraus *et al.*, 2003), or inherent features of the target enzyme. We did note a modest increase in susceptibility of the *ags1* Δ cells to several other antifungal compounds currently in use (Table 1), but this probably reflects the general reduction in cell wall integrity and health of this mutant.

A unique feature of the *ags1* Δ mutant is that the surface display and secretion of capsule polysaccharides have been unlinked. This may shed light on the individual roles in pathogenesis of these two populations of capsule material. Because of the temperature sensitivity of the *ags1* Δ mutant, these studies cannot be carried out in mammals, but we did examine the behavior of the mutant and parental strains in a *C. elegans* model of infection. The mutant strain was predominantly lysed upon ingestion, but nonetheless caused accelerated death of the worms. This was similar to the phenotype we previously described for a strain that is mutated in the *CAP59* gene (Mylonakis *et al.*, 2002). Strains deleted for *CAP59* lack capsule as assessed by India Ink staining (Chang and Kwon-Chung, 1994), and their surfaces do not react with antibody to glucuronoxylomannan (GXM), the dominant capsule component (Fig. 3). However, such cells do contain limited GXM that is detectable by ELISA assay upon cell lysis

(Garcia-Rivera et al., 2004). Point mutants in *CAP59* also produce the second major capsule component, galactoxylomannan (Vaishnav et al., 1998), although deletion strains have not been specifically tested for this compound. Further studies, perhaps using other low temperature experimental models of infection (London et al., 2006; Mylonakis and Fuchs, 2006), are clearly needed to define the key determinants of toxicity in the *C. elegans* model. While these determinants may include the presence of one or both capsule polysaccharides, it is clear that neither surface presentation of GXM nor an intact yeast cell is required. In the future it will be important to identify additional *C. neoformans* mutants that shed capsule components without displaying them on their surfaces, yet survive at mammalian body temperatures. Such mutants will help to extend our understanding of how the mode of capsule presentation affects cryptococcal virulence.

EXPERIMENTAL PROCEDURES

Reagents

Media components were from Difco, nourseothricin (ClonNat) was from Werner BioAgents (Germany), 5-fluoroorotic acid was from Midwest Scientific, and oligonucleotides were from Integrated DNA Technologies, Inc. and Invitrogen Life Technologies. Reagents for DNA isolation and biolistic transformation were from BioRad, DH5 α cells and some restriction enzymes were from Invitrogen, and other restriction enzymes were from New England Biolabs. Specialty reagents for electron microscopy were from Polysciences Inc. or Ted Pella, Inc, and colloidal gold-conjugated antibody was from Jackson Immunoresearch. Mice for virulence studies were from Jackson Laboratories and 100 mm tissue culture plates for worm studies were from Falcon. Other reagents were from Sigma Chemical Co.

Strains and culture conditions

Encapsulated *Cryptococcus neoformans* strains used in this work included JEC21 [serotype D *MAT α* (Kwon-Chung et al., 1992)], JEC43 [serotype D *MAT α ura5* (Wickes et al., 1997)], and JEC34 [serotype D *MAT α ura5* (Wickes et al., 1997)] from Joseph Heitman (Duke University Medical Center), as well as H99 [serotype A *MAT α* (Perfect et al., 1993)] and H99R [serotype A *MAT α ura5* (Gorlach et al., 2002)] from Gary Cox (Duke University Medical Center). Acapsular strains were provided by June Kwon-Chung (National Institutes of Health). These were *cap59* [serotype D *MAT α cap59* (Chang and Kwon-Chung, 1994)], in which the *CAP59* gene is deleted, and B4131 [also called Cap67; serotype D *MAT α cap59* (Jacobson et al., 1982)], which has a point mutation in the same gene. Cells were grown at 30 °C with shaking in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% dextrose) or in minimal medium as indicated. Strains in which the *AGS1* gene was partially replaced by the *URA5* gene (see below) are termed *ags1 Δ* , and these strains complemented by exogenous integration of the wild type *AGS1* gene are designated *ags1 Δ AGS1*.

Sequences and databases

To search for a cryptococcal homolog of the *Schizosaccharomyces pombe* α (1–3) glucan synthase, we performed a TBLASTN-type Basic Local Alignment Search Tool (BLAST (Altschul et al., 1997)) search against the 9x sequence coverage of serotype D strain JEC21 that was available in February 2003 from The Institute of Genomic Research (TIGR). The resulting genomic DNA sequence (gDNA from contig 502641, chromosome 7) was PCR-amplified, sequenced, and aligned with a gene model predicted by TWINSKAN (Tenney et al., 2004) to obtain predicted coding sequence (AY436751). Full sequence information is available at TIGR

(
http://www.tigr.org/tigr-scripts/euk_manatee/shared/seq_display.cgi?db=cna1&orf=177.m03284
) (Loftus et al., 2005). Final annotation predicts that the protein contains 10 amino acids at the

N-terminus that were not in our complementing construct, but our results indicate that the shorter protein is functional. The full protein sequence is available with GenBank accession number XP_572121.

Preparation of nucleic acids

Genomic *C. neoformans* DNA was prepared from JEC43 as described by Nelson *et al.* (Nelson *et al.*, 2001). Total RNA was isolated from JEC43 *C. neoformans* according to published methods (Missall *et al.*, 2005), with the modifications that 15 ml conical tubes capable of high speeds (Falcon #352059) were used for centrifugation and DEPC-treated water was used for the final suspension. First strand cDNA synthesis was accomplished with the SuperScript™ First-Strand System (Invitrogen).

Polymerase chain reaction conditions

For amplification of most nucleic acid sequences of 2 kb or less, the following conditions were used with a RoboCycler® Gradient 96 Temperature Cycler with Hot Top Assembly (Stratagene): initial denaturation of 2 min at 94 °C; 30 cycles of 1 min denaturation at 94 °C, 1 min annealing at 55 °C, and 2 min elongation at 68 °C; and one final 7 min extension at 68 °C. For larger segments, the conditions were altered as indicated in the text.

Strain disruption

We used fusion PCR (Davidson *et al.*, 2002) to generate an *AGS1* disruption construct with the cryptococcal *URA5* gene (Edman and Kwon-Chung, 1990) as a selectable marker, flanked by portions of the *AGS1* gene. The construct was designed so the marker replaced 203 bp of the *AGS1* sequence from the proposed hydrolase domain (Hochstenbach *et al.*, 1998). First, a 981 bp upstream *AGS1* gene segment (5' *AGS1*, corresponding to genomic nucleotides 1399–2380) was amplified by primers AJR-O11 and AJR-O14 (Table 2); a 1021 bp downstream *AGS1* gene segment (5' *AGS1*, corresponding to genomic sequence 2583–3572) was amplified by primers AJR-O15 and AJR-O12; and a 1931 bp segment containing the *URA5* promoter and gene from plasmid pRCD69 (courtesy of R. Davidson, (Davidson *et al.*, 2002)) was amplified with primers AJR-O13 and AJR-O16. Each of these three segments was then gel purified and combined as template with primers AJR-O11 and AJR-O16 to produce a 3933 bp product (PCR conditions were modified to annealing temperature of 58 °C and extension time of 4 min). This final product was gel purified and introduced into JEC43 (a serotype D *ura*-strain) using a BioRad biolistic PDS-1000/He gene gun (Toffaletti *et al.*, 1993). We screened transformants that grew on medium lacking uracil for the expected disruption, using PCR and DNA blotting as previously described (Griffith *et al.*, 2004). For PCR, primers were designed to regions of *AGS1* within and flanking the planned deletion, and to the *URA5* coding sequence. PCR reactions confirmed the absence of the expected portion of *AGS1* and the presence of *URA5* in its place. Gel analysis of PCR products from primers flanking the replacement also demonstrated the size increase expected because a 203 bp sequence was replaced with one of 1931 bp (not shown). For DNA blots, total genomic DNA was digested with *NheI* and *SacI*, transferred to nylon membranes, and blotted with a radiolabeled PCR product corresponding to either the deleted segment or a portion of *URA5*. The blots confirmed the gene replacement and showed that no additional insertions of the marker had occurred at other genomic sites (not shown). Details of these experiments are available from the authors. Following confirmation of the serotype D *ags1Δ* strain, primers AJR-O11 and AJR-O16 were used to amplify the disruption cassette from this strain. The resulting DNA fragment was gel purified and used in similar biolistic transformation of H99R (a serotype A *ura*-strain), to derive a serotype A *ags1Δ* strain.

Strain complementation

The disruption strains from both serotypes were complemented with the entire cDNA corresponding to *AGS1*. Full length *AGS1* cDNA was amplified from JEC43 total RNA using primers AJR-O143 and AJR-O144 (Table 2) to incorporate flanking 5' *Xba*I and 3' *Sac*II restriction sites. The cryptococcal actin promoter (*ACT1*p, (Gorlach *et al.*, 2002)) was similarly amplified from plasmid pGMC200 from Gary Cox, Duke University Medical School) using primers AJR-O53 and AJR-O54 to incorporate flanking *Spe*I and *Xba*I restriction enzyme sites. This *ACT1*p was then cloned into the appropriate sites of pGMC200 downstream of the nourseothricin N-acetyl-transferase (*NAT1*) resistance marker (McDade and Cox, 2001) in that plasmid. The resulting plasmid pGMC200-*ACT1*p was transformed into DH5 α cells (GIBCO), the DNA extracted by Quantum Prep Plasmid Miniprep (BioRad) and the plasmid construction confirmed by PCR and restriction digests (not shown). The amplified *AGS1* cDNA was then cloned in-frame downstream of the *ACT1* promoter sequence, between the *Xba*I and *Sac*II sites, and confirmed as above. The final 12,733 bp plasmid (pAJR-B16) was transformed into DH5 α cells. Purified DNA was linearized with *Xmn*I and *Pme*I and the resulting 11.8 kb fragment gel purified and introduced by biolistics into both *ags1* Δ strains. Transformants were plated on YPD medium, incubated for 24 h at 30 °C, and then replated onto YPD plates containing 50 μ g/ml nourseothricin with 1 M sorbitol for osmotic support. Nourseothricin-resistant transformants were further screened by PCR and DNA blotting. These studies demonstrated the presence of wild type *AGS1* and *NAT1* sequences in the complemented mutant strain, each present in only one copy.

Strain growth studies

Strain growth rates in liquid culture were assessed by serial hemacytometer counts of cells from freshly grown overnight cultures that were sub-cultured to $1 - 5 \times 10^5$ cells per ml into room temperature YPD medium. Counts were performed in quadruplicate from two samples from each time point, and experiments were repeated. Growth on solid media was tested by spotting 5 μ l of 5-fold serial dilutions of cells onto the appropriate plates, starting from 1×10^6 cells per ml. For antibiotic sensitivity, cells were grown in YPD at 30 °C for 48 h, then inoculated into Sensititre YeastOne Susceptibility plates (TREK Diagnostic Systems) as directed by the manufacturer and incubated for 72 h at the same temperature. The MIC was recorded as the lowest concentration of an antifungal agent that prevented the development of a red or purple color in a well that was initially blue.

Immunofluorescence studies

Cells were stained as previously described (Pierini and Doering, 2001) with anti-capsular monoclonal antibody 1255 (from Tom Kozel, University of Nevada at Reno) and an Alexafluor 546-tagged anti-mouse antibody (Molecular Probes). Samples were visualized on a Zeiss Axioskop2 MOT Plus microscope (Carl Zeiss Inc, Thornwood, NJ) under phase-contrast and fluorescence-filtered conditions. To evaluate capsule transfer, conditioned medium (CM) from wild type cells was prepared as described previously (Reese and Doering, 2003). CM from other strains was prepared similarly, but without a dialysis step. For capsule transfer assays, 2.5×10^6 cells (of either acapsular strain *cap59* or the *ags1* Δ mutant) were washed twice in phosphate buffered saline (PBS), resuspended in 0.5 ml of PBS with 1 μ l of CM, rotated for one hour at 23 °C, and then washed twice with PBS before imaging.

Immunoelectron microscopy

Immunoelectron microscopy was performed at the Molecular Microbiology Imaging Facility at Washington University School of Medicine. Cells were grown in 50 ml YPD medium at 30 °C with shaking until they reached log phase and subjected to centrifugation (5 min, room temperature, RT, 1500 x g) in 50 ml conical tubes. the supernatant fractions were discarded,

and the cells were fixed in 2% glutaraldehyde in 100 mM phosphate buffer, pH 7.2 for 2 h at 4 °C. Cells were washed in phosphate buffer and post-fixed in 1% osmium tetroxide for 1 h at 4 °C. Samples were then rinsed in buffer followed by dehydration and embedding as above. 70–90 nm sections were blocked with 5% fetal calf serum, 5% normal goat serum for 30 min, and then incubated with a mouse monoclonal antibody to $\alpha(1\text{--}3)$ glucan for 1 h (Sugawara *et al.*, 2003). After washing with blocking buffer, samples were probed with 18 nm colloidal gold-conjugated anti-mouse secondary antibody for 1 h. Sections were washed in phosphate buffer and rinsed in water before examination as above. Parallel controls omitting the primary antibody were consistently negative at the concentration of secondary reagent used in these studies.

Electron microscopy

The fixation conditions described above (for immunoelectron microscopy) are not effective on cells with thick walls. This effectively selects against imaging of such cells because they are not well preserved. Further, the cell wall is a low contrast structure in images from cells prepared in this way, making it difficult to resolve details of its structure and to detect its outer boundary. For these reasons, we developed an alternate procedure to specifically examine cell walls. Cells were grown and sedimented as above, supernatant fractions were discarded, and the cell pellets were rapidly resuspended in 1 ml of pre-fixation mix (0.1 M sorbitol, 1 mM MgCl_2 , 1 mM CaCl_2 , 2% glutaraldehyde in 0.1M PIPES, pH 6.8). The cell suspension was transferred to microfuge tubes for centrifugation (0.5 min, room temperature (RT), top speed), and the cell pellets were resuspended in 1 ml of fresh pre-fixation mix and fixed overnight at 4 °C. Cells were next pelleted (as above), washed 3 times in water (each wash involved resuspension of the pellet followed by a 10 min incubation at RT), transferred to borosilicate tubes, pelleted (5 min, RT, 1500 x g), and resuspended in 2% KMnO_4 in water (Wright, 2000). The final spin and resuspension was repeated, and the cells were then post-fixed for 45 min at RT before being washed repeatedly in water until no purple color was visible (Wright, 2000). The samples were next transferred to microcentrifuge tubes and dehydrated for 10 min intervals in increasing concentrations of ethanol in water (once each with 30%, 50%, 70%, 90%, and 95%; then three times with 100%). The ethanol-substituted samples were next substituted in propylene oxide (PO; twice for 30 min each), and then infiltrated in PO and finally embedded in Eponate 12 resin. Samples were sectioned with a Leica Ultracut UCT ultramicrotome. 60–80 nm sections were stained with uranyl acetate and lead citrate and viewed with a JEOL 1200EX transmission electron microscope.

Strain crossing

For complete cell wall analysis, we required a control strain without capsule, to avoid potential contamination with capsule polysaccharides. To allow comparison of strains that were isogenic except at the *AGS1* locus, we crossed *ags1Δ* with an acapsular strain. We preceded this cross with intermediate steps to incorporate markers into the strains of interest and simplify progeny selection and screening. All strains used were of serotype D, and all crosses were performed on freshly poured V8 medium plates as in (Kwon-Chung, 1994). Briefly, we first crossed B4131 (*MATa cap59*) with JEC34 (*MATa ura5*), selecting for dull (acapsular) colonies that survived on 5-fluoroorotic acid (5-FOA) medium. The resulting *cap59 ura5* strains were tested for mating type by crossing to known α or $\mathbf{a}$ strains and observing filamentation, and the presence of the *cap59* point mutation from strain B4131 (Chang and Kwon-Chung, 1994) was confirmed by sequencing genomic DNA. In parallel, we crossed our *MATa ags1Δ* strain to a *MATa ade2 ura5* strain, and plated the resulting spores on minimal plates lacking uracil to select against the *MATa* parent (Edman and Kwon-Chung, 1990). Colonies that were both pink (*ade2*) and dull (probably *ags1*) were selected, tested by PCR and temperature sensitivity for the presence of the *ags1* mutation, and checked for mating type. Finally, a *cap59 ura5* strain product of the first cross was mated to an *ags1 ade2* strain from the second cross, and dull

white colonies were selected from spores plated on minimal medium lacking both uracil and adenine. These candidates were tested by PCR and growth at 37 °C to confirm the presence of the *ags1* mutation, by sequencing to confirm the presence of the *cap59* mutation, and by crosses to assign mating type. Confirmed MAT α *ags1 cap59* and MAT α *cap59* strains were then analyzed for cell wall composition.

Cell wall analysis

For analysis of total cell wall composition, cells were grown in YPD for 8 h, washed with water, and resuspended at 1-2.5 OD₆₀₀ units per 500 ml of minimal medium with 2% glucose. Cells were further grown at 30 °C with shaking for two days, at which point volumes corresponding to 1200 OD₆₀₀ units of each strain were sedimented, resuspended in 10 ml of 50 mM Tris HCl pH 7.5, and broken at 1100 psi in a French pressure cell. The lysed cells were washed 6 times in distilled water, frozen on dry ice, and then lyophilized to yield ~100 mg of cell wall material. Dried cell walls were boiled twice in 50 mM Tris-HCl, pH 7.4 containing 50 mM EDTA, 2% SDS, and 40 mM β -mercaptoethanol, and extensively washed with water. The alkali-soluble (AS) and alkali-insoluble (AI) fractions were extracted as described (Beauvais *et al.*, 2005). Determination of total hexose was performed by the phenol sulfuric acid procedure, with glucose as a standard (DuBois *et al.*, 1956). Total hexosamine was measured after hydrolysis with 4 N HCl for 4 h at 100 °C (Johnson *et al.*, 1971). Monosaccharides were determined by gas chromatography after hydrolysis, reduction, and peracetylation of the AI and AS fractions (Mouyna *et al.*, 1998). Aliquots of each fraction (15 – 30 μ g) were treated with recombinant glucanases for 24 h at 45 °C, and the reducing sugars that were released by digestion were measured by the *p*-aminohydroxybenzoic acid hydrazide method as previously described (Beauvais *et al.*, 2005). For α -1,3-glucanase digestion, incubation was with 0.2 μ g of Mutanase (from Claus Crone Fuglsang, Novozymes A/S, Bagsvaerd, Denmark) in 100 μ l of 50 mM sodium acetate buffer (pH 5.6). For β -1,3-glucanase digestion, incubation was with 2 ng of recombinant *Thermoyoga neapolita* LamA, purified from *E. coli* bearing a plasmid provided by Vladimir Zverlov (Institute of Molecular Genetics, Moscow) (Zverlov *et al.*, 1997), in 100 μ l of 50 mM sodium phosphate citrate buffer (pH 6.2).

Virulence studies

***C. elegans* killing assays**—*C. elegans* worms were monitored for survival on *Cryptococcus neoformans* lawns, essentially as described previously (Mylonakis *et al.*, 2002). *C. neoformans* serotype A strains H99R and *ags1 Δ AGS1* were inoculated in 2 ml of YPD with kanamycin (45 μ g/ml), ampicillin (100 μ g/ml), and streptomycin (100 μ g/ml), and grown with shaking at 30 °C for 24 h; the slower-growing *C. neoformans* serotype A *ags1 Δ* strain was grown similarly for 48 h. Lawns were prepared by spreading 10 μ l of each culture on 35-mm tissue-culture plates containing brain heart infusion (BHI) agar with the same antibiotics. The plates were incubated at 30 °C for 24 hours and then at 25 °C for 24 h. Approximately 70 nematodes at the L4 stage were transferred from Nematode Growth Media (NGM) seeded with *E. coli* OP50 to each of three BHI plates per *C. neoformans* strain. The plates were incubated at 25 °C and the worms were examined for survival at 24 h intervals with a Nikon SMZ645 dissecting microscope. At each interval, dead worms were counted and removed.

***C. elegans* progeny quantification**—*C. neoformans* lawns for progeny quantification were prepared as described above, and experiments were performed as in Tang *et al.*, 2005. One nematode at the L4 stage was moved from NGM plates seeded with *E. coli* OP50 to each of 16 lawns per *Cryptococcus* strain. At 24 h intervals living worms were transferred to new lawns for each yeast strain, and at 72 h, the living worms were removed and each BHI agar pad was inverted onto a 100 mm tissue culture plate containing NGM agar with streptomycin (100 μ g/ml) and seeded with *E. coli*. The plates were incubated at 25 °C for 48 h and viable

progeny, defined as larvae at or beyond first-stage, were counted. Only worms that survived all three days were included in calculating the total progeny laid over the course of 72 h.

Growth of *C. neoformans* in *C. elegans*—The number of *C. neoformans* colony forming units in *C. elegans* were quantified essentially as described (Garsin *et al.*, 2001), but with 10 worms at the L4 stage in each group and only one 48 h time-point of exposure to *Cryptococcus* lawns prepared on BHI plates as above. After exposure, worms were washed twice in 8 μ l drops of M9 medium on a BHI agar plate containing antibiotics as above, in order to remove surface cryptococci. Each group of 10 worms was then placed in an 1.5 ml microcentrifuge tube containing 40 μ l of M9 medium with 1% Triton X-100, and ground with a pestle. The volume was adjusted to 300 μ l with M9 medium containing 1% Triton X-100, and the final suspension was serially diluted and plated on YPD agar containing the same antibiotics. The plates were incubated for 48 h at 30°C, and colonies were counted.

Virulence in mice— $2-5 \times 10^4$ cryptococcal cells in 50 μ l of deionized water were delivered intranasally into 4–6 week old C57Bl/6 mice. Groups of three and five mice were humanely sacrificed at 1 h and 7 days after inoculation, respectively. The lungs were harvested, homogenized in water, serially diluted, and plated for colony-forming units as described previously (Sommer *et al.*).

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Figure 1.

C. neoformans Ags1p is closely related to other fungal alpha glucan synthases. The level of identity between the predicted protein sequences of *C. neoformans* Ags1p (AAR97368), *S. pombe* Ags1p (or Mok1p; AAC31430) and *A. fumigatus* Ags1p (AAL28129) is indicated by shading of each 50 amino acid segment: 0–20%, white; 21–40%, light gray; 41–60%, dark gray; 61–80%, black. Putative domains are indicated based on the model of Hochstenbach *et al.* (Hochstenbach *et al.*, 1998). The horizontal black bar from amino acids 946–1013 indicates the segment replaced by a marker in the *C. neoformans ags1Δ* strain, and the asterisk indicates the location of the mutation in the temperature sensitive *S. pombe* strain *ags1-1* (Hochstenbach *et al.*, 1998).

Figure 2.
ags1Δ cells lack $\alpha(1-3)$ glucan and capsule fibers. Shown are representative immuno-EM images of serotype D wild type (JEC43; wt), mutant (*ags1Δ*), and complemented (*ags1Δ*/wt) strains probed with monoclonal antibody specific for $\alpha(1-3)$ glucan. All panels are to the same scale; scale bar = 0.5 μ m.

Figure 3.

ags1Δ cells have no surface capsule, although they shed capsule material. Upper panel, the strains indicated (serotype A for all except *cap59*) were stained with monoclonal anti-GXM antibody 1255 and visualized by DIC (left image of each pair) or indirect immunofluorescence (right image of each pair). Lower panel, capsule transfer assays were performed as described in Experimental Procedures, using serotype D strains as acceptor cells (indicated before the slash) and sources of conditioned media (indicated after the slash). Image pairs are arranged as in the upper panel. Image contrast in both panels was adjusted in Photoshop, with identical changes made to each image, and all photographs are to the same scale (scale bar = 0.5 μ m).

Figure 4. *ags1Δ* cells are temperature sensitive. Growth of the indicated serotype D strains on YPD medium, without or with 0.05% SDS, is shown.

Figure 5.

ags1Δ mutants have poorly formed and hypertrophic cell walls, as well as difficulty separating. Panels A–C, serotype D: Panel A, JEC21; panel B, *ags1Δ*; panel C, *ags1Δ AGS1*. Panels D–E, serotype A: Panel D, H99R; panel E, *ags1Δ*. Upper image of each pair, scale bar 1 μm. Lower image of each pair, an expanded portion of the cell wall from the parental cell above, all to the same scale, scale bar 200 nm. Note that this fixation method does not preserve capsule.

Figure 6.

Relative to *AGS1* cells, *ags1Δ* cells lack alpha glucan, show redistribution of beta glucan to the alkali-insoluble fraction of the cell wall, and have increased content of chitin/chitosan. Open bars, *AGS1*; closed bars, *ags1Δ*. Both strains bear a point mutation in *CAP59*, as described in the text. It is possible that this mutation alters baseline cell wall composition relative to wild type, but use of strains in an acapsular background is necessary to enable cell wall analysis without contamination by capsule polysaccharides. The methods used do not distinguish between chitin and chitosan.

Figure 7.

C. elegans worms exposed to serotype A wild type or *ags1Δ* cryptococci. Upper panels, anterior (left) and posterior (right) portions of a nematode grown on a lawn of wild type (wt) cells show intact cryptococci distending the gut (demarcated by white arrows) after passage through the pharyngeal grinder (black arrows). Lower panels, similar portions of a nematode grown on a lawn of *ags1Δ* cells. All images are to the same scale.

An early bud of the pathogenic fungus *Cryptococcus neoformans*, demonstrating the polysaccharide capsule (meshwork at upper right) linked to the cell wall via $\alpha(1-3)$ glucan.

Table 1

Minimal inhibitory concentrations of antifungal agents.

Compound	<i>ags1Δ</i>	<i>ags1Δ AGS1</i>	H99
Caspofungin	4.0 [□]	4	8
Amphotericin B	0.12	0.25	0.25
5-Flucytosine	0.12	0.25	0.25
Fluconazole	8.0 ^a	32	32
Itraconazole	0.06	0.25	0.25
Ketoconazole	0.06	0.25	0.25
Voriconazole	0.06 ^a	0.25	0.25

Results of three independent experiments are shown, with values in µg/ml.

^a In one of the three experiments this result was twice the value listed.

Table 2

PCR Primers

Primer	Sequence
AJR-O11	5' CCAAGTTGAAACGTTTGGTTGTCTACC
AJR-O12	5' AGATAATTTGCACTCTGAGCGTTGGGC
AJR-O13	5' ACCAGTCAACAGGAAGGTCACGGATCCACTAGTAACGGCC
AJR-O14	5' GGCCGTTACTAGTGGATCCGTGACCTTCCTGTTGACTGGT
AJR-O15	5' GCCGAATTCTGCAGATATCCTCCCTATGAGAATTACACCC
AJR-O16	5' GGGTGTAATTCTCATAGGGAGGATATCTGCAGAATTCGGC
AJR-O143	5' <u>TCTAGA</u> ATGTTATTATGTCCAAGAGGAGGGGCGAGAATATCG (<i>Xba</i> 1)
AJR-O144	5' <u>CCGCGG</u> TTATGGCTTGCTCAGCTGTCCGCGTCGGAAGAA (<i>Sac</i> II)
AJR-O53	5' <u>ACTAG</u> TAACCGCTGCGAGGATGTGAG (<i>Spe</i> I)
AJR-O54	5' <u>TCTAGAC</u> GCCATAGACATGTTGGGCG (<i>Xba</i> 1)