Unusual galactofuranose modification of a capsule polysaccharide in the pathogenic yeast *Cryptococcus neoformans*

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Running title: Gal\textsuperscript{f} modifies a *Cryptococcus neoformans* capsule polysaccharide

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Supplementary data: Figures S1- S3, Table S1.

Background: Galactofuranose, the five-membered ring form of galactose, occurs in the encapsulated pathogenic fungus *Cryptococcus neoformans* but not in humans.

Results: We established the position of galactofuranose within a capsule polysaccharide and characterized cryptococci lacking this modification.

Conclusion: Galactofuranose occurs in an unusual linkage, but is not required for growth or virulence.

Significance: This work fills a gap in knowledge about a pathogen-specific modification.

SUMMARY

Galactofuranose (Gal\textsuperscript{f})\textsuperscript{8} is the five-membered ring form of galactose. Although it is absent from mammalian glycans, it occurs as a structural and antigenic component of important cell surface molecules in a variety of microbes, ranging from bacteria to parasites and fungi. One such organism is *Cryptococcus neoformans*, a pathogenic yeast that causes lethal meningoencephalitis in immunocompromised individuals, particularly AIDS patients. *C. neoformans* is unique among fungal pathogens in bearing a complex polysaccharide capsule, a critical virulence factor reported to include Gal\textsuperscript{f}. Notably, how Gal\textsuperscript{f} modification contributes to the structure and function of the cryptococcal capsule is not known. We have determined that Gal\textsuperscript{f} is β1,2-linked to an unusual tetrasubstituted galactopyranose of the glucuronoxylomannogalactan (GXMGal) capsule polysaccharide. This discovery fills a longstanding gap in our understanding of a major polymer of the cryptococcal capsule. We also engineered a *C. neoformans* strain that lacks UDP-galactopyranose mutase; this enzyme forms UDP-Gal\textsuperscript{f}, the nucleotide sugar donor required for Gal\textsuperscript{f} addition. Mutase activity was required for the incorporation of Gal\textsuperscript{f} into GXMGal, but was dispensable for vegetative growth, cell integrity, and virulence in a mouse model.

Glycans are critical components of the cell surface, defining the interface between cells and their environment. The monosaccharide components of glycans primarily occur in cyclic forms,
with aldohexoses forming either five- or six-membered rings (1). Galactose, for example, occurs as either a six-membered galactopyranose (Galp) or five-membered galactofuranose (Galf) ring. Galp occurs ubiquitously across all domains of life, but Galf is curiously restricted to simpler organisms that include prokaryotes (2-4), protozoa (5), and fungi (6-8), as well as certain algae (9), archaea (10), and invertebrates (11). Particularly intriguing is the absence of Galf from mammalian glycans, despite its prevalence in the pathogenic microbes that infect mammals (12). The role of Galf in microbial biology and pathogenesis is thus a subject of considerable interest.

Pathogenic microbes incorporate Galf into diverse extracellular molecules (13). In Mycobacterium tuberculosis the galactose portion of arabinogalactan (the material that links the external mycolic acid layer of the cell envelope to the peptidoglycan wall) is composed of β-linked Galf. Inability to synthesize Galf compromises cell integrity in this system, resulting in severely decreased viability and attenuated survival in the host (14). Kinetoplastids incorporate Galf into a protective glycoalyx that enhances establishment of disease and promotes survival of these parasites within their insect vectors (8). In Leishmania, loss of Galf synthesis or of the key galactofuranosyltransferase required to synthesize surface lipophosphoglycan results in severe defects in survival, both in the insect and in the early stages of mammalian infection (15-18). In the pathogenic fungus Aspergillus fumigatus, the presence of Galf in secreted glycoproteins, glycolipids, and cell wall polysaccharides (7,8) improves cell integrity (19-21) and reduces adhesion to mammalian epithelial and endothelial surfaces by shielding underlying mannan structures (20). Galf may also play a role in immune recognition of pathogenic microbes. Many mammals, including humans, produce intelectins that recognize monomeric Galf and Galf-containing molecules such as arabinogalactan (22). Galf is also a potent antigenic determinant in diverse extracellular molecules, including the lipopolysaccharides of Gram-negative bacteria (23-25) and the galactomannans of aspergilli (7).

The pathogenic yeast C. neoformans is responsible for lethal meningoencephalitis in over 600,000 immunocompromised individuals worldwide each year (26) and ranks among the main causes of mortality in patients with AIDS (27). The cryptococcal cell wall is surrounded by a highly immunogenic and antiphagocytic polysaccharide capsule that is required for fungal virulence (28). Historical classification of cryptococcal strains into four serotypes was based on immunoreactivity of this material with polyclonal rabbit sera (29), although more recent classification is based on molecular typing (30). The capsule is assembled from two polysaccharides: glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal, historically called GalXM as discussed in (31)). GXM is the more abundant capsule component; cells without it are acapsular and are avirulent in animal models of cryptococcal infection (32). GXMGal comprises less of the capsule mass, but also exhibits potent immunomodulatory properties (33-37) and may regulate capsule expansion (38).

Both capsule polysaccharides consist of repeating structural units and share several components, although their overall molecular structures are quite distinct. GXM, typically 1-7 MDa (39,40), is a linear α1,3-linked mannopyranose (Manp) polymer substituted with β1,2- or β1,4-xylopyranose (Xylp) and β1,2-glucopyranuronic acid (GlcAp) (41,42). GXMGal, typically ~0.1 MDa (39,40), has a backbone of α1,6-linked Galp with α1,3-Manp-α1,4-Manp-β1,3-Galp side chains; the side chain residues are additionally modified with variable amounts of β1,2- and β1,3-Xylp and β1,3-GlcAp (Fig. 1A) (31,43). Compositional analyses have further suggested that GXMGal contains Galf (43,44), although neither the site of this modification, nor its functional significance, has been determined.

The occurrence of Galf in human pathogens but not their hosts suggests that, in cases where this moiety is important in pathogen biology, it could be a potential target for selective therapy. We therefore wished to determine the contribution of Galf to C. neoformans capsule structure and virulence. We first established the linkage of Galf within GXMGal, analyzing capsular material from mutants with defects in capsule synthesis to make this problem more tractable. We next engineered a strain lacking Galf by deleting the gene encoding UDP-galactopyranose mutase (UGM), the enzyme that catalyzes the formation of the Galf donor molecule, UDP-Galf. The eukaryotic gene encod-
ing UGM, GLF, was discovered by bioinformatic analysis in *Leishmania* and an ortholog was identified in *Cryptococcus*; the activity corresponding to both sequences was demonstrated by heterologous expression in prokaryotes (45). Here we show that as expected, the GXMGal from glfΔ mutants was devoid of Galf. Nonetheless, the absence of this moiety did not affect overall capsule morphology or the ability of cryptococci to proliferate in a mouse model of cryptococcal infection. These findings establish the structure of a complex and biologically important polysaccharide, and suggest that Galf is dispensable for cryptococcal virulence in mice although it may play biological roles under conditions not included in our analysis.

**EXPERIMENTAL PROCEDURES**

*Strains and growth conditions* — All strains used in this study (Table S1) were constructed in serotype D *C. neoformans* var. *neoformans* CAP67 (46) or JEC21 (47). Cells were grown with continuous shaking at 30°C in YPD medium (1% w/v yeast extract, 2% w/v peptone, 2% w/v dextrose) or at 30°C on YPD agar plates (YPD medium with 2% w/v agar). Media were supplemented as appropriate with 100 µg/ml of nourseothricin (from Werner BioAgents).

*Gene deletion* — We previously identified the gene (GLF; Genbank locus number CNG00060) encoding UDP-galactopyranose mutase in the genome of *C. neoformans* var. *neoformans* JEC21 (45). In this study, we replaced the genomic coding sequence of GLF with a nourseothricin resistance marker (*NAT*) by homologous recombination. To assemble the deletion cassette, we first amplified the GLF locus from cryptococcal genomic DNA (prepared as described in Ref. (48)) by PCR using primers HL1 (5’-TTCCATGGCCTTTGGGAGATTCTTCTC-3’) and HL2 (5’-CGACG-TACTTCCGCTTTTGGAATAGGC-3’), and TA-cloned the amplicon into pCR2.1-TOPO (from Invitrogen) to form plasmid pGLF1. This plasmid was then digested with *Sma*I and *Ssp*I to release the GLF genomic coding sequence along with 995 bp upstream of the translation start site and 53 bp downstream of the translation stop site. The *NAT* sequence was released from plasmid pGMC200 (49) using *Hpa*I and *Eco*RV, and ligated to the remaining portion of pGLF1 such that the marker was oriented in the opposite direction relative to GLF and flanked on each side by 1 kb of genomic DNA. The resulting cassette was amplified by PCR using primers H1 and H2, and used to biolistically transform both JEC21 and CAP67 cells as described (50). Nourseothricin-resistant colonies were restreaked twice on solid YPD medium, and several independently-generated transformants with the desired gene replacement (assessed by PCR and DNA-blotting, not shown) were maintained.

*GXMGal purification* — GXMGal was purified from culture supernatants as described (51). Briefly, CAP67 or CAP67-derived mutant cells were grown in YPD medium and collected by centrifugation. Non-GXMGal components were partially removed from filtered and concentrated culture supernatant fluid by multiple passes over a Concanavalin A column. The column flow-through was dialyzed extensively, lyophilized, redissolved, bound to DE-52 resin, and eluted with a NaCl gradient. The first eluted carbohydrate peak was dialyzed extensively, dried, redissolved, and further purified by Sephacryl S-300 gel filtration. The peak of carbohydrate that eluted from the gel filtration step was again dialyzed and then lyophilized for analysis. All structural analyses were performed on GXMGal from the CAP67 cxt1Δcxt2Δ mutant strain (see text) except where indicated otherwise.

*Synthesis of the methyl galactofuranoside standard* — Methyl galactofuranoside was synthesized as in Ref. (52). Briefly, 30 mg of galactose were stirred in 2 ml of 0.5% w/w methanolic hydrogen chloride (MeOH-HCl) at 25°C until complete dissolution (16 h). The solution was then neutralized with excess pyridine and concentrated 10-fold by evaporation. The galactofuranoside was acetylated by adding an equal volume of acetic anhydride solution containing pyridine (Ac₂O-pyridine) and incubating overnight at room temperature. The mixture was then combined with an excess of ice water, and after one hour the acetylated galactofuranoside was extracted with dichloromethane. Following evaporation of the solvent, the residue was dissolved in 1 ml of 20 mM NaOMe in methanol, and the next day the solution was evaporated to dryness, treated with an aqueous suspension of Dowex 50W H⁺, filtered, and evaporated to yield methyl galactofuranoside.

*Methylation analysis* — For methylation analysis, each sample was suspended in dimethyl sul-
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foxide (DMSO), then permethylated by treatment with sodium hydride and methyl iodide in anhydrous DMSO. Excess methyl iodide was evaporated under a stream of nitrogen, and the permethylated sample was extracted with dichloromethane, concentrated by evaporation, and hydrolyzed with 2 M trifluoroacetic acid (by incubation at 121°C for 2 h in a sealed tube). Released monosaccharides were reduced with NaBD₄ and acetylated with a solution of acetic anhydride and trifluoroacetic acid. The resulting partially methylated alditol acetates were analyzed on a Hewlett-Packard 5890 gas chromatograph interfaced to an Agilent 5970 mass selective detector in electron impact ionization mode. Separation was performed on a 30-m Supelco 2330 bonded-phase fused silica capillary column.

Microscopy — For light microscopy, cells from an overnight culture in YPD medium were washed twice with Dulbecco’s Modified Eagle’s Medium (DMEM; from Sigma-Aldrich) and adjusted to 10⁶ cells/ml in DMEM. To induce capsule, 1-ml aliquots of this cell suspension were transferred to a 24-well tissue-culture plate (from Techno Plastic Products) and incubated at 37°C in the presence of 5% CO₂ for 48 h. To visualize capsule, 10⁶ cells were collected by centrifugation, mixed with 8 µl of water and 1.5 µl of India ink, and spotted on a glass microscope slide. All samples were imaged with identical acquisition settings on a Zeiss Axioskop 2 MOT Plus wide-field fluorescence microscope.

For quick-freeze deep-etch electron microscopy, samples were grown overnight in YPD medium, washed in water, and processed as in Ref. (53) with minor modifications. Briefly, a 10-µl aliquot of the cell pellet was pipetted onto a 3x3x1-mm cushion of glutaraldehyde-fixed, water-washed mouse lung and immediately frozen by forceful impact against a pure copper block cooled to 4 K with liquid helium. Frozen samples were mounted in a nitrogen-cooled Balzers 400 vacuum evaporator and warmed to -105 °C, fractured, and deep-etched for 2 min. Rotary shadowing of approximately 2 nm of platinum was deposited from a 20° angle above the horizontal, followed immediately by application of an ~10-nm stabilization film of pure carbon deposited from an 85° angle. Replicas were immersed in chromo-sulfuric acid cleaning solution overnight and then transferred through several rinses of distilled water, picked up on formvar coated copper grids, and photographed using a JEOL 1400 microscope with attached AMT digital camera.

Evaluation of phagocytic uptake — Interactions of C. neoforms and THP-1, a monocyct cell line, were assessed as described (54). Briefly, fungal cells grown in YPD were serum-opsonized and their cell walls labeled with Lucifer Yellow dye before exposure to differentiated THP-1 cells for 1 h. After the exposure interval samples were washed thoroughly, permeabilized, labeled with DAPI (nuclei) and CellMask (host cytosol), and analyzed by automated microscopy to assess the extent of fungal adherence and internalization.

Growth in mice — To assess fungal survival in the mouse lung, cryptococcal cells were cultured overnight in YPD medium, washed three times in PBS, and adjusted to 2.5 × 10⁵ cells/ml in PBS. For each strain tested, eight 4-6 week-old female C57BL/6 mice (from Jackson Laboratories) were anesthetized with a combination of ketaset-HCl and xylazine, and inoculated intranasally with 50 µl of the prepared cell suspension. Three animals from each group were sacrificed 1 h after inoculation, and the remaining five were sacrificed after 7 days. Following sacrifice, the lungs were removed, homogenized in PBS, and serial dilutions of the homogenate were plated on solid YPD medium to determine colony-forming units (CFU). Initial inocula were also plated to confirm CFU. All studies were performed in compliance with institutional guidelines for animal experimentation.

Statistical calculations — Where indicated in the text, statistical significance was calculated using a two-tailed, unpaired Student’s t-test.

NMR analysis — One-dimensional and two dimensional NMR analyses were performed as described previously (31).

RESULTS

Early studies of capsule polysaccharides suggested that Gal/f was a terminal substituent of GXMGal (44,55). The site of this glycosyl modification was never determined, however, so Gal/f is not included in the currently accepted GXMGal structure (Fig. 1A). To address this gap in knowledge, we analyzed GXMGal from the C. neoforms serotype D strain CAP67 (strains are listed in Supplementary Table 1). We used this strain for structural studies because it does not produce
GXM; this simplifies GXMGal purification and reduces potential contamination with the more abundant GXM polysaccharide (31,43,51,56,57). One-dimensional $^{13}$C-NMR analysis of CAP67 GXMGal (data not shown) showed the anomeric signal at 110 ppm that was originally attributed to Galp (43,44). Unexpectedly, heteronuclear single quantum correlation (HSQC) NMR experiments showed that this carbon anomeric signal was associated with two signals in the proton dimension, one at 5.22 and the other at 5.17 ppm (Fig. 2A, lower left). The location of these cross peaks is consistent with the presence of two distinct Galf residues, both in the $\beta$ configuration (58). For further investigation, we designated the furanosyl whose H-1 resonates further downfield as Galf$^\alpha$, and the other furanosyl as Galf$^\beta$ (Fig. 2A and Table I).

Our HSQC study suggested that Galf was linked at two different positions in GXMGal. We could not resolve these linkages by correlation spectroscopy techniques, however, probably because the H-1 of Galf$^\alpha$ and Galf$^\beta$ (Table I) resonate at frequencies very similar to those of the H-1 of $\beta$1,3-xyllosylated $\alpha$1,3-Manp and terminal $\alpha$1,3-Manp, respectively (31). Since the latter residues are much more abundant than Galf in GXMGal (31,43), the more intense mannose signals may obscure the spectroscopic correlations associated with galactofuranose; such masking would explain why previous studies could not identify the position of Galf. To circumvent this problem, we took advantage of two of our previous observations regarding capsule biosynthesis. First, we had found that cryptococci lacking the $\beta$1,2-xyllosyltransferase Cxt1p do not add $\beta$1,3- or $\beta$1,2-Xylp to GXMGal; GXMGal from these cells therefore does not contain $\beta$1,3-xyllosylated $\alpha$1,3-Manp and has a simpler NMR profile (31,51). Second, we had noticed that the intensity of both Galf anomeric signals rises in GXMGal from CAP67 cells lacking both Cxt1p and a close homolog with the same in vitro activity (Cxt2p; Fig. 2B and data not shown). The ratio of the HSQC-NMR H-1 integrals of $\beta$-Galf to those of $\alpha$-Galp in mutant GXMGal was 0.40, compared to 0.11 for the parent strain. The 3.5-fold increase in signal in the mutant strain probably reflects the greater abundance of Galf (see Discussion).

As a first step in studying GXMGal from cxt1$\Delta$ cxt2$\Delta$ cells, we performed methylation analysis. Consistent with previous results from the parent CAP67 strain (31,43), our studies indicated the presence of a terminal hexofuranose (Fig. 3A and Fig. S1), whose derivative eluted with the same retention time as that of a terminal Galf standard (Fig. 3B). We confirmed this identification by mixing the Galf standard and the sample prior to GC-MS analysis (Fig. 3C), and concluded that GXMGal of the CAP67 cxt1$\Delta$ cxt2$\Delta$ strain indeed contains terminal Galf. In agreement with our NMR observations, this moiety was ~3-fold more abundant in the cxt1$\Delta$ cxt2$\Delta$ strain than in the CAP67 parent (Table II).

The absence of $\beta$1,3-xyllosylated $\alpha$1,3-Manp in CAP67 cxt1$\Delta$ cxt2$\Delta$, together with the increased abundance of Galf in this strain, revealed spectroscopic correlations that were previously obscured. First, total correlation spectroscopy (TOCSY) analysis confirmed our initial finding that Galf$^\alpha$ and Galf$^\beta$ correspond to independent furanosyl spin systems (Table I). We next used nuclear Overhauser effect spectroscopy (NOESY) to identify a glycosidic linkage between each Galf residue and other GXMGal components. We detected an NOE cross peak between H-1 of Galf$^\alpha$ and a proton that resonates at 4.01 ppm (Fig. 4A). Correlation spectroscopy (COSY) and TOCSY analysis (Fig. 4B) revealed that this proton corresponded to H-2 of a previously unassigned residue with an H-1 that resonates at 5.06 ppm (Fig. 4D). The chemical shifts corresponding to H-1 through H-4 of this residue could be assigned from COSY, TOCSY, and HSQC experiments, and were consistent with a Galp in the $\alpha$ anomeric configuration (Table I, entry iii). We also identified an NOE cross peak between H-1 of Galf$^\beta$ and a proton that resonates at 3.98 ppm (Fig. 4A). Analysis of this proton signal by COSY and TOCSY (Fig. 4B) demonstrated that it belonged to H-2 of an $\alpha$-Galp residue with H-1 at 5.05 ppm (Table I, entry iv). These spectroscopic correlations suggest that Galf$^\alpha$ and Galf$^\beta$ are linked to position 2 of distinct Galp residues within the GXMGal repeating unit. We accordingly designated the residue linked to Galf$^\alpha$ as $\alpha$-Galp$^A$, and the residue linked to Galf$^\beta$ as $\alpha$-Galp$^B$.

*Klutts and Doering, manuscript in preparation
To confirm our deduced linkage positions for Galf, we inspected the carbon chemical shifts of the Galf-modified α-Galp residues by HSQC spectroscopy. We observed that C-2 of α-GalpA and C-2 of α-GalpB both demonstrate a downfield shift (Table I) expected from a glycosyl modification (59). HMBC experiments (Fig. 4C) further identified a cross peak between H-1 of GalfA and C-2 of α-GalpA, as well as a cross peak between H-1 of GalfB and C-2 of α-GalpB. The HMBC analysis thus supports the same substitution pattern that we deduced from NOESY, and our conclusion that Galf modifies position 2 of two different α-Galp residues in GXMGal.

Finally, we sought to identify the positions of the Galf-modified α-Galp residues within the GXMGal repeating unit. According to the currently accepted GXMGal structure (Fig. 1A), α-Galp residues only occur within the α1,6-linked galactose backbone, as all other galactose residues of this polysaccharide are in the β anomeric configuration. Supporting the conclusion that Galf is linked directly to the backbone, NOESY analysis (Fig. S2) revealed a cross peak between H-1 of each Galf-modified α-Galp residue and H-6 of a backbone galactose. This H-6 could correspond to either the 6-linked (non-branching) or the 3,6-linked (branching) backbone galactose, as H-6 of these two residues could not be resolved by TOCSY. However, C-3 of α-GalpA and C-3 of α-GalpB both resonate around 79 ppm (Table I), indicating the presence of an additional oligosaccharyl modification on O-3 of these residues. We therefore conclude that α-GalpA and α-GalpB are 3,6-linked (branching) backbone galactose residues substituted with Galf at position 2, and propose the structure depicted in Fig. 1B.

According to our proposed revision of the GXMGal structure, the backbone residues modified with Galf at position 2 already bear a side chain at position 3 and are linked α1,6. Based on this observation, we expected to detect 2,3,6-substituted Galp (2,3,6-Galp) by methylation analysis. Indeed, we did observe the 4-O-methylgalactitol acetate derivative that corresponds to 2,3,6-Galp in analysis of GXMGal from CAP67 (Table II). The abundance of this residue rose approximately 3-fold in GXMGal from cxt1Δ cxt2Δ cells, consistent with the 3-fold greater abundance of Galf in this strain noted above. HSQC analysis (Fig. 2B) similarly showed a 2,3,6-Galp anomic resonance signal of greater intensity in cxt1Δ cxt2Δ cells compared to the parent strain. (Other residues in Fig. 2B showed changes from CAP67 that were expected from the lack of xylose, as we previously described (31,51).) Overall, both Galf and 2,3,6-Galp exhibited coordinated changes in abundance that did not occur in other GXMGal components, supporting our proposed structure (Fig. 1B).

To further test our revised GXMGal structure, and to assess the biological role of Galf, we engineered cryptococci that are unable to add Galf to this polysaccharide. Since the galactofuranosyltransferase responsible for this modification of GXMGal is not known, we instead targeted the gene encoding the cryptococcal homolog of UDP-galactopyranose mutase (GLF; see Methods and reference (60)). In the absence of this enzyme, cryptococci do not synthesize UDP-Galf (the Galf donor) and should therefore produce GXMGal lacking Galf. We deleted the GLF gene in two serotype D strains: the CAP67 strain that lacks the GXM capsule polysaccharide and the serotype D reference strain, JEC21. As mentioned above, the CAP67 background allows optimal GXMGal purification for structural studies, while the reference background allows direct comparison to wild type for testing the effects of Galf absence on capsule morphology and cryptococcal virulence.

We first confirmed that deletion of GLF eliminated the incorporation of Galf into GXMGal, using polysaccharide purified from CAP67 glfΔ. As anticipated, we detected no terminal Galf by methylation analysis (Table II) and neither Galf residue was detected in HSQC experiments (Fig. 2C). Furthermore, 2,3,6-substituted galactose, which reflects Galf modification of the polymer backbone, was undetectable by HSQC analysis (Fig. 2C) and its abundance was reduced to below 0.2% of total signal in methylation analysis (Table II). (The residual amount is likely due to undermethylation of 3,6-linked Galp, which is much more abundant.) Together, these results demonstrate the absence of Galf in the CAP67 glfΔ mutant, support GLF as the sole route to Galf synthesis in C. neoformans, and confirm our proposed structure (Fig. 1B).

We next examined the contribution of Galf to cryptococcal biology and virulence, using strains in the JEC21 background, which makes normal
capsule. Cryptococci lacking GLF were morpho- logically indistinguishable from wild type, forming similarly mucoid colonies (Fig. S3) and exhibiting identical flocculating behavior (data not shown), suggesting that capsule structure was not grossly altered by the lack of Galf. Wild type and glf/Δ cells also produced capsules of comparable size after incubation in capsule-inducing conditions (Fig. 5A). Closer inspection by quick-freeze deep-etch electron microscopy (Fig. 5B) also showed that capsule assembly and attachment to the cell wall were unchanged in the glf/Δ mutant.

We next tested whether Galf influenced the ability of C. neoformans to withstand challenges to cell integrity. These studies showed no change in the growth of glf/Δ compared to wild type at 25 °C, 30 °C, or 37 °C on rich medium; medium supplemented with Congo red (0.003%), SDS (0.005%), or NaCl (1 M); or medium adjusted to acidic or basic pH (Fig. S3).

Inhibition of phagocytosis is a major function of the cryptococcal capsule. Given the known contribution of Galf to phagocytic clearance of other microbial pathogens (61,62), we tested whether the absence of Galf from the capsule would influence the interactions of cryptococci with phagocytes (Fig. 6A). We found that cryptococci lacking GLF were internalized as efficiently as wild type (p > 0.118) by a human monocytic cell line, and that both strains exhibited comparable adherence to phagocytic cells (p > 0.839). Finally, we used a mouse model of cryptococcal infection to directly test the effect of Galf absence on cryptococcal growth within a mammalian host. At one week post-inoculation, the number of colony-forming units isolated from the lungs of mice intranasally infected with the glf/Δ mutant was indistinguishable from the result for mice infected with wild type cryptococci (Fig. 6B; p > 0.675), and lung inflammation in both groups was comparable (data not shown). Acapsular cryptococci typically do not proliferate within this period (63).

**DISCUSSION**

Pursuing suggestions first published several decades ago, we have found Galf to be a component of the GXMGal polysaccharide of C. neoformans. We have further productively applied a strategy of using specific mutants to reduce the structural complexity of this polysaccharide; this enabled us to determine the linkage position of Galf, which was previously unknown. Finally, we probed the role of this moiety in cryptococcal biology, using a mutant strain unable to generate the Galf donor.

Previous attempts to identify the site of Galf modification were hindered by several obstacles, one of which is the low abundance of this moiety. Further complicating analysis, the signals from (much more abundant) mannose residues of GXMGal obscure key spectroscopic correlations that support the linkage position of Galf. We used cryptococcal mutants defective in various aspects of glycan synthesis to circumvent both challenges. For analysis of GXMGal we used cxt1Δ cxt2Δ cells, in which the overall abundance of Galf is increased and the lack of xylose (and therefore of β1,3-xylosylated Manp) exposes relevant NMR crosspeaks that allow structural interpretation. We further supported our structure through analysis of GLF mutant cells, which lack Galf in GXMGal.

We determined that Galf modifies position 2 of branched backbone galactose residues in serotype D GXMGal (Fig. 1B); we believe this also occurs in serotype A polysaccharide (based on our unpublished data). Our NMR results suggest that 11% of these galactose residues are modified with Galf in wild type cells, a value that rises to 40% for the cxt double mutant. Our conclusion as to specific linkage is supported by NMR spectroscopic correlations indicating the presence of Galf on backbone Galp residues (Table I and Fig. 4), and by the coordinated changes in abundance of Galf and 2,3,6-Galp that we observed by methylation analysis (Table II). In particular, the absence of 2,6-substituted Galp by methylation analysis demonstrates that Galf is only present on backbone residues that already carry a side chain. We suspect that differences in the substitution pattern of the side chains account for the two discrete Galf spin systems detected by NMR (Table I). Since only two Galf anemic signals are observed, the presence or absence of only one non-stoichiometric sugar substituent (either Xylp or GlcAp) likely determines the Galf H-1 chemical shift. Being nearest the branching Galp, the GlcAp residue would influence the Galf H-1 chemical shifts more significantly than Xylp. Interestingly, the degree of substitution with GlcAp is ~0.4 (31), which approximates the Galf8 fraction of the total Galf.
We did consider an alternate model for GXMGal structure, in which both Galf residues are linked to the non-branching galactose units of the GXMGal backbone. In this model, the relevant backbone residue would be glycosylated on position 2 by GalfB and on position 3 by GalfA. Although both this configuration and our proposed structure (Fig. 1B) would produce similar methylation analysis profiles, the NMR data do not support the alternate model. First, the relevant TOCSY cross peaks connect the signals at 3.98 and 4.01 ppm with distinctly different anomeric protons at 5.05 and 5.06 ppm, respectively (Fig. 4B). Second, the 1-D proton spectrum (Fig. 4D) shows these anomeric signals as a complex peak and not as a simple doublet, as would be expected if both Galf substituents were attached to the same residue. Our proposed structure is therefore more consistent with the results of our analysis. It is also more parsimonious in terms of potential glycosyltransferase activities required to generate the GXMGal, because both Galf residues occur in the same linkage and would presumably be attached by the same enzyme.

We observed increased abundance of Galf in a strain that lacks cryptococcal β1,2-xylosyltransferases Cxt1p (64) and Cxt2p (J. S. Klutts and T. L. Doering, manuscript in preparation). The increase was 3.5-fold based on NMR data (see above), a value that was supported by methylation analysis results showing a 2.8-fold increase in t-Galf and 3.0-fold increase in 2,3,6-Galp. We did not expect this rise; it is an intriguing observation that will clearly require further investigation. Notably, other aspects of our analysis support a relationship between the addition of xylose and of Galf, for example, the almost two-fold reduction in xylose abundance in GXMGal from glfA cells (Table II). Direct linkage between xylose and Galf is unlikely, as NMR does not support spatial proximity between these residues. It may be that the glycosyltransferases responsible for adding these moieties to capsule polysaccharides physically interact or are otherwise interde-
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ENDNOTES

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8The abbreviations used are: CXT1, cryptococcal xylosyl transferase 1; CXT2, cryptococcal xylosyltransferase 2; Galp, galactofuranose; Galp, galactopyranose; GlcA, glucopyranuronic acid; HSQC, Heteronuclear Single Quantum Correlation; NOESY, Nuclear Overhauser Effect Spectroscopy; Manp, manno-pyranose; Xylp, xlyopyranose; GXM, glucuronoxylomannan; GXMGal, glucuronoxylomannogalactan; TOCSY, Total Correlation Spectroscopy; UDP-Galp, UDP-galactopyranose mutase

FIGURE LEGENDS

FIGURE 1. Models of the GXMGal repeating unit. Panel A represents the currently accepted structure, which does not include Galf. Panel B shows the structure proposed in this study, with Galf shown in bold. Each panel shows the two extremes of side branch substitution; intermediate structures also occur (44,83).

FIGURE 2. Two different Galf residues are present in GXMGal. Shown are the anomeric regions of partial heteronuclear single quantum correlation (HSQC) 2-D NMR spectra of GXMGal from the indicated strains. The anomeric peaks from residues involved in Galf linkage are shown in red.

FIGURE 3. Identification of Galf in GXMGal by methylation analysis. Partial gas chromatograms of methylated alditol acetates are shown. Panel A, analysis of GXMGal from a cxt1Δcxt2Δ strain in the CAP67 background, showing the presence of terminal Manp (t-Manp), Galf (t-Galf), and Galp (t-Galp). Panel B, analysis of terminal galactoside standards. Panel C, analysis of a mixture of the samples shown in panels A and B. Panels A and C are normalized to the abundance of t-Manp.

FIGURE 4. Galf modifies position 2 of branched backbone Galp residues in GXMGal. Partial 2-D NMR correlation spectra of GXMGal from the CAP67 cxt1Δcxt2Δ mutant are shown, indicating linkage of both Galf moieties to two different backbone α-Galp residues. Numbering is as in Table I.
FIGURE 5. Cryptococcal capsule morphology. Panel A, cryptococci from the indicated strains were incubated for 48 h in capsule-inducing conditions (37°C in DMEM, 5% CO₂) and negatively stained with India ink to reveal the capsule as halos of ink particle exclusion surrounding individual cryptococci. Scale bar, 10 µm. Panel B, quick-freeze deep-etch electron micrographs of cryptococci grown in rich medium, showing the ultrastructural morphology of the capsule (C), cell wall (W), and intracellular (I) regions. Scale bar, 100 nm.

FIGURE 6. Galf is dispensable for phagocytic uptake of cryptococci and cryptococcal survival within the mammalian host. Panel A, THP-1 cells were exposed to cryptococci of the indicated strains for 1 h, washed, fixed, and the fraction of total cryptococci that remained adherent (white bars) or had been internalized (black bars) was determined. The mean values from three experiments ± S.E are plotted. Panel B, mice were intranasally infected with the indicated strains, and colony-forming units (CFU) were isolated from the lungs after 1 h (white bars) or 7 days (black bars). Mean total lung CFU ± S.E. values are plotted.
TABLES

Table I. NMR chemical shifts and spectroscopic correlations of galactose residues involved in Galf linkage.

| Residue                  | Chemical shift (ppm) | NOE / HMBC |
|--------------------------|----------------------|------------|
|                          | 1        | 2     | 3     | 4     | 5     | 6     |
| i β1,2-Galf<sub>A</sub> | H        |       |       |       |       |       |
|                          | 5.22     | 4.19  | 4.07  | 4.07  | 3.85  | 3.73/3.66 |
|                          | C<sup>13</sup> | 110   | 82.6  | 78.0  | 84.1  | 72.0  | 63.8  |
| ii β1,2-Galf<sub>B</sub>| H        |       |       |       |       |       |
|                          | 5.17     | 4.17  | 4.02  | 4.01  | n.d.  | n.d.  |
|                          | C<sup>13</sup> | 110   | 82.6  | 77.2  | 83.08 | n.d.  | n.d.  |
| iii →2,3,6)-α-Galp<sub>A</sub> | H      |       |       |       |       |       |
|                          | 5.06     | 4.01  | 3.97  | 4.17  | n.d.  | n.d.  |
|                          | C<sup>13</sup> | 99.2  | 77.2  | 79.3  | 70.7  | n.d.  | n.d.  |
| iv →2,3,6)-α-Galp<sub>B</sub>| H        |       |       |       |       |       |
|                          | 5.05     | 3.98  | 3.97  | 4.18  | n.d.  | n.d.  |
|                          | C<sup>13</sup> | 99.2  | 75.7  | 79.3  | 70.7  | n.d.  | n.d.  |

<sup>a</sup> n.d., not determined.

<sup>b</sup> Although the H6 and C6 could also correspond to →3,6)-α-Galp, this interpretation would be incompatible with other structural data (see Results and Discussion).
Table II. Methylation analysis of GXMGal from the indicated strains.

| Residue | Glycosidic linkage | O-methyl-alditol acetate | Relative detector response |
|---------|-------------------|--------------------------|---------------------------|
|         |                   |                          | CAP67 | CAP67 | CAP67 |
|         |                   |                          |       |       |       |
|         |                   |                          |       |       |       |
| Xylopyranose | t-Xylp<sup>f</sup> | Xylp-(1,→)                | 2,3,4-Me<sub>3</sub>-xylitol | 13.2<sup>d</sup> | -- | 7.1 |
| Mannopyranose | 2,3-Manp | →2,3)-Manp-(1,→)          | 4,6-Me<sub>2</sub>-mannitol | 14.9 | -- | 17.3 |
|                 | 3-Manp | →3)-Manp-(1,→)             | 2,4,6-Me<sub>3</sub>-mannitol | 12.5 | 18.0 | 13.6 |
|                 | t-Manp | Manp-(1,→)                 | 2,3,4,6-Me<sub>4</sub>-mannitol | 6.5 | 18.2 | 5.2 |
| Galactofuranose | t-Galf | Galf-(1,→)                 | 2,3,5,6-Me<sub>4</sub>-galactitol | 3.9 | 11.0 | -- |
| Galactopyranose | 2,3,6-Galp | →2,3,6)-Galp-(1,→) | 4-Me<sub>1</sub>-galactitol | 1.2 | 3.5 | 0.2 |
|                 | 3,6-Galp | →3,6)-Galp-(1,→)           | 2,4-Me<sub>2</sub>-galactitol | 14.6 | 15.9 | 18.2 |
|                 | 6-Galp | →6)-Galp-(1,→)             | 2,3,4-Me<sub>3</sub>-galactitol | 16.2 | 16.5 | 21.4 |
|                 | 3,4-Galp | →3,4)-Galp-(1,→)           | 2,6-Me<sub>2</sub>-galactitol | 4.6 | 6.0 | 4.2 |
|                 | 4-Galp | →4)-Galp-(1,→)             | 2,3,6-Me<sub>3</sub>-galactitol | 11.6 | 10.0 | 11.7 |

<sup>a</sup> Species that do not correspond to linkages in Fig. 1 are not listed; none of these exceeded 1% of the total signal with the exception of glucose (a common contaminant).

<sup>b</sup> t, non-reducing terminal

<sup>c</sup> 2,3,4-Me<sub>3</sub>-xylitol denotes 1,5-O-acetyl-2,3,4-tri-O-methyl-xylitol, etc.

<sup>d</sup> All species corresponding to linkages in Fig. 1 are listed, with values normalized to total 100% for purposes of comparison.

<sup>e</sup> --, none detected
Figure 1.

A

\[
\begin{array}{c}
\beta-D-Xylp(1\rightarrow3)-\alpha-D-Manp \\
1 \\
\downarrow \\
3 \\
\beta-D-Xylp(1\rightarrow2)-\alpha-D-Manp \\
1 \\
\downarrow \\
1 \\
\beta-D-GlcAp(1\rightarrow3)-\beta-D-Galp \\
1 \\
\downarrow \\
1 \\
[6]-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6) \_n
\end{array}
\]

B

\[
\begin{array}{c}
\beta-D-Xylp(1\rightarrow3)-\alpha-D-Manp \\
1 \\
\downarrow \\
3 \\
\beta-D-Xylp(1\rightarrow2)-\alpha-D-Manp \\
1 \\
\downarrow \\
1 \\
\beta-D-GlcAp(1\rightarrow3)-\beta-D-Galp \\
1 \\
\downarrow \\
1 \\
[6]-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6)-\alpha-D-Galp(1\rightarrow6) \_n
\end{array}
\]
Figure 2.
Figure 3.
Figure 4.
Figure 6.
Unusual galactofuranose modification of a capsule polysaccharide in the pathogenic yeast Cryptococcus neoformans

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