Unusual Galactofuranose Modification of a Capsule Polysaccharide in the Pathogenic Yeast Cryptococcus neoformans

Received for publication, December 11, 2012, and in revised form, February 11, 2013. Published, JBC Papers in Press, February 13, 2013, DOI 10.1074/jbc.M112.441998

Christian Heiss‡, Michael L. Skowrya†, Hong Liu§,3, J. Stacey Klutta†,4, Zhirui Wang†, Matthew Williams§, Deepa Srikanta§, Stephen M. Beverley†, Parastoo Azadi§, and Tamara L. Doering§,2

From the Complex Carbohydrate Research Center, University of Georgia, Athens, Georgia 30602 and Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri 63110

Galactofuranose (Gal\(^{f}\)) 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 meningitis/encephalitis 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\(^{f}\). Notably, how Gal\(^{f}\) is incorporated into capsule polysaccharide and characterized cryptococci lacking this modification.

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.

**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.

Galactofuranose (Gal\(^{f}\)) 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 meningitis/encephalitis 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\(^{f}\). Notably, how Gal\(^{f}\) modification contributes to the structure and function of the cryptococcal capsule is not known. We have determined that Gal\(^{f}\) is β,1,2-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-Galf, the nucleotide sugar donor required for Gal\(^{f}\) addition. Mutase activity was required for the incorporation of Gal\(^{f}\) into glucuronoxylomannogalactan 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 (Gal\(^{p}\)) or five-membered galactofuranose (Gal\(^{f}\)) ring. Gal\(^{f}\) occurs ubiquitously across all domains of life, but Gal\(^{f}\) 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 Gal\(^{f}\) from mammalian glycans despite its prevalence in the pathogenic microbes that infect mammals (12). The role of Gal\(^{f}\) in microbial biology and pathogenesis is thus a subject of considerable interest.

Pathogenic microbes incorporate Gal\(^{f}\) 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 Gal\(^{f}\). Inability to synthesize Gal\(^{f}\) compromises cell integrity in this system, resulting in severely decreased viability and attenuated survival in the host (14). Kinetoplastids incorporate Gal\(^{f}\) into a protective glycocalyx that enhances establishment of disease and promotes survival of these parasites within their insect vectors (8). In *Leishmania*, loss of Gal\(^{f}\)/synthesis or of the key galactofuranosyltransferase required to synthesize surface lipopolysaccharide 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 Gal\(^{f}\) in secreted glycoproteins, glycolipids, and cell wall polysaccharides (7, 8) improves cell integrity (19–21) and reduces adherence...
Galf Modifies a C. neoformans Capsule Polysaccharide

The pathogenic yeast Cryptococcus 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: glucuronoxylan (GXM) and glucuronoxylanomalactan (GXMGal; historically called GalXM as discussed in Ref. 31). GXM is the more abundant capsule component; cells without it are acapsular and are avirulent in animal models of cryptococcal infection (28). GXMGal comprises less of the capsule mass but also exhibits potent immunomodulatory properties (32–36) and may regulate capsule expansion (37).

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 (38, 39), is a linear α1,3-linked mannoxyranose (Manp) polymer substituted with β1,2- or β1,4-xylpyranose (Xylp) and β1,2-glucopyranuronic acid (GlcAp) (40, 41). GXMGal, typically ∼0.1 MDa (38, 39), 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 (see Fig. 1A) (31, 42). Compositional analyses have further suggested that GXMGal contains Galp (42), although neither the site of this modification nor its functional significance has been determined.

The occurrence of Galp in human pathogens but not in 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, the enzyme that catalyzes the formation of the Galf donor molecule, UDP-Galf. The eukaryotic gene encoding UDP-galactopyranose mutase, GLF, was discovered by bioinformatics analysis in Leishmania, and an ortholog was identified in Cryptococcus; the activity corresponding to both sequences was demonstrated by heterologous expression in prokaryotes (43). 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 (supplemental Table S1) were constructed in serotype D C. neoformans var. neoformans CAP67 (44) or JEC21 (45). 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 nourseothricin (Werner BioAgents).

Gene Deletion—We previously identified the gene (GLF; GenBankTM locus number CNG00060) encoding UDP-galactopyranosyluraminate in the genome of C. neoformans var. neoformans JEC21 (43). 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. 43) by PCR using primers HL1 (5′-TCCATGCTTGGAGATTCTTCTC-3′) and HL2 (5′-CGACGTACTCCTGGATTTGAGCC-3′) and inserted the amplicon into pCR2.1-TOPO (from Invitrogen) to form plasmid pGLF1. This plasmid was then digested with SnaBI and Spel 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 (46) using Hpal and EcoRV 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 HL1 and HL2 and used to biologically transform both JEC21 and CAP67 cells as described (47). 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 (48). 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 an 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 extΔ extΔ mutant strain (see text) except where indicated otherwise.
Galf Modifies a C. neoformans Capsule Polysaccharide

Synthesis of the Methyl Galactofuranoside Standard—Methyl galactofuranoside was synthesized as in Ref. 49. 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 and then transferred from an 85° angle. Replicas were immersed in chloroform (16 h). The solution was then neutralized with excess pyridine hydrogen chloride (MeOH-HCl) at 25 °C until complete dissolution, and hydrolyzed with 2M 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 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.

Methylation Analysis—For methylation analysis, each sample was suspended in dimethyl sulfoxide (DMSO) and then permethylated by treatment with sodium hydroxide 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; Sigma-Aldrich) and adjusted to 10⁶ cells/ml in DMEM. To induce capsule formation, 1-ml aliquots of this cell suspension were transferred to a 24-well tissue culture plate (Techno Plastic Products) and incubated at 37 °C in the presence of 5% CO₂ for 48 h. To visualize the 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. 50 with minor modifications. Briefly, a 10-µl aliquot of the cell pellet was pipetted onto a 3 × 3 × 1-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, warmed to −105 °C, fractured, and deeply etched for 2 min. Rotary shadowing of ~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 chromosulfuric 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 digital camera.

Evaluation of Phagocytic Uptake—Interactions of C. neoformans and THP-1, a monocytic cell line, were assessed as described (51). Briefly, fungal cells grown in YPD were serum-opsonized, and their cell walls were 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.

RESULTS

Early studies of capsule polysaccharides suggested that Galf was a terminal substituent of GXMGal (42, 53). The site of this glycosyl modification was never determined, however, so Galf is not included in the currently accepted GXMGal structure (Fig. 1A). To address this gap in knowledge, we analyzed GXMGal from C. neoformans serotype D strain CAP67 (strains are listed in supplemental Table S1). 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, 42, 48, 54). One-dimensional ¹³C NMR analysis of CAP67 GXMGal data (not shown) showed the anomeric signal at 110 ppm that was originally attributed to Galf (42). Unexpectedly, heteronuclear single quantum correlation (HNSQC) 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 β configuration (56). For further investigation, we designated the furanose whose H-1 resonates further downfield as Galf₁ and the other furanose as Galf₂ (Fig. 2A and Table 1).

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 anomeric protons of Galf₁ and Galf₂ (Table 1)
resonate at frequencies very similar to those of the anomeric protons of \(\beta 1,3\)-xylosylated \(\alpha 1,3\)-Manp and terminal \(\alpha 1,3\)-Manp, respectively (31). Because the latter residues are much more abundant than Gal\(f\) in GXMGal (31, 42), 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 Gal\(f\).

To circumvent this problem, we took advantage of two of our previous observations regarding capsule biosynthesis. First, we had found that cryptococci lacking the \(\beta 1,2\)-xylosyltransferase

![FIGURE 1. Models of the GXMGal repeating unit.](image)

\(\alpha\) represents the currently accepted structure, which does not include Gal\(f\) shown in bold. Each panel shows the two extremes of side branch substitution; intermediate structures also occur (31, 42).

![FIGURE 2. Two different Gal\(f\) residues are present in GXMGal.](image)

Shown are the anomeric regions of partial HSQC two-dimensional NMR spectra of GXMGal from the following strains in the CAP67 genetic background: A, wildtype; B, cryptococci lacking CXT1 and CXT2; and C, cryptococci lacking GLF. The anomeric peaks from residues involved in Gal\(f\) linkage are shown in red.

**TABLE 1**

NMR chemical shifts and spectroscopic correlations of galactose residues involved in Gal\(f\) linkage

<table>
<thead>
<tr>
<th>Residue</th>
<th>Chemical shift (ppm)</th>
<th>Chemical shift (ppm)</th>
<th>Chemical shift (ppm)</th>
<th>NOE/HMBC&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>i) (\beta 1,2)-Gal(f_a)</td>
<td>1H 5.22 4.19 4.07 4.07 3.85 3.73/3.66</td>
<td>13C 110 82.6 78.0 84.1 72.0 63.8</td>
<td>iii (H-2)</td>
<td></td>
</tr>
<tr>
<td>ii) (\beta 1,2)-Gal(f_b)</td>
<td>1H 5.17 4.17 4.02 4.01 ND ND</td>
<td>13C 110 82.6 77.2 83.0 83.08 ND ND</td>
<td>iv (C-2)</td>
<td></td>
</tr>
<tr>
<td>iii) (-2,3,6)-(\alpha)-Gal(p_a)</td>
<td>1H 5.06 4.01 3.97 4.17 ND ND</td>
<td>13C 99.2 77.2 79.3 70.7 ND ND</td>
<td>iii (H-2)</td>
<td></td>
</tr>
<tr>
<td>iv) (-2,3,6)-(\alpha)-Gal(p_b)</td>
<td>1H 5.05 3.98 3.97 4.18 ND ND</td>
<td>13C 99.2 73.7 79.3 70.7 ND ND</td>
<td>iv (C-2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> ND, not determined.

<sup>b</sup> Heteronuclear multiple bond correlation.

<sup>c</sup> Although the H-6 and C-6 could also correspond to \(-3,6\)-\(\alpha\)-Gal\(p\), this interpretation would be incompatible with other structural data (see "Results" and "Discussion").
Cxt1p do not add β1,3- or β1,2-Xylp to GXMGal; GXMGal from these cells therefore does not contain β1,3-xylosylated α1,3-Manp and has a simpler NMR profile (31, 48). 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 β-Galf to those of α-Galp in mutant GXMGal was 0.40 compared with 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 cxt1Δ cxt2Δ cells, we performed methylation analysis. Consistent with previous results from the parent CAP67 strain (31, 42), our studies indicated the presence of a terminal hexofuranose (Fig. 3A and supplemental 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 cxt1Δ cxt2Δ strain indeed contains terminal Galf. In agreement with our NMR observations, this moiety was ~3-fold more abundant in the cxt1Δ cxt2Δ strain than in the CAP67 parent (Table 2).

The absence of β1,3-xylosylated α1,3-Manp in CAP67 cxt1Δ cxt2Δ 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 GalfA and GalfB correspond to independent furanose spin systems (Table 1). 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 GalfA 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 α anomeric configuration (Table 1, entry iii). We also identified an NOE cross-peak between H-1 of GalfB 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 α-Galp residue with H-1 at 5.05 ppm (Table 1, entry iv). These spectroscopic correlations suggest that GalfA and GalfB are linked to position 2 of distinct Galp residues within the GXMGal repeating unit. We accordingly designated the residue linked to GalfA as α-Galp and the residue linked to GalfB as α-GalpB.

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 1) expected from a glycosyl modification (57). Heteronuclear multiple bond correlation 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 heteronuclear multiple bond correlation 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 (supplemental 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...
We first confirmed that deletion of GLF eliminated the incorporation of Gal f into GXMGal using polysaccharide purified from CAP67 glfΔ. As anticipated, we detected no terminal Gal f by methylation analysis (Table 2), and neither Gal f residue was detected in HSQC experiments (Fig. 2C). Furthermore, 2,3,6-substituted galactose, which reflects Gal f 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 2). (The residual amount is likely due to undermethylation of 3,6-linked Gal p, which is much more abundant.) Together, these results demonstrate the absence of Gal f in the CAP67 glfΔ mutant, support GLF as the sole route to Gal f synthesis in C. neoformans, and confirm our proposed structure (Fig. 1B).

We next examined the contribution of Gal f to cryptococcal biology and virulence using strains in the JEC21 background, which makes normal capsule. Cryptococci lacking GLF were morphologically indistinguishable from wild type, forming similarly mucoid colonies (supplemental Fig. S3) and exhibiting identical flocculating behavior (data not shown), suggesting that capsule structure was not grossly altered by the lack of Gal f. Wild type and glfΔ 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 glfΔ mutant.

We next tested whether Gal f influenced the ability of C. neoformans to withstand challenges to cell integrity. These studies showed no change in the growth of glfΔ compared with wild type at 25, 30, 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 (supplemental Fig. S3).

Inhibition of phagocytosis is a major function of the cryptococcal capsule. Given the known contribution of Gal f to phagocytic clearance of other microbial pathogens (58, 59), we tested whether the absence of Gal f from the capsule would influence the interactions of cryptococci with phagocytic cells (Fig. 6A). We found that cryptococci lacking GLF were internalized as efficiently as wild type (p > 0.118) by a human monocyte cell line and that both strains exhibited comparable adher-

### Table 2

<table>
<thead>
<tr>
<th>Residue</th>
<th>Glycosidic linkage</th>
<th>O-Methylalditol acetate</th>
<th>Relative detector response</th>
<th>CAPEP glf</th>
<th>CAPEP cxt1Δ glfΔ</th>
<th>CAPEP cxt2Δ glfΔ</th>
<th>CAPEP glfΔ</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-Xylp</td>
<td>Xylp—(1,→)</td>
<td>2,3,4-Me3-Xylitol</td>
<td>13.2</td>
<td>1.00</td>
<td>0.97</td>
<td>0.97</td>
<td>0.97</td>
</tr>
<tr>
<td>2,3-Manp</td>
<td>→(2,3)-Manp—(1,→)</td>
<td>4,6-Me2-Manitol</td>
<td>14.9</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>3-Manp</td>
<td>→(3,-)</td>
<td>2,3,4-Me3-Manitol</td>
<td>12.5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>t-Manp</td>
<td>Manp—(1,→)</td>
<td>2,3,4,6-Me4-Manitol</td>
<td>6.5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>t-Galf</td>
<td>Galf—(1,→)</td>
<td>2,3,5,6-Me4-galactitol</td>
<td>3.9</td>
<td>11.0</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>2,3,6-Galp</td>
<td>→(2,3,6)-Galp—(1,→)</td>
<td>4,Me2-galactitol</td>
<td>1.2</td>
<td>3.5</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>4-Galp</td>
<td>→(4,-)</td>
<td>2,3,4-Me4-galactitol</td>
<td>14.6</td>
<td>15.9</td>
<td>1.00</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>3,4-Galp</td>
<td>→(3,4)-Galp—(1,→)</td>
<td>2,6-Me3-galactitol</td>
<td>16.2</td>
<td>16.5</td>
<td>21.4</td>
<td>21.4</td>
<td>21.4</td>
</tr>
<tr>
<td>6-Galp</td>
<td>→(6,-)</td>
<td>2,3,4-Me4-galactitol</td>
<td>4.6</td>
<td>6.0</td>
<td>4.2</td>
<td>4.2</td>
<td>4.2</td>
</tr>
<tr>
<td>4-Manp</td>
<td>→(4,-)</td>
<td>2,3,6,Me3-galactitol</td>
<td>11.6</td>
<td>10.0</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
<tr>
<td>t-Galp</td>
<td>Galp—(1,→)</td>
<td>2,3,4,6,Me4-galactitol</td>
<td>0.8</td>
<td>0.9</td>
<td>1.1</td>
<td>1.1</td>
<td>1.1</td>
</tr>
</tbody>
</table>

*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).

t, non-reducing terminal.

2,3,4-Me4-galactitol denotes 1,5-O-acetyl-2,3,4-tri-O-methylgalactitol, etc.

*All species corresponding to linkages in Fig. 1 are listed with values normalized to total 100% for purposes of comparison.

*—, none detected.
ence 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 1 week postinoculation, the number of colony-forming units isolated from the lungs of mice intranasally infected with the glf/ 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 (60).

**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...
Galf Modifies a C. neoformans Capsule Polysaccharide

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 \( \text{cxt}^1 \Delta \text{cxt}2 \Delta \) cells in which the overall abundance of Galf is increased and the lack of xylose (and therefore of \( \beta_1,3 \)-xylosylated Manp) exposes relevant NMR cross-peaks that allow structural interpretation. We further supported our structure through analysis of Galf 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; this value rises to 40% for the \( \text{cxt} \) double mutant. Our conclusion as to specific linkage is supported by NMR spectroscopic correlations indicating the presence of Galf on backbone Galf residues (Table 1 and Fig. 4) and by the coordinated changes in abundance of Galf and 2,3,6-Galp that we observed by methylation analysis (Table 2). In particular, the absence of 2,6-substituted Galf 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 1). Because only two Galf anomeric 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 Galfp, the GlcAp residue would influence the Galf/H-1 chemical shifts more significantly than Xylp. Interestingly, the degree of substitution with GlcAp is \( \sim 0.4 \) (31), which approximates the Galf/H 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 Galf and on position 3 by Galf. 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 one-dimensional 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 \( \beta_1,2 \)-xylosyltransferases Cxt1p (61) and Cxt2p. Based on NMR data (see above), the increase was 3.5-fold, a value that was supported by methylation analysis results showing a 2.8-fold increase in terminal 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 2-fold reduction in xylose abundance in GXMGal from glf/\( \Delta \) cells (Table 2). 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 interdependent, although future studies will be needed to explore this interesting direction.

We were surprised that the absence of Galf did not perturb cryptococcal capsule architecture, growth, and virulence in a mouse model; these findings contrast with the antigenic and structural contribution of this moiety in other pathogenic microbes. The lack of a change in capsule morphology does have precedent, however, as eliminating xylose from GXMGal similarly fails to produce morphological defects in the capsule (48). Galf is also a relatively low abundance component of GXMGal (Table 2); its low density may influence its potential for interacting with host pattern recognition molecules (62). Finally, it is possible that Galf plays a role under conditions not included in our analysis. C. neoformans is an extremely adaptable environmental yeast that can withstand a variety of harsh conditions (63–65) and is capable of living in close association with numerous organisms, including plants (66, 67), birds (68, 69), predatory amoebae (70), and mammals (71, 72), notably humans (73, 74). The cryptococcal capsule in particular is a dynamic structure whose morphology and architecture change with growth conditions (75–77) and the local environment (52, 55). It may be that specific capsular features are only required in a subset of the diverse ecological niches inhabited by this yeast. In support of this hypothesis, Galf has been detected in GXMGal of multiple serotypes (31, 42, 53), and UDP-galactopyranose mutase appears to be conserved in all cryptococcal species that have been sequenced to date (not shown). Galf is thus unlikely to be a vestigial modification, although future studies will be required to test this hypothesis. Meanwhile, our identification of the linkage position of Galf in GXMGal significantly builds on earlier reports that suggested the presence of this moiety and further refines the intricate structure of this complex polymer. Such knowledge will advance future efforts aimed at addressing the properties of this capsule polysaccharide whose importance in cryptococcal pathogenesis has only recently begun to emerge.

Acknowledgments—We thank Pardeep Kumar for preparation of GXMGal from serotype A and members of the Doering laboratory for helpful discussions of this project. We also thank Robyn Roth and John Heisser from the Department of Cell Biology and Physiology at Washington University School of Medicine for preparing the electron micrographs.

REFERENCES

\* C. Heiss, P. Kumar, and T. L. Doering, unpublished work.
Galf Modifies a C. neoformans Capsule Polysaccharide


Galf Modifies a C. neoformans Capsule Polysaccharide


April 19, 2013 • Volume 288 • Number 16
Legends to supplementary figures.

**Figure S1. MS analysis supports the presence of terminal Galf in GXMGal from CAP67 cxt1Δ cxt2Δ cells.** Partially methylated alditol acetates derived from GXMGal were resolved by gas chromatography as in Fig. 3A and analyzed by MS. Panel A, the alditol acetate derivative corresponding to terminal Manp (Fig. 3A) yields a mass of 161 amu (*), which supports the assignment of this compound as a terminal pyranose based on the predicted fragmentation pattern shown in the inset. Panel B, the alditol acetate derivative assigned to t-Galf (Fig. 3A) produces a mass of 278 amu (*), which is characteristic of a terminal furanose residue as indicated by the predicted fragmentation pattern diagrammed in the inset. This assignment is consistent with the comigration of this derivative with a terminal Galf standard by GC (Fig. 3).

**Figure S2. NOESY analysis.** Shown is the 2-D NOESY NMR spectrum of GXMGal from the cxt1Δ cxt2Δ double mutant, acquired at 600 MHz and 70 °C as in reference (31). The arrow indicates the cross peak from H-1 of 2,3,6-α-Galp to H-6 of 6-α-Galp.

**Figure S3. Cryptococcal growth and cell integrity are not affected by the absence of Galf.** The strains listed at the right were grown overnight in YPD medium to an OD$_{600}$ = 1 and diluted 10-fold in water. 5-μl aliquots of the initial cell suspension and of three 5-fold serial dilutions were spotted on YPD modified as indicated at the left and grown for 2-4 days in the temperatures shown. The mucoid character of encapsulated strains
(those in the JEC21 background) in comparison to the dull surface of colonies in the acapsular (CAP67) background is particularly apparent on the last two panels.
Figure S1.
Figure S2.
Figure S3.
Table S1.

The *C. neoformans* var. *neoformans* strains used in this study.

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>JEC21</td>
<td>(1)</td>
</tr>
<tr>
<td>JEC21 <em>glf</em>&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>This study.</td>
</tr>
<tr>
<td>CAP67</td>
<td>(2)</td>
</tr>
<tr>
<td>CAP67 <em>glf</em>&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>This study.</td>
</tr>
<tr>
<td>CAP67 <em>cxt1</em>&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>(3)</td>
</tr>
<tr>
<td>CAP67 <em>cxt2</em>&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>(4)</td>
</tr>
<tr>
<td>CAP67 <em>cxt1</em>&lt;sup&gt;Δ&lt;/sup&gt; <em>cxt2</em>&lt;sup&gt;Δ&lt;/sup&gt;</td>
<td>(4)</td>
</tr>
</tbody>
</table>

<sup>a</sup> All strains are *MAT*<sup>α</sup>.


(4) J. S. Klutts and T. L. Doering, manuscript in preparation.