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Zhuo A. Wang
Washington University School of Medicine in St. Louis

Cara L. Griffith
Washington University School of Medicine in St. Louis

Michael L. Skowyra
Washington University School of Medicine in St. Louis

Nichole Salinas
Washington University School of Medicine in St. Louis

Matthew Williams
Washington University School of Medicine in St. Louis

See next page for additional authors

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Authors
Zhuo A. Wang, Cara L. Griffith, Michael L. Skowyra, Nichole Salinas, Matthew Williams, Ezekiel J. Maier, Stacey R. Gish, Hong Liu, Michael R. Brent, and Tamara L. Doering
Cryptococcus neoformans Dual GDP-Mannose Transporters and Their Role in Biology and Virulence

Zhao A. Wang,* Cara L. Griffith,*** Michael L. Skowyra,*** Nichole Salinas,* Matthew Williams,* Ezekiel J. Maier,*d Stacey R. Gish,* Hong Liu,* Michael R. Brent,*** Tamara L. Doering*

Departments of Molecular Microbiology* and Genetics*** and The Center for Genome Sciences,* Washington University School of Medicine, St. Louis, Missouri, USA; Department of Computer Science, Washington University, St. Louis, Missouri, USA.

Cryptococcus neoformans is an opportunistic yeast responsible for lethal meningoencephalitis in humans. This pathogen elaborates a polysaccharide capsule, which is its major virulence factor. Mannose constitutes one-half of the capsule mass and is also extensively utilized in cell wall synthesis and in glycosylation of proteins and lipids. The activated mannose donor for most biosynthetic reactions, GDP-mannose, is made in the cytosol, although it is primarily consumed in secretory organelles. This compartmentalization necessitates specific transmembrane transporters to make the donor available for glycan synthesis. We previously identified two cryptococcal GDP-mannose transporters, Gmt1 and Gmt2. Biochemical studies of each protein expressed in Saccharomyces cerevisiae showed that both are functional, with similar kinetics and substrate specificities in vitro. We have now examined these proteins in vivo and demonstrate that cells lacking Gmt1 show significant phenotypic differences from those lacking Gmt2 in terms of growth, colony morphology, protein glycosylation, and capsule phenotypes. Some of these observations may be explained by differential expression of the two genes, but others suggest that the two proteins play overlapping but nonidentical roles in cryptococcal biology. Furthermore, gmt1 gmt2 double mutant cells, which are unexpectedly viable, exhibit severe defects in capsule synthesis and protein glycosylation and are avirulent in mouse models of cryptococcosis.

Mannose is a dominant component of fungal glycoconjugates, in contrast to its lesser role in parallel structures of higher organisms. For example, in both mammals and fungi N-glycosylation of proteins begins with the transfer to asparagine of a conserved structure consisting of two N-acetylglucosamine and eight mannose residues in the endoplasmic reticulum. Mammals typically process this initial structure to forms with only three mannose residues, elaborating the modified core with other moieties such as galactose, N-acetylgalactosamine, and N-acetylneuraminic acid in the Golgi apparatus (1). In contrast, yeasts maintain most or all of the initial mannose-rich structure and frequently elaborate it to various extents with additional mannose residues, ranging from the relatively limited elongation seen in C. neoformans to the extensive modifications observed in Saccharomyces cerevisiae (2, 3). As another example, mammalian O-glycans typically contain fucose, xylose, and N-acetylglucosamine (1). In contrast, fungal O-glycans are initiated by the addition of mannose to serine/threonine residues and are generally elongated with several more mannoses (2, 4). Proteins extensively modified with mannose (mannoproteins) are important constituents of fungal cell walls, so that mannose constitutes 30 to 50% of the cell wall mass of the model yeast S. cerevisiae (5). Mannoprotein further occurs as a component of fungal glycolipids (2), GPI anchors (6), and capsules (see below).

Glycan biosynthetic reactions require high-energy monosaccharide donors; in the case of mannose, this compound is the nucleotide sugar GDP-mannose. Its synthesis is catalyzed by the action of GDP-mannose phosphorylase on GTP and mannose-1-phosphate; the latter is generated in the cytosol from mannose-6-P (7). Some GDP-mannose is used at the cytosolic face of the endoplasmic reticulum (ER) to form lipid-linked oligosaccharide precursors of N-glycans (7) or dolichol-P-mannose. These products are subsequently flipped into the ER for further modification or for use as mannose donors in glycosylation reactions, respectively (8). Most GDP-mannose, however, is used as a substrate for glycosylation reactions in the lumen of the Golgi complex (7). To enter the Golgi, this negatively charged compound requires a specific nucleotide sugar transporter (9, 10). GDP-mannose transport activity was first identified and characterized through in vitro studies of S. cerevisiae (11). VRG4, the gene encoding the GDP-mannose transporter, was subsequently identified in S. cerevisiae (12, 13) and other fungi (see below). GDP-mannose transporters have also been reported in plants (14–16) and the protozoan parasite Leishmania donovani (17, 18). Notably, mammalian cells lack GDP-mannose transporters, since they do not perform mannolysis in the Golgi.

As expected from its role in GDP-mannose transport, the S. cerevisiae VRG4 protein is localized to the Golgi apparatus and required for normal Golgi functions (12, 13); the corresponding VRG4 gene is essential (12). Viable cells that express partly functional mutant Vrg4 have been reported, but these strains show defects in both N- and O-linked protein glycosylation and are more sensitive than their wild-type counterparts to cell wall stress (12, 13, 19). Single Vrg4 homologs have been identified in multiple fungi, including Pichia pastoris (20), Aspergillus niger (21), etc.
Aspergillus fumigatus (22), Candida glabrata (23), and Candida albicans (24); all of them complement S. cerevisiae vg4 mutants. While most of these genes, like the S. cerevisiae VG4 gene, are essential, it is notable that A. fumigatus genes lacking GMTA are viable although significantly impaired (22). The GDP-mannose transporter of the protozoan parasite L. donovani is also not essential for cell viability, although it is required for parasite virulence (17, 18). Finally, the Aspergillus nidulans genome contains two VG4-like genes, gmtA and gmtB (25). Both of these are thought to encode functional GDP-mannose transporters since the reduced cell surface mannosylation of a gmtA mutant can be corrected by overexpression of gmtB (26); a double mutant has not been reported.

Our studies focus on the encapsulated fungal pathogen Cryptococcus neoformans, which causes severe disease in immunocompromised individuals and over 600,000 deaths per year worldwide (27). Pathogenic members of the Cryptococcus genus have historically been classified by seroreactivity of their capsule polysaccharides (28); serotypes A and D are the main ones responsible for opportunistic infections. The glycoconjugates of C. neoformans include mannose-containing glycoproteins and glycolipids (3, 4, 29, 30). Mannan is also a major component of this organism’s extensive polysaccharide capsule (31), which is essential for fungal virulence. The capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal; also termed GalXM [28]), along with a small amount of mannanproteins (31). GXM accounts for about 90% of the capsule mass and consists of a mannose backbone modified with xylose, glucuronic acid, and acetyl groups (32). Mutants lacking GXM are avirulent in animal models, suggesting that it plays an important role in pathogenesis (33). We have reported that GXM is made within the secretory pathway (34), which would require the translocation of GDP-mannose into lumenal compartments. The second capsule polymer, GXMGal, is less well understood but is also associated with cryptococcal virulence (35). GXMGal is based on a linear polymer of galactose, and its side chains contain mannose residues along with galactose, glucuronic acid, and xylose (36–38).

We previously identified two C. neoformans genes encoding GDP-mannose transporters, GMT1 and GMT2, which encode proteins that are 63% identical at the amino acid level (39). Each of these genes effectively complements the S. cerevisiae Vrg4 mutant, and the two proteins demonstrate similar GDP-mannose transport activity in vitro (39). Strains lacking either gene are viable, although the mutants do have distinct phenotypes, with gmt1 cells exhibiting a severe capsule defect while gmt2 cells produce normal capsules (39). In addition, transcriptional analysis suggests that the two genes are not coordinately regulated and therefore may have distinct biological functions in C. neoformans (39).

To investigate potential functional differences between the two cryptococcal GDP-mannose transporters, we generated a double mutant strain and evaluated mannose-related phenotypes of the parental strain. We also assessed phenotypes and GMT transcription in strains in which the coding sequence of each gene was expressed from the usual chromosomal location of the other. Finally, we determined that loss of both transporters has significant impact on capsule synthesis and protein glycosylation and completely abrogates fungal virulence.

### MATERIALS AND METHODS

#### Cell growth.
All C. neoformans strains (Table 1) were grown at 30°C if not otherwise indicated. Cells were cultured in YPD medium (1% [wt/vol] yeast extract, 2% [wt/vol] peptone, 2% [wt/vol] dextrose) with shaking (230 rpm) or grown on YPD agar plates (YPD medium with 2% [wt/vol] agar); for strains marked with drug resistance cassettes, 100 μg/ml nourseothricin (Werner BioAgents) or Geneticin (G418; Invitrogen) and/or 150 μg/ml hygromycin B (Roche) was included in the medium.

#### Immunofluorescence localization.
Strains were cultured in minimal medium, consisting of 0.17% yeast nitrogen base without amino acids and 2% yeast extract, 2% (wt/vol) peptone, 2% (wt/vol) dextrose (31). GXM accounts for about 90% of the capsule mass and consists of a mannose backbone modified with xylose, glucuronic acid, and acetyl groups (32). Mutants lacking GXM are avirulent in animal models, suggesting that it plays an important role in pathogenesis (33). We have reported that GXM is made within the secretory pathway (34), which would require the translocation of GDP-mannose into lumenal compartments. The second capsule polymer, GXMGal, is less well understood but is also associated with cryptococcal virulence (35). GXMGal is based on a linear polymer of galactose, and its side chains contain mannose residues along with galactose, glucuronic acid, and xylose (36–38).

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#### Strain construction (serotype D).
To make a gmt1 gmt2 double mutant, the gmt2 deletion construct described previously (39) was biolistically transformed into a gmt1 mutant strain (39). Genomic DNA was extracted from drug-resistant transformants and screened by PCR to confirm replacement of GMT2 by the appropriate marker. DNA blotting (not shown) confirmed the presence of both gene replacements and the absence of additional ectopic insertions of drug markers.

For GMT2 localization, the coding sequence of the serotype D gene, designated here GMT2(D), was PCR amplified from JEC21 genomic DNA (4) using primers GMT2-1 and GMT2-2 to generate a 2,914-bp GMT2(D) DNA fragment, GMT2-3 and GMT2-4 to generate a DNA fragment consisting of the 26-bp FLAG epitope and 336 bp 3’ to the GMT2 gene, and GMT2-7 and GMT2-8 to generate a 1,142-bp GMT2(D) downstream DNA fragment. A 2,821-bp hygromycin resistance cassette was amplified from plasmid pHYG7-KB1 (40) using primers GMT2-5 and GMT2-6 to generate a DNA fragment, GMT2-3 and GMT2-4 to generate a DNA fragment consisting of the 26-bp FLAG epitope and 336 bp 3’ to the GMT2 gene, and GMT2-7 and GMT2-8 to generate a 1,142-bp GMT2(D) downstream DNA fragment. A 2,821-bp hygromycin resistance cassette was amplified from plasmid pHYG7-KB1 (40) using primers GMT2-5 and GMT2-6 to generate a DNA fragment, GMT2-3 and GMT2-4 to generate a DNA fragment consisting of the 26-bp FLAG epitope and 336 bp 3’ to the GMT2 gene, and GMT2-7 and GMT2-8 to generate a 1,142-bp GMT2(D) downstream DNA fragment. A 2,821-bp hygromycin resistance cassette was amplified from plasmid pHYG7-KB1 (40) using primers GMT2-5 and GMT2-6 to generate a DNA fragment, GMT2-3 and GMT2-4 to generate a DNA fragment consisting of the 26-bp FLAG epitope and 336 bp 3’ to the GMT2 gene, and GMT2-7 and GMT2-8 to generate a 1,142-bp GMT2(D) downstream DNA fragment.

#### Table 1. C. neoformans strains used in these studies.

<table>
<thead>
<tr>
<th>C. neoformans strain*</th>
<th>Serotype</th>
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</thead>
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<tr>
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</tr>
<tr>
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<td>This study</td>
</tr>
<tr>
<td>KN99 gmt2</td>
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<tr>
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<tr>
<td>JEC21 GMT1-HA</td>
<td>D</td>
<td>4</td>
</tr>
<tr>
<td>JEC21 GMT1-HA GMT2-FLAG</td>
<td>D</td>
<td>This study</td>
</tr>
</tbody>
</table>

* All strains are MATa.

* In these strains, only the coding region was moved to a new location; flanking regions retained the native sequences.
tance cassette was released from pGMT2(D)-Flag-G418 by digestion with Clal and Smll. An overlapping fragment consisting of GMT2(D) downstream sequence and the 5’ portion of the Genetin resistance cassette was released from the same plasmid by digestion with PstI and Kpnl. To replace the chromosomal copy of the GMT2 gene with GMT2-Flag, the two overlapping DNA fragments were mixed at an equimolar ratio and biolistically transformed into a strain expressing Gmt1-HA (4). Genomic DNA was prepared from Genetin-resistant transformants and screened by PCR. GMT2-Flag protein expression was confirmed by immunostaining.

We wanted to generate strains in which only one GMT gene was expressed and that gene was expressed from the opposite locus. To express the coding sequence of GMT1 from the GMT2 locus, maintaining the GMT2 promoter and terminator, we again used fusion PCR and a split marker strategy to modify the gmt1 strain. We first used JEC2 genomic DNA as a template to amplify a 1,776-bp fragment consisting of the GMT1(D) coding sequence with 3’-terminal addition of a 20-bp fragment of the GMT2(D) downstream sequence using primers ZAW-117 and ZAW-118; a 910-bp fragment of sequence upstream of GMT2(D) using primers ZAW-115 and ZAW-116; and a 937-bp fragment of sequence downstream of GMT2(D) using primers ZAW-119 and ZAW-120. We also amplified an 867-bp portion of a nourseothricin resistance cassette with 5’-terminal addition of a 20-bp fragment of the GMT2(D) downstream sequence from plasmid pGMC200 (43) using primers ZAW-114 and ZAW-078; an overlapping 1,392-bp fragment of the same cassette was amplified using primers GMT1CB-E and GMT1CB-F. The 1,776-bp, 910-bp, and 851-bp amplicons were next assembled by fusion PCR and cloned into the pcR2.1-TOPO vector (Invitrogen) to form the plasmid pGMY1R2-5; this construct was confirmed by DNA sequencing. The 937-bp and 1,392-bp amplicons were similarly assembled, and the product was mixed at a 1:1 molar ratio with the fragment released by XbaI and Sphl digestion of plasmid pGMY1R2-5. The mixed DNA was biolistically transformed into the serotype D gmt1 strain as described above. Transformants were identified by nourseothricin resistance and verified by PCR analysis and DNA sequencing (not shown).

To express the coding sequence of GMT2 from the GMT1 locus, maintaining the GMT1 promoter and terminator, we used overlap PCR and a split marker strategy to modify the gmt2 strain. We used JEC2 genomic DNA as the template to generate a 1,613-bp fragment consisting of the GMT2(D) coding sequence with 3’-terminal addition of a 21-bp fragment of the GMT1(D) downstream sequence using primers ZAW-128 and ZAW-129; an 820-bp fragment of sequence upstream of GMT1(D) using primers ZAW-126 and ZAW-127, and an 841-bp fragment of sequence downstream of GMT1(D) using ZAW-134 and ZAW-135. We also amplified a 1,725-bp segment of a Genetin resistance cassette from plasmid pPB103 (44) with 5’-terminal addition of a 21-bp fragment of GMT1(D) downstream sequence using primers ZAW-130 and ZAW-131 and an overlapping 1,554-bp fragment using primers ZAW-132 and ZAW-133. The 1613-bp, 820-bp, and 1,725-bp amplicons were next assembled by fusion PCR and cloned into the pcR2.1-TOPO vector (Invitrogen) to form plasmid pGMT2R1-5; this construct was checked by DNA sequencing. The 841-bp and 1,554-bp amplicons were similarly assembled, and the product was mixed at a 1:1 molar ratio with the fragment released from the plasmid pGMY2R1-5 by digestion with XbaI and Sphl. The mixed DNA was biolistically transformed into the serotype D gmt2 strain as described above. Transformants were identified by Genetin resistance and verified by PCR analysis and DNA sequencing (not shown).

**Virulence studies (serotype A).** For use in mouse infections, mutant strains were generated in the more virulent serotype A background. For gmt1, a 1,248-bp fragment upstream of the serotype A gene, designated here GMT2(A), was amplified from KN99a genomic DNA using primers Gmt1-1 and Gmt1-4b, and a 1,329-bp fragment downstream of GMT1(A) was amplified using primers Gmt1-3b and Gmt1-8. A 2,930-bp hygromycin resistance cassette was amplified from plasmid pHYG-KB, using primers Gmt1-3b and Gmt1-6. The three amplicons were next assembled by fusion PCR, and the final construct was biolistically transformed into KN99a cells. For gmt2, a 1,295-bp fragment upstream of the serotype A gene, designated here GMT2(A), was amplified from KN99a genomic DNA using primers Gmt2-7 and Gmt2-4, and a 1,294-bp fragment downstream of GMT2(A) was amplified using primers Gmt2-5b and Gmt2-8. A 1,868-bp nourseothricin resistance cassette was amplified from plasmid pGMC200 using primers Gmt2-3 and Gmt2-6. The three amplicons were next assembled by fusion PCR, and the final construct was biolistically transformed into KN99a cells. Transformed cells were allowed to recover for 24 h on YPD medium, transferred onto YPD plates containing hygromycin or nourseothricin as appropriate, and incubated at 30° for 3 to 5 days. For the gmt1 gmt2 double mutant, the gmt2 deletion construct described above was biolistically transformed into the serotype A gmt1 mutant strain. For all strains, genomic DNA was extracted from drug-resistant transformants and screened by PCR to confirm replacement of GMT1(A) or GMT2(A) by the appropriate markers. DNA blotting was used to confirm gene replacements and that any marker cassette was present only once in the genome, with no additional ectopic insertions.

To complement the serotype A gmt1 strain at the original locus using overlap PCR and a split marker strategy, a 2,819-bp GMT1(A) sequence with its flanking sequences was PCR amplified from KN99a genomic DNA using primers GMT1CB-A and GMT1CB-B; a 985-bp fragment of sequence downstream of GMT1(A) was amplified from the same DNA using GMT1CB-C and GMT1CB-D; an overlapping 1,392-bp fragment of the same cassette was amplified using primers GMT1CB-E and GMT1CB-F. The 2,891-bp and 851-bp amplicons were next assembled by fusion PCR and cloned into the pcR2.1-TOPO vector (Invitrogen) to form the plasmid pGMT1CB-1-5. The 985-bp and 1,392-bp amplicons were similarly assembled and cloned to form the plasmid pGMT1CB-3. The two fused fragments were released from their respective vectors by digestion with XbaI and Sphl, mixed at a 1:1 molar ratio, and biolistically transformed into the disruption strain as described above. Transformants were identified by nourseothricin resistance, and complementation was verified by PCR analysis and reversion of phenotypes (not shown).

**Strains to be tested were cultured overnight in YPD medium, collected by centrifugation, washed in phosphate-buffered saline (PBS), and diluted to 2 × 10° cells/ml in PBS. For each strain, 10 4 to 6-week-old female A/Jcr mice (from the National Cancer Institute) were anesthetized with a combination of ketamine hydrochloride (Ketaset) and xylazine and inoculated intranasally with 100 μl of the preserved preparation of C. neoformans. The animals were weighed the day of inoculation and were sacrificed if weight decreased to a value below 80% of peak weight on any subsequent day (an outcome which in this protocol precedes any signs of disease) or upon completion of the study. Initial inocula were plated to confirm CFU. All studies were performed in compliance with institutional guidelines for animal experimentation.**

**Immunoblotting.** Cells from a 3-ml overnight culture in YPD medium were harvested by centrifugation (2,500 × g, 10 min, 4°C) and washed in 500 μl of cold lysis buffer (50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, and 1% SDS) with protease inhibitors (chymostatin, leupeptin, antipain, and pepstatin A, each at 10 μg/ml final concentration, and 0.2 mM phenylmethylsulfonyl fluoride). The suspension was then vigorously vortex mixed with 200 μl of 0.5-mm glass beads (Biospec Products, Bartlesville, OK) for 1-min intervals alternating with 2 min on ice until ~80% breakage was observed under a light microscope. The lysate was centrifuged (6,000 × g, 1 min, at room temperature [RT]), and the supernatant was reserved. Protein concentration of each sample was determined by Bio-Rad protein assay (Bio-Rad). Equal amounts of protein from each fraction were boiled in SDS-PAGE sample buffer and resolved by SDS-PAGE on a 12% gel. Standard methods were used for transfer and immunoblotting, using dilutions of 1:1,000 for rabbit polyclonal anti-α-1,6-mannose antibody (45) and rabbit polyclonal anti-cell wall protein antibody (CWP) (46), and 1:5,000 for chicken polyclonal anti-β-elimin-
nated mannoprotein antibody (from S. Levitz, Boston University School of Medicine, Boston, MA) and secondary antibodies (anti-chicken IgG conjugated to horseradish peroxidase from Sigma; anti-rabbit IgG conjugated to horseradish peroxidase from GE Healthcare). Detection was with the Western Lighting chemiluminescence reagent (PerkinElmer Life Sciences).

Capsule induction. For capsule induction, single colonies were inoculated into 50 ml of YPD medium and shaken overnight at 30°C. Approximately 10⁹ cells were collected by centrifugation (2,500 × g, 10 min, 4°C), washed twice with 1 ml Dulbecco’s modified Eagle medium (DMEM; Sigma), and resuspended in DMEM at 10⁸/ml. The washed cell suspension was then transferred into a 24-well tissue culture plate (TPP; 1 ml/well) and incubated at 37°C in a 5% CO₂ atmosphere. After 48 h, the cells were collected by centrifugation, resuspended in 8 µl PBS, and mixed with 1.5 µl India ink. A 5-µl volume of the suspension was spotted onto a microscope slide, and cells were viewed with a ZEISS Axioskop2 MOT Plus microscope (Carl Zeiss, Thornwood, NJ) with a 40× objective. Random fields were photographed and printed for manual measurement of capsule radius and cell diameter.

RNA-Seq. Wild-type cells were cultured overnight in YPD followed by growth in capsule-inducing conditions (DMEM, 37°C, 5% CO₂). RNA isolation, and transcriptome sequencing (RNA-Seq) analysis as described in reference 47. RNA was isolated at 0, 1.5, 3, 8, and 24 h of culture.

qRT-PCR. For quantitative reverse transcriptase PCR (qRT-PCR), cells were cultured overnight in YPD and then either harvested directly or grown under capsule-inducing conditions as above for 3 h. RNA was prepared from 10⁸ cells and analyzed as described in reference 39 using the following primers: ZAW-117 and ZAW-166 for GMT1; ZAW-063 and ZAW-137 for GMT2; and ZAW-171 and ZAW-172 for ACT1 (for normalization).

Microscopy. Capsule immunofluorescence was performed as described in reference 39 using 50 µg/ml anti-capsular monoclonal antibody 3C2 (from T. Kozel, University of Nevada at Reno) followed by 200 ng/ml of Alexa Fluor 488-tagged anti-mouse antibody (Invitrogen). Transmission electron microscopy was performed exactly as described in reference 48.

For protein localization, sample preparation for microscopy was done as described in reference 4. After blocking, slides were treated with either a high-affinity rat anti-hemagglutinin (anti-HA) monoclonal antibody (Roche Applied Science; 20 ng/ml in blocking buffer) together with a mouse anti-FLAG monoclonal antibody (Sigma; 80 ng/ml in blocking buffer) or blocking buffer alone. Secondary antibody treatment was with Alexa Fluor 594-tagged goat anti-rat IgG and Alexa Fluor 488-tagged goat anti-mouse IgG (Invitrogen; 1 µg/ml in blocking buffer) as appropriate.

RESULTS
In our earlier studies of GDP-mannose transport in C. neoformans, we deleted the genes encoding Gmt1 and Gmt2 in a serotype D strain background (39). The resulting gmt1 and gmt2 single mutants grew like the wild type in rich medium at both 30°C and 37°C (reference 39 and data not shown), although gmt1 cells showed a modest growth defect at 37°C in the presence of the cell wall stressor calcofluor white (Fig. 1). To further define the roles of the two cryptococcal transporters in the biology of this fungal pathogen, we generated a double mutant strain in the same background. The double mutant showed only mildly reduced viability at 30°C (Fig. 1). This was surprising, given the essentiality of VRG4, the gene encoding the single GDP-mannose transporter in S. cerevisiae (12) (see Discussion). The double mutant was not viable at 37°C, however, either in the presence of 80 µg/ml calcofluor white (Fig. 1) or its absence (not shown).

GDP-mannose transport is important in fungi for the synthesis of cell wall polysaccharides (22) and mannoproteins (24), so defects in this process generally correlate with defects in cell wall integrity (12). To test this property in our strains, we examined the growth of gmt mutants at 30°C in the presence of SDS concentrations ranging from 0.0001% to 1% (Fig. 2). The single mutants responded like wild-type cells to this stressor. In contrast, the gmt1 gmt2 double mutant was significantly more sensitive, showing only half as much growth as the other strains at 0.0025% SDS, a concentration at which the wild type and single mutants grew normally. The double mutant was also more sensitive to caffeine, sorbitol, ruthenium red, and hygromycin B (data not shown). (Addition of sorbitol to provide osmotic support did not reduce sensitivity to the other stressors [data not shown].) In all of these conditions, each single mutant behaved like the wild type.

Based on the growth defects of the double mutant, we suspected that it might have reduced virulence in a mouse model of cryptococcal infection. To test this, we generated gmt1 and gmt2 single and double mutants in the more virulent serotype A background (see Materials and Methods). As suspected, the double mutant was completely avirulent, although single mutants had normal virulence (Fig. 3 and data not shown).

Our results to this point suggested that Gmt1 and Gmt2 are redundant proteins involved in a key cellular process. Each protein can presumably compensate for loss of the other, but in the
double mutant function is so compromised that it alters critical
phenotypes. This model is consistent with our in vitro
studies that showed similar activities of the two proteins when each was ex-
pressed individually in S. cerevisiae (39) and suggests that they
have the same cellular function. One observation, however, led us
to question this model: we had noted a growth defect in
gmt1 cells at 37°C in the presence of 80
/ml calcofluor white (Fig. 1) that
was not evident in gmt2. To probe potential specific functions of
the two transporters, we therefore explored additional pheno-
types.

Beyond its occurrence in capsule and cell wall, mannose is a
major component of both N- and O-linked glycans in yeast (2–4).
We broadly surveyed protein N-glycosylation in gmt mutants by
performing immunoblotting experiments with an antibody spe-
cific for terminal α-1,6-mannose, which is added to N-glycans in
the Golgi complex (2). The gmt1 gmt2 double mutant showed a
striking defect in this modification; similar results were observed
when we used antiserum made against beta-eliminated cryptococ-
cal mannoproteins (lacking O-glycans) (Fig. 4A). We also noted a
modest but consistent effect on protein glycosylation in the ab-
sence of GMT1; several polypeptides demonstrated increased gel
migration, consistent with reduced glycosylation, and the overall
signal was slightly reduced compared to that of the wild type (Fig.
4B). We obtained qualitatively similar results when we probed
blots with antiserum made against an S. cerevisiae cell wall protein
(46) that cross-reacts with several cryptococcal polypeptides (data
not shown). Notably, the absence of GMT2 did not significantly
change the glycosylation profile from that of the wild type in par-
allel studies with each antibody (data not shown). These results
suggested that while both Gmt proteins act in protein glycosyla-
tion, Gmt1 plays a more important role.

We found evidence for a dominant role for Gmt1 in several
contexts beyond protein glycosylation. In the course of our
growth studies, we had noticed that gmt1 gmt2 double mutant
cells tended to form clumps in liquid medium. We observed a
mild version of this phenotype in cultures of gmt1 cells, although not in
cultures of gmt2 (Fig. 5, top panel). We also noticed a difference in
colony morphology (Fig. 5, bottom panel); the gmt1 mutant col-
ony surface was duller than that of the wild type, while the gmt2
mutant showed no obvious defect. Both cell clumpiness and dull
colony morphology are suggestive of a defect in capsule produ-
cion, as in the acapsular cap67 mutant shown for comparison (Fig.
5). The colonies of the double mutant not only were dull but also
showed a distinctly altered morphology (Fig. 5, bottom panel).
The cell wall defects of this strain that are suggested by its SDS
sensitivity (Fig. 2) may contribute to this phenotype.

Mannoprotein is a major component of both of the cryptococcal
capsule polysaccharides (GXM and GXMGal [32, 36, 37]), and

FIG 3 The gmt1 gmt2 double mutant is severely attenuated for virulence. Mice
were monitored for 50 days after inhalational inoculation with 2 × 10^9 cells of
the indicated serotype A strains. Animals exhibiting loss of greater than or
equal to 20% of body weight were humanely sacrificed.

FIG 4 Single gmt mutations have modest effects on protein glycosylation.
Total protein fractions of the strains indicated were resolved by SDS-PAGE
and immunoblotted with antibody specific for terminal α-1,6-mannose res-
idues or mannoproteins, as indicated below. Panel A, 10% gel; panel B, 12%
gel. *, examples of bands that migrate faster in gmt1 cells; the wild type was
loaded twice to facilitate comparison. For each panel, the same amount of
protein was loaded in all lanes.

FIG 5 Cells lacking Gmt proteins have altered morphology. (Top) The indi-
cated strains were grown in YPD, and the fraction of cells occurring as single
cells (open bars), in small aggregates (2 or 3 cells, gray bars), and clumps (>3
cells, black bars) was determined. A representative experiment is shown, where
at least 200 cells of each strain were categorized. (Bottom) Photographs of
representative colonies of the indicated strains, grown on YPD. The light re-
fection seen on wild-type and gmt2 cells indicates normal encapsulation,
manifested as shiny colony morphology.
earlier studies have shown that the absence of GDP-mannose synthesis severely curtails capsule production (49). We previously demonstrated that GXM is synthesized in the secretory pathway (34), likely in the Golgi complex. Together, these findings imply that capsule synthesis requires the transport of GDP-mannose into the Golgi complex and therefore would need an appropriate transporter. To probe the capsule defect suggested by the dull colony and clumpy growth morphologies that we had observed, we stained our panel of mutants with a monoclonal anticapsule antibody. We noted that the gmt1 fibers were markedly more sparse than those of the wild type, although the complemented gmt1 mutant and gmt2 cells looked normal in this respect. Consistent with our observations by light microscopy, we detected no capsule fibers on the surfaces of gmt1 gmt2 double mutant cells.

Our results suggested that in contrast to cell wall integrity phenotypes, where the two transporters seem to effectively compensate for one another, normal growth at 37°C, protein N-glycosylation, and capsule synthesis all rely more heavily on the activity of Gmt1 and are dramatically impared in the absence of both proteins (see Discussion). Because of the similar in vitro activities of these proteins (39), we suspected that the apparent difference in protein function was due to levels of expression. We therefore evaluated gene expression by RNA-Seq at various time points of capsule induction. This study showed significantly higher expression of GMT1, which ranged from roughly 3-fold that of GMT2 in rich medium (YPD, zero time in Fig. 7) to over 25-fold after 1 day under capsule-inducing conditions (24 h in Fig. 7).

To confirm our hypothesis that the phenotypic differences between gmt1 and gmt2 reflected the distinct expression levels of their remaining GMT genes, we performed a gene swap experiment, diagrammed in Fig. 8 (panel A). To do this, we replaced the wild-type GMT gene that remained in each single mutant with the absent coding sequence. This generated pairs of strains expressing the same gene from different genomic contexts (GMT2 in strains 2 and 5; GMT1 in strains 3 and 4). When we measured GMT gene expression in these strains by qRT-PCR, we noticed that absence of either GMT did not significantly alter expression of the other (Fig. 8C; compare strains 2 and 3 to strain 1). This suggests that there is no mechanism for compensatory upregulation of either GMT upon loss of the other. We also noted that movement of GMT1 from its native locus (strain 3) to the GMT2 locus (strain 4) reduced its expression roughly 25-fold, consistent with the RNA-Seq expression differences between these loci that we observed in wild-type cells under the same inducing conditions (Fig. 7). The change in GMT2 expression between the two sites, however, was significantly less (roughly 3-fold; compare strains 2 and 5), although in the expected direction.

We next assessed capsule induction by negative staining after growth under inducing conditions. We found that moving GMT1 to the usual site of GMT2 caused reduced GMT1 expression and dramatic reduction in capsule thickness (Fig. 8B and C; compare strains 3 and 4). Furthermore, moving GMT2 to the usual site of GMT1 caused an increase in gene expression and capsule size (compare strains 2 and 5). These data are consistent with a model of interchangeable transporters, which differ only in expression under capsule-inducing conditions. We then used negative staining with India ink to visualize the capsules and measured them to quantitate induction (Fig. 6B). Consistent with the experiments presented above, gmt1 mutant cells displayed significantly reduced capsule thickness compared to that of the wild type (1.5 ± 0.2 μm versus 3.7 ± 0.1 μm; P < 0.05); complementation of this mutant with the wild-type GMT1 gene restored it to wild-type thickness (3.6 ± 0.2 μm; P = 0.4). Capsules of gmt2 cells were also similar to those of wild-type cells (3.9 ± 0.1 μm; P = 0.2), while the capsules of gmt1 gmt2 double mutant cells were undetectable, even under these inducing conditions.

The thickness of the capsule, which reflects fiber length (50, 51), was clearly reduced in gmt1 mutants, but these studies did not address fiber density. To investigate this, we performed electron microscopy (Fig. 6C). We noted that gmt1 fibers were markedly thicker compared to those of the wild type, although the complemented gmt1 mutant and gmt2 cells looked normal in this respect. Consistent with our observations by light microscopy, we detected no capsule fibers on the surfaces of gmt1 gmt2 double mutant cells.
level. However, comparison of strains with different genes in the same site as their sole GMT sequences suggests otherwise. For example, expressing GMT2 from the GMT1 locus (strain 5) yielded consistently smaller capsules than when the endogenous gene was at that site (strain 3); even expressed at levels similar to those expected for GMT1, GMT2 did not functionally replace it.

The capsule thickness of each strain (Fig. 8B) generally correlated with total expression of GMT sequences (Fig. 8C), and the two strains with the lowest GMT expression (and least capsule) showed cell and colony morphology consistent with clumpy acapsular cells (Fig. 8B and D, strains 4 and 6). To our surprise, however, the growth of the various strains on rich medium (Fig. 8D) did not similarly correlate with GMT expression under those growth conditions (see Fig. S1 in the supplemental material, top panel). In particular, colonies of strain 5, where GMT2 was expressed from a nonnative locus, grew slowly (note the small colony size in Fig. 8D) despite robust GMT2 expression in this medium (see Fig. S1 in the supplemental material, top panel); it may be that this level of GMT2 expression is toxic to the cells (see Discussion). Furthermore, both of the swapped strains (4 and 5) were highly sensitive to SDS stress, resembling the double mutant in rich medium (see Fig. S1 in the supplemental material, bottom panel). This was the case even though GMT1 expression from strain 4 was close to GMT2 expression in strain 2 and GMT2 expression in strain 5 exceeded GMT1 expression in strain 3 (see Fig. S1 in the supplemental material, top panel). These observations held in multiple independent transformants.

The results of our gene swapping study suggested that factors beyond gene expression influence Gmt function: despite similar activity in vitro in an exogenous system, these proteins do not appear to function identically in vivo even when expressed from

FIG 7 GMT gene expression in wild-type cells under capsule-induction conditions. Wild-type KN99α cells were grown under capsule-inducing conditions, and RNA-Seq analysis was performed at 0, 1.5, 3, 8, and 24 h. The expression values plotted (median ± standard deviation) were calculated from three separate experiments, each including RNA prepared from three biological replicate cultures.

FIG 8 Genomic location does not explain the functional differences between GMT1 and GMT2. (A) Schematic representation of the GMT strain variants listed at left, indicating the coding sequence present at each locus (colored boxes): orange, GMT1; green, GMT2; dark gray, Geneticin resistance marker (G418) replacing GMT1 (gmt1::G418); light gray, nourseothricin resistance marker (NAT) replacing GMT2 (gmt2::NAT). Promoter and terminator sequences of each locus (colored lines) were not altered. Strains: 1, wild type; 2, gmt1 mutant; 3, gmt2 mutant; 4 and 5, new “swapped” strains; 6, gmt1 gmt2 double mutant. (B) The capsule thickness of each strain under capsule-inducing conditions was examined with India ink as described in Materials and Methods. Scale bar, 5 μm. (C) GMT expression levels under capsule-inducing conditions determined by RT-PCR, plotted relative to ACT1. (D) Growth on rich medium. Aliquots (5 μl) of a stock cell suspension (2 × 10⁶ cells/ml) and three 10-fold serial dilutions were spotted on YPD plates and grown for 1 day at 30°C.
the same locus at similar levels. One possible explanation is that the proteins are differentially localized. Because both Gmt1 and Gmt2 complement the loss of *S. cerevisiae* Vrg4p (39), which is known to be a Golgi protein (13), we had assumed them to be similarly localized. However, there is precedent for subcompartmentalization of Golgi functions (52) and for association of nucleotide sugar transporters with specific glycosyltransferases (53).

To examine the localization of the Gmt1 and Gmt2 proteins by immunofluorescence microscopy, we modified the chromosomal copies of *GMT1* and *GMT2* to encode C-terminally epitope-tagged proteins (Gmt1-HA and Gmt2-FLAG); we know from our previous studies that C-terminally modified GMTs are still functional (39). When we probed the resulting doubly tagged strain with both anti-HA and anti-FLAG antibodies, we observed staining of large puncta in a perinuclear distribution, consistent with Golgi localization (4,13). Neither protein colocalized with BiP, a marker of the endoplasmic reticulum (not shown). To improve resolution, we used AxioVision 4.8 software (Carl Zeiss) for image deconvolution. Images processed by an inverse filter algorithm (Fig. 9, first row) showed that the two Gmt proteins generally colocalized, although occasional areas where only one was stained were also detectable (Fig. 9 and data not shown). Application of a fast iterative algorithm resulted in sharper images but with lower total signal intensity (Fig. 9, second row, and data not shown). In such images, we could clearly appreciate the membrane localization of the two Gmt proteins, as well as regions where they did not completely colocalize (examples indicated by arrowheads); interestingly, the latter consistently gave the impression that regions of Gmt1 staining surrounded those of Gmt2 localization (Fig. 9 and data not shown). This result held even when the secondary antibody fluorophores were swapped to rule out potential artifacts due to staining or fluorophore properties (Fig. 9, third and fourth rows).

**DISCUSSION**

*C. neoformans* has two functional GDP-mannose transporters, which show the same biochemical activity *in vitro* (39). Surprisingly, cells lacking both of these proteins grow well, although they are somewhat compromised in growth under stress (Fig. 1 and 2). This contrasts with findings in *S. cerevisiae* and most other fungi that have been tested, where GDP-mannose transport is essential (12, 21, 23, 24). *A. fumigatus* is notably also viable without its single GDP-mannose transporter, but the mutant is severely defective in growth and cannot spread or sporulate (22). There are several possible explanations for the robustness of *C. neoformans* gmt1 gmt2 cells. One is that Golgi glycan synthetic reactions involving mannose, such as the extension of protein-linked glycans, are not needed for cryptococcal viability or virulence. This is con-

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**FIG 9** Gmt proteins are located in the Golgi complex. Chromosomally expressed Gmt1-HA and Gmt2-Flag were immunolocalized and imaged as detailed in the text. Shown are single-channel and merged images of signal derived from staining with DAPI (4′,6-diamidino-2-phenylindole) (nucleus) and with antibodies to the two tags as indicated. Arrowheads indicate examples of regions where Gmt1 and Gmt2 do not colocalize; broken white lines indicate the cell edge as observed under bright-field microscopy. Scale bar, 5 μm.
sistent with the findings of Park et al. in studies of C. neoformans mutants unable to initiate Golgi α-1,6-mannose branching (3). In this regard, it is also notable that cryptococcal N-glycan extension is more limited than that of S. cerevisiae or C. albicans (2); this is also the case for A. fumigatus (54). Another possibility is that other C. neoformans nucleotide sugar transporters that are localized to the Golgi complex have sufficiently relaxed specificity to transport some amount of GDP-mannose. Our finding that protein N-glycans in gmt1 gmt2 cells are apparently devoid of the terminal α-1,6-mannose modification that is normally acquired in the Golgi complex (Fig. 4A) argues against this, but additional glycan analysis will be required to resolve this question.

Gmt1 and Gmt2 behave similarly in terms of in vitro assays and complementation of an S. cerevisiae mutant defective in GDP-mannose transport (39). However, the gmt1 and gmt2 single mutants have distinct phenotypes, with greater or lesser differences from the wild type depending on the biological process queried. In some cases, both single mutants resemble the wild type, as with cell wall integrity (Fig. 2) or virulence (Fig. 3 and data not shown). In others, the two single mutants exhibit modest differences; an example of this is protein glycosylation, where a size shift suggesting reduced terminal glycan modification is seen in a subset of gmt1 polypeptides but not seen in gmt2 polypeptides (Fig. 4B and data not shown). There are also striking differences between the two mutants. For example, gmt1 cells show significant reduction in capsule thickness and fiber density compared to the wild type. These capsule defects are reflected in colony appearance and cell clumping in culture and cannot be overcome by capsule-inducing conditions. In contrast, capsule is normal in gmt2 cells by all of these tests. Distinct phenotypes have also been observed in A. nidulans mutants lacking one of a pair of GDP-mannose transporter genes (25, 26), although a double mutant in that system has not been reported.

We initially postulated that the activities of Gmt1 and Gmt2 are simply redundant, which would be consistent with the more extreme defects of cells lacking both transporters. In this case, the different single mutant phenotypes could reflect differential expression of the two proteins, as has been suggested for A. nidulans (25, 26). In line with this idea, our RNA-Seq results indicate that GMT1 mRNA expression is severalfold higher than that of GMT2 in rich medium and rises to 1 order of magnitude higher under capsule-inducing conditions. Immunostaining and immunoblotting studies (not shown) also suggested that Gmt1 is also more abundant at the protein level, although in those experiments the two proteins bore different epitope tags, so the comparison is not definitive. In this model, the lack of the more abundant protein, Gmt1, would be expected to have more dramatic consequences, as we do observe for several phenotypes that we tested. The differing effects on various biosynthetic processes seen in each mutant might then reflect cellular prioritization of glycan synthetic pathways in the context of enzymatic competition for use of a limited resource (Golgi complex-localized GDP-mannose). Alternatively, the differing extents of perturbation of various cellular processes could parallel the overall demand for GDP-mannose: capsule production under inducing conditions, for example, likely requires more mannose than does N-glycan outer chain modification.

Although a model of completely redundant activities is consistent with many of our results, it raises the question of why C. neoformans and its sister species Cryptococcus gattii, which diverged over 45 million years ago, have each maintained two copies of this transporter. Furthermore, simple movement of each gene to the genomic location of the other did not yield the phenotypes that would be expected from the resulting modulation of RNA expression levels if the gene products behaved identically (Fig. 8); overexpression of GMT2 also did not complement the defects in gmt1 mutant cells (data not shown). These studies suggest an alternative model, whereby the two transporters in fact play different roles, although they can compensate for each other to some extent in the setting of single mutants. This hypothesis is supported by the distinct expression patterns of the two genes across multiple growth conditions (39), including opposite responses to capsule-inducing conditions (Fig. 7). The higher substrate affinity of Gmt2, which has a $K_m$ for GDP-mannose that is twice that of Gmt1 (39), also suggests distinct functions of the two proteins, as does our observation that when GMT2 is expressed at GMT1 levels (Fig. 8, strain 5), it has adverse effects on cell growth (Fig. 8D and Fig. S1 in the supplemental material); potentially Gmt2 directs substrate to less productive biosynthetic pathways. Interestingly, the two Gmt proteins in A. nidulans also demonstrate different expression patterns that suggest distinct roles in development (26).

Our imaging studies (Fig. 9) hint that although the two transporters largely colocalize, they may also occur in distinct locations within the secretory pathway. This could in turn enable association with distinct glycosyltransferases or synthetic complexes that mediate specific cellular functions and may also influence protein stability. Reports from studies of mammals, yeasts, insects, and plants suggest that subcompartmentalization contributes to efficient organization of glycan synthesis in eukaryotes (52, 55, 56). Defining such compartments in C. neoformans, however, will require resolution beyond those of the methods that we have employed (standard immunofluorescence with deconvolution software) because of the limits imposed by the size of this yeast and its organelle structure; newer microscopy approaches, such as stochastic optical reconstruction microscopy (STORM) (57), may be required for these studies.

We did consider the possibility that Gmt1 and Gmt2 interact with each other. The Vrg4 protein in S. cerevisiae dimerizes, which is important for its GDP-Man transport activity (58, 59), and the LPG2 GDP-mannose transporter of Leishmania functions as a homodimer (60). Preliminary studies (not shown) did not reveal such interactions. Furthermore, the differing regulation and relative abundance of the two transcripts and the similar phenotypes of wild type and gmt2 cells argue against this model. Nonetheless, we cannot strictly rule out this possibility.

It is notable that gmt1 cells do not show a significant defect in virulence, despite reduced capsule and several underglycosylated proteins. It appears that the mutant yeast cells, which do maintain some capsule, are able to sufficiently balance glycan synthetic processes in vivo to compensate for reduced mannose utilization. The double mutant strain, in contrast, clearly demonstrates the critical role of mannose glycosylation in C. neoformans. This strain is sensitive to stress and temperature, has incomplete N-glycans, is essentially acapsular, and is avirulent in a mouse model of infection. The lack of virulence of the double mutant, coupled with the fact that humans and other mammalian hosts of C. neoformans do not express GDP-mannose transporters, suggests these proteins as potential targets for antifungal chemotherapy.
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