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Perspective

Synthesis of cell envelope glycoproteins of *Cryptococcus laurentii*

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Abstract—Fungi of the genus *Cryptococcus* are encapsulated basidiomycetes that are ubiquitously found in the environment. These organisms infect both lower and higher animals. Human infections that are common in immune-compromised individuals have proven difficult to cure or even control with currently available antimycotics that are quite often toxic to the host. The virulence of *Cryptococcus* has been linked primarily to its polysaccharide capsule, but also to cell-bound glycoproteins. In this review, we show that *Cryptococcus laurentii* is an excellent model for studies of polysaccharide and glycoprotein synthesis in the more pathogenic relative *C. neoformans*. In particular, we will discuss the structure and biosynthesis of O-linked carbohydrates on cell envelope glycoproteins of *C. laurentii*. These O-linked structures are synthesized by at least four mannosyltransferases, two galactosyltransferases, and at least one xylosyltransferase that have been characterized. These glycosyltransferases have no known homologues in human tissues. Therefore, enzymes involved in the synthesis of cryptococcal glycoproteins, as well as related enzymes involved in capsule synthesis, are potential targets for the development of specific inhibitors for treatment of cryptococcal disease.

Keywords: *Cryptococcus laurentii*; *Cryptococcus neoformans*; O-Linked glycoprotein synthesis; Synthesis; Sugar nucleotides; Mannosyltransferase; Xylosyltransferase; Galactosyltransferase; AIDS

1. Introduction

Cryptococcus species are encapsulated basidiomycetes that are ubiquitously found in the environment. Several species of this yeast infect animals ranging from insects[1] to man.[2] Human infections have proven difficult to cure or even control with currently available antimycotics that are quite often toxic to the host. Pigeons are well-known carriers of pathogenic Cryptococcoideae, and human contact with pigeon droppings may cause severe lung disease and meningitis, especially in immunosuppressed individuals.[3–5] The spores and antigenic debris from these and related Cryptococcoideae in pigeon droppings are common components of city dust and may be a causative agent for allergies and asthma.[6,7] *Cryptococcus neoformans*, the most severe pathogen of this genus, is one of the major disease-determining factors in AIDS.[2,8] The virulence of *C. neoformans* has been linked both to its polysaccharide capsule and to cell envelope-bound glycoproteins.[2,9,10] This organism can, however, also infect immunocompetent hosts. The medical importance of *Cryptococcus* is shown by the large number of recent publications dealing with its pathogenicity[11,12] and provided the impetus for the recent sequencing of the *C. neoformans* genome.[13] With few exceptions, most other Cryptococcoideae are believed to be non-virulent in humans even though they also produce large and voluminous capsules very similar in composition and structure to those produced by *C. neoformans*. This paradigm requires reappraisal, however, because in recent years increasing numbers of case reports demonstrate that other species of encapsulated yeast, that is, *C. laurentii* and *C. albidus*, can also infect humans and cause disease despite their apparent temperature sensitivity in cell culture.[14,15] Some investigators have suggested that cryptococcal infections may represent an emerging disease.[14,16,17] Since the carbohydrate structure surrounding microbial organisms determines

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their immunological interaction with the host, structural differences of these components in different *Cryptococcus* species might alter the intensity of their virulence and antigenicity.[18] For examples, *C. neoformans* serotypes A, B, C, and D, although having similar capsule structures, express differences in virulence that can be correlated with subtle variations in the arrangement of xylosyl, glucuronosyl, and acetyl substituents on their capsular antigens. More recently, cell wall derived glycoproteins (mannoproteins) have also been implicated in *C. neoformans* pathogenicity although little is known about their individual carbohydrate structures.[12,19,20]

In this review, we show that *C. laurentii* is an excellent model for structural and biosynthetic studies of polysaccharide and glycoprotein synthesis in the *Cryptococcodiae*, including *C. neoformans*. As shown in the established work and in recent studies, these two species share common glycosidic linkages in their extracellular and capsular polysaccharides, as well as common pathways for the biosynthesis of sugar nucleotide precursors. Studies of sugar nucleotide biosynthetic enzymes and glycosyl transferases have already revealed identical or similar enzymatic properties and specificities for both organisms. We will emphasize biosynthetic features of O-linked oligosaccharides on cell envelope glycoproteins of *C. laurentii* that have unique carbohydrate structures yet to be described in *C. neoformans*.[21,22] These oligosaccharides are synthesized by distinct glycosyltransferases, most of which have no known homologues in human tissues. Such enzymes, as well as those responsible for capsule synthesis, are promising targets for drug intervention in the treatment of cryptococcal disease. This approach represents a major new development, since only a few effective antifungal drugs are currently available, and these often cause severe side effects, especially in immune compromised patients.

2. Structural studies of cryptococcal polysaccharides

2.1. Neutral glycoproteins containing Gal, Man, Glc, and Xyl

A number of extracellular glycoproteins have been described in *C. neoformans*,[12,19,23,24] but except for carbohydrate composition, few detailed studies of oligosaccharide structures have been carried out. The presence of sialic acids, including the 9-O-acetylated species, on some of the yeast glycoproteins[25] may suggest the presence of complex type mammalian protein-bound N-linked oligosaccharide chains, but this has yet to be established and the sialic acid could occur on O-linked oligosaccharides. Most of the sialic acid residues of *C. neoformans* appear to be (α2-6)-linked to Gal.[12] Recent work[19] has identified genes for over 40 putative mannoproteins that contain potential N-glycosylation sites and many potential sites for O-linkages in a Ser/Thr-rich domain (S/T-region) near the C-terminus, which is followed by C-terminal glycosylphosphatidylinositol (GPI) anchor motifs. Although the S/T-region is proposed as an attachment site for *O*-glycosidic linkages, there is currently only limited information on O-linked oligosaccharide structures of *C. neoformans* glycoproteins. The N-glycan structures of cryptococcal glycoproteins also remain to be determined. Extracellular glucomannan and galactoglucoxylomannan proteins have been described in *C. laurentii*.[22,26,27] The glycoproteins contain O-linked Man, mannobiose, and mannotetraose as well as more complex oligosaccharides composed of Man, Glc, Xyl, and Gal. One oligosaccharide structure consists of an (α1-6)-linked Man backbone with numerous side chains consisting of Man, or Man di-, tri-, and tetrasaccharides connected by (α1-2)- and (α1-3)-linkages. These structures are not present in mammalian types of O-glycans.[28]

However, insoluble protein-bound neutral heteroglycans have been identified in *C. laurentii* membrane preparations and in the isolated cell envelope shell.[29] The glycoproteins were solubilized by ethylene diamine extraction, and acidic capsular polymer was removed by cetavlon precipitation. Both solubilized preparations contained Man, Gal, and Xyl and varying amounts of Glc. Results of acetolysis and partial acid hydrolysis identified (α1-2)-, (α1-3)-, and (α1-6)-Man-Man linkages. Further structural analysis of some of these glycoprotein substituents was carried out concurrently with biosynthetic studies that are described here.[21,30,31] The membrane-bound glycoproteins were shown to contain unique tri-, penta-, and dodeca-saccharide chains of different composition and structure that are O-linked to protein through Ser and Thr residues.[21,22,30,31] Structures of these O-linked oligosaccharides are shown in Table 1. The individual transferases will be further discussed in Section 3.

In order to elucidate the oligosaccharide structures, the endogenous glycoproteins were labeled by incubating membrane–enzyme preparations with GDP-[^{14}C]Man.[21] Radioactive products were solubilized from the membrane preparation with Pronase and then purified by chromatography on columns of DEAE-cellulose and Sephadex G-200, followed by chromatography on Sepharose 4B (Fig. 1). The radioactivity eluted in a single symmetrical peak, in which the [^{14}C]Man-label coeluted with protein and carbohydrate. When the enzyme

Table 1. Oligosaccharides bound to *C. laurentii* envelope glycoprotein

I	Man(α1-2)Man(α1-2)Man-Ser/Thr
II	Man(α1-2)Man(α1-6)Man(α1-3) [Xyl(β1-2)]Man-Ser/Thr
III	{Gal(α1-6)}$_{10}$Galβ-Man-Ser/Thr

The linkage between Man and Ser/Thr has not yet been established, but based on studies in other species it should be Manα-O-Ser/Thr.

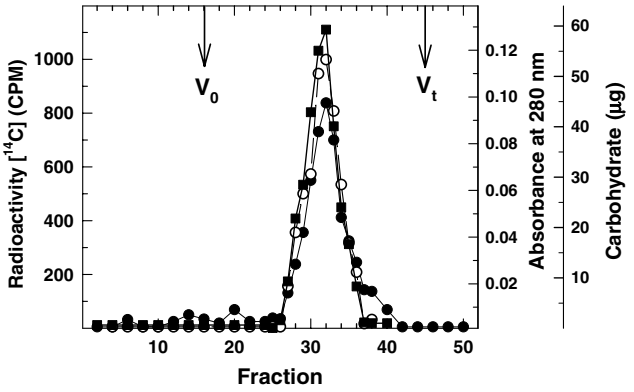

Figure 1. Sepharose 4B column chromatography of [^{14}C] Man-labeled endogenous product. *C. laurentii* membrane enzyme was incubated with GDP-[^{14}C]Man and the radioactive product was solubilized with Pronase, purified on Sephadex G-200 and DEAE-cellulose and then applied to a column of Sepharose 4B.[21] Eluted radioactivity (●-●) in cpm, carbohydrate (■-■) expressed as µg sugar, and protein (○-○) measured as absorbance at 280 nm are shown. Man was used as the standard, which elutes in the included volume. The arrows show the void volume V_0 and the total column volume V_t. (Data from Ref. 21.)

preparation was incubated with both GDP-[^{14}C]Man and UDP-[^{3}H]Gal,[21] both labels were incorporated into the insoluble endogenous products. The doubly labeled radioactive product was solubilized from the membrane preparation with Pronase, purified by chromatography on columns of DEAE-cellulose and Sephadex G-200 as above, and then applied to a column of Sepharose 4B (Fig. 2). The radioactivity eluted in a single symmetrical peak, in which both labels coincided and co-eluted. When this material was applied to a column of Concanavalin A (ConA)-Sepharose, which binds Man residues, both the [^{14}C]Man and [^{3}H]Gal-labels were retained (Fig. 3a) and were co-eluted with α-methylglucoside, which specifically releases Man-oligosaccharides from ConA. The doubly labeled material was then subjected to β-elimination, which reductively cleaves O-linked sugars from proteins, and again applied to a ConA column (Fig. 3b). In this experiment, the [^{14}C]Man label (which resides in side chains I and II, Table 1) was retained on the column, while the [^{3}H]Gal label (side chain III) did not bind and was quantitatively eluted in the void volume. The [^{14}C]Man-labeled oligosaccharides were eluted with α-methylglucoside. This demonstrates that oligosaccharide III, originally bound to ConA, was linked to the same polypeptide as oligosaccharide chains I and II. The identification of [^{3}H]Ala and [^{3}H]α-aminobutyrate after β-elimination and reduction using NaB^3H_4 further supported the presence of covalent bonds of the carbohydrate chains to protein via the hydroxyl groups of Ser and Thr.[31] The report that over 40 putative cryptococcal glycoproteins have long C-terminal S/T-rich sequences[19] suggests that the *Cryptococci* contain numerous potential membrane-bound proteins that are suitable as carriers of oligosaccharide structures I, II, III, and probably several others.

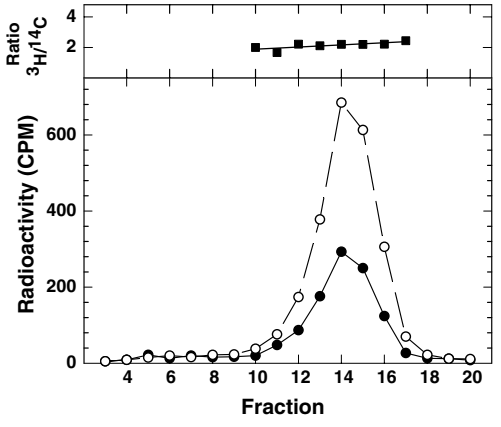

Figure 2. Sepharose 4B column chromatography of [^{14}C]Man and [^{3}H]Gal-labeled endogenous product from *C.laurentii.* Membrane enzyme was incubated with GDP-[^{14}C]Man and UDP-[^{3}H]Gal and the doubly labeled radioactive product was solubilized by incubation with Pronase. Following purification of Sephadex G-200 and DEAE-cellulose, the solubilized radioactivity was applied to a column of Sepharose 4B and eluted.[21] The figure shows the ratio of [^{3}H]-label to [^{14}C]-label in each fraction (top) and the elution of the [^{3}H]-label (○-○) and [^{14}C]-label (●-●) (bottom). The void volume of the column was in fraction 5 and V_t was in fraction 18. (Data from Ref. 21.)

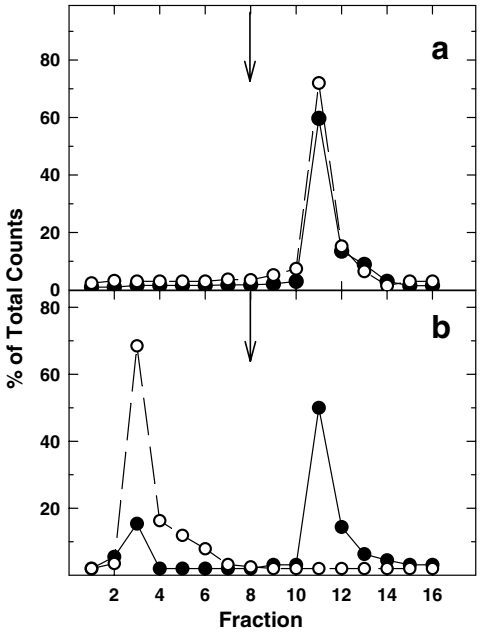

Figure 3. Concanavalin A-Sepharose column chromatography of solubilized [^{14}C]Man and [^{3}H]Gal doubly labeled endogenous product. The radioactivity in the purified endogenous product (from Fig. 2) is shown before (a) and after (b) incubation with NaOH and $NaBH_4$ (β-elimination reaction).[21] Doubly labeled radioactive product(s) containing [^{14}C]Man (●-●) and [^{3}H]Gal (○-○) was applied to a column of Concanavalin A-Sepharose (15 × 0.5 cm) and the column was eluted with buffer and then with 0.1 M α-methyl-glucoside (at the arrows). Radioactivity in each fraction was plotted as the percentage of total counts. (Data from Ref. 21.)

2.2. Acidic capsular and extracellular polysaccharides of *Cryptococcus* containing GlcA, Man, and Xyl

In addition to cell-bound and extracellular glycoproteins and mannoproteins, the distinguishing characteristic of the *Cryptococci* is the production of copious capsular and extracellular polysaccharides. The main capsular component of cryptococcal cells is a high molecular weight acidic polysaccharide composed of an (α1-3)-linked mannosyl backbone with Xyl and GlcA side chains and various *O*-acetyl substituents on the Man.[32,33] The distributions and linkages of the side chains on this glucuronoxylomannan (GXM) are species and strain dependent. The molecular masses of the polysaccharides are also species- and serotype-dependent and range from 1.7 to 7×10^6 Da[34] The capsules are antiphagocytic and contribute to the intracellular survival of the yeast. Soluble acidic polysaccharides with corresponding structures are also found in the extracellular growth medium of cryptococcal cell cultures under appropriate conditions of pH and aeration. The question remains as to whether the soluble polysaccharides are unique products or represent excess capsule shed into the medium. A recent report[35] suggests that (α1-3)-glucan is involved in anchoring GXM to the cell, but the nature of the linkage or association has not been established.

The structure of a capsular polysaccharide of a cryptococcal species isolated from an AIDS patient, originally identified as *C. laurentii*, but later reclassified as *C. flavescens*, was found to contain the linear backbone of (α1-3)-linked mannan common to all GXM isolates. Every second Man residue was substituted at C-2 with GlcA in β-linkage, and in addition, C-6 of the same or the unsubstituted Man residue contained either a β-linked Man(β1-4)Xyl-disaccharide or a Man(β1-4)-Xyl(β1-4)Xylβ-trisaccharide side chain.[16] This structure resembles that reported for the *C. laurentii var. flavescens* laboratory strain NRRL Y-1401, except for the absence of Man-capped oligosaccharide branches.[36] Membrane–enzyme preparations from *C. laurentii* do contain, however, a mannosyltransferase (ManT) that catalyzes the formation of a Man-Xyl linkage[37] and could potentially synthesize this structure in the capsule (see Section 4.1). Man(β1-4)Xyl and Man-Xyl-Xyl structures have also yet to be reported in the *C. neoformans* GXM, in which most (α1-3)-linked Man residues of the backbone are substituted by monosaccharide units of Xyl(β1-2), GlcA(β1-2) or Xyl(β1-4). Although there are correlations between serotype and infection, it has also been reported that the structures of the capsular polysaccharides can vary depending on the environment, the site of infection, and may even vary among cells in a population or during prolonged infections.[38–44] Moyrand et al.[45] have recently identified a series of genes (*Cas*) that are homologous to a gene (*Cap64*) of unknown function, but is required for capsule synthesis. The *Cas* genes are involved in Xyl branching and/or acetylation of the capsule. It is therefore possible that Xyl branching and specific acetylation reactions are regulated by yet to be identified control mechanisms that would affect overall structure and serotype of the capsular polysaccharide. Thus, under limiting growth conditions unique populations might be selected that are better able to survive in the local environment or in tissues of the infected host. Interestingly, when *C. laurentii* is grown in culture under optimal growth conditions, extracellular acidic polysaccharide production begins only in late log phase, and maximal synthesis continues long after stationary phase is reached[46] (Fig. 4). In fact, GXM synthesis continues until all of the Glc in the medium is exhausted.

2.3. Neutral capsular and extracellular polysaccharides of *Cryptococcus* containing Gal, Xyl, and Man

Capsular preparations of *Cryptococcus* contain a second more complex neutral heteropolysaccharide (GalXM) composed of Gal, Xyl, and Man. It has an (α1-6)-linked Gal backbone with side chains attached to C-3 on each second Gal residue in the backbone.[47] At this position, Man(α1-3)Man(α1-4)Gal side chains are attached in β-linkage. Side chain Man and Gal residues may also be capped by Xyl residues in β- linkage. The Xyl linkage to the terminal Man as well as to the internal Gal is (β1-3), and the linkage between Xyl and the penultimate Man is (β1-2). The average molecular mass of GalXM is 1×10^5 Da, considerably smaller than that for GXM. Although the total weight of GXM in the capsule is larger, GalXM is present in molar excess due to its smaller molecular weight.[34] The role of GalXM in cryptococcal

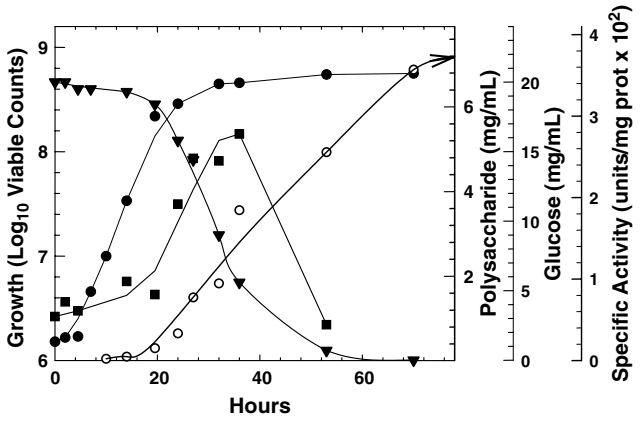

Figure 4. Growth and acidic polysaccharide production by *C. laurentii*. A *Cryptococcus* culture was grown as described.[29] At the indicated times samples were withdrawn to determine viable count (●-●) expressed as $\log_{10}$ viable count, polysaccharide concentration in the culture medium (○-○) in mg/ml, glucose remaining in culture medium (▼-▼) in mg/ml, and specific activity of UDP-GlcA decarboxylase (■-■) in μmol/min (units)/mg protein $\times 10^2$. (Data from Ref. 46.)

infection is still undefined, but it has been shown to act as an immunomodulator and induces apoptosis in T-cells through the Fas receptor and subsequent activation of caspase-8.[48] GalXM is quite different from the protein-linked neutral dodecasaccharide of *C. laurentii* (Table 1, structure III).

3. Glycosyltransferases of *C. laurentii*

3.1. Biosynthesis of substrates for glycosyltransferases

When the biosynthetic studies were initiated, *C. laurentii* was one of the few eukaryotic organisms outside the plant kingdom known to contain Xyl as a component of its acidic polysaccharide structure. Some of the sugar nucleotide substrates for capsule synthesis, including GDP-Man, UDP-Glc, and UDP-Xyl, were isolated from cell extracts of *C. laurentii*,[49] but the catalytic mechanism and the pathway leading to the biosynthesis of the Xyl-donor had not been described in fungi or other microorganisms. Studies by Ankel and Feingold[50,51] demonstrated activities for UDP-Glc dehydrogenase and UDP-GlcA decarboxylase in soluble fractions of *C. laurentii* extracts showing that the pathway led from UDP-Glc to UDP-GlcA to UDP-Xyl via decarboxylation of UDP-GlcA. The enzymatic, kinetic, and allosteric properties of these enzymes were then thoroughly characterized. In retrospect, one should have expected a dynamic control mechanism for the enzymatic steps leading from UDP-Glc to UDP-Xyl because decarboxylation of UDP-GlcA is an irreversible reaction due to the release of CO_2. Without control of the pathway, UDP-GlcA would be completely converted to UDP-Xyl and no longer available for glucuronosyl transfer. Likewise, UDP-Xyl would accumulate if it were not removed during xylosyltransfer to heteroglycan acceptors. Feedback inhibition of the dehydrogenase by UDP-Xyl represents a well-suited mechanism to counteract the unfavorable equilibrium of UDP-GlcA decarboxylation.

In fact, UDP-Glc dehydrogenase was found to be allosterically inhibited by UDP-Xyl with a Hill constant of 1.7, indicating the presence of at least two cooperative sites or subunits on the enzyme. UDP-Xyl binds at these multiple sites for feedback control by the end product of the pathway. This enzyme is also under further control by the intracellular NAD/NADH ratio and through inhibition by its own product, further preventing accumulation of UDP-GlcA. An enzyme with similar properties was reported in *C. neoformans*,[52] but inhibition by UDP-Xyl was reported as competitive rather than allosteric. More recently, UDP-Glc dehydrogenase has been cloned and purified from *C. neoformans*,[53,54] but the type of inhibition by UDP-Xyl was not reported. Deletion mutants lacking the dehydrogenase did not synthesize capsule,[45,54] which is a logical expectation, since the mutants lack the required glycosyl donors UDP-GlcA and UDP-Xyl. Although polyclonal antibodies were prepared[53] against the *C. neoformans* UDP-Glc dehydrogenase, cross reactivity studies with the *C. laurentii* enzyme were not carried out to compare the enzyme structures.

Ankel and Feingold[51] also demonstrated the next enzyme in the pathway, UDP-GlcA decarboxylase, which is responsible for the decarboxylation of UDP-GlcA to yield UDP-Xyl. The *C. laurentii* enzyme has an absolute requirement for exogenous NAD as a co-factor[51] and is inhibited by $NADH_2$. A reaction mechanism was proposed involving intramolecular oxido-reduction of the enzyme-bound substrate at C-4 of the GlcA residue that would explain the role of NAD. This enzyme-bound intermediate, representing a β-keto acid structure, would easily release CO_2 by β-elimination. The resulting 4-keto-xylosyl moiety would then be stereospecifically reduced by intermittently formed NADH to yield the final product, UDP-Xyl. This reaction mechanism was confirmed in studies by Schutzbach and Feingold.[55] The need of an absolute requirement for NAD was established that explained the catalytic isotope effect noted during the conversion of UDP-[4-^{3}H]GlcA to UDP-[4-^{3}H]Xyl with stereoselective re-addition of the hydrogen at C-4 from the enzyme-bound NAD[^{3}H]. An inversion of configuration at C-5, with complete retention of label, when the starting substrate was UDP-[5-^{3}H]GlcA, showed that the approaching proton was introduced onto the side opposite to the leaving carboxyl, prior to the stereospecific reduction at C-4. All of these reactions must occur while the substrate remains enzyme-bound because of the strict stereo-specificity of the reaction steps. Thus no reaction occurs in the absence of added NAD, in contrast to observations described for the cloned enzyme from *C. neoformans*.[56]

Kinetic studies[46,57] demonstrated that UDP-GlcA decarboxylases from both plants and *Cryptococcus* are allosteric enzymes activated by the substrate UDP-GlcA. Thus at very low concentrations of UDP-GlcA, enzyme activity is essentially null, but activity increases in a sigmoidal fashion as substrate concentration increases. Thus in vivo, as the UDP-Xyl concentration is reduced, the dehydrogenase produces more UDP-GlcA, which in turn activates the decarboxylase to synthesize more UDP-Xyl. The Hill constant for the yeast decarboxylase was found to be 1.9, suggesting at least two interacting sites or subunits in the native protein that could bind UDP-GlcA. The enzyme has an apparent K_M for UDP-GlcA of 0.2 mM so that enzyme assays below this concentration should give curved lines on Lineweaver–Burk plots.[46] Although the plant enzyme is strongly inhibited by UDP-Xyl, the yeast enzyme is less inhibited by product, but instead is inhibited by UDP-Glc. In *C. laurentii*, the intracellular concentra-

tions for substrates in the pathway in early log phase were found to be 0.3 mM for UDP-Glc, 0.006 mM for UDP-GlcA, and 0.09 mM for UDP-Xyl.[46] At these concentrations, the activity of the decarboxylase would be strongly regulated by nucleotide sugar concentrations in the cytoplasm, not even taking into account regulation by the $NAD/NADH_2$ ratio.[51] It should be noted that the specific activity of the decarboxylase is maximal shortly after the cell culture enters stationary phase (Fig. 4) when GXM synthesis as well as the requirement for Xyl is at a maximum.

UDP-GlcA decarboxylase activity has been demonstrated in *C. neoformans*[58] with no mention of the enzyme's allosteric properties, although the *C. neoformans* enzyme was shown to be inhibited by UDP-Xyl and UDP-Glc. More recently, the enzyme from *C. neoformans* was cloned in *Escherichia coli* and purified.[56] Interestingly, the purified cloned enzyme, which contained a His-tag for purification purposes, was active as a monomer, but did not appear to demonstrate allosteric properties at substrate concentrations that should clearly have shown substrate activation. Perhaps the His-tag on the protein sterically prevents the formation of dimers or higher oligomers of the enzyme, thereby negating the allosteric effect. Important information about controls of this enzyme may be lost if one studies only the recombinant protein. The cloned *C. neoformans* enzyme may also bind some *E. coli* derived NAD more tightly since it appears to have activity in the absence of added NAD. These results may indicate that there are significant differences between the *C. laurentii* and *C. neoformans* enzymes, or alternatively between the cloned enzyme and the mammalian one,[59] since the latter is also allosterically activated by UDP-GlcA. If these differences are to be exploited for the development of specific inhibitors for the *C. neoformans* enzyme, then a need for clarification of these discrepancies seems indicated.

3.2. Glycosyltransferases in *C. laurentii*

The complex variety of structures found in cryptococcal oligosaccharides and heteroglycans suggests that a large number of different glycosyltransferases are required for the synthesis of the various glycosyl linkages. In studies utilizing microsomes from *C. laurentii* as a source of enzymes,[29–31,37,60,61] glycosyltransfer was shown from nucleotide sugars to both endogenous and to well defined exogenous acceptors. By this approach it was possible to characterize and differentiate four mannosyltransferases (ManT), two xylosyltransferases (XylT), and two galactosyltransferases (GalT) based on pH optima, metal ion requirements, heat stability, and especially substrate specificity and products formed (Table 2). Each enzyme was specific for the formation of a single well-defined linkage. Furthermore, the role of glycosyltransferases in the stepwise biosynthesis of three major O-linked oligosaccharide components of cell membrane glycoproteins was established. These oligosaccharide structures are shown in Table 1, and the proposed biosynthetic pathways in the synthesis of oligosaccharides I, II, and III are shown in Figure 5. In the following, the biochemical properties of the individual enzymes will be described.

3.3. Synthesis of the Man-O-Ser/Thr linkage

The synthesis of the Man-O-Ser/Thr linkage[62–64] has not yet been established in *Cryptococcus*. However, other yeasts, mycobacteria, mammals, and other species have been shown to have the gene encoding PMT (or POMT in mammals), an *O*-mannosyltransferase that adds the first Man residue in O-linkage to the protein backbone (Fig. 5, reaction a). The yeasts *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Candida albicans* express orthologues of the PMT family. Three genes resembling those encoding PMTs have been found in *C. neoformans*,[65] but the protein-*O*-mannosyltransferase activity has not yet been demonstrated. For full activity in *S. cerevisiae*, several members of this enzyme family have to be present to form hetero- and homomeric protein complexes.[62–64] Based on the studies of protein O-mannosylation in other species, PMT is expected to span the ER membrane several times, with the active site oriented toward the ER lumen, utilizing dolichyl-phospho-Man as the donor substrate.

3.4. Formation of Man(α1-2)Man linkages by α2-ManT

α2-ManT[29,37] (Table 2) is a distinct transferase of the membrane enzyme complex of *C. laurentii* that transfers Man from GDP-Man directly to C-2 of free Man or α-methyl-Man with the formation of a Man(α1-2)Man linkage (Fig. 5a, reaction b). The enzyme can subsequently transfer another Man residue to yield a Man(α1-2)Man(α1-2)Man trisaccharide, which has the structure of a trisaccharide found on *C. laurentii* envelope glycoprotein (Table 1).[21,29]

The enzyme recognizes free Man as an acceptor as well as the non-reducing Man residues of (α1-2)-linked mannobiose, (α1-2)-linked mannotriose, Man α-linked to a methyl group (Manα-CH_3) (Table 3), and also the Manα- residues bound to the alcohol groups of Ser and/or Thr residues on a polypeptide chain.[21] Thus α2-ManT appears capable of catalyzing the de novo formation of tri- and tetrameric Man(α1-2)-linked oligomers from GDP-Man (Fig. 5a, reaction b). The di- and trisaccharide structures that are potential acceptors were shown to be present in acetolysates of glycoproteins of *C. laurentii*.[22,29] K_M and V_{max} values for the exogenous acceptors are compared in Table 3. Based on these values, the rate of mannosyltransfer is 40-times

Table 2. Glycosyltransferases of *C. laurentii*

Enzyme (reaction)[a]	Acceptor	Donor K_M (mM)	Linkage formed[b]	pH optimum	Divalent cation (optimal concn mM)	Activity after heating[c] (%)
PMT (a)	Peptide	Dol-P-Man[e]	Manα-Ser/Thr[e]	nd	nd	nd
α2-ManT (b)	Man Man(α1-2)Man Manα-CH_3	GDP-Man (0.15)	Man(β1-2)Man	7.0–7.5	Mn^{2+} (6–12)	50–57
α3-ManT (c)	Manα-CH_3	GDP-Man (0.4)	Man(α1-3)Man	7.5–8.5	Mg^{2+} (60) Co^{2+}, Mn^{2+}	49
β2-XylT (d)	Man(α1-3)Man Man(α1-3)Manα-CH_3	UDP-Xyl (1.4)	Xyl(β1-2)Man	6.0–6.5	None	60, 7[d]
α6-ManT	Man(α1-6)Man Man(α1-6)Man(α1-6)Man	GDP-Man (0.4)	Man(α1-6)Man	7.5	Mn^{2+} (12)	15
β-GalT (g)	Man	UDP-Gal (nd)	Galβ-Man	nd	Mn^{2+}	nd
α6-GalT (h)	Endogenous GP	UDP-Gal (0.12)	Gal(α1-6)Gal	nd	Mn^{2+} (15)	nd
ManT	Xylα-CH_3	GDP-Man (0.5)	Man-Xyl	7.5–8.5	Mn^{2+} (3)	49
XylT	Acidic capsule	UDP-Xyl (0.3)	Xylβ1-	7.5–8.0	None	100, 100[d]

Dol, dolichol; GP, glycoprotein; nd, not determined. The data have been obtained from published reports.[29–31,37,60,61].

[a] The letter in parentheses refers to the glycosyltransfer reaction shown in Figure 5. All assays were carried out at 25 °C.

[b] The linkages formed by glycosyltransferases are found in *C.laurentii* glycoproteins, with the exceptions of ManT and XylT that synthesize linkages found in GXM.

[c] The remaining activity after heat inactivation is shown. Heat inactivation was carried out at 38 °C for 5 min.

[d] The enzyme was heated at 38 °C for 30 min.

[e] The substrates and linkage formed have been established in *S. cerevisiae*.

faster with Man or a disaccharide acceptor than with the corresponding trisaccharide, although the affinity for the trisaccharide acceptor is increased 10-fold compared to disaccharide. This suggests a self-limiting control mechanism, in which the length of the polymer determines the overall fate of the polymerization reaction. Transfer to monomers and dimers is optimal, whereas one additional Man on the acceptor drastically decreases the reaction velocity. Thus the chance of adding still another Man residue becomes less likely as the polymer lengthens. The 10-fold lower K_M value for the trimer might signify a lower off rate for the product from the enzyme, likewise limiting further Man addition. This hypothesis could explain why in vivo side chain I is mainly a trimer and not a higher oligomer. An α2-ManT with the same specificity and similar properties has been described in *C. neoformans* using Man(α1-2)Man-CH_3 as an acceptor.[66]

3.5. Formation of Man(α1-3)Man linkages by α3-ManT

α3-ManT[37] (Table 2) catalyzes the formation of a Man(α1-3)Man linkage found in cell envelope glycoproteins (Table 1 and Fig. 5b), but can also utilize Manα-CH_3 as an alternative exogenous acceptor.[21,30,37] The α3-ManT is optimally active in the presence of 60 mM Mg^{2+}. Taking the reaction rate with 10 mM Mg^{2+} as 100%, the activity with Co^{2+} is 68%, and with Mn^{2+} is 41%. This is in contrast to other ManT (Table 2) that require Mn^{2+} and have minimal activity in the presence of Mg^{2+} (Table 2). The α3-ManT clearly synthesizes the formation of the Man(α1-3)Man linkage in oligosaccharide II (Table 1 and Fig. 5b, reaction c), but does not further elongate the disaccharide product. The disaccharide, however, is the substrate for β2-XylT described below. The α3-ManT activity has more recently been described in *C. neoformans*.[67,68] Although it was proposed as being involved in synthesis of the (α1-3)-linked backbone of the acidic capsular polysaccharide because Man(α1-3)Man was utilized as an exogenous acceptor, only a single Man was added to the exogenous acceptor, which was used in relatively high concentrations. In the second study,[68] the exogenous acceptor was a fluorescent anthranilic acid derivative of the disaccharide that has an open chain at the reducing end and thus does not resemble the (α1-3)-linked backbone of the acidic capsule polysaccharide. The *C. laurentii* and *C. neoformans* enzymes are essentially identical in all properties and our results show that they are not involved in capsule synthesis. Sommer et al.,[68] however, did purify and clone the gene encoding α3-ManT, which was named Cmt1 protein. They were able to express the activity in *Saccharomyces* and prepare deletion mutants in *C. neoformans*. They found that deletion of the activity reduced capsule synthesis in some strains of *C. neoformans* while in other strains deletions of the *Cmt1*

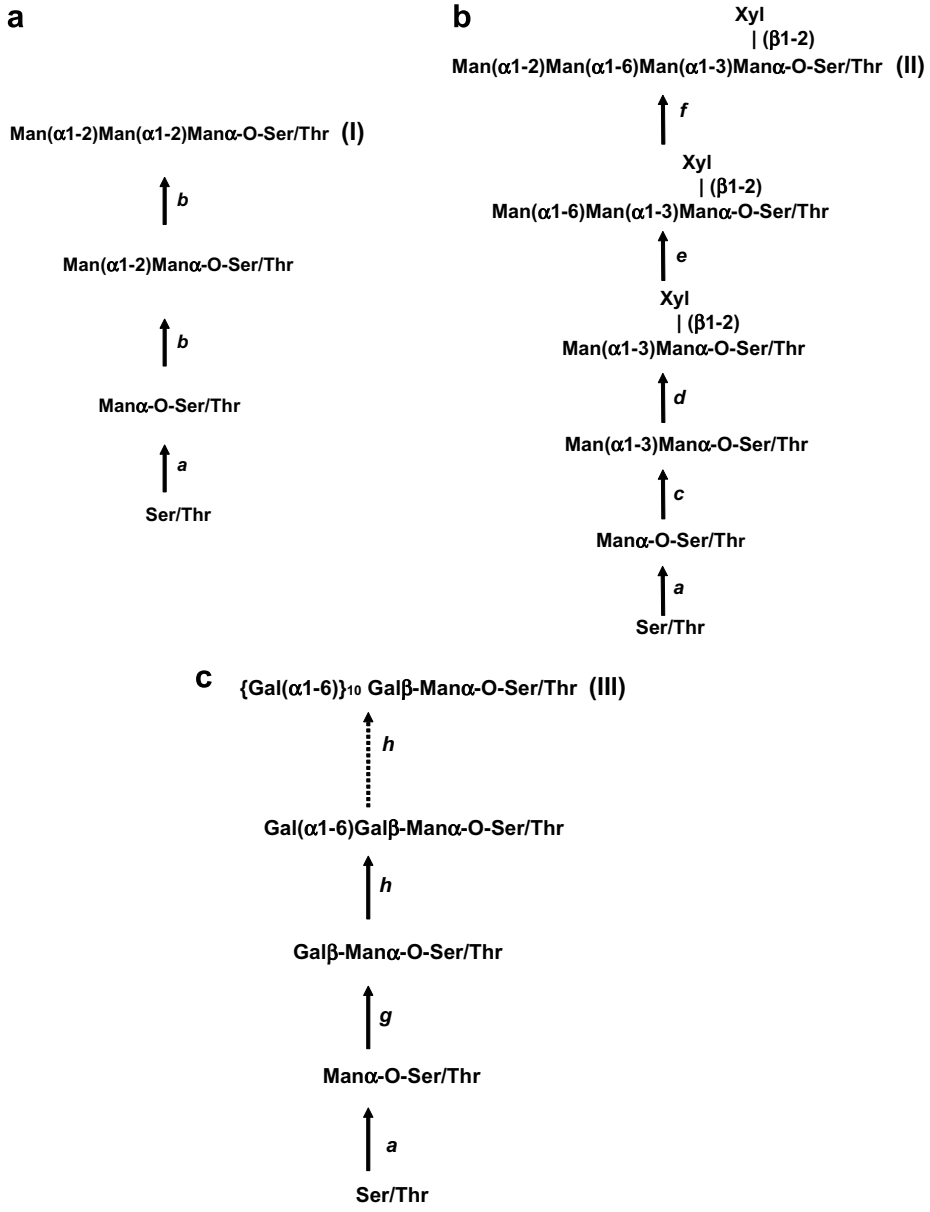

Figure 5. Proposed pathways for the biosynthesis of O-Man-linked oligosaccharides of *Cryptococcus* glycoproteins. (a) Synthesis of oligosaccharide I: Reaction a, Protein O-Man-transferase (PMT); reaction b, α1,2-Man-transferase (α2-ManT) that synthesizes the disaccharide as well as the trisaccharide (I). (b) Synthesis of oligosaccharide II: Reaction a, Protein O-Man-transferase (PMT); reaction c, α1,3-Man-transferase (α3-ManT); reaction d, β1,2-Xyl-transferase (β2-XylT); reaction e, α1,6-Man-transferase that acts after the Xyl has been added; reaction f, α1,2-Man-transferase that completes the synthesis of oligosaccharide II. Further properties of the enzymes catalyzing reactions e and f have not been characterized. (c) Synthesis of oligosaccharide III: Reaction a, Protein O-Man-transferase (PMT); reaction g, β-Gal-transferase (β-GalT), the linkage of the product has not yet been established; reaction h, processive α1,6-Gal-transferase (α6-GalT) that adds 10 Gal residues to form oligosaccharide III. Specific enzymes and their properties are listed in Table 2.

gene did not affect capsule synthesis.[68] It is possible that glycosyltransferases, or other proteins, involved in capsule synthesis are themselves glycoproteins that require the α3-ManT to process their O-linked oligosaccharides and thus maintain activity. Based on all of these results, it is our conclusion that α3-ManT is primarily involved in the synthesis of the O-linked pentasaccharide (Table 1) found on cell envelope glycoproteins.

3.6. Formation of Xyl(β1-2)Man linkages by β2-XylT

The β2-XylT[60] (Table 2) catalyzes xylosyltransfer from UDP-Xyl to both endogenous acceptor and to the exogenous substrates Man(α1-3)Man or Man(α1-3)Manα-CH_3 resulting in the formation of a branched trisaccharide (Fig. 5b, reaction d). Structural studies showed that the Xyl was transferred to the Man residue at the reduc-

Table 3. Kinetic properties of *C. laurentii* mannosyltransferases

Enzyme	Substrate	K_M (mM)	V_{max} (nmol/h/mg protein)
α2-ManT	Man	160	25
	Manα-CH_3	27	50
	Man(α1-2)Man	31	25
	Man(α1-2)Man(α1-2)Man	3	0.63
α6-ManT	Man(α1-6)Man	16	33
	Man(α1-6)Man(α1-6)Man	8	5

Data from Ref. 37.

Table 4. Acceptor specificity of β2-XylT from *C. laurentii*

Active substrates	Inactive substrates
Man(α1-3)Man	Man
Man(α1-3)Manα-Me	Manα-Me
Man(α1-3)Man(α1-2)Man	Man(α1-2)Man
Man(α1-3)Man(α1-3)Man	Man(α1-6)Man
Man(α1-3)Man(α1-2)Man(α1-2)Man	Acidic *Cryptococcus* capsule
Endogenous glycoprotein acceptor	

Data from Ref. 60.

ing end of the disaccharide to form a (β1-2)-linkage. The enzyme has no metal ion requirements for activity and is active in the presence of EDTA. The substrate specificity for exogenous substrates is given in Table 4. The enzyme can utilize tri- and tetrasaccharide acceptors, but requires the presence of a Man(α1-3)Man structure and transfers Xyl only to the Man unit penultimate to the non-reducing end of the oligosaccharide. This enzyme is responsible for the incorporation of Xyl into structure II (Table 1) found O-linked to cell envelope glycoprotein.[21,30] Transfer of Xyl to endogenous acceptor is greatly stimulated by prior incubation of the enzyme/membrane/substrate complex with GDP-Man and Mg^{2+}, which synthesizes new terminal Man(α1-3)Man structures linked to protein that can serve as acceptor sites for the β2-XylT. A brief description of the identical enzyme activity in *C. neoformans* has recently been reported.[65] Based solely on the linkage formed, however, the *C. neoformans* enzyme was proposed to be involved in the synthesis of the capsular polysaccharide GXM.

3.7. Formation of Man(α1-6)Man linkages by α6-ManT

α6-ManT[37] (Table 2) catalyzes mannosyltransfer from GDP-Man to (α1-6)-linked mannobiose and mannotriose with the formation of a new Man(α1-6)Man linkage. The enzyme is distinct from other ManT based on heat stability, K_M for GDP-Man, and differential inhibition,[37] but its role in capsule or glycoprotein synthesis has not yet been established. In the trisaccharide product of the α6-ManT reaction, using GDP-[^{14}C]Man as the glycosyl donor, only the non-reducing Man residues are radioactive, showing that a single Man is transferred to the non-reducing terminal Man residue of the acceptor. No de novo synthesis of a polymeric structure takes place. Likewise, with exogenous Man(α1-6)Man(α1-6)-Man trisaccharide as an acceptor, the only reaction product is a tetrasaccharide. Kinetic properties for both acceptors are shown in Table 3. As described above for α2-ManT, the trisaccharide has a lower K_M value than the disaccharide acceptor, but α6-ManT also has a 6-fold lower V_{max} for the trisaccharide acceptor compared to the disaccharide. Although these differences are not as pronounced as for α2-ManT, the stepwise addition of one Man residue at a time disfavors the addition of another Man residue and prevents polymerization of the saccharide chains. This α6-ManT could potentially synthesize the Man(α1-6)-linkage (Fig. 5b, reaction e) found in the O-linked protein-bound heterosaccharide (structure II in Table 1). However, synthesis of that linkage requires the branched trisaccharide Man(α1-3)-[Xyl(β1-2)]Man-R as an optimal acceptor, suggesting the possibility of another α6-ManT enzyme (Fig. 5b, reaction e). No similar α6-ManT activities have as yet been described in *C. neoformans*. The α2-ManT (Fig. 5b, reaction f) that completes the synthesis of oligosaccharide II has a unique substrate specificity, which suggests that this α2-ManT activity is distinct from the enzyme discussed in Section 3.4.[30,37]

3.8. Formation of Galβ-Man linkages by β-GalT

The β-GalT[31] (Table 2) catalyzes formation of the Galβ-Man linkage at the reducing end Man of oligosaccharide III (Table 1) and can utilize either endogenous Man-Ser/Thr or exogenous Man as an acceptor (Fig. 5c, reaction g).[31] The Galβ-Man disaccharide was isolated from reaction mixtures in which membrane enzyme complex was incubated with UDP-[^{14}C]Gal, Mn^{2+}, and free Man. No detailed studies of this enzyme have been carried out, but it was noted that no higher degree of polymerization of oligo-galactosides occurred. The next enzyme in the pathway is the processive α6-GalT (Section 3.9), which may have a substrate requirement for protein O-linked Galβ-Man as an acceptor, since it does not appear to utilize free Galβ-Man disaccharide as an acceptor.

3.9. Formation of {Gal(α1-6)}$_n$ Gal linkages by α6-GalT

The membrane enzyme complex of *C. laurentii* was shown to also contain an α6-GalT (Table 2) that catalyzes the processive transfer of multiple Gal residues from UDP-[^{14}C]Gal to an endogenous acceptor (Fig. 5c, reaction h)[31] The newly formed radioactive oligosaccharide remained particle-bound, but could be solubilized by exhaustive Pronase treatment, suggesting that it was protein-bound. Column chromatography of the solubilized product on Sepharose 4B resulted in a retarded peak in

which radioactivity, endogenous carbohydrate, and protein eluted together. The product was also retained on DEAE-cellulose and could be eluted with 0.5 N NaCl. After β-elimination, however, [^{14}C]Gal-labeled oligosaccharide was no longer retained on DEAE-cellulose. Chromatography on a calibrated Sephadex 25 column revealed a symmetrical radioactive peak of MW 2000. Structural studies of the oligosaccharide after β-elimination showed it to be a linear oligosaccharide consisting of ten (α1-6)-linked Gal residues β-linked to a Gal β-linked to mannitol (Table 1, structure III). This indicates that the Man residue was O-linked to Ser/Thr residues in the original glycopeptide. That no partially completed oligogalactosyl chains were found suggests a processive polymerase type reaction mechanism that is different from the reactions catalyzed by the ManT described above which transfer only one or two residues to either endogenous or exogenous acceptors.

4. Assembly of capsule polysaccharides

Although polysaccharide biosynthesis has been studied in *Cryptococcus* for over four decades, there are still few definitive results demonstrating the mechanisms or the enzymes involved in de novo synthesis of the yeast capsules. Enzymes that transfer single sugars with formation of linkages found in the capsular polysaccharides have been reported,[21,29–31,37,52,58,60,66,69] but with minor exceptions, the glycosyltransferases have either not been well characterized, or are more likely involved in the synthesis of O-linked glycoproteins (this review). More recently, the entire genome of *C. neoformans* has been sequenced[13] and investigations have focused on the identification of genes involved in capsule synthesis, but only a few of these have been identified.[45,65,70] Some of the *C. neoformans* enzymes involved in the synthesis of nucleotides sugars and in glycosyltransfer have been cloned,[54,65,68] but reactions involved in de novo capsule synthesis have not been clearly elucidated. Specific enzyme activities have been deleted with various measurable effects on capsule synthesis or virulence, but these studies have also not delineated the mechanism(s) of capsule synthesis.[45,71] Two enzyme activities likely to be involved in capsular polysaccharide synthesis are described below.

4.1. Formation of Man-Xyl linkages

ManT[37] (Table 2) catalyzes the transfer of Man from GDP-Man to Xylα-CH_3.[37] Free Xyl is not an acceptor for this enzyme under standard assay conditions. The linkage formed was resistant to both α- and β-mannosidases that were available at the time the studies were carried out, and the linkage was not further characterized. Several criteria, including pH optimum, heat lability, and differences in inhibition by sugars demonstrated that this ManT (Table 2) is distinct from the α2-ManT, α3-ManT, and α6-ManT (Table 2) described above. A Man(β1-4)Xyl linkage has been reported on the side chains of a *Cryptococcus* capsular polysaccharide[16] and it is possible that the ManT is responsible for synthesis of this linkage.

4.2. Formation of Xyl linkages of acidic capsules

Cohen and Feingold[61] were the first to demonstrate and describe a sugar nucleotide-dependent glycosyltransferase in *C. laurentii* that might actually be involved in capsule synthesis. They were able to show that a membrane-bound enzyme complex catalyzed the transfer of Xyl residues from UDP-Xyl to exogenously added partially dexylosylated capsular polysaccharide derived from *C. laurentii*. This XylT (Table 2) was solubilized with digitonin and shown to attach terminal Xyl residues to acceptors from *C. laurentii*, *C. neoformans*, and *Tremella mesenterica*. The enzyme was able to replace about 20% of the Xyl removed in preparation of the acceptor. The XylT did not require divalent cation, and the reaction was inhibited by UDP, UTP, and a number of other nucleoside di- and triphosphates. The polysaccharide XylT is distinctly different in specificity and properties from β2-XylT (Table 2).[60] The two enzymes differ in acceptor specificity, K_M for UDP-Xyl, pH optimum, and inhibition by nucleotides. Most conclusive are their different responses to elevated temperature. The XylT that utilizes dexylosylated polysaccharide retained 100% of its activity after 30 min at 38°, while β2-XylT lost over 90% of its activity (Table 2). A XylT with similar specificity to the *C. laurentii* XylT has been described in extracts from *C. neoformans*.[69] The *C. neoformans* enzyme activity was shown to be stimulated severalfold by Mg^{2+} ions. The enzyme did have activity in the absence of added Mg^{2+}, but the effect of EDTA on enzyme activity was not tested.

5. Conclusions

The *Cryptococci* comprise a group of encapsulated yeast that synthesize complex extracellular and capsular polysaccharides, including GXM and GalXM. These polysaccharides comprise a wide variety of different sugar linkages and contain acetyl substituents attached at various sites. In addition, the *Cryptococci* synthesize soluble and membrane-bound glycoproteins that have been less well characterized, but have also been shown to include a large number of different glycosyl linkages. The *Cryptococci* synthesize the oligosaccharides of membrane GPI anchors[72] that are potentially found on many cell-bound glycoproteins[19] and also synthesize dolichol linked and other lipid linked saccharide struc-

tures.[29,67,69,73] Cryptococcal cell wall structures contain α-linked glucans and the organisms also synthesize starch and glycogen intracellularly depending on the growth conditions of the culture.[74] Thus these eukaryotic microorganisms must contain a wide battery of glycosyltransferases that synthesize each of the numerous linkages found in their glycoconjugates, as well as enzymes necessary for the synthesis of the required sugar nucleotides and co-factors, but only a few of these enzymes have been characterized at either the biochemical or the genetic level.

We have reviewed the relevant literature for some of the enzymes that synthesize glycoprotein O-linked oligosaccharides, because this area has been neglected in most recent reviews that have instead focused on capsule synthesis. Most of the enzymes described in this review are glycosyltransferases that synthesize specific linkages shown to be present in O-linked oligosaccharide side chains of glycoproteins rather than being involved in capsular polysaccharide synthesis. It is important to note that some of the glycosyltransferases more recently reported in *C. neoformans* closely resemble the enzymes described in *C. laurentii* and are probably also involved in glycoprotein synthesis rather than in capsule synthesis. This further demonstrates that *C. laurentii* is a good model system for studies of oligosaccharide and glycoprotein biosynthesis in the *Cryptococci*, and that the results obtained carry over to more pathogenic species. Our analyses also raise some precautions, however, as results obtained using recombinant technology may not provide a complete characterization of biochemical and regulatory properties of the native enzyme forms.

It is important to note that *C. laurentii* has a large number of other glycosyltransferases that remain to be studied and characterized. For example, *C. laurentii* can synthesize Man-containing lipids by transfer from GDP-Man to endogenous acceptor lipid.[29] Although it is likely that the product(s) of this reaction is dolichyl-phospho-Man, or larger dolichyl-linked oligosaccharides, enzymes involved in the formation of these intermediates of N-linked oligosaccharides on cryptococcal glycoproteins and on phosphatidylinositol anchors have not been characterized. It is also important to reiterate that *C. laurentii* and other *Cryptococcus* species produce large amounts of acidic capsule and extracellular polysaccharides, but to date, none of the enzyme mechanisms involved in de novo synthesis of the (α1-3)-linked Man backbone have been reported. Thus capsule synthesis in the *Cryptococci* remains an enigma waiting to be solved. Finally recent work on fungal mannoproteins has opened a new window to study the immunological significance of these membrane components.[19,20,75] Evolving insights into their interactions with lectin-like surface receptors on macrophages and dendritic cells (macrophage mannose receptor MMR) point to selective targeting of oligomannoside structures on fungal pathogens. Such interaction will lead to endocytosis, degradation, and antigen presentation, followed by T-cell activation, proliferation and excretion of various lymphokines and cytokines such as interleukin 2, interferon, and others. As immune cells express several mannose receptors in addition to MMR, knowledge of their structural requirements for defined oligosaccharides on cryptococcal membrane glycoproteins will allow a broader understanding of their significance in the immune response of the human host. Since *Cryptococcosis* is one of the major lethal consequences of AIDS, the ability to fight *Cryptococcus* infection before it is established in lungs, and spreads to the brain and the nervous tissue, would be a hallmark in the prevention of death in HIV infected patients.

6. Note added in proof

C. laurentii has recently been isolated from the guano of Canada Geese that presented three of the common virulence factors of *C. neoformans* including growth at 37°. Filion, T.; Kidd, S.; Aguirre, K. *Mycopathologia* **2006**, *162*, 363–368.

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