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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 mannnose 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 *uxs1*Δ 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 ktr3*Δ 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)[3] 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*-acetylglucosamine 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 model 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

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[3] The abbreviations used are: ER, endoplasmic reticulum; PMT, protein *O*-mannosyltransferase; cwMP, cell wall mannoprotein; 2-AA, 2-aminobenzoic acid; α1,2-MNS, α1,2-mannosidase; α1,6-MNS, α1,6-mannosidase; CFW, calcofluor white; L-DOPA, L-3,4-dihydroxyphenylalanine; YPD, yeast extract-peptone-dextrose.

TABLE 1
Strains and plasmids used in this study

	Genotype/description	Parent	Source/reference
Strain			
C. neoformans H99	*MATα* (serotype A)		Ref. 28
*uxs1*Δ	*MATα uxs1*::*NAT*#159[a]	H99	Ref. 28
*hxl1*Δ	*MATα hxl1*::*NAT*#229	H99	Ref. 33
*cac1*Δ	*MATα cac1*::*NAT*#159	H99	Ref. 36
*ktr3*Δ	*MATα ktr3*::*NAT*#159	H99	Ref. 28
*ktr3*Δ/*KTR3*	*MATα ktr3*::*NAT*#159/*KTR3*::*NEO*	*ktr3*Δ	This study
*hoc1*Δ	*MATα hoc1*::*NAT*#159	H99	Ref. 28
*hoc2*Δ	*MATα hoc2*::*NAT*#159	H99	Ref. 28
*hoc3*Δ	*MATα hoc3*::*NAT*#159	H99	This study
*hoc4*Δ	*MATα hoc4*::*NAT*#159	H99	This study
*hoc1*Δ::*HOC1*	*MATα hoc1*::*NAT*#159 *HOC1*::*NEO*	*hoc1*Δ	This study
*hoc3*Δ::*HOC3*	*MATα hoc3*::*NAT*#159 *HOC3*::*NEO*	*hoc3*Δ	This study
JEC21	*MATa* (serotype D)		Ref. 53
C. gattii R265	*MATα* (serotype B)		Ref. 54
Plasmid			
pNAT-STM#159	*NAT*-resistant marker vector for gene disruption		Ref. 28
pJAFS1	Modified pJAF12 vector containing *NEO*[r] as a selection marker		Ref. 33
pJAFS1-*KTR3*	pJAFS1 containing the *KTR3* ORF		This study
pJAFS1-*HOC1*	pJAFS1 containing the *HOC1* ORF		This study
pJAFS1-*HOC3*	pJAFS1 containing the *HOC3* ORF		This study

[a] Each number of *NAT* (#) indicates the unique signature tag of the Nat[r] marker.

opportunistic human pathogen, five multifunctional mannosyltransferases of the *KRE2/MNT1* family have been reported. *C. albicans* Mnt1p and Mnt2p have redundant α1,2-mannosyltransferase activities, and they add the second and third α1,2-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 α1,2-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, α1,2-linked mannobiose and mannotriose (17). Unlike *S. cerevisiae*, the *O*-glycans of *S. pombe* are decorated with α1,2-linked and/or α1,3-linked galactose residues, which are added by the *gma12* and *otg2* gene products (18, 19). The *O*-linked glycans of the methylotrophic yeast *Pichia pastoris* were shown to consist of α1,2-linked mannose chains of up to four residues (20) with β1,2-linked mannobioses 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 basidiomycete that causes fatal cryptococcal meningoencephalitis in immunocompromised individuals such as AIDS patients. Several extracellular 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 *ktr3*Δ 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*.

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_{NAT}, or YPD plates containing 200 μg/ml G418 disulfate (Duchefa, Haarlem, The Netherlands), referred to as YPD_{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 (*MAT**α***), 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 genomic DNA in the corresponding mutant strains using pJAFS1 (33) with a G418 resistance marker. The systematic PCR analysis confirmed the integration of *HOC1* and *HOC3* 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 $CaCl_2$, and 1 mM $MnCl_2$). 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 μg) 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 saturated $NaHCO_3$, mixed with 10 μl of $(CH_3CO)_2O$, and incubated on ice for 30 min. *O*-Glycans were purified by using Dowex 50WX8–400 resins (H^+ form; Sigma) to remove other contaminants and dried in a vacuum evaporator. Next, the isolated *O*-glycans were dissolved in 50 μl of fresh 1% (w/v) sodium acetate·$3H_2O$ and then labeled with 100 μl of 2-aminobenzoic acid (2-AA) solution (30 mg of 2-AA and 30 mg of $NaBH_3CN$ in 1 ml of 4% sodium acetate·$3H_2O$ and 2% boric acid in methanol) at 80 °C for 45 min. Labeled *O*-glycans were cleaned up using a SampliQ Cyano cartridge (100 mg; Agilent) to remove excess 2-AA.

Exoglycosidase Treatment Experiments—2-AA-labeled *O*-glycans were reacted with 1 μl of jack bean mannosidase (150 milliunits/μl; Sigma), α1,2-mannosidase (α1,2-MNS; 0.1 milliunits/μl; Prozyme), or α1,6-mannosidase (α1,6-MNS; 40 units/μl; New England Biolabs) in 20 mM ammonium acetate buffer (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 A_{600} of 0.6, spotted onto an L-DOPA plate (7.6 mM L-asparagine monohydrate, 5.6 mM glucose, 22 mM KH_2PO_4, 1 mM $MgSO_4·7H_2O$, 0.5 mM L-DOPA, 0.3 mM thiamine-HCl, and 20 nM 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 × 10^6 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 10^5 cells of the wild-type, *ktr3*Δ, and *ktr3*Δ/*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 (Man_2–Man_4; M2–M4), our HPLC profile of wild-type *O*-glycans displayed

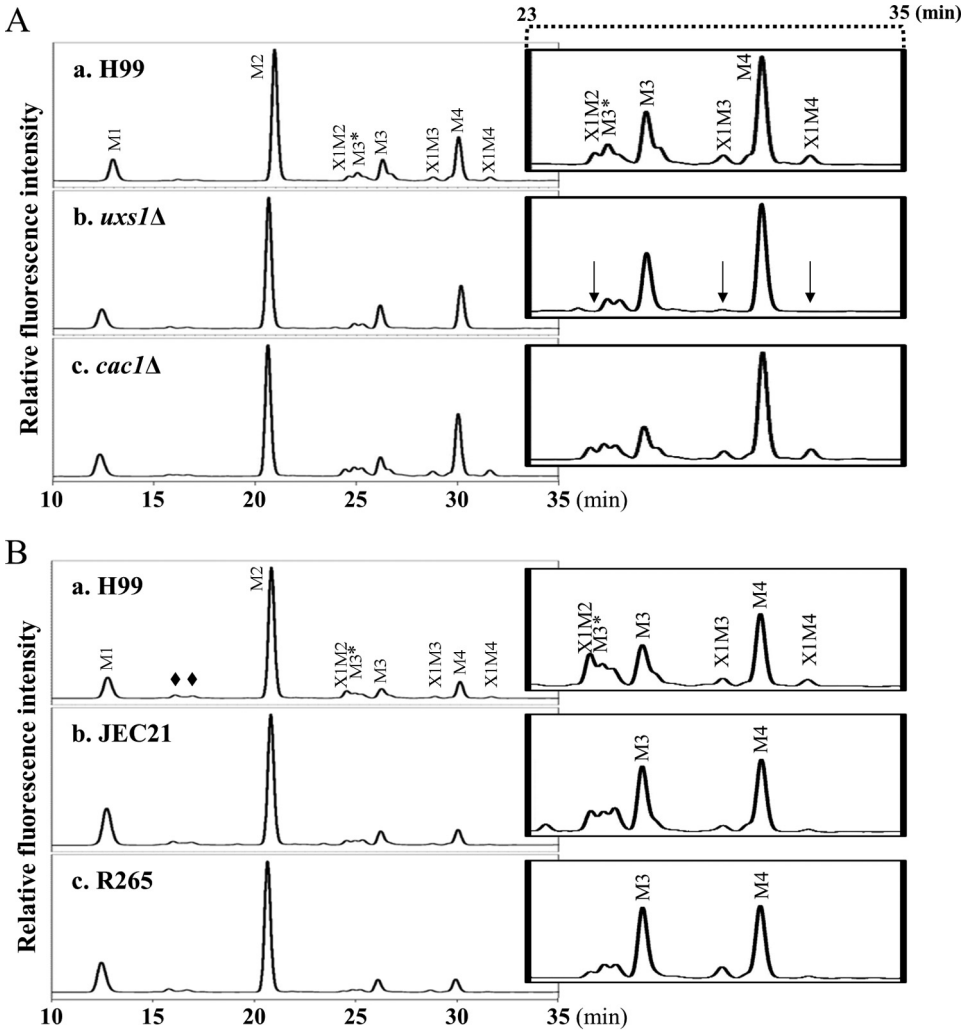

FIGURE 1. ***O*-Linked glycan profiles of *C. neoformans* serotype A, B, and D strains.** *A*, *O*-linked glycans derived from the cwMPs of serotype A wild-type (H99) (*a*), *uxs1*Δ (*b*), and *cac1*Δ (*c*) strains were labeled with 2-AA and subjected to HPLC analysis. *B*, the *O*-glycans from serotype A (H99) (*a*), serotype D (JEC21) (*b*), and serotype B (R265) (*c*) wild-type strains were analyzed for comparison. 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. The *arrows* indicate the peaks that disappeared in the *uxs1*Δ mutant. *M3**, α1,2-Mannotriose; ♦, unknown peaks.

major peaks at the M2–M4 positions (Fig. 1*A*, *a*). The profile of the *O*-glycans from the *uxs1*Δ mutant, which is defective in the synthesis of UPD-xylose (35), lacked three minor peaks (Fig. 1*A*, *b*), confirming the presence of a xylose residue in minor species of cryptococcal *O*-glycans (Xyl_1Man_2–Xyl_1Man_4; 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 *cac1*Δ 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. 1*A*, *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. 1*B*, *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 α-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-

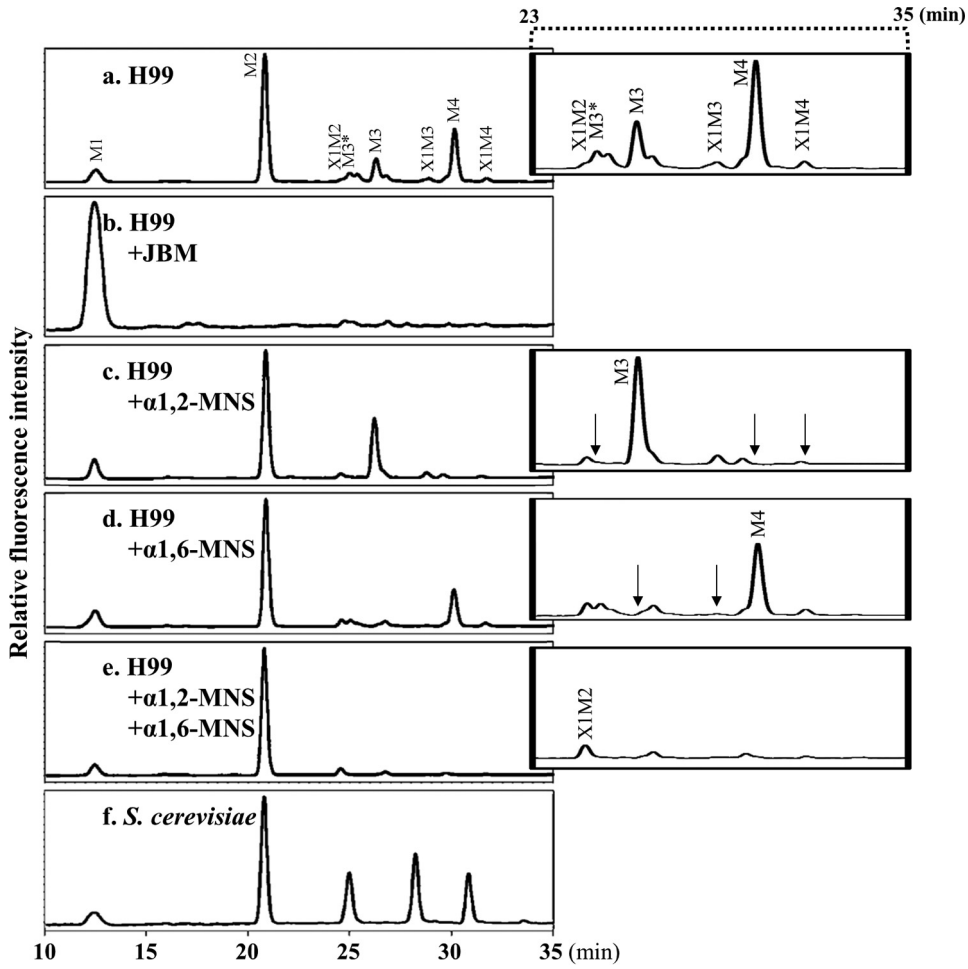

FIGURE 2. **Analysis of exoglycosidase-treated *O*-glycans of *C. neoformans*.** *O*-Linked glycans from the cell wall mannoproteins of the serotype A wild-type strain were purified, labeled with 2-AA, and then analyzed by HPLC. *a*, untreated *O*-glycans. *b*, jack bean mannosidase (*JBM*)-treated *O*-glycans. *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.

due is connected to the second mannose via a different linkage. Given that *Cryptococcus laurentii* was suggested to have not only α1,2-linked mannose but also α1,6-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 an α1,6-linked mannose residue. Indeed, α1,6-MNS treatment abolished the M3 peak without changing the M4 peak, suggesting the presence of an α1,6-linkage between the M2 and M3 residues (Fig. 2*d*). Moreover, sequential treatment with α1,6-MNS after α1,2-MNS converted all of the major M3 and M4 peaks, including the minor peak of the X1M3 oligosaccharide, to M2 and X1M2 peaks (Fig. 2*e*), strongly supporting our speculation that the third mannose of *C. neoformans* *O*-glycans is attached to the second mannose primarily via an α1,6-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 (Manα1–2Manα1–2Manα1). The minor M3 peak disappeared after α1,2-MNS treatment (Fig. 2, *c* 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 mannobiose. 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-mannobiose (Sigma-Aldrich) and α1,3-mannobiose (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. 3*A*), 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-

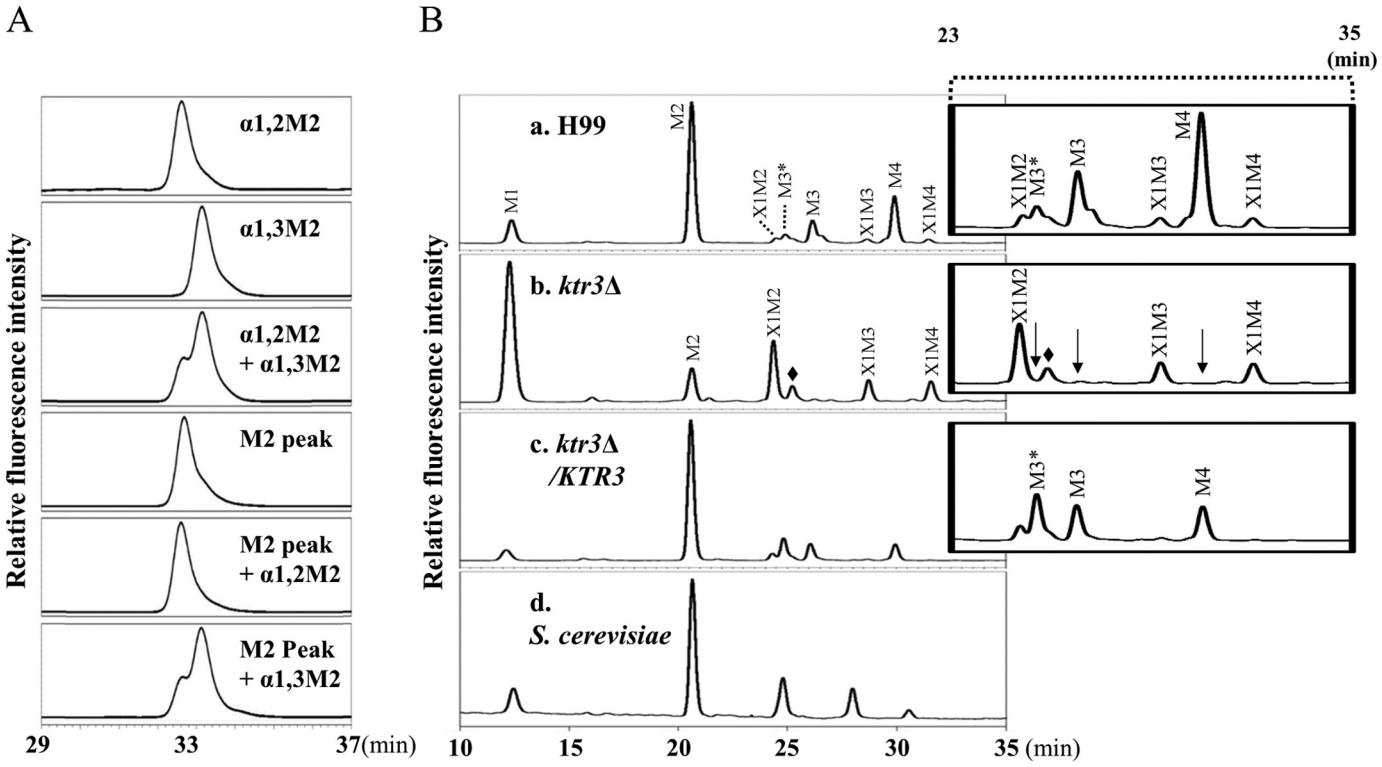

FIGURE 3. **Oligosaccharide profiles of the *O*-linked glycans of the *C. neoformans ktr3*Δ mutant strain.** *A*, comparative analysis of HPLC elution pattern of *C. neoformans* wild-type M2 with the standard α1,2- and α1,3-mannobioses (*α1,2M* and *α1,3M*). HPLC analysis was conducted under the conditions described under "Experimental Procedures," except using an Asahipak NH2P-50 4E column (0.46 × 25 cm, 5 μm; Shodex) instead of a TSKgel Amide-80 column. *B*, the *O*-linked glycan profiles from the cwMPs of the *C. neoformans* wild-type (*a*), *ktr3*Δ mutant (*b*), and *ktr3Δ/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**, α1,2-mannotriose; ♦, an unknown peak. The *arrows* indicate the peaks that are absent in the *ktr3*Δ mutant strain but present in the *ktr3Δ/KTR3* strain.

dues are added via an α1,2-linkage and the third mannose residue is attached via an α1,6-linkage and that minor species are present as xylosylated forms (X1M2–X1M4 oligomannoses).

The results of our previous study indicated that *KTR3*, the only member of the *KRE2/MNT1* family encoding putative α1,2-mannosyltransferases in *C. neoformans*, is not involved in *N*-glycosylation but is associated with *O*-glycan synthesis (28). To characterize the specific roles of *KTR3* in *O*-glycan extension in the Golgi of *C. neoformans*, we analyzed the *O*-glycan profiles of the *ktr3*Δ mutant and the *ktr3Δ/KTR3* reintegrated strains by HPLC. As shown in Fig. 3*B*, the *O*-glycan profile of the *ktr3*Δ mutant (Fig. 3*B*, *b*) 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. 3*B*, *a*). Interestingly, it was notable that the minor xylose-containing species was more detectable in the *O*-glycans of the *ktr3*Δ strain. Moreover, despite the dramatic decrease in the M2 peak, the low M2 peak still remained in the *ktr3*Δ strain (Fig. 3*B*, *b*). This suggests that Ktr3p plays an important role in the addition of the second α1,2-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 *KTR3* gene into the *ktr3*Δ mutant restored the M2–M4 peaks and dramatically decreased the M1 peak (Fig. 3*B*, *c*), strongly suggesting that Ktr3 is an α1,2-mannosyltransferase that adds the second mannose residue to most cryptococcal *O*-linked glycans. It is also noteworthy that the minor M3 peak, corresponding to α1,2-mannotriose, was increased in the *ktr3Δ/KTR3* strain (Fig. 3*B*, *c*, indicated as *M3**), indicating that Ktr3p could also add an α1,2-linked mannose residue sequentially after the addition of the second mannose in the *O*-glycan biosynthetic pathway to generate α1,2-mannotriose. We observed the overexpression of *KTR3* in the complemented *ktr3*Δ strain, probably due to the ectopic integration of *KTR3* into an unidentified genomic locus. It is speculated that the overexpression of *KTR3* might increase the synthesis of M3* while relatively reducing the synthesis of major M3 and M4 oligomannoses carrying an α1,6-linked mannose residue in the *ktr3Δ/KTR3* strain.

Analysis of the secreted proteins derived from the wild-type and *ktr3*Δ strains showed the presence of a set of proteins in the *ktr3*Δ strain with a significantly faster migration pattern (Fig. 4). The migration pattern of the proteins from the complemented *ktr3*Δ 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. 3*B*. After removal of the *N*-glycans by treatment with PNGase F, lectin blotting of the protein sample from the *ktr3*Δ strain showed a significant decrease of the detection signals compared with that of the wild-type strain (Fig. 4*B*), strongly supporting the lesser degree of protein *O*-mannosylation in the *ktr3*Δ strain.

*Analysis of the Glycan Profile of the C. neoformans hoc*Δ *Mutant Strains*—Our glycan structure analysis data indicated that the biosynthesis of *C. neoformans O*-glycans probably involves α1,6-mannosyltransferases that can catalyze the addition of a third

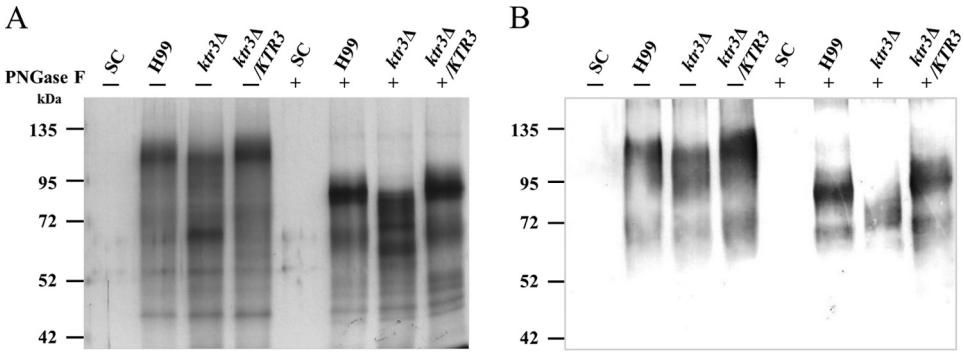

FIGURE 4. **Glycosylation pattern analysis of secreted proteins of *C. neoformans* wild-type and *ktr3*Δ mutant strains.** Silver staining (*A*) and lectin blotting (*B*) of the TCA-precipitated culture supernatants of the wild-type and *ktr3*Δ strains. Yeast cells were cultivated in synthetic complete (*SC*) medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and complete supplement mixture of amino acids) for 24 h. Then the culture supernatants were separated and analyzed. *SC*, the TCA samples of the culture medium only. *Galanthus nivalis* agglutinin, recognizing terminal α1,2-, α1,3-, and α1,6-linked mannose residues, was used for lectin blotting.

TABLE 2

***C. neoformans* and *S. cerevisiae* genes belonging to the yeast *OCH1* family**

S. cerevisiae			*C. neoformans*		
Gene name	Function	Reference	Gene name (H99 locus)	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[a]	This study
			HOC3 (CNAG_00158)	α1,6-Elongating mannosyltransferase in biosynthesis of *O*-glycans	This study
			HOC4 (CNAG_04449)	ND	This study

[a] ND, not defined yet.

α1,6-linked mannose residue to the second mannose 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 *hoc3*Δ and *hoc4*Δ 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 *hoc2*Δ and *hoc4*Δ mutants (Fig. 5, *b* and *c*) compared with the wild-type profile (Fig. 5*a*), the *hoc1*Δ mutant clearly lacked the peaks for the minor X1M3-X1M4 oligosaccharides (Fig. 5*d*). Moreover, the *hoc3*Δ mutant showed a glycan profile with no peaks for the major M3-M4 oligosaccharides (Fig. 5*f*). 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 *hoc3*Δ and *hoc4*Δ mutants (data not shown), as was also the case for the *hoc1*Δ and *hoc2*Δ 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 mannose 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 ktr3*Δ 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 *ktr3*Δ 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 *ktr3*Δ 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. 6*A*). The slightly retarded growth of *ktr3*Δ*/KTR3* strain at 39 °C, compared with the wild-type strain, might reflect the subtle change of glycan profiles of the *ktr3*Δ*/KTR3* strain (Fig. 3*B*), 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

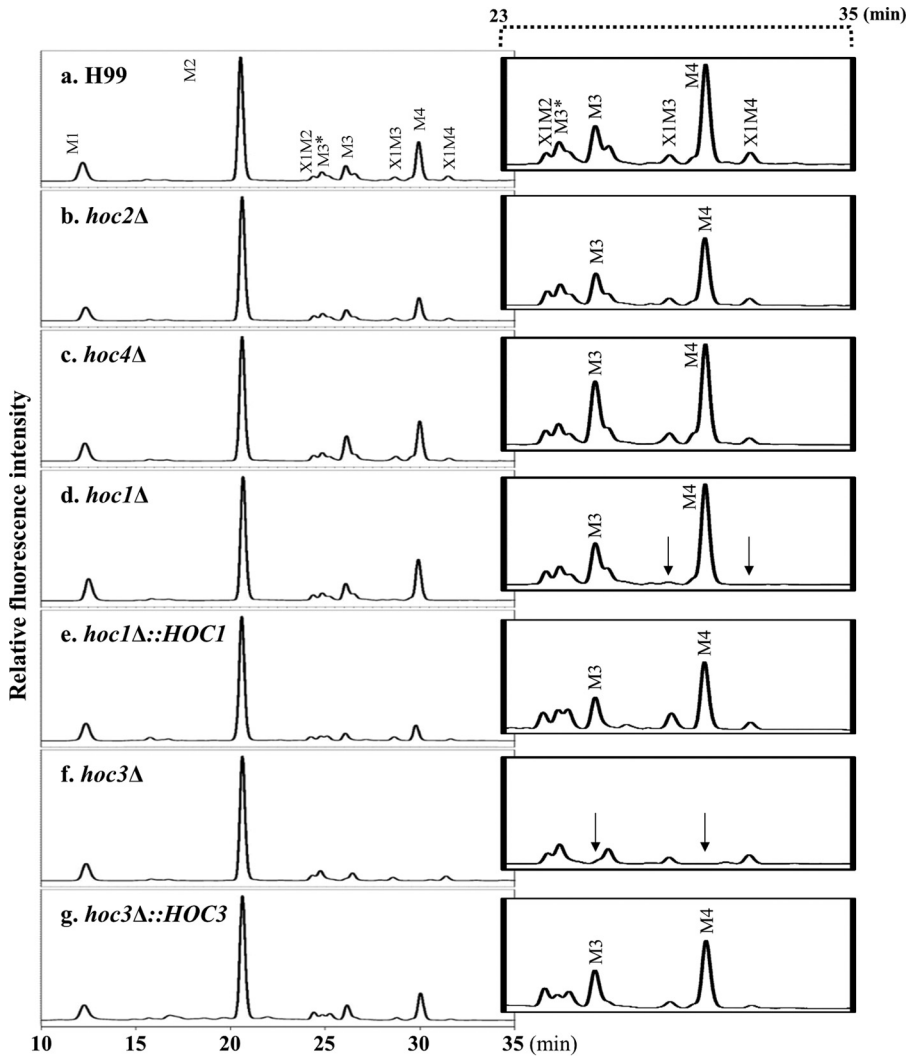

FIGURE 5. **Oligosaccharide profiles of the *O*-linked glycans of *C. neoformans hoc*Δ mutants.** The *O*-linked glycans from the cwMPs of the wild-type (*a*), *hoc2*Δ (*b*), *hoc4*Δ (*c*), *hoc1*Δ (*d*), *hoc1*Δ::*HOC1* (*e*), *hoc3*Δ (*f*), and *hoc3*Δ::*HOC3* (*g*) strains were purified, labeled with 2-AA, and subjected to HPLC analysis. 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. The *arrows* indicate the peaks that are absent in the *hoc1*Δ and *hoc3*Δ strains but present in the *hoc1*Δ::*HOC1* and *hoc3*Δ::*HOC3* strains, respectively.

conditions generated by the presence of hygromycin B, vanadate, CFW, or Congo red (data not shown). The *hoc1*Δ, *hoc2*Δ, and *hoc3*Δ mutants were slightly more sensitive to SDS than the wild-type strain, whereas the *hoc1*Δ and *hoc2*Δ mutants were more sensitive to NaCl (Fig. 6*B*). It is intriguing that the *hoc2*Δ 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*Δ *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*Δ 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*Δ 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 *ktr3*Δ mutant was severely attenuated compared with the wild-type strain (Fig. 7*C*). Reintroduction of the wild-type *C. neoformans KTR3* gene into the *ktr3*Δ 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

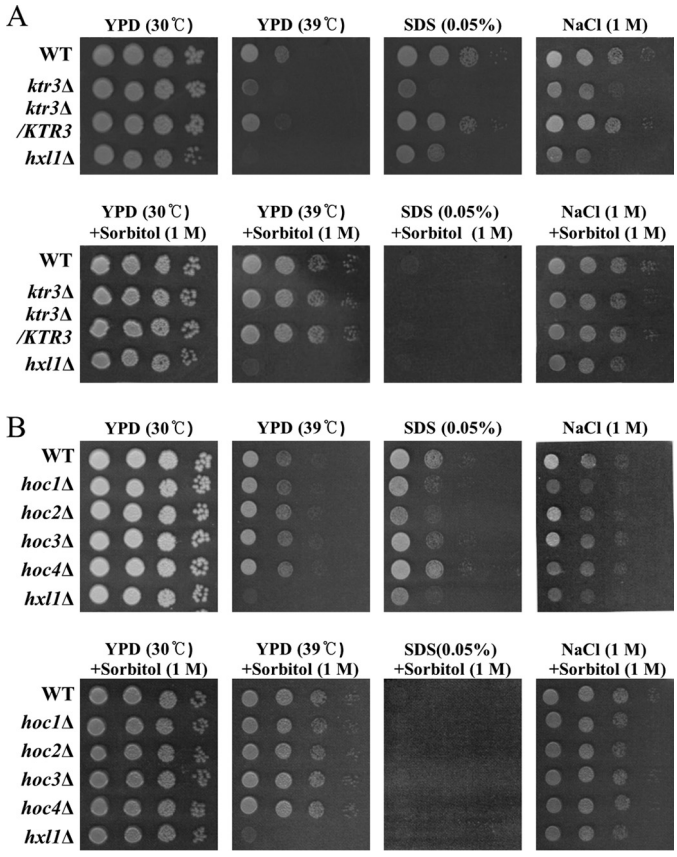

FIGURE 6. **Growth phenotypes of the *C. neoformans* wild-type, *ktr3*Δ, and *hoc* null mutant strains.** Yeast cells were cultivated overnight on YPD, adjusted to an A_{600} of 1, and then serially diluted 10-fold. Diluted cells were spotted on YPD plates with or without 1 M sorbitol under different 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 *ktr3*Δ mutant. *B*, phenotype analysis of *hoc1*Δ, *hoc2*Δ, *hoc3*Δ, and *hoc4*Δ mutants. The *hxl1*Δ mutant (33) was included as a control strain showing increased sensitivity under the tested conditions.

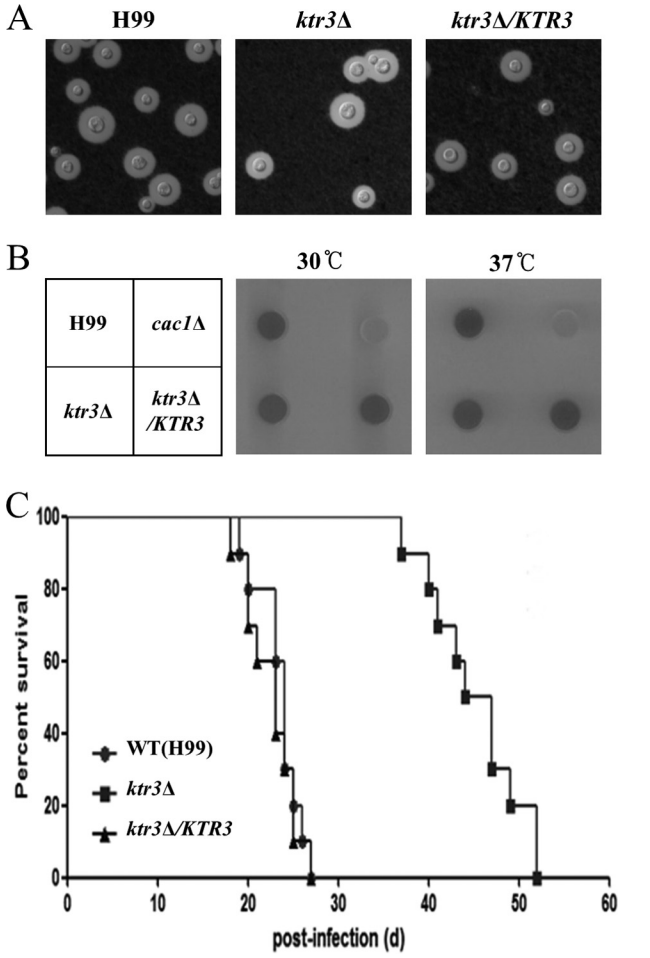

FIGURE 7. **Analysis of virulence-associated phenotypes of the *ktr3*Δ mutant strain.** *A*, analysis of the capsule formation. *B*, analysis of the melanin synthesis capacity. The *cac1*Δ mutant strain (36) was included as a control strain showing defective melanization on L-DOPA medium. *C*, virulence test of *C. neoformans* H99 wild-type, *ktr3*Δ, and *ktr3*Δ*/KTR3* strains.

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 $Gal_{0-2}Man_{1-3}$ 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, Manα1–6-Manα1–2Man mannotriose and Manα1–2Manα1–6-Manα1–2Man 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 *O*-glycans, Man_1–Man_4, and the minor species, Xyl_1Man_1–Xyl_1Man_4, 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 Manα1–6-Manα1–2Man 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 *ktr3*Δ strain revealed the presence of minor Man_2 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 Man_2 species might have Manα1–3Man structure. Considering the previous report that the β1,2-xylosyltransferase activity of *C. laurentii* requires Manα1–3Man as active substrate, but neither Manα1–2Man nor Manα1–6Man, in the xylose transfer reaction (37), it can be speculated that the remaining Man_2 species having Manα1–3Man structure could be the substrate of *C. neoformans* xylosyltransferase(s).

Although the gene products involved in the synthesis of the minor Man_2 and Xyl_1Man_2 species in the cryptococcal Golgi

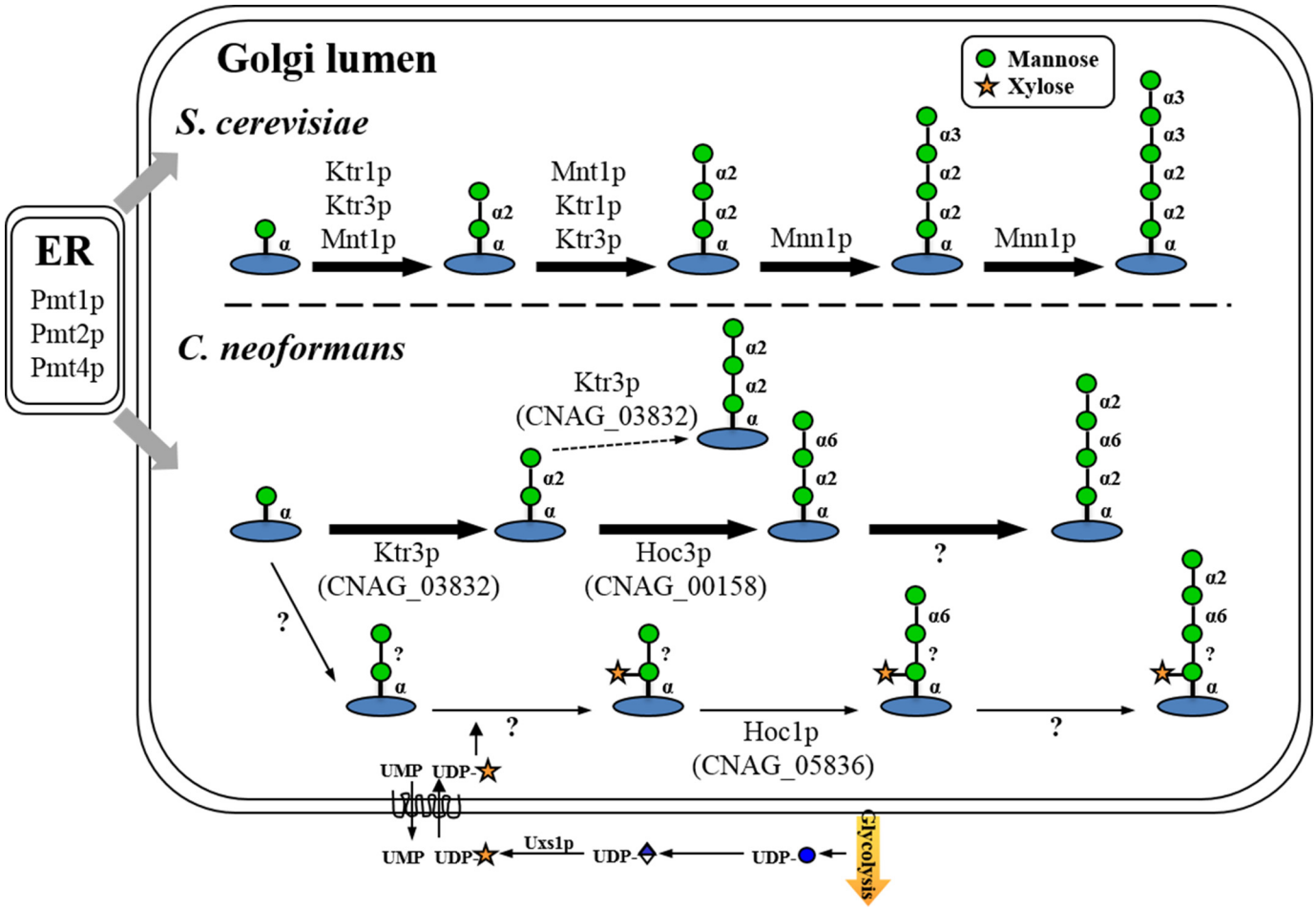

FIGURE 8. **Proposed cryptococcal *O*-glycans biosynthetic pathway in the Golgi.** The *top* and *bottom panels* depict the *O*-glycan biosynthetic pathways in the Golgi of *S. cerevisiae* and *C. neoformans*, respectively. Steps for mannose and xylose phosphorylation are not included.

were not determined, we showed that Hoc1p catalyzes a reaction that adds an α1,6-linked mannose residue to Xyl_1Man_2, generating Xyl_1Man_3. 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 *pmt1*Δ and *pmt4*Δ 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 ktr3*Δ 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 *ktr3*Δ mutant or the *pmt*Δ 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-

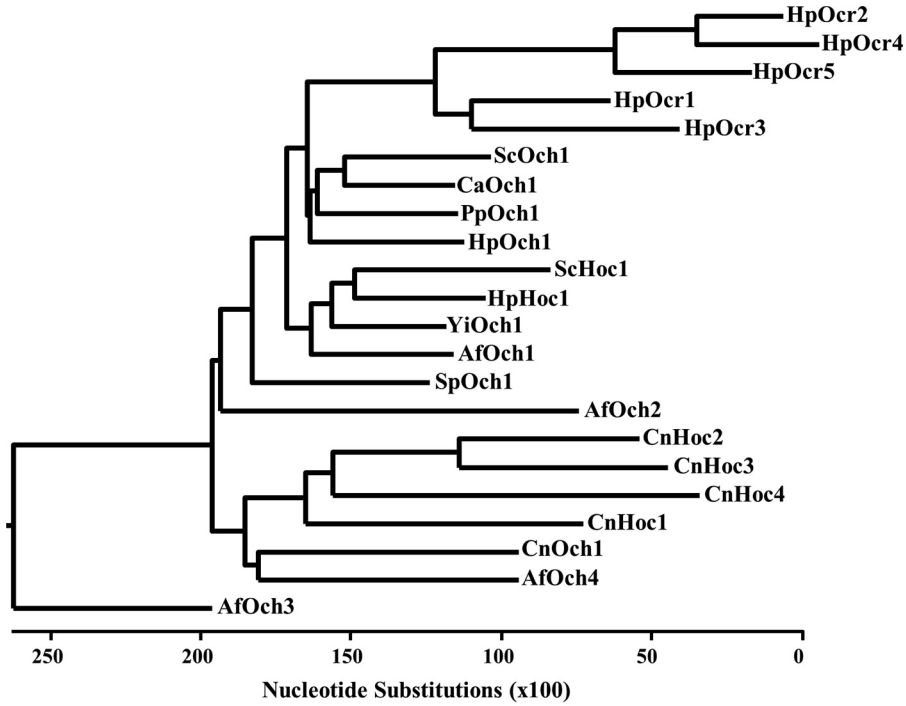

FIGURE 9. **Phylogenetic analysis of fungal Och1 and Hoc1 proteins.** Protein sequences were aligned using ClustalW. The phylogenetic tree was constructed using Cladogram in the DNASTAR MegAlign program. The branch distances in the phylogenetic tree correspond to sequence divergence. Sequences were derived from genome databases. *Sc*, *S. cerevisiae* Och1 (NP_011477.1), Hoc1 (NP_012609.3); *Ca*, *C. albicans* Och1 (XP_716632); *Pp*, *P. pastoris* Och1 (XP_002489596.1); *Hp*, *H. polymorpha* Och1 (AAS77488.1), Hoc1 (AAQ11191.1), Ocr1 (AAQ06408.1), Ocr2 (ABB83802.1), Ocr3 (ABB83803.1), Ocr4 (ABB83804.1), Ocr5 (ABB83805.1); *Yl*, *Yarrowia lipolytica* Och1 (CAD91643.1); *Sp*, *S. pombe* Och1 (CAD24818.1); *Af*, *A. fumigatus* Och1 (XP_753779.1), Och2 (XP_751278.1), Och3 (XP_747002.1), Och4 (XP_749888.1); *Cn*, *C. neoformans* Och1 (CNAG_00744), Hoc1 (CNAG_05836), Hoc2 (CNAG_01214), Hoc3 (CNAG_00158), Hoc4 (CNAG_04449).

uated the virulence of *C. neoformans* in a mouse model of cryptococcosis. The attenuated virulence of the *ktr3*Δ 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 glycosylphosphatidylinositol-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*Δ mutant showed no growth phenotypes, a *cda1*Δ*cda2*Δ*cda3*Δ 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*Δ 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 mannoprotein-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*Δ 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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