A β-1,2-Xylosyltransferase from Cryptococcus neoformans Defines a New Family of Glycosyltransferases*†§

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J. Stacey Klutts*†§, Steven B. Levery*§, and Tamara L. Doering†

From the Departments of *Molecular Microbiology and §Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri 63110 and the †Department of Chemistry, University of New Hampshire, Durham, New Hampshire 03824

Cryptococcus neoformans is an opportunistic fungal pathogen characterized by a prominent polysaccharide capsule that envelops the cell. Although this capsule is dispensable for in vitro growth, its presence is essential for virulence. The capsule is primarily made of two xylose-containing polysaccharides, glucuronoxylomannan and galactoxylomannan. There are likely to be multiple xylosyltransferases (XTs) involved in capsule synthesis, and the activities of these enzymes are potentially important for cryptococcal virulence. A β-1,2-xylosyltransferase with specificity appropriate for capsule synthesis was purified ~3000-fold from C. neoformans, and the corresponding gene was identified and cloned. This sequence conferred XT activity when expressed in Saccharomyces cerevisiae, which lacks endogenous XT activity. The gene, termed CXTI for cryptococcal xylosyltransferase 1, encodes a 79-kDa II membrane protein with an N-linked glycosylation site and two DXD motifs. These latter motifs are believed to coordinate divalent cation binding in the activity of glycosyltransferases. Site-directed mutagenesis of one DXD motif abolished Cxt1p activity, even though this activity does not depend on the addition of a divalent cation. This may indicate a novel catalytic mechanism for glycosyl transfer. Five homologs of Cxt1p were found in the genome sequence of C. neoformans and 34 within the sequences of other fungi, although none were found in other organisms. Many of the homologous proteins are similar in size to Cxt1p, and all are conserved with respect to the essential DXD motif. These proteins represent a new family of glycosyltransferases, found exclusively within the fungal kingdom.

The basidomycetous fungus Cryptococcus neoformans causes a variety of maladies, including an often fatal meningoencephalitis (1). Currently available chemotherapeutic agents are unable to completely clear the infection, necessitating long-term treatment in those patients that survive (1). The main virulence factor of this opportunistic pathogen is the large polysaccharide capsule that envelops the cell. Strains of C. neoformans that lack this capsule are avirulent in animal models (2), suggesting that the synthesis of this structure could be targeted therapeutically (3). Two xylose-containing polysaccharides, termed glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), make up ~97% of the mass of the capsule, with the remaining mass contributed by mannoproteins (3).

The biochemical structures of both GXM and GalXM have been determined. GXM, with a molecular mass of 1–7 megadaltons (4), is composed of a backbone of α-1,3-linked mannan substituted with glucuronic acid and xylose residues (5) (Fig. 1). GalXM is a smaller polymer (100 kDa), consisting of an α-1,6-galactan backbone with galactomannan side chains that are decorated with xylose residues (6) (Fig. 2).

There are four serotypes of C. neoformans (A–D) (7) that differ with respect to virulence and the amount of xylose present within GXM (Fig. 1, panels A–D). Serotype D GXM has the simplest xylosylation pattern, with only one β-1,2-linked xylose residue (Fig. 1, panel D), and is also the least virulent of the serotypes. Serotype A GXM has two β-1,2-linked residues (Fig. 1, panel A), and this serotype is more virulent than serotype D strains (8). The GXM of serotypes B and C, which can cause disease in immunocompetent patients, contains additional β-1,4-linked xylose residues (Fig. 1, panels B and C). The structure of GalXM has only been studied in serotype D strains (6).

GXM mediates a variety of detrimental effects on the host immune response that allow for the in vivo survival of C. neoformans (9). In addition to the correlation between increased GXM xylosylation and virulence among the four serotypes described above, there is further evidence that capsular xylose participates in these immunological effects. A mutant strain of C. neoformans lacking UDP-xylose, the activated donor for xylose transfer, has a general defect in xylosylation (10, 11). This strain lacks capsular xylose, has an aberrant capsule appearance, and is avirulent in mice (11, 12). In addition, Fries and co-workers (13, 14) have identified phenotypic switching in C. neoformans. Three phenotypic variants of one C. neoformans strain differ with respect to virulence and the arrangement of xylose moieties within GXM (15), further implicating these residues in cryptococcal pathogenesis.

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‡ To whom correspondence should be addressed: Dept. of Molecular Microbiology, Washington University School of Medicine, 660 South Euclid Ave., Campus Box 8230, St. Louis, MO 63110-1093. Tel.: 314-747-5597; Fax: 314-362-1232; E-mail: doering@wustl.edu.
§ The abbreviations used are: GXM, glucuronoxylomannan; GalXM, galactoxylomannan; XT, xylosyltransferase; TOCSY, total correlation spectroscopy; PMMA, partially methylated alditol acetate; MS, mass spectrometry; HPLC, high pressure liquid chromatography.

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Our current understanding of capsule biosynthesis is limited. By screening mutagenized C. neoformans cells for defects in capsule formation, Kwon-Chung, Janbon, and their co-workers (16–21) identified a number of genes that appear to be involved in capsule synthesis. Disruption of any one of the four CAP genes (CAP10, CAP59, CAP60, and CAP64) yields an acapsular phenotype, suggesting that these each play a central role in capsule synthesis (16–19). Five homologs of CAP10 (CAP1–5) have also been identified, but the biochemical functions of the CAP genes and these homologs have remained undefined (22).

Glycosyltransferases catalyze the specific transfer of a monosaccharide from an activated donor, such as a nucleotide disphosphosugar, to an acceptor. These enzymes are typically 30–50-kilodalton type II membrane proteins (23). Glycosyltransferases have been organized into families based on sequence similarity (24) and can be broadly divided into two superfamilies (GT-A and GT-B) based on structural patterns. Most members of the GT-A group contain a DXD motif that is involved in the binding of a divalent cation cofactor important for catalytic activity (25–31). Although a few GT-A enzymes lack the DXD motif and cation requirement, this is more typical of the GT-B glycosyltransferases.

The synthesis of large glycans such as GXM or GalXM generally requires the sequential action of several glycosyltransferases. Thus, it is likely that a number of these enzymes, including up to seven xylosyltransferases, are actively involved in capsule synthesis. It is also reasonable to suggest that some of the previously identified capsule synthesis associated genes may encode glycosyltransferases, especially as one of them, CAP59, encodes a homolog to a known mannosyltransferase (32). However, no glycosyltransferase with a defined role in capsule synthesis has been identified.

Capsule synthesis offers a potential target for antifungal chemotherapy. To understand this process, we must identify the glycosyltransferases involved in GXM and GalXM production. Because of the importance of xylose residues within these structures, we have focused on identifying xylosyltransferases (XTs) involved in capsule biosynthesis. As no homologs to known mammalian or plant XTs exist within the XTs involved in capsule biosynthesis. As no homologs to known mammalian or plant XTs exist within the C. neoformans data base, we took a biochemical approach to this question. Here we describe the discovery, purification, characterization, and expression of Cxt1p, a β-1,2-XT from C. neoformans with activity appropriate for the synthesis of either GXM or GalXM. Cxt1p is a large, apparently cation-independent glycosyltransferase that defines a new family of glycosyltransferases. This family includes C. neoformans Cap10p and its homologs and is exclusive to fungi.

**EXPERIMENTAL PROCEDURES**

**Materials**—Unless otherwise noted, all chemicals were from Sigma.

**Strains and Cell Growth**—C. neoformans wild-type strain JEC21 (serotype D MATα) was provided by Joseph Heitman (Duke University). C. neoformans ags1Δ strain was generated in our laboratory (serotype D MATα ags1Δ) (33), and Saccharomyces cerevisiae strain TDY172 (RSY620; MATa ade2-1 trp1-1 leu2-3,112 ural3-1 his3-11,15 pep4-::TRP1) was from Randy Schekman (University of California, Berkeley). For activity analysis, 50 ml of minimal medium minus uracil (URA−) (34) was inoculated with a single colony of JEC21 or ags1Δ and incubated at 30 °C with continuous shaking (200 rpm) until the A600 was between 1 and 2. For purification, such a culture of ags1Δ was then inoculated into 3 liters of the same medium and grown at 30 °C with continuous shaking for 3–4 days until the A600 was between 4 and 5. TDY172 was grown at 30 °C with continuous shaking in 50 ml of minimal broth media supplemented with uracil to an A600 of 1–2.

**Xylosyltransferase (XT) Enzyme Assays**—Activity was assayed by monitoring the transfer of [14C]xylose from UDP-[U-14C]xylose (264.4 mCi/mmol; PerkinElmer Life Sciences) to α-1,3-mannobiose (α-1,3-Man2; Carbohydrate Synthesis, Oxford, UK). Standard assay mixtures (50 μl) included 15 μl of protein sample, 8.5 mm α-1,3-Man2 (0.43 μmol), 57 nmol of UDP-[U-14C]xylose (15 nCi), and 100 mm Tris, pH 6.5. The reaction was incubated for 4 h at 20 °C and terminated by applying the assay mixture to a small disposable column containing 0.6 ml of AG2X-50 resin (Bio-Rad), followed by 30 μl of deionized water to ensure the sample was fully loaded into the resin. 600 μl of deionized water was then applied to the column, and the eluate collected and spun at 13,000 × g for 5 min to remove particulate materials, and the supernatant fraction removed to another tube. 14C-Labeled trisaccharide product was detected by scintillation counting or TLC. For the latter, all or part of the supernatant was dried under N2 at 50 °C, resuspended in 15 μl of 40% n-propyl alcohol, and applied to a dried (70 °C for 30 min) 20 × 20-cm Silica Gel-60 TLC plate (EMD Chemicals, Gibbstown, NJ). The plate was developed in 5:4:2 n-propyl alcohol:acetone:water for 2 h, dried, and then developed for an additional 2 h in the same solvent. After drying, the standards were visualized by spraying with 0.2% orcinol in 75:15:10 ethanol:sulfuric acid:water and incubating for 5–10 min at 70 °C. The sample lanes were sprayed with Enhance surface autoradiography spray (PerkinElmer Life Sciences) and allowed to dry, and the radioactive products were visualized by autoradiography.

**Production and Purification of Trisaccharide Product for Analysis**—To generate product for structural analysis, XT reactions were scaled up. Ten 70-μl reactions (20 μl of partially purified enzyme (post Sephacryl S-300, see below), 12.8 mM UDP-xylose (0.9 μmol) (Carbosource Services, Athens, GA), 57 nmol of UDP-[U-14C]xylose (0.015 μCi), and 147 mm Tris, pH 6.5) were incubated at 20 °C for 30 h and applied to AG2X-50 columns as above. Each column was eluted with 700 μl of deionized water, and the eluates were pooled, lyophilized, resuspended in 100 μl of 40% n-propyl alcohol, streaked onto two Silica Gel 60 TLC plates, and developed as above. The radioactive trisaccharide was localized using a TLC plate scanner (System 200A imaging scanner; Bioscan Inc., Washington, D.C.), and the corresponding area of silica was recovered. The product was eluted from the silica powder by vortexing for 1 min with 5 ml of water and allowing the mixture to remain for 1 h at room temperature. The silica was sedimented (10,000 × g, 10 min, room temperature), and the supernatant was lyophilized and resuspended in 1 ml of water. The product was then purified by solid phase


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extraction using a protocol adapted from Ref. 35. Briefly, the suspension was applied to a 0.25 g (1 ml) Envi-Carb solid phase extraction column (Supelco, Bellefonte, PA) that had been pre-conditioned with 3 ml of 80% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid followed by 2 ml of water. The loaded column was washed with 3 ml of water, and the product was eluted with 3 ml of 25% acetonitrile in water (v/v). The acetonitrile in the eluate was removed by evaporation under N₂ at 50 °C, and the remainder of the sample was lyophilized.

α-Mannosidase Treatment of the XT Product—XT product was partially purified by TLC as described above. The product was then incubated with jack bean α-mannosidase (Oxford Glycosystems, Oxford, UK) for 18 h at 37 °C in buffer supplied by the manufacturer.

One-dimensional ¹H and Two-dimensional ¹H-¹H Nuclear Magnetic Resonance Spectroscopy—Samples of the XT product and the α-1,3-Man₉ substrate (~0.3–1.0 mg) were deuterium-exchanged by repeated lyophilization from D₂O and then dissolved in 0.5 ml of D₂O for NMR analysis. One-dimensional ¹H NMR, two-dimensional ¹H-¹H-gCOSY, two-dimensional ¹H-¹H-TOCSY, and one-dimensional ¹H-¹H nuclear Overhauser effect spectra were acquired at 25 °C on a Varian Unity Inova 500-MHz spectrometer (Department of Chemistry, University of New Hampshire), using standard acquisition software available in the Varian VNMR software package. Proton chemical shifts are referenced to internal acetone (δ = 2.225 ppm).

Permethylation and Linkage Analysis—A portion of the XT product was permethylated using the method of Ciucanu and Kerek (36) with the modification of Ciucanu and Costello (37). An aliquot of the permethylated product was further treated (by hydrolysis, reduction, and per-O-acetylation) for analysis of partially methylated alditol acetates (PMAAs), using the protocols described by Lavery and Hakomori (38). The PMAAs were analyzed on an Rtx-5MS-bonded phase-fused silica capillary columns described by Levery and Hakomori (38). The PMAAs were (by hydrolysis, reduction, and per-

Magnetic Resonance Spectroscopy

TABLE 1

Buffers used in xylosyltransferase purification

| Buffer | Component | Concentration |
|--------|-----------|---------------|
| A      | 100 mM Tris 8.0, 0.1 mM EDTA |             |
| B      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.05% Triton X-100, 50 mM NaCl |             |
| C      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.05% Triton X-100, 2 mM NaCl |             |
| D      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.05% Triton X-100, 100 mM NaCl |             |
| E      | 20 mM Tris 7.5, 0.05% Triton X-100, 400 mM NaCl, 1 mM CaCl₂, 1 mM MnCl₂ |             |
| F      | 20 mM Tris 8.0, 0.05% Triton X-100, 400 mM NaCl, 0.01 mM EDTA, 200 mM αMM³ |             |
| G      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.01% Triton X-100 |             |
| H      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.01% Triton X-100, 80 mM NaCl |             |
| I      | 20 mM Tris 8.0, 0.1 mM EDTA, 0.01% Triton X-100, 225 mM NaCl |             |
| J      | 20 mM Tris 6.5, 0.1 mM EDTA, 0.01% Triton X-100 |             |
| K      | 20 mM Tris 6.5, 0.1 mM EDTA, 0.01% Triton X-100, 20 mM NaCl |             |
| L      | 20 mM Tris 6.5, 0.1 mM EDTA, 0.01% Triton X-100, 150 mM NaCl |             |

³αMM indicates α-methyl mannoside.

Q-Sepharose Chromatography—The SolS fraction (2–3 ml) was filtered using 0.45-μm spin filters and promptly applied to a 20-ml HiPrep 16/10 Q-Sepharose Fast Flow column (GE Healthcare) that had been pre-equilibrated with Buffer B. The column was then washed with 200 ml of Buffer B, and activity was eluted with a 200-ml gradient from Buffer B to Buffer C. 4-ml fractions were assayed as above, and the activity peak was pooled and concentrated to ~500 μl (SolQ) using a 15-ml Amicon Ultra 10-kDa molecular mass cutoff spin concentrator.

Sephacryl S-300 Gel Filtration Chromatography—The Sol Q fraction was filtered and applied to a 316-ml HiPrep 26/60 Sephacryl S-300 gel filtration column (GE Healthcare) that was pre-equilibrated with Buffer B. The column was then eluted with 253-ml (0.8 column volume) isocratic gradient of Buffer D, with MS operated in electron ionization mode, and gas chromatography programmed as described previously (38). The derivatives were mass spectra compared with standards (39–41).

Concanavalin A Chromatography—The SolS fraction was diluted to 10 ml with Buffer E, concentrated to 1 ml, and diluted back to 2.5 ml with Buffer E. This was then applied to a 2.5-ml column of concanavalin A-Sepharose 4B (GE Healthcare) that had been pre-equilibrated with Buffer E. The column
was capped and allowed to rock at 4 °C overnight. The flow-through was then collected and the column washed with 60 ml of Buffer E. 50 ml of Buffer F was then added to the column. After 10 ml of Buffer F had passed through and been collected, the column was capped and allowed to rock for 30 min at 4 °C. The remainder of the Buffer F was then allowed to elute, pooled with the first 10 ml, and concentrated to 1 ml (SolC).

DEAE Chromatography—To desalt SolC, it was diluted to 10 ml with Buffer G, concentrated to 1 ml, rediluted to 10 ml, and concentrated to 0.8 ml. The final solution was filtered and applied to a 1-ml HiTrap DEAE-Fast Flow column (GE Healthcare). The column was washed with 15 ml of Buffer H and eluted with a 20-ml gradient from Buffer H to Buffer I. 1-ml fractions were assayed, and the activity peak was concentrated to 1 ml (SolD).

α-1,3-Man₂ Affinity Chromatography—The SolD fraction was diluted to 15 ml with Buffer G and extensively desalted with three successive concentrations and dilutions, to a final volume of 1 ml. This was mixed with 1 ml of Buffer J and loaded onto a column containing 5 ml of a custom-synthesized α-1,3-Man₂-agarose resin (Carbohydrate Synthesis, Oxford, UK) that had been pre-equilibrated with Buffer J. The column was capped, allowed to rock at 4 °C for 30 min and then at 25 °C for 90 min, and then washed with 30 ml of Buffer K at 25 °C. The column was then moved to 4 °C and eluted with a 44-ml gradient from Buffer K to Buffer L, collecting 0.85-ml fractions. The activity peak was combined into three pools that were each concentrated to 25 μl; one pool that consisted of the shoulders of the peak and the other the middle of the peak.

Protein Analysis—The two pools from the α-1,3-Man₂ column were resolved by SDS-PAGE on a 12% gel, which was stained with SYPRO Ruby (Bio-Rad) as per the manufacturer’s instructions and visualized on a Bio-Rad Molecular Imager FX. An ~90-kDa band of interest was excised and submitted to the Protein and Nucleic Acid Chemistry Laboratory at Washington University School of Medicine for trypsin digestion followed by HPLC separation and mass spectrometry analysis of the resulting fragments. The mass spectra were compared with the C. neoformans protein data base, and the protein sequence corresponding to the band of interest was then analyzed using the Prosite domain prediction server and the TMpred transmembrane domain prediction server.

Expression in S. cerevisiae—CXT1 was amplified from JEC21 cDNA by PCR using primers Exp-sense and Exp-antisense (Table 2) to incorporate a His₆ tag at the C terminus, as well as HindIII and BamHI sites at the 5’ and 3’ ends, respectively. The product was cloned into a TOPO pCR2.1 vector (Invitrogen) and sequenced (Protein and Nucleic Acid Chemistry Laboratory, Washington University School of Medicine). A Topo-CXT1 clone with the correct sequence was then digested with HindIII and BamHI, and the released fragment was cloned into a 2-μm yeast expression vector between the promoter and terminator of phosphoglycerate kinase (plasmid pPGK (43); from K. Blumer, Washington University School of Medicine). The resulting plasmid and empty vector were transformed into TDY172 by electroporation, and transformants containing the URA3 marker were selected by plating onto URA− medium. To assay XT activity, two independent transformants, bearing either pPGK-CXT1 or empty pPGKm were grown overnight in 50 ml of URA− medium, and membranes were prepared and assayed as above. Expression was confirmed by immunoblotting with anti-His antibody (1:5000) using standard methods. Expressed, solubilized Cxt1p-His₆ was partially purified using a Talon Superflow metal affinity resin (Clontech). Briefly, 500 μl of solubilized membranes were applied to 2 ml of resin that was equilibrated with 50 mM Tris 7.5, 0.01% Triton X-100, 200 mM NaCl. The column was washed with 5 ml of the same buffer, and then with 15 ml of the same buffer containing 250 mM imidazole. Protein was eluted with 15 ml of the same buffer containing 250 mM imidazole, and the eluate was collected and concentrated.

Site-directed Mutagenesis—The CXT1 sequence in pPGK-CXT1 was mutated using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) with the primers listed in Table 2. The mutagenized sequences were confirmed by DNA sequencing, and correct plasmids were electroporated into TDY172 and analyzed as above.

RESULTS

Development of a Xylosyltransferase Activity Assay—To detect an XT with activity appropriate for capsule synthesis, we developed an assay for xylose transfer to an α-1,3-linked disaccharide of mannose (α-1,3-Man₂). This substrate resembles both the backbone of GXM and the dimannose moiety of GalXM (see Figs. 1 and 2). Cryptococcal membranes were incubated with this substrate in the presence of UDP-[¹⁴C]xylose; unutilized UDP-[¹⁴C]xylose was removed by anion exchange, and the radiolabeled products formed were separated by thin layer chromatography and detected by autoradiography. As
shown in Fig. 3, a radio-labeled product, which migrated more slowly than the α-1,3-Man₂ standard, was detected. Appearance of this product was dependent on α-1,3-Man₂ addition, time, and membrane protein concentration (data not shown). Notably, the activity was not dependent on the addition of divergent cations, nor could activity be reduced with up to 10 mM EDTA (data not shown).

Analysis of the Oligosaccharide Product of the XT Assay—We hypothesized that the XT product was a trisaccharide, as it migrated in this TLC solvent system similar to other trisaccharide standards (data not shown). To confirm this, the product was digested with jack bean α-mannosidase. This resulted in its quantitative conversion to a radio-labeled product that migrated close to the α-1,3-Man₂ standard (Fig. 3). Because this α-mannosidase enzyme only cleaves terminal, nonreducing mannose residues, this suggested that the product of the XT assay was a trisaccharide with the radioactive xylose moiety linked to the reducing mannose of the α-1,3-Man₂.

To verify our conclusions about the assay product and to determine the linkage of xylose attachment, purified product was subjected to structural characterization by MS, NMR, and methylation linkage analysis. In positive mode electrospray ionization-MS in a quadrupole ion trap (ThermoFinnigan LCQ), we observed a molecular salt/adduct at nominal, monoisotopic m/z 17894, consistent with [M + Na]⁺ for a trisaccharide consisting of one pentose and two hexose residues (spectrum not shown). Following permethylation, the molecular adduct [M + Na]⁺ was observed strongly in electrospray ionization MS at a nominal mono-isotopic m/z 637 consistent with the same composition. MS² fragmentation in the ion trap yielded a trio of major product ions consistent with loss of either the nonreducing pentose residue (m/z 463, m/z 431, [m/z 463 – MeOH]) or one hexose residue (m/z 419) (spectrum not shown). Loss of a permethylated hexose residue from either the reducing or nonreducing end may produce product ions of the same m/z, so further MS³ analysis would not determine to which hexose residue the pentose was attached. To address this question, NMR spectroscopic analysis was performed.

Fig. 4 compares the one-dimensional ¹H-NMR spectra of the α-1,3-Man₂ substrate (panel A) and XT product (panel B). The latter clearly shows changes consistent with glycosylation by another sugar residue, including a pair of additional δ-linked H-1 resonances of unequal amplitude at 4.543 ppm (δ₂₁,₂ = 7.7 Hz) and 4.412 ppm (δ₂₁,₂ = 7.8 Hz). This spectrum also shows significant chemical shift changes for the four downfield resonances corresponding to H-1 of both mutarotatory forms of the original Manα-1,3Manα/β disaccharide acceptor, which are in the same relative proportions as the two additional δ-configured H-1 signals. Assignment of the product spectrum by two-dimensional NMR methods showed both of the new β H-1 signals to be coupled into two respective 6-proton spin systems, as shown in the TOCSY spectrum (Fig. 4, panel C); sequential analysis of their J-coupling patterns confirmed that they belong to two distinct δ-Xyl spin systems, consistent with their incorporation into a product undergoing slow mutarotatory interconversion. Furthermore, the large magnitude of the chemical shift differences corresponding to the two δ H-1 resonances, along with the large magnitude of the shift changes for the two H-1 signals corresponding to the α and β forms of the reducing end Man residue (5.245 ppm, δ₁₁,₂ = 1.7 Hz, and 4.981 ppm, δ₁₁,₂ = 0.9 Hz, respectively), are consistent with
FIGURE 4. 500-MHz $^1$H NMR spectra (D$_2$O, 25 °C) of the XT acceptor substrate and product. Panel A, one-dimensional NMR spectrum of the Man$\alpha$-1,3-Man$\alpha$/$\beta$ substrate; panel B, one-dimensional NMR spectrum of the product; panel C, partial two-dimensional TOCSY spectrum (200-ms mixing time) of the product. Two clear H-1 signals for each residue in the disaccharide acceptor and the trisaccharide product, corresponding to the slowly equilibrating $\alpha$- and $\beta$-anomeric configurations of the reducing Man residue, are marked $3\text{Man}^\alpha$ H-1 or $2,3\text{Man}^\alpha$ H-1 and $3\text{Man}^\beta$ H-1 or $2,3\text{Man}^\beta$ H-1, respectively (panels A and B). Assignments of H-1 and other protons of nonreducing residues in the two forms of each oligosaccharide are denoted by superscripts $\alpha$ and $\beta$. The TOCSY spectrum (panel C) shows only those correlations arising directly from the monosaccharide anomic protons (4.3–5.4 ppm in F1 dimension). Assignment strategy also incorporated TOCSY spectra with lower mixing times, as well as gCOSY spectra giving only 3-bond correlations (not shown). In the TOCSY spectrum, the prefix H is omitted from proton designations for clarity, and complete spin systems for the $\beta$-Xyl residue(s) are visible and marked; for Man residues, additional correlations are visible in other parts of the spectrum.
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| TABLE 3 |
|---|
| A representative β-1,2-xylosyltransferase purification |

| Fraction | Specific activity | Purification | Yield |
|---|---|---|---|
| Crude membranes | 3 | 1 | 100 |
| 1% Triton X-100 extract | 9 | 3 | 97 |
| QFF anion exchange | 200 | 67 | 82 |
| S-300 gel filtration | 260 | 87 | 40 |
| Concanavalin A affinity/DEAE | 315 | 105 | 17 |
| Man-α-1,3-Man affinity | 500 | 175 | 32 |
| S-300 resolution | 8900 | 2967 | 3 |

* A unit of activity was defined as the amount of enzyme that transfers 100 cpm of [14C]Xyl from UDP-[14C]Xyl to α-1,3-dimannoside per min.

Glycosylation of the interconverting reducing end Man residue. The large chemical shift difference between the two β H-1 resonances also suggests glycosylation at a site very near to the anomeric carbon, i.e. at O-2. By contrast, the other pair of H-1 resonances, at 5.149 ppm (3 J/1,2 = 1.8 Hz) and 5.136 ppm (3 J/1,2 = 1.5 Hz) experience little change with respect to their positions in the spectrum of the acceptor substrate; these only differ from each other by a small increment because of long range effects of mutarotation of the reducing end monosaccharide.

Our structural assessments were confirmed by one-dimensional 1H-1H nuclear Overhauser effect difference spectra acquired following selective pre-irradiation of the two β-Xyl H-1 signals (supplemental Fig. 1). The magnitude of signals appearing in nuclear Overhauser effect difference spectra is indicative of spatial proximity (less than 5 Å) between the irradiated and nearby nuclei, because of cross-relaxation highly dependent on internuclear distance (and other parameters, including relative geometry and molecular motion). Pre-irradiation of the major β-Xyl H-1 resonance yielded selective time-dependent enhancements, not only for other resonances within the same sugar ring (β-Xyl H-2, H-3, and H-5ax), but also strong inter-residue enhancements for the reducing Man α H-1 and H-2 signals (supplemental Fig. 1, panel B). Upon pre-irradiation of the minor β-Xyl H-1, only two enhancements could be clearly observed, probably due to the lower abundance of this configuration. These were an intra-residue enhancement of β-Xyl H-3 and an inter-residue enhancement of reducing Man β H-2 signal (supplemental Fig. 1, panel C). Construction of molecular models for both forms of the trisaccharide product show that these patterns of enhancements are consistent only with linkage of the β-Xyl residue to O-2 of the reducing Man.

Additional confirmation of xylose linkage to O-2 of the reducing mannose was provided by linkage analysis of the XT product following permethylation, depolymerization, reduction, and peracetylation to produce partially methylated PMAAs (data not shown). In the subsequent gas chromatography-MS analysis, PMAA derivatives were detected corresponding to nonreducing terminal Xyl, nonreducing terminal Man, and 2(→3)-linked Man. Because the Man residues in the acceptor substrate were joined by a 3-linkage, it follows that the added Xyl residue occupied the 2-position of the di-substituted reducing Man in the intact XT product.

Purification and Identification of the β-1,2-Xylosyltransferase — We were interested that the product formed in our XT assay corresponded to structures present in GalXM (Fig. 2) and GXM of all serotypes (Fig. 1). We therefore proceeded to purify the protein responsible for this activity. We were aided by the fact that the product of interest was the dominant radiolabeled species in our assay (Fig. 3, − lane). This allowed us to use scintillation counting as a method of detection, rendering the assay more rapid, less laborious, and appropriate for use in XT
purity. As described under “Experimental Procedures,” we prepared membranes from the serotype D bags1/H9004 strain (33). XT activity in this strain is equivalent to wild type (data not shown), but the cells were considerably easier to mechanically disrupt. We then used both conventional and affinity resins to enrich the β-1,2-XT ~3,000-fold from washed cryptococcal membranes (Table 3). Notably, the XT activity eluted from the S-300 gel filtration column at the position of a 90-kDa protein.

Final enrichment of the XT enzyme was not to homogeneity but did lead to a significant reduction in the number of bands on an SDS-polyacrylamide gel. Chromatography using α-1,3-Manα-agarose affinity resin was a particularly effective step. Fractions from the XT activity peak observed upon elution of this resin were combined in two pools, with the middle of the activity peak separated from the combined shoulders of the peak. Each pool was concentrated and assayed for XT activity, which indicated that there was 2.5 times more activity in the middle “peak” pool. We next analyzed these two fractions by SDS-PAGE, seeking a protein band that was severalfold more abundant in the peak fraction and of roughly 90 kDa. One band (Fig. 5, arrow) displayed those characteristics. This band was excised and submitted for trypsin/HPLC/MALDI analysis, which yielded peptide masses matching a 694-amino acid (79 kDa) predicted protein from C. neoformans serotype D genome sequence. This sequence had been previously named CAP3 based on homology to CAP10, but it had no known function.

Because we had purified the XT from membranes and the activity bound to a concanavalin A resin (Table 3), we expected that the identified protein would have a transmembrane domain and N-linked glycosylation site. As shown in Fig. 6, the predicted protein did have these features, in addition to two DxD motifs commonly found in glycosyltransferases.

Expression of the Putative Xylosyltransferase in Yeast—To confirm that the sequence we identified encoded the enzyme catalyzing the XT activity, we expressed this hypothetical protein in S. cerevisiae, which lacks any XT activity (Fig. 7, left lanes). Sequence encoding a C-terminal His6-tagged version of the open reading frame was cloned into 2-μ plasmid, and expression was confirmed by immunoblotting with anti-His antibodies (data not shown). Expression of the protein did indeed lead to the appearance of substrate-dependent XT activity (Fig. 7, middle lanes), and the product co-migrated with authentic product produced by C. neoformans (Fig. 7, right lanes). This confirmed that the gene we had

![FIGURE 8. Site-directed mutagenesis of Cxt1p. The XT activity from membrane preparations of S. cerevisiae expressing empty vector, wild type (WT) Cxt1p, or the indicated mutant was assayed in the absence (gray bars) or presence (black bars) of the Manα2 substrate.](image)

![FIGURE 9. Alignment of Cxt1p homologs. Black bar, DxD motif at position 550 of Cxt1p. Residues showing identity in all homologs are shaded in red; blue indicates those that display strong similarity but not identity. The number in parentheses indicates the position of the first residue of the sequence shown.](image)
C. neoformans β-1,2-Xylosyltransferase

identified was correct. Furthermore, XT activity could be partially purified from solubilized membranes of S. cerevisiae expressing this protein using a Co2⁺ metal affinity column (data not shown). Upon this confirmation of activity, we renamed the gene CXT1 for cryptococcal xylosyltransferase 1 (GenBank™ accession number 905015).

Site-directed Mutagenesis of CXT1—We wondered whether N-glycosylation and both DXD motifs were important for the XT activity. To address this, we separately mutated each of these loci of the pPGK-CXT1 expression plasmid, and we expressed the mutated proteins in S. cerevisiae. None of the mutations altered protein expression (immunoblotting data not shown). However, when the N-glycosylation site was mutated (N141Q), the apparent gel mobility of Cxt1p did shift to the molecular weight predicted for the unmodified protein sequence, suggesting that the protein is normally N-glycosylated at that site. Despite this mutation, we saw little change in XT activity (Fig. 8). In contrast, mutation of either DXD motif to AXD led to a dramatic reduction in activity, with complete loss of activity upon mutagenesis of the first DXD motif, at position 550 (Fig. 8).

Identification of Cxt1p Homologs—We were intrigued by the size and apparent cation independence of Cxt1p and searched for homologous proteins in C. neoformans and other organisms. Within C. neoformans (serotype D), we found five homologs with identities ranging from 20 to 30% and similarities from 30 to 42%. This group consisted of CAP10 and its four homologs (supplemental Table S-1). We also discovered 34 additional homologs in other fungal organisms, with identities ranging from 12 to 23% and similarities from 21 to 32%. The homologs were generally large in size, with only five being less than 60 kDa (supplemental Table S-1). Although neither the DXD motif at position 659 nor the N-glycosylation site of Cxt1p was conserved (data not shown), the activity-dependent DXD motif at position 550 of Cxt1p is conserved in all of these homologs (Fig. 9).

Finally, Cxt1p was also present in the C. neoformans serotype A (H99) genome sequence. This sequence had 93% identity and 95% similarity to the serotype D sequence and was conserved with respect to both DXD motifs and to the N-glycosylation site (not shown). Furthermore, a robust, apparently identical XT activity was also detected in the H99 serotype A strain (data not shown).

DISCUSSION

In the course of our investigations of capsule synthesis in C. neoformans, we have detected and purified a β-1,2,3-Man-α-1,3-Man motif formed by CXT1 in the GXM of all serotypes, even the simplest serotype D strains (Fig. 1). Furthermore, this structure is also present in GalXM (Fig. 2). Thus, the activity of Cxt1p is consistent with a role in the synthesis of either GXM or GalXM, and we would expect this enzyme to be present and active in all serotypes. So far, we have shown activity in serotypes D and A.

Although our overall interests focus on cryptococcal capsule synthesis, it is possible that CXT1 is involved in the synthesis of other glycans. For example, the Xyl-β-1,2-Man-α-1,3-Man motif is also found in glycolipids (45) and O-glycans (46). Furthermore, a β-1,2-xylosyltransferase activity with similar characteristics has also been described in the nonpathogenic fungus Cryptococcus laurentii (47). This C. laurentii XT activity was believed to be involved in O-glycan synthesis based on the structure of the product formed in vitro (48). However, this enzyme was not purified, and thus its role in protein glycosylation has not been definitively established. Defining the precise role of Cxt1p in cryptococcal biology must await the generation and analysis of mutant strains, work now in progress.

3 B. Henrissat, personal communication.
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