Unraveling the Novel Structure and Biosynthetic Pathway of O-Linked Glycans in the Golgi Apparatus of the Human Pathogenic Yeast Cryptococcus neoformans*

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Background: Information on the structure of cryptococcal O-glycans is limited.

Results: C. neoformans O-glycans are short manno-oligosaccharides, extended mostly by α1,2-linkages but containing an α1,6-linkage.

Conclusion: Ktr3p adds a second α1,2-linked mannose to major O-glycans, whereas Hoc1p and Hoc3p transfer a third α1,6-linked mannose residue to O-glycans with and without xylose, respectively.

Significance: This work reports novel features of the cryptococcal O-glycan biosynthesis pathway.

Cryptococcus neoformans is an encapsulated basidiomycete causing cryptococcosis in immunocompromised humans. The cell surface mannoproteins of C. neoformans were reported to stimulate the host T-cell response and to be involved in fungal pathogenicity; however, their O-glycan structure is uncharacterized. In this study, we performed a detailed structural analysis of the O-glycans attached to cryptococcal mannoproteins using HPLC combined with exoglycosidase treatment and showed that the major C. neoformans O-glycans were short manno-oligosaccharides that were connected mostly by α1,2-linkages but connected by an α1,6-linkage at the third mannose residue. Comparison of the O-glycan profiles from wild-type and ues1Δ mutant strains strongly supports the presence of minor O-glycans carrying a xylose residue. Further analyses of C. neoformans mutant strains identified three mannosyltransferase genes involved in O-glycan extensions in the Golgi. C. neoformans KTR3, the only homolog of the Saccharomyces cerevisiae KRE2/MNT1 family genes, was shown to encode an α1,2-mannosyltransferase responsible for the addition of the second mannose residue via an α1,2-linkage to the major O-glycans. C. neoformans HOC1 and HOC3, homologs of the Saccharomyces cerevisiae OCH1 family genes, were shown to encode α1,6-mannosyltransferases that can transfer the third mannose residue via an α1,6-linkage, to minor O-glycans containing xylose and to major O-glycans without xylose, respectively. Moreover, the C. neoformans ktr3Δ mutant strain, which displayed increased sensitivity to SDS, high salt, and high temperature, showed attenuated virulence in a mouse model of cryptococcosis, suggesting that the extended structure of O-glycans is required for cell integrity and full pathogenicity of C. neoformans.

Protein O-mannosylation is a post-translational modification commonly found in bacteria and eukaryotes from yeasts to humans that plays important roles in protein function, localization, secretion, and host-pathogen protein-protein interactions (1–5). In yeast and higher eukaryotes, protein O-mannosylation is initiated in the endoplasmic reticulum (ER) by protein O-mannosyltransferases (PMTs), which transfer a mannose from Dol-P-Man to the serine or threonine residues of secretory proteins (6). The first step of mannose addition by PMTs in the ER is highly conserved; however, the subsequent processing of O-glycans in the Golgi apparatus is quite diverse among eukaryotes. In mammalian cells, the inner O-linked mannose is elongated first with the addition of an N-acetylgalactosamine and then various sugars (7). In the case of yeast, further elongation of the mannose residues occurs on the luminal side of the Golgi apparatus and is catalyzed by several Golgi mannosyltransferases that transfer a mannose from GDP-Man. In the budding yeast Saccharomyces cerevisiae, members of the KRE2/MNT1 family have overlapping roles in the addition of the second and third α1,2-linked mannose residues (8–10), and members of the MNN1 family are responsible for the addition of the fourth and fifth mannose residues on the O-linked carbohydrate chain via α1,3-linkages, generating Manα1–3Manα1–3Manα1–2Manα1–2Manα1–Ser/Thr (11–13). Although S. cerevisiae cells with a single, double, or triple disruption of the KRE2/MNT1 family genes show no apparent growth defects under normal conditions (8, 10), these mutants exhibit increased resistance to K1 killer toxin, which requires an O-mannosylated cell wall receptor for function (9).

Studies on the structure of O-glycans and the genes involved in the O-mannosylation biosynthetic pathway in fungi have revealed both common and species-specific glycan structures. In most yeasts and filamentous fungi, an α1,2-linked mannotriose, Manα1–2Manα1–2Manα1–Ser/Thr, is present in the Golgi as the major common core structure of O-glycans, which is differentially expanded according to species (14). In Candida albicans, an
opportunistic human pathogen, five multifunctional mannosyltransferases of the KRE2/MNT1 family have been reported. *C. albicans* Mnt1p and Mnt2p have redundant α,1,2-mannosyltransferase activities, and they add the second and third α,1,2-mannose residues and may add the fourth and fifth moieties to the O-linked glycan (15, 16). In the fission yeast *Schizosaccharomyces pombe*, six *omh* (O-glycoside α,1,2-mannosyltransferase homolog) genes, which are homologous to the *S. cerevisiae* KRE2/MNT1 family genes, have been identified; however, only *Omh1p* was shown to catalyze the addition of a mannose residue to linear, α,1,2-linked mannobiose and mannotriose (17). Unlike *S. cerevisiae*, the O-glycans of *S. pombe* are decorated with α,1,2-linked and/or α,1,3-linked galactose residues, which are added by the gna12 and *otg2* gene products (18, 19). The O-linked glycans of the methylotrophic yeast *Pichia pastoris* were shown to consist of α,1,2-linked mannosene chains of up to four residues (20) with β,1,2-linked mannobiose as terminal residues (21). The O-glycans of the major airborne fungal pathogen *Aspergillus fumigatus* were reported to contain β-galactofuranose, which has not been observed in other fungal O-linked oligosaccharides. These novel β-galactofuranose-containing O-linked oligosaccharides in *A. fumigatus* serve as the immunodominant epitopes in this medically important fungus (22).

*Cryptococcus neoformans* is an encapsulated basidioymete that causes fatal cryptococcal meningococcal infections in immunocompromised individuals such as AIDS patients. Several extra-cellular factors that are produced by pathogenic *Cryptococcus* species complex are important for complete virulence, including an extensive polysaccharide capsule, secreted hydrolytic enzymes, and melanin pigment (23). Cell-bound or secreted cryptococcal mannoproteins have been reported to be associated with *C. neoformans* pathogenicity (24) and are involved in the stimulation of host T-cell responses (25, 26). *C. neoformans* has been predicted to contain more than 50 putative mannoproteins, which typically contain potential N-glycosylation sites, putative Ser/Thr-rich regions for O-glycosylation, and a glycosylphosphatidylinositol anchor (26, 27). However, the structure and biosynthetic pathway of cryptococcal oligosaccharides assembled on mannoproteins have only been partially characterized. We previously reported the unique features of the biosynthetic pathway and the structure of cryptococcal N-linked glycans containing xylose and xylosyl phosphate residues (28). A recent report by Reilly et al. (29) showed that *C. neoformans* O-linked glycans mostly consist of 1–4 mannose residues, whereas minor species contain xylose and xylosyl phosphate residues. It was reported that *C. neoformans* contains three putative PMT genes (*PMT1*, *PMT2*, and *PMT4*), encoding protein O-mannosyltransferases that are involved in the addition of the first mannose in O-glycan biosynthesis (30, 31). However, information on the detailed structure of O-linked glycans and the proteins responsible for the extension of O-linked glycans in *C. neoformans* has not yet been made available. In this study, we performed a systematic analysis of the O-glycans in *C. neoformans* species complex based on HPLC analysis combined with exoglycosidase treatment to obtain detailed information on the O-glycan linkages in *C. neoformans*. In addition, we performed a functional analysis of the KTR3, HOC1, and HOC3 genes to define the specific roles of their gene products in O-glycan extension in the Golgi apparatus. Moreover, by analyzing the virulence of the ktr3Δ mutant in a mouse model of systemic cryptococcosis, we demonstrated that the extended structure of O-glycan is required for the full pathogenicity of *C. neoformans*.

**TABLE 1**

| Strain  | Genotype/description               | Parent | Source/reference |
|--------|-----------------------------------|--------|------------------|
| *C. neoformans* H99 | MATa (serotype A) | H99 | Ref. 28 |
| aex1Δ | MATa aex1::NAT#159ARYG63D | H99 | Ref. 28 |
| hxl1Δ | MATa hxl1::NAT#229 | H99 | Ref. 33 |
| cac1Δ | MATa cac1::NAT#159 | H99 | Ref. 36 |
| ktr3Δ | MATa ktr3::NAT#159 | H99 | Ref. 28 |
| ktr3Δ/KTR3 | MATa ktr3::NAT#159/KTR3::NEO | ktr3Δ | This study |
| hoc1Δ | MATa hoc1::NAT#159 | H99 | Ref. 28 |
| hoc2Δ | MATa hoc2::NAT#159 | H99 | This study |
| hoc3Δ | MATa hoc3::NAT#159 | H99 | This study |
| hoc2Δ/KOC1 | MATa hoc2::NAT#159/KOC1::NEO | hoc1Δ | This study |
|hoc3Δ::HOC3 | MATa hoc3::NAT#159/HOC3::NEO | hoc3Δ | This study |
| JEC21 | MATa (serotype D) | Ref. 53 |
| C. *gattii* R265 | MATa (serotype B) | Ref. 54 |

| Plasmid | Genotype/description | Source/reference |
|---------|----------------------|------------------|
| pNAT-STM#159 | NAT-resistant marker vector for gene disruption | Ref. 28 |
| plAFS1 | Modified pAF12 vector containing NEO as a selection marker | Ref. 33 |
| plAFS1-KTR3 | plAFS1 containing the KTR3 ORF | This study |
| plAFS1-HOC1 | plAFS1 containing the HOC1 ORF | This study |
| plAFS1-HOC3 | plAFS1 containing the HOC3 ORF | This study |

* Each number of NAT(+) indicates the unique signature tag of the Nat' marker.

**EXPERIMENTAL PROCEDURES**

**Strains, Plasmids, and Media—**The *C. neoformans* strains and plasmids used in this study are listed in Table 1. Yeast cells were generally grown at 30 °C in yeast extract–peptone–dextrose (YPD) medium (1% (w/v) yeast extract, 2% (w/v) peptone, and 2% (w/v) glucose) with shaking. *C. neoformans* transformants were selected on YPD plates containing 100 μg/ml nourseothricin (Werner BioAgents, Jena, Germany), referred to as YPD$_{\text{NAT}}$, or YPD plates containing 200 μg/ml G418 disulfate (Duchefa, Haarlem, The Netherlands), referred to as YPD$_{\text{G418}}$. Phenotypic analyses were conducted on YPD solid medium containing 0.05% SDS and 1 M NaCl without or with 1 M sorbitol.
Mutant and Complemented Strain Construction—In the *C. neoformans* serotype A strain H99 (MATα), genes were disrupted by biolistic transformation as described previously using double joint-PCR strategies (32). Transformants were isolated on YPD_NAT, and gene disruptions were screened by PCR (data not shown). To generate complemented strains, genomic DNA fragments containing KTR3, HOC1, and HOC3 were amplified by PCR and introduced into their respective native genomic loci, respectively, but confirmed the ectopic integration of KTR3 into an unidentified locus (data not shown).

Preparation of Cell Wall Mannoproteins—Briefly, cells from a 24-h 100-ml culture in YPD medium were washed in water and incubated twice in 5 ml of DMSO for 30 min at room temperature to reduce the possibility of capsular polysaccharide contamination. After removing the DMSO by centrifugation, the cell pellets were washed with water and then resuspended in 0.1 M citrate buffer (pH 7.0) and autoclaved at 121 °C for 120 min. The autoclaved cells were centrifuged at 4,000 × g for 30 min at 4 °C, and then the supernatants were mixed with three volumes of ethanol and incubated at 4 °C overnight. Crude cell wall mannoproteins (cwMPs) were collected by centrifugation at 4,000 × g for 30 min at 4 °C and then dissolved in 9 ml of concanavalin A binding buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂). After incubating the dissolved cell wall proteins with 1 ml of concanavalin A-Sepharose beads (GE Healthcare) in a column for 4 h with slow rotation, the beads were washed with 10 ml of concanavalin A binding buffer, and then the cwMPs were collected by adding 5 ml of 1 M methyl-α-D-mannopyranoside. The eluted glycoproteins were dialyzed in water for 2 days and dried using a vacuum evaporator (Hanil Scientific, Incheon, Korea).

Preparation of O-Glycans for HPLC Analysis—The O-linked oligosaccharides were released from the cryptococcal cwMPs by modified hydrazinolysis as described previously (34). Briefly, dried cwMPs (100 mg) were resuspended in 100 µl of hydrazine monohydrate (Tokyo Chemical Industry Co.), and the mixture was incubated at 60 °C for 4 h. After cooling and desiccation of the reactant without heating, the pellets were dissolved in 100 µl of 1,6-mannosidase (150 units/µl; Sigma) to remove other glycan and monitored twice in 5 ml of DMSO for 30 min at room temperature to reduce the possibility of capsular polysaccharide contamination. After removing the DMSO by centrifugation, the cells were washed with water and then resuspended in 0.1 M citrate buffer (pH 7.0) and autoclaved at 121 °C for 120 min. The autoclaved cells were centrifuged at 4,000 × g for 30 min at 4 °C, and then the supernatants were mixed with three volumes of ethanol and incubated at 4 °C overnight. Crude cell wall mannoproteins (cwMPs) were collected by centrifugation at 4,000 × g for 30 min at 4 °C and then dissolved in 9 ml of concanavalin A binding buffer (20 mM Tris-HCl, pH 7.4, 0.5 M NaCl, 1 mM CaCl₂, and 1 mM MnCl₂). After incubating the dissolved cell wall proteins with 1 ml of concanavalin A-Sepharose beads (GE Healthcare) in a column for 4 h with slow rotation, the beads were washed with 10 ml of concanavalin A binding buffer, and then the cwMPs were collected by adding 5 ml of 1 M methyl-α-D-mannopyranoside. The eluted glycoproteins were dialyzed in water for 2 days and dried using a vacuum evaporator (Hanil Scientific, Incheon, Korea).

Profiles of Total *C. neoformans* O-Glycans—To dissect the structure of the neutral O-glycans of the pathogenic *Cryptococcus* species complex, the O-linked oligosaccharides assembled on cwMPs derived from serotype A strain H99 (α-mating type) were obtained by hydrazinolysis, labeled with 2-AA, and then analyzed using HPLC with fluorescence detection (Fig. 1). Consistent with a previous mass spectrometry analysis (29), which reported that *C. neoformans* O-linked oligosaccharides are mostly composed of 2–4 mannose residues (Man₂-Man₄; M2–M4), our HPLC profile of wild-type O-glycans displayed (pH 5.0) for 18 h at 37 °C. After enzymes were removed using a 10 K Microcon (Millipore), the glycans were dried in a vacuum evaporator and reconstituted with 50 µl of water for HPLC analysis. The α1,2-MNS-treated glycan was successively treated with 1 μl of α1,6-MNS for 18 h at 37 °C.

**O-Glycan Profile Analysis by HPLC**—The HPLC analysis of the O-glycan profile was conducted on a TSKgel Amide-80 column (0.46 × 25 cm, 5 µm; Tosoh, Tokyo, Japan) at a flow rate of 1.0 ml/min. The column was equilibrated with a solution containing 90% solvent A (2% acetic acid and 1% tetrahydrofuran in acetonitrile) and 10% solvent B (5% acetic acid, 3% triethylamine, and 1% tetrahydrofuran in water). After sample injection, solvent B was maintained at 10% for 5 min and then increased linearly to 90% over 60 min. 2-AA-oligosaccharides were detected with a 474 fluorescence detector at excitation and emission wavelengths of 360 and 425 nm, respectively. Data were collected using Empower TM2 chromatography data software (Waters).

**Capsule Formation and Melanin Analysis—** *C. neoformans* cells grown on liquid Sabouraud dextrose medium (Difco) for 16 h at 30 °C were inoculated in 10% Sabouraud dextrose medium (buffered to pH 7.3 with 50 mM MOPS) and incubated at 30 °C for 2 days. After incubation, the cells were concentrated by centrifugation, and the capsule was stained with India ink. A Zeiss Axioscope (A1) equipped with an AxioCam MRM digital camera was used to visualize the India ink-stained *C. neoformans* cells. For the melanin assay, *C. neoformans* cells were incubated for 16 h at 30 °C in YPD, diluted in phosphate-buffered saline (PBS) to an A₄₀₀ of 0.6, spotted onto an L-DOPA plate (7.6 mM L-asparagine monohydrate, 5.6 mM glucose, 22 mM KH₂PO₄, 1 mM MgSO₄·7H₂O, 0.5 mM L-DOPA, 0.3 mM thiamine-HCl, and 20 mM biotin), and then incubated at 30 °C and 37 °C for 2–4 days.

**Animal Study**—Virulence assays were performed as described previously (33). 4–6-week-old female A/Jcr mice (Japan SLC Inc., Hamamatsu, Japan) were utilized in this study. For infection, the *C. neoformans* strains were grown in YPD medium at 30 °C for 18–24 h, washed with sterile PBS, and then resuspended in sterile PBS at a density of 2 × 10⁶ cells/ml. Serially diluted cells were plated onto YPD medium and incubated at 30 °C to determine viability. Ten mice were infected via intranasal instillation with 10⁵ cells of the wild-type, ktr3Δ, and ktr3Δ/KTR3 strains. Following infection, mice were weighed and monitored twice daily and then sacrificed when they lost 30% of original body weight. Kaplan-Meier survival curves were generated by using Prism version 5.02 (GraphPad Software).

**RESULTS**

Profiles of Total *C. neoformans* O-Glycans—To dissect the structure of the neutral O-glycans of the pathogenic *Cryptococcus* species complex, the O-linked oligosaccharides assembled on cwMPs derived from serotype A strain H99 (α-mating type) were obtained by hydrazinolysis, labeled with 2-AA, and then analyzed using HPLC with fluorescence detection (Fig. 1). Consistent with a previous mass spectrometry analysis (29), which reported that *C. neoformans* O-linked oligosaccharides are mostly composed of 2–4 mannose residues (Man₂-Man₄; M2–M4), our HPLC profile of wild-type O-glycans displayed...
major peaks at the M2–M4 positions (Fig. 1A, a). The profile of the O-glycans from the uxs1Δ mutant, which is defective in the synthesis of UPD-xylose (35), lacked three minor peaks (Fig. 1A, b), confirming the presence of a xylose residue in minor species of cryptococcal O-glycans (Xyl1Man2–Xyl1Man4; X1M2–X1M4). The results also indicated that UDP-xylose was utilized as a xylose donor for O-glycan biosynthesis in C. neoformans.

We also analyzed the glycan profile of the cac1Δ mutant, which is deficient in capsule production due to deletion of the adenylyl cyclase gene (36) and found that is identical to that of the wild-type profile, excluding the possibility of capsule oligosaccharide contamination during preparation of the O-linked glycans (Fig. 1A, c). Because cryptococcal N-glycans show serotype-specific structures with variation in length and in the presence of xylose (28), we also analyzed the O-glycan profiles of serotype B (R265) and serotype D (JEC21) strains. Both the JEC21 and R265 strains showed O-glycan profiles with minor O-glycan species containing xylose, which were nearly identical to those of the H99 strain, except that the proportions of M4 and X1M4 were slightly reduced in the B and D serotype strains compared with the A serotype (Fig. 1B, b and c). The results indicate that, in contrast to N-glycans, cryptococcal O-glycans do not exhibit serotype-specific structures.

Presence of an α1,6-Mannose Linkage in C. neoformans O-Linked Glycans—In contrast to the O-glycan profile of S. cerevisiae, the M3-M4 peaks in the C. neoformans profile migrate differently, although the M1 and M2 peaks migrate at the same elution time, indicating a difference in the linkages beyond the second mannose residue. To obtain more detailed information on the mannose linkages in cryptococcal O-glycans, we carried out exoglycosidase treatment experiments. Digestion of O-glycans with jack bean mannosidase, which can cleave three types of α1,6-mannose linkages (α1,2-, α1,3-, and α1,6-), resulted in the convergence of most peaks to a single mannose M1 peak with a elution time corresponding to an authentic 2-AA-labeled mannose (Fig. 2, a and b). However, interestingly, treatment with α1,2-MNS did not convert all of the peaks to a single M1 peak; instead, it only shifted a major M4 peak to an M3 peak (Fig. 2, c). This suggested that in the cryptococcal O-glycans, the fourth mannose residue is connected to the third mannose via an α1,2-linkage, whereas the third resi-
due is connected to the second mannose via a different linkage. Given that Cryptococcus laurentii was suggested to have not only 1,2-linked mannose but also 1,6-linked mannose residues in the O-linked glycans derived from cell envelope glycoproteins (37), we speculated that O-linked glycans from C. neoformans cwMPs might also contain a 1,6-linked mannose residue. Indeed, 1,6-MNS treatment abolished the M3 peak without changing the M4 peak, suggesting the presence of an 1,6-linkage between the M2 and M3 residues (Fig. 2d). Moreover, sequential treatment with 1,6-MNS after 1,2-MNS converted all of the major M3 and M4 peaks, including the minor peak of the X1M3 oligosaccharide, to M2 and X1M2 peaks (Fig. 2e), strongly supporting our speculation that the third mannose of C. neoformans O-glycans is attached to the second mannose primarily via an 1,6-linkage. It is noteworthy that there is a minor M3 peak that matches the M3 peak of S. cerevisiae O-glycans, an α1,2-linked mannotriose (Manα1–2Manα1–2Man1). The minor M3 peak disappeared after α1,2-MNS treatment (Fig. 2c and f, indicated as M3*), suggesting the presence of an α1,2-linked mannotriose as a minor form in C. neoformans O-glycans.

Analysis of the Glycan Profile of C. neoformans ktr3Δ Mutant Strains—We observed that the M2 peaks of the O-glycans from several yeast species were not shifted by treatment with linkage-specific mannosidases, including α1,2-MNS, α1,6-MNS, and α1–2,3-MNS, probably due to inefficient utilization of M2 as a substrate by these exoglycosidases (data not shown). The M2 peak of C. neoformans O-glycans showed an elution time that was identical to that of the M2 peak from S. cerevisiae O-glycans, suggesting that the M2 peak of C. neoformans O-glycans is likely to be an α1,2-linked mannobiase. To specifically define the linkage between the first and second mannose residues in C. neoformans O-glycans, we isolated and analyzed the M2 peak from the wild-type strain under HPLC conditions that separate standard α1,2-mannobiase (Sigma-Aldrich) and α1,3-mannobiase (Santa Cruz Biotechnology, Inc.). Comparison of the HPLC elution profile strongly indicated that the second mannose residue in the major O-glycans of C. neoformans is also connected by an α1,2-linkage (Fig. 3A), as was reported in other yeast and fungal O-glycans. Together, these results indicate that the major species of C. neoformans O-glycans are M2–M4 oligomannoses in which the second and fourth mannose resi-

**FIGURE 2.** Analysis of exoglycosidase-treated O-glycans of C. neoformans. O-Linked glycans from the cell wall mannoproteins of the serotype A wild-type strain were purified, labeled with 2-AA, and then analyzed by HPLC. a, untreated O-glycans. b, jack bean mannosidase (JBM)-treated O-glycans. c, α1,2-MNS-treated O-glycans. d, α1,6-MNS-treated O-glycans. e, α1,6-MNS-treated O-glycans after α1,2-MNS-treatment. f, O-linked glycans from the cwMPs of an S. cerevisiae wild-type strain. To distinguish small peaks more clearly, the HPLC profile, in which the x and y axes from 23 to 35 min were 2-fold expanded, was inserted. M3*, α1,2-mannotriose. The arrows indicate the peaks that disappeared after mannosidase treatment.
Structure and Biosynthetic Pathway of \( C. \) neoformans O-\( \text{Glycans} \)

![Diagram](image-url)

**FIGURE 3. Oligosaccharide profiles of the \( O \)-linked glycans of the \( C. \) neoformans \( \text{ktr3} \Delta \) mutant strain.** A, comparative analysis of HPLC elution pattern of \( C. \) neoformans wild-type M2 with the standard \( \alpha_{1,2} \)- and \( \alpha_{1,3} \)-mannobioses (\( \alpha_{1,2} \)M2 and \( \alpha_{1,3} \)M2). HPLC analysis was conducted under the conditions described under “Experimental Procedures,” except using an Asahipak NH2P-50 4E column (0.46 \( \times \) 25 cm, 5 \( \mu \)m; Shodex) instead of a TSKgel Amide-80 column. B, the \( O \)-linked glycan profiles from the cvMPs of the \( C. \) neoformans wild-type (a), \( \text{ktr3} \Delta \) mutant (b), and \( \text{ktr3} \Delta /\text{KTR3} \) complemented (c) strains and an \( S. \) cerevisiae wild-type strain (d). To distinguish small peaks more clearly, the HPLC profile, in which the x and y axes from 23 to 35 min were −2-fold expanded, was inserted. M3*, \( \alpha_{1,2} \)-mannotriose; \( \bullet \), an unknown peak. The arrows indicate the peaks that are absent in the \( \text{ktr3} \Delta \) mutant strain but present in the \( \text{ktr3} \Delta /\text{KTR3} \) strain.

Dues are added via an \( \alpha_{1,2} \)-linkage and the third mannose residue is attached via an \( \alpha_{1,6} \)-linkage and that minor species are present as xylosylated forms (X1M2–X1M4 oligomannoses).

The results of our previous study indicated that \( \text{KTR3} \), the only member of the \( \text{KRE2/MNT1} \) family encoding putative \( \alpha_{1,2} \)-mannosyltransferases in \( C. \) neoformans, is not involved in \( N \)-glycosylation but is associated with \( O \)-glycan synthesis (28). To characterize the specific roles of \( \text{KTR3} \) in \( O \)-glycan extension in the Golgi of \( C. \) neoformans, we analyzed the \( O \)-glycan profiles of the \( \text{ktr3} \Delta \) mutant and the \( \text{ktr3} \Delta /\text{KTR3} \) reintegrated strains by HPLC. As shown in Fig. 3B, the \( O \)-glycan profile of the \( \text{ktr3} \Delta \) mutant (Fig. 3B, a) displayed dramatically reduced M2, M3, and M4 peaks and a remarkably increased M1 peak, which was distinct from the profile of the H99 wild-type strain (Fig. 3B, a). Interestingly, it was notable that the minor xylose-containing species was more detectable in the \( O \)-glycans of the \( \text{ktr3} \Delta \) strain. Moreover, despite the dramatic decrease in the M2 peak, the low M2 peak still remained in the \( \text{ktr3} \Delta \) strain (Fig. 3B, b). This suggests that Ktr3p plays an important role in the addition of the second \( \alpha_{1,2} \)-mannose residue in the biosynthetic pathway of the major \( O \)-glycans but not in the pathway of the minor xylosylated \( O \)-glycans. Reintroduction of the wild-type \( \text{KTR3} \) gene into the \( \text{ktr3} \Delta \) mutant restored the M2–M4 peaks and dramatically decreased the M1 peak (Fig. 3B, c), strongly suggesting that Ktr3 is an \( \alpha_{1,2} \)-mannosyltransferase that adds the second mannose residue to most cryptococcal \( O \)-linked glycans. It is also noteworthy that the minor M3 peak, corresponding to \( \alpha_{1,2} \)-mannotriose, was increased in the \( \text{ktr3} \Delta /\text{KTR3} \) strain (Fig. 3B, c, indicated as M3*), indicating that Ktr3p could also add an \( \alpha_{1,2} \)-linked mannose residue sequentially after the addition of the second mannose in the \( O \)-glycan biosynthetic pathway to generate \( \alpha_{1,2} \)-mannotriose. We observed the overexpression of \( \text{KTR3} \) in the complemented \( \text{ktr3} \Delta \) strain, probably due to the ectopic integration of \( \text{KTR3} \) into an unidentified genomic locus. It is speculated that the overexpression of \( \text{KTR3} \) might increase the synthesis of M3* while relatively reducing the synthesis of major M3 and M4 oligomannoses carrying an \( \alpha_{1,6} \)-linked mannose residue in the \( \text{ktr3} \Delta /\text{KTR3} \) strain.

Analysis of the secreted proteins derived from the wild-type and \( \text{ktr3} \Delta \) strains showed the presence of a set of proteins in the \( \text{ktr3} \Delta \) strain with a significantly faster migration pattern (Fig. 4). The migration pattern of the proteins from the complemented \( \text{ktr3} \Delta \) strain was nearly identical to that of the wild-type strain, indicating that the faster migration of these secreted proteins might be attributed to the truncated \( O \)-glycan chains assembled on them, as indicated in Fig. 3B. After removal of the \( \text{N} \)-glycans by treatment with PNGase F, lectin blotting of the protein sample from the \( \text{ktr3} \Delta \) strain showed a significant decrease of the detection signals compared with that of the wild-type strain (Fig. 4B), strongly supporting the lesser degree of protein \( O \)-mannosylation in the \( \text{ktr3} \Delta \) strain.

Analysis of the Glycan Profile of the \( C. \) neoformans hoc\( \Delta \) Mutant Strains—Our glycan structure analysis data indicated that the biosynthesis of \( C. \) neoformans \( O \)-glycans probably involves \( \alpha_{1,6} \)-mannosyltransferases that can catalyze the addition of a third
**Structure and Biosynthetic Pathway of C. neoformans O-Glycans**

![FIGURE 4. Glycosylation pattern analysis of secreted proteins of C. neoformans wild-type and ktr3Δ mutant strains.](image)

**TABLE 2**

| S. cerevisiae | C. neoformans |
|--------------|--------------|
| **Gene name** | **Function** | **Reference** | **Gene name** | **Function** | **Reference** |
| OCH1 | α1,6-Initiating mannosyltransferase in outer chain biosynthesis of N-glycans | Ref. 38 | OCH1 (CNAG_00744) | α1,6-Initiating mannosyltransferase in outer chain biosynthesis of N-glycans | Ref. 28 |
| HOC1 | α1,6-Elongating mannosyltransferase in outer chain biosynthesis of N-glycans | Ref. 39 | HOC1 (CNAG_05836) | α1,6-Elongating mannosyltransferase in biosynthesis of O-glycans | This study |
| HOC2 (CNAG_01214) | ND | This study |
| HOC3 (CNAG_00158) | ND | This study |
| HOC4 (CNAG_04449) | ND | This study |

* ND, not defined yet.

α1,6-linked mannoside residue to the second mannoside residue. Previously, we discovered two C. neoformans homologs of the S. cerevisiae HOC1 gene encoding α1,6-elongating mannosyltransferase (39), HOC1 and HOC2, and showed that they are not involved in the outer chain elongation of N-glycans (28). In the present study, we found two additional Cryptococcus genes encoding proteins homologous to S. cerevisiae Hoc1p, CNAG_00158 and CNAG_04449, which we designated HOC3 and HOC4 (Table 2). Sequence analysis revealed that C. neoformans Hoc1p–Hoc4p shared low sequence identities (14–19%) with S. cerevisiae Hoc1p. To analyze the function of C. neoformans Hoc3p and Hoc4p, we constructed hoc3Δ and hoc4Δ mutants and examined the O-glycan profiles of these deletion strains by HPLC. Although no apparent differences were detected in the O-glycan profiles of the hoc2Δ and hoc4Δ mutants (Fig. 5, b and c) compared with the wild-type profile (Fig. 5a), the hoc1Δ mutant clearly lacked the peaks for the minor X1M3–X1M4 oligosaccharides (Fig. 5d). Moreover, the hoc3Δ mutant showed a glycan profile with no peaks for the major M3–M4 oligosaccharides (Fig. 5f). Reintroduction of the wild-type HOC1 and HOC3 genes into the corresponding null mutant strains, respectively, restored the missing oligosaccharide peaks to the levels observed in the wild-type profile (Fig. 5, e and g). We did not observe any change in the N-glycan profiles of the hoc3Δ and hoc4Δ mutants (data not shown), as was also the case for the hoc1Δ and hoc2Δ mutants (28). These observations suggest that in C. neoformans, Hoc1p and Hoc3p play important roles in O-glycan synthesis by attaching the third α1,6-linked mannoside residue to the X1M2 and M2 oligosaccharides, respectively. These data further support our notion that the biosynthesis of O-glycans in C. neoformans is mediated by two pathways, a major pathway without xylose addition and a minor pathway with xylose addition, and that the two pathways employ separate sets of mannosyltransferases.

**Growth Phenotypes of the C. neoformans O-Glycan Mutant Strains**—Yeast mutants with defects in protein glycosylation have been shown to display increased sensitivities to cell wall-disturbing reagents and high temperatures (40). Although the C. neoformans ktr3Δ mutant has been previously shown not to have any detectable change in sensitivity to some cell wall-disturbing reagents, including hygromycin B, vanadate, Calcofluor white (CFW), and Congo red, the mutant did display increased sensitivity to SDS and high temperature (39 °C) (28). Additionally, we observed that the ktr3Δ mutant exhibited increased sensitivity to osmotic stress induced by NaCl, and this sensitivity could be recovered in the presence of 1 M sorbitol, an osmotic stabilizer. The restoration of sensitivity to high temperature and NaCl in the ktr3Δ mutant in the presence of sorbitol implies that the growth defects might be derived from defects in cell integrity. Unexpectedly, the addition of 1 M sorbitol in the presence of SDS severely inhibited the growth of even the wild-type C. neoformans strain. All of the observed defective growth phenotypes were restored by the reintroduction of C. neoformans KTR3 (Fig. 6A). The slightly retard growth of ktr3Δ/KTR3 strain at 39 °C, compared with the wild-type strain, might reflect the subtle change of glycan profiles of the ktr3Δ/KTR3 strain (Fig. 3B), which could affect cell wall integrity. Conversely, all of the C. neoformans hoc null mutants grew similar to the wild-type strain on YPD under normal conditions, at high temperature, and under some cell wall stress.
conditions generated by the presence of hygromycin B, vanadate, CFW, or Congo red (data not shown). The hoc1/H9004, hoc2/H9004, and hoc3/H9004 mutants were slightly more sensitive to SDS than the wild-type strain, whereas the hoc1/H9004 and hoc2/H9004 mutants were more sensitive to NaCl (Fig. 6B). It is intriguing that the hoc2/H9004 mutant exhibited increased sensitivity to SDS and NaCl, although no apparent changes in the N- and O-glycosylation patterns were detected. This suggests that Hoc2p might play a role in glycosphingolipid biosynthesis, such as the synthesis of glycosylinositolphosphoceramide, in C. neoformans.

Analysis of the Virulence-associated Phenotypes of C. neoformans ktr3/H9004 Mutant Strains—To determine whether truncation of the major O-glycans affects the virulence of C. neoformans, we examined the virulence-associated phenotypes of the C. neoformans ktr3/H9004 mutant strain, which produced truncated O-glycans with only two mannose residues and displayed the most severe growth defect among the O-glycan mutants tested. Capsule formation and melanin synthesis of the ktr3/H9004 mutant did not differ significantly from that of the wild-type strain (Fig. 7, A and B). However, in a murine model of systemic cryptococcosis, the virulence of the ktr3Δ mutant was severely attenuated compared with the wild-type strain (Fig. 7C). Reintroduction of the wild-type C. neoformans KTR3 gene into the ktr3/H9004 mutant restored virulence, indicating that the extended structure of the O-glycans is required for the full pathogenicity of C. neoformans.

DISCUSSION

O-mannosylation is a vital type of protein glycosylation that is conserved from bacteria to humans. In all yeasts and filamentous fungi studied thus far, the reducing terminal mannose residue of O-mannosyl glycans is α-linked to Ser or Thr in the ER and then mostly extended to form an α1,2-linked mannotriose (Manα1–2Manα1–2Manα1-Ser/Thr) in the Golgi (14). This core structure is further processed, in various ways according to species, by different mannosyltransferases that belong to separate families of glycosyltransferases. In S. cerevisiae, mannose residues are added via an α1,3-linkage to form short linear oligosaccharides that are up to five mannosyl residues long and can be modified by mannosyl phosphate. In C. albicans and P. pastoris, O-glycans consist of α1,2-linked mannose polymers.
of up to five or six residues; however, some α1,2-Man3 and α1,2-Man4 structures can be capped by a Manβ1–2Manβ1–2-disaccharide (41). In *S. pombe*, linear α1,2-linked mannobiose and mannotriose are extended by the addition of up to two galactosyl residues to generate branched O-linked oligosaccharides of the Gal0–2Man1–3 structure (14). Moreover, in *A. fumigatus*, branched O-linked oligomannoses containing galactose, glucose, and galactopentose have been described (42).

Here, we reported the novel structures of the *C. neoformans* O-glycans, Man1–6-Man1–2Man mannotriose and Man1–2Man1–6-Man1–2Man mannotetraose, in which the third mannose is attached via an α1,6-linkage instead of an α1,2-linkage, which are distinct from those in most other fungal O-glycans. More interestingly, we presented data indicating that the biosynthesis of the major species of *C. neoformans* O-glycans, Man1–Man4, and the minor species, Xyl1Man1–Xyl1Man4, might be processed by independent pathways containing different mannosyltransferases (Fig. 8). In the cryptococcal ER, the biosynthesis of O-glycans is initiated by the addition of the first mannose residue to secretory proteins by three PMT proteins, Pmt1p, Pmt2p, and Pmt4p. In the cryptococcal Golgi, extension of the major O-glycans begins with the addition of a second α1,2-linked mannose residue to the first mannose, which is catalyzed by Ktr3p. Subsequently, Hoc3p adds the third α1,6-linked mannose residue to the α1,2-linked mannobiose chain, generating Man1–6-Man1–2Man mannotriose. The fourth mannose residue is connected via an α1,2-linkage that is catalyzed by an unknown mannosyltransferase. In contrast, in the biosynthetic pathway of minor species of *C. neoformans* O-glycans, containing a xylose residue attached to the first mannose residue, glycan biosynthesis appears to be mediated by separate sets of mannosyltransferases. The O-glycan profile in the ktr3Δ strain revealed the presence of minor Man2 species, indicating that *C. neoformans* might have another yet unknown mannosyltransferase that is responsible for the addition of a second mannose residue to the O-glycan in the absence of Ktr3p. Our preliminary experiments indicated the possibility that the minor Man2 species having Man1–3Man structure could be the substrate of *C. neoformans* xylosyltransferase(s).

Although the gene products involved in the synthesis of the minor Man2 and Xyl1Man2 species in the cryptococcal Golgi

FIGURE 6. Growth phenotypes of the *C. neoformans* wild-type, ktr3Δ, and hoc null mutant strains. Yeast cells were cultivated overnight on YPD, adjusted to an *A*<sub>600</sub> of 1, and then serially diluted 10-fold. Diluted cells were spotted on YPD plates with or without 1 M sorbitol under different conditions, such as high temperature (39 °C), the presence of 0.05% SDS, and the presence of 1 M NaCl. A, phenotype analysis of the ktr3Δ mutant. B, phenotype analysis of hoc1Δ, hoc2Δ, hoc3Δ, and hoc4Δ mutants. The hxl1Δ mutant (33) was included as a control strain showing increased sensitivity under the tested conditions.

FIGURE 7. Analysis of virulence-associated phenotypes of the ktr3Δ mutant strain. A, analysis of the capsule formation. B, analysis of the melanin synthesis capacity. The cac1Δ mutant strain (36) was included as a control strain showing defective melanization on L-DOPA medium. C, virulence test of *C. neoformans* H99 wild-type, ktr3Δ, and ktr3Δ/KTR3 strains.
were not determined, we showed that Hoc1p catalyzes a reaction that adds an α1,6-linked mannose residue to Xyl1Man2, generating Xyl1Man3. To the best of our knowledge, our finding of the roles of Hoc1p and Hoc3p in the extension of *C. neoformans* O-glycans is the first report on the involvement of **OCH1** family members, encoding α1,6-mannosyltransferases, in fungal O-glycan biosynthesis. Our analysis confirmed that Hoc1p and Hoc3p do not participate in the outer chain elongation of N-glycans. We previously reported the role of the **OCR1** gene, a novel member of the **OCH1** family implicated in both N- and O-linked glycosylation, in the methylotrophic yeast *Hansenula polymorpha*. However, in this organism, **OCR1** encodes an α1,2-mannosyltransferase that is involved not only in the outer chain elongation of N-glycans but also in the extension of O-glycans via α1,2-mannose addition (43). Phylogenetic analysis of several yeast and filamentous fungal genes belonging to the **OCH1** family suggests that the *C. neoformans* **HOC** genes are clustered as a subfamily that is separate from other fungal **OCH** family members (Fig. 9). The minor species of *C. neoformans* O-glycans have been reported to contain a xylose-phosphate residue that is added by Xpt1p. However, the relative level of total acidic O-glycans is extremely low (less than 1% of total O-glycans) (29). In the present study, we could not clearly detect the presence of acidic O-glycans using HPLC, probably due to the extremely low level of acidic glycans, which was below the threshold for detection.

Functional analysis of O-mannosylation in the eukaryotic model yeast *S. cerevisiae* showed that O-mannosyl glycans are important for the stability, localization, and/or function of various secretory and membrane proteins, which affects numerous important cellular processes, including cell wall integrity (44). Recent studies on the human pathogenic yeast *C. albicans* and the plant pathogenic fungus *Ustilago maydis* also demonstrated that O-mannosylation is required for adherence, invasion of host tissues, and virulence (45, 46). A previous study on the function of the PMT family proteins in *C. neoformans* reported that *pmt1Δ* and *pmt4Δ* mutants displayed defects in cell morphology and severely attenuated virulence in a murine inhalation model of cryptococcosis (31). In the present study, we also observed that the *C. neoformans* ktr3Δ mutant was sensitive to SDS and NaCl, indicating that elongation of the O-glycans in the Golgi is important for cell integrity. Considering that cell wall-destabilizing agents, such as Congo red and CFW, which affect microfibril assembly in the cell wall, had no significant effect on the growth of the ktr3Δ mutant or the *pmt5Δ* mutant strains (31), O-mannosylation appears to be more closely associated with cell membrane integrity than with cell wall assembly in *C. neoformans*. Deletion of **KTR3** also significantly atten-
uated the virulence of *C. neoformans* in a mouse model of cryptococcosis. The attenuated virulence of the *ktr3*/*H9004* mutant strain might be due in part to defects in the function of cell surface proteins and secretory proteins, particularly those associated with host infection, as a result of truncated O-glycans. *C. neoformans* was reported to contain 55 potential glycosyl-phosphatidylinositol-anchored proteins that are subjected to intensive modification by glycosylation (26). For example, the Cda2/MP98 protein is a homologue of chitin deacetylases that is heavily mannosylated during secretion. Although a single *cda2*/*H9004* mutant showed no growth phenotypes, a *cda1*/*H9004* *cda2*/*H9004* *cda3*/*H9004* triple mutant strain was sensitive to SDS and NaCl but not Congo red, CFW, or caffeine (47) and exhibited dramatically attenuated virulence (48), as was observed with the *ktr3*/*H9004* mutant. Another mannosylated protein, cytokine-inducing glycoprotein (CNAG_01653), was reported to be abundant during cerebrospinal fluid infection, indicating its possible involvement in cryptococcosis (49). Therefore, we can speculate that the loss of *KTR3* function probably affects the stability and/or function of several O-glycosylated proteins necessary for complete virulence, which leads to attenuated virulence. This suggests the potential of Ktr3p, a *C. neoformans*-specific mannosyltransferase that is not present in mammals, as an ideal target for the development of antifungal drugs.

In *C. neoformans*, ~50% of the secreted proteins are thought to be mannosylated (50). In recent years, several studies have shown that O-linked mannosylation is more important than N-linked mannosylation for T-cell responses and optimal mannosprotein-mediated protection (51, 52). For example, chemical elimination of the O-glycans in Cda2/MP98 protein was shown to reduce its effectiveness in the T-cell response. In contrast, enzymatic N-deglycosylation of MP98 affects immune activation, indicating that extensive O-mannosylation facilitates recognition by mannose receptors on antigen-presenting cells, particularly dendritic cells. We observed some abundant secreted mannoproteins at 95–120 kDa in the wild-type strain, whereas their sizes appeared to be reduced significantly in the *ktr3*/*H9004* mutant. Thus, it will be intriguing to determine how truncation of the O-glycans on cell wall and secretory mannoproteins affects their functions in the host immune response and to identify virulence factors that require O-mannosylation. The O-mannosylation mutants constructed in this study could be valuable tools for determining the respective roles of cryptococcal O-glycans carrying different mannosides during their interaction with host cells. These *C. neoformans* mutants could be employed to systematically dissect the effect of structural alterations of O-glycans on host immunological interactions during cryptococcal infection, which will provide useful information for the development of a cryptococcal vaccine and effective therapies based on glycans.

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