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How Sweet it is! Cell Wall Biogenesis and Polysaccharide Capsule Formation in *Cryptococcus neoformans*

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Abstract

Cryptococcus neoformans is a pathogenic fungus responsible for severe opportunistic infections. The most prominent feature of this yeast is its elaborate polysaccharide capsule, a complex structure that is required for virulence. The capsule is intimately associated with the cell wall, which underlies the capsule and offers the organism strength and flexibility in potentially hostile environments. Both structures are primarily composed of polysaccharides, offering a glimpse of the tremendous variation inherent in natural carbohydrate structures and their multiple biological functions. The steps in cell wall and capsule biosynthesis and assembly pose fascinating questions of metabolism, enzymology, cell biology, and regulation; the answers have potential application to treatment of a deadly infection. This article reviews current knowledge of cryptococcal cell wall and capsule biosynthesis and outstanding questions for the future.

Keywords

cryptococcosis; glycan biosynthesis; glycosyltransferase; fungal virulence

INTRODUCTION

Surface structures are critical to microbial pathogenesis. In some cases, these molecules provide the pathogen with physical protection; in others, they determine whether invading microbes are detected by the host or deceive it by camouflage or mimicry. Surface components may also confer tissue specificity and guide dissemination within the host, mediate pathogen binding to or uptake by host cells, or stimulate and modulate host immune responses. Any one of these functions can determine the outcome of an infection.

The external structures of microbes include viral capsids and envelopes, parasite surface coats, and capsules and cell walls of bacteria and fungi. These protein, lipid, or carbohydrate assemblies range from relatively simple oligomers to complex macromolecules or extensive polymers. They may incorporate unique moieties not found in the host, as with core oligosaccharides of bacterial lipopolysaccharide, or at the other extreme they may mimic host structures, as with the hyaluronan capsule of group A *Streptococcus*.

How can we productively generalize about this astounding variety of pathogen surface molecules? One approach is to step back from their myriad forms and functions and instead

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examine their biosynthesis, in search of common themes that may lead to a broader biological understanding of these structures and potentially exploitable aspects of their biogenesis. Such themes include the provision of precursors for surface molecule production, the sites and topology of the synthetic machinery, the pathways for surface component biosynthesis, and the final assembly of external structures. This review examines these biosynthetic themes as they apply to the pathogenic fungus *Cryptococcus neoformans*.

Like other yeasts, *C. neoformans* is bounded by a plasma membrane and polysaccharide-based cell wall. Beyond these, it displays an extensive polysaccharide capsule that is closely associated with the wall (Figure 1) and is required for virulence (23,55,116,137); this structure distinguishes *C. neoformans* from other pathogenic fungi. This article explores biosynthesis of the cell wall and capsule to review current knowledge and stimulate consideration of the many intriguing yet unanswered questions in this fascinating area of research.

CRYPTOCOCCUS NEOFORMANS AND CRYPTOCOCCOSIS

Features of *C. neoformans*

The *C. neoformans* species complex, with subspecies *C. gattii* and *C. neoformans* (17,88,92), likely diverged from the lineage of ascomycetes such as *Saccharomyces cerevisiae* ~1000 mya (68). Historically, *Cryptococcus* isolates were categorized by serotype, based on a defined set of capsule-reactive immune sera (28). *C. gattii* includes serotypes B and C, while strains classified as serotypes A, D, or AD hybrids make up *C. neoformans*. Serotypes A and D have also been classified as varieties of *C. neoformans*, var. *grubii* and var. *neoformans*, respectively (52), although molecular typing allows more definitive strain classification (17,88,92). Genome sequence information for serotypes A and D is available (see Related Resources).

C. gattii occurs in subtropical regions and generally infects immunocompetent mammals. *C. neoformans* occurs ubiquitously in the environment and causes opportunistic infections in patients who are deficient in cell-mediated immunity. Most *C. neoformans* disease is caused by serotype A strains (154). Although *C. neoformans* came to broad attention as the cause of an AIDS-defining illness (extrapulmonary cryptococcosis), in recent years disease in HIV-negative patients has increased and presents new clinical challenges (28).

Cryptococcal Disease

Cryptococcosis is contracted upon inhalation of an infectious particle, either a spore or desiccated yeast cell (16,28,92). In the context of a healthy individual the invading microbe is either cleared, without significant symptoms but leaving immunological evidence of its presence (62), or perhaps sequestered to emerge later in the event of immune compromise (57,130). In immunocompromised hosts, however, *C. neoformans* causes a pulmonary infection that can disseminate widely, most commonly to the brain (Figure 2) and the skin (28). The former leads to a meningoencephalitis that is fatal without treatment and is the most devastating manifestation of cryptococcal disease.

C. neoformans has a substantial impact on human health; recent estimates suggest almost 1 million cases per year among HIV-positive individuals leading to over 600,000 deaths annually (115). Regions with poor access to health care experience extremely high mortality attributed to cryptococcosis in HIV-positive patients (35), with median survival after diagnosis of less than one month (54). In developed countries incidence and mortality have fallen dramatically since the early AIDS epidemic (95,101), but new groups of susceptible patients have appeared due to therapies that alter immunity (28). Furthermore, even the best current therapy does not completely clear cryptococcal infection. This necessitates long-term treatment with antifungal drugs, which exacerbates existing problems of cost, adverse reactions, and drug resistance.

Established antifungal drugs interfere with the synthesis of fungal membranes, protein, and nucleic acids. The newest compounds impair cell wall synthesis and are highly effective against some fungal infections, although not against cryptococcosis. In *C. neoformans* the capsule has attracted attention as a potential drug target because of its central role in virulence. Powerful incentive to study the biosynthesis of the cell wall and capsule thus comes from their fascinating biology combined with the grim impact of cryptococcal disease and the need for effective and available antifungal therapy.

THE CRYPTOCOCCAL CELL WALL

Fungal cell walls provide strength and rigidity, protecting cells from environmental stress and osmotic lysis while maintaining cell shape and regulating permeability (65,82). Remarkably, these powerful structures are also flexible and dynamic, allowing dramatic morphologic changes during mating, budding, and other cellular interactions and enabling cells to transition between environments as different as ecological niches and the mammalian bloodstream. Notably, mammalian cells have no analogous structures, making the cell wall a potential Achilles' heel of fungal pathogens.

Cryptococcal cell walls include glucan, chitin, and glycosylated protein. They are further enhanced by the presence of melanin (63,151), which may influence wall porosity (73), and immunogenic membrane-derived glucosylceramides (9,124). Morphologically, the wall is a two-layered structure (Figure 1): The striated inner layer has fibers arranged parallel to the plasma membrane, while the outer layer appears more particulate (122,131).

Precursors for Cell Wall Glycosylation

Like other glycans, cell wall carbohydrates are synthesized in a template-independent process by the coordinated action of glycosyltransferases, with their final structures depending on enzyme activities, the levels of sugar donor molecules, and the availability of acceptor substrates. Nucleotide sugars serve as the activated donors for this synthesis, including UDP-glucose for glucan, UDP-*N*-acetylglucosamine for chitin, and GDP-mannose for protein modification. Synthesis of the two UDP sugars has not been directly examined in *C. neoformans*; GDP-mannose synthesis is discussed below.

Cell Wall Glucans

The cryptococcal wall contains abundant α -glucans, mainly 1,3 linked (78) and likely generated from UDP-glucose by a membrane-bound α -glucan synthase, Ags1p (121). Ags1p homologs in other fungi are cell surface proteins thought to function in priming, polymerization, and translocation of glucan (67). Analysis of *ags1* Δ mutants, together with immuno-electron microscopy (EM) of normal cells, suggests that the inner layer of the cryptococcal wall consists of an alkali-insoluble meshwork of β -glucan and chitin and that the outer layer corresponds to an alkali-soluble fraction containing mainly α - and β -glucans; loss of the α 1,3-glucan decimates this structure. *ags1* Δ cryptococci partially compensate with increased chitin and β -glucan redistribution (122), but their cell walls are still malformed, hypertrophic, and fragile. These cells display no surface capsule, although they still shed capsule polysaccharides (122). This indicates a required association of capsule with wall α 1,3-glucan, although we do not yet know whether this interaction is direct or indirect via another cellular component. A recent report suggests that chitin or chitosan participates in capsule association with the cell wall (126); the relationship between these observations remains to be determined.

Significant β 1,3-glucan is also present in cryptococcal cell walls (78). In other yeast this occurs as polymers of ~1500 residues branched by β 1,6-linkages (123). Its synthesis from cytosolic UDP-glucose is mediated by a plasma membrane-localized complex that includes a regulatory

subunit, Rho1p, and a likely catalytic subunit, Fks1p. Glucan synthase activity is effectively inhibited by echinocandin compounds, which are now used to treat multiple fungal infections (81). Unfortunately, these compounds are not effective in *C. neoformans* (1). This is puzzling because the single Fks1p in *C. neoformans* is essential (142) and is susceptible to these inhibitors in vitro (96). Further study may enable the design of related compounds or combination therapy to allow use of these well-tolerated drugs for cryptococcal infection (37,120). Until then, the echinocandins still provide a powerful example of extracellular polysaccharide synthesis as a suitable drug target in fungi.

A third glucan present in fungi, including *C. neoformans*, is β 1,6 linked. This glucan is typically shorter and more highly branched than the others. It can be covalently linked to β 1,3-glucan, chitin, and cell wall proteins, integrating these cell wall components via multiple physical connections that confer wall elasticity and strength (65,82). Cryptococcal β 1,6-glucan is more abundant relative to β 1,3-glucan than in other yeasts (10,78) and has been observed in covalent linkage to protein (135). β 1,6-glucan synthesis is complex and depends on multiple genes; in several ascomycetes a dominant role is played by *KRE5* (89,134). Deletion of the cryptococcal homolog of this gene eliminates soluble β 1,6-glucan and severely compromises cell integrity (N.M. Gilbert & J.K. Lodge, unpublished data).

Cell Wall Chitin and Chitosan

Chitin is a relatively minor cell wall component, but its hydrogen-bond-stabilized association into microfibrils strengthens the wall structure (65). This linear polymer of β 1,4-*N*-acetylglucosamine is generated from cytosolic UDP-*N*-acetylglucosamine by membrane-associated synthases that translocate the growing chain across the cell membrane. *C. neoformans* encodes eight putative chitin synthases and three potential regulatory proteins, presumably coordinated to spatially and temporally regulate chitin deposition (6). Systematic gene deletion showed that although no single gene of this group is essential for viability, one synthase (Chs3p) and one regulator (Csr2p) play a dominant role in cell integrity and cell wall function; deletion of either gene yields stress-sensitive cells with morphological alterations and the inability to retain melanin (6).

Chitin can be deacetylated to produce chitosan, a more soluble and flexible polymer. *C. neoformans* has unusually high levels of chitosan during vegetative growth compared with model yeasts; chitosan content may exceed chitin content by up to 10-fold (6). In *chs3Δ* and *csr2Δ* mutants the chitosan fraction is significantly reduced, although an increase in chitin partly compensates for this change. It may be that only chitin formed by specific synthases may be subsequently deacetylated (6). Genes encoding three putative chitin deacetylases occur in *C. neoformans*. A triple mutation (*cda1Δ cda2Δ cda3Δ*) is required to eliminate chitosan; this mutant has increased chitin and exhibits defects in cell integrity and separation (5).

Cell Wall Proteins

Several classes of cell wall glycoproteins occur in fungi. One is composed of glycosylphosphatidylinositol (GPI)-linked proteins that have been transferred, with part of their anchor glycan, to covalent linkage with β 1,6-glucan (64). A second group of proteins crosslinks β 1,3-glucan, while members of a third group associate with other wall proteins via disulfide binding or charge interactions (156). Studies in *C. neoformans* have focused on the GPI-anchored proteins.

Nascent glycoproteins destined for the cell wall are glycosylated as they transit the endoplasmic reticulum (ER) and Golgi (reviewed for *C. neoformans* in References 85 and 91). Some proteins receive GPI anchors, which are similar in *C. neoformans* and *S. cerevisiae* (50). In silico analyses of the cryptococcal genome (94) predict over 50 GPI-modified proteins (39,91).

Twenty-nine of these were identified in proteomic analysis of wall polypeptides released by protease or β -glucanase digestion (45); the latter treatment presumably released proteins that were covalently linked to glucan via a GPI remnant. This was demonstrated for a phospholipase B1 important for cryptococcal virulence (29): Treating cells with β 1,3-glucanase released the protein modified with β 1,6-glucan. The naturally secreted phospholipase was similarly modified, suggesting wall linkage as an intermediate in protein release (135). Another notable GPI-anchored protein in *C. neoformans* is the homolog of *S. cerevisiae* Gas1p. Gas1p remodels β 1,3-glucan, consistent with cell wall localization (91).

In addition to GPI anchoring, cell wall proteins are extensively modified with O-linked and N-linked glycans. Fungal O-glycan synthesis is initiated when protein mannosyltransferases (Pmts) modify serine or threonine residues, often present in clusters (150). Three cryptococcal Pmts have been identified; the disruption of one of these yielded stress-sensitive mutants with abnormal morphology, defects in cell separation, decreased protein mannosylation, and reduced virulence (112). Although *C. neoformans* O-glycans have not been directly characterized, biochemical studies in a related nonpathogenic species, *Cryptococcus laurentii*, identified three classes: linear α 1,2-linked trimannose structures, oligomannose chains modified with xylose, and extended chains of α 1,6-galactose linked to a single mannose (133). Given the variability of O-glycosylation across fungi (60) it is hard to predict what structures will be found in *C. neoformans*.

Genome analysis suggests that the core N-glycan added to proteins in the cryptococcal ER consists of nine mannose and two N-acetylglucosamine residues, a truncated structure lacking the usual distal glucose residues (132). Once attached, fungal N-glycans may be core modified, typically generating structures of ~15 residues, or more extensively outer chain modified to form mannan. Surprisingly, genes encoding the machinery for outer chain synthesis are lacking in *C. neoformans* (85,91). Although this may indicate that cryptococcal N-glycans are not extended, more interesting possibilities are that either the synthetic machinery or the structures themselves are unique. In support of the latter, cryptococcal proteins may be modified with sialic acid (127) or xylose (M.C. Reilly & T.L. Doering, unpublished data), neither of which is present in model yeast. Structural analysis of mature cryptococcal N-glycans will be needed to resolve this issue.

Major interest in cell wall mannoproteins derives from their critical role in stimulating T-cell responses (91). One such protein, MP-98 (90), is the chitin deacetylase Cda2p (5); another, MP84, is also a polysaccharide deacetylase (14). These roles in polysaccharide modification are consistent with the wall location of mannoproteins (147).

The cell wall is a dynamic structure that undergoes constant remodeling through variation in the distribution of components and their cross-linking. This allows cells to maintain integrity through the extraordinary morphologic transformations of mating and cell division and to withstand challenges from external stresses such as variations in temperature and osmotic conditions. In *C. neoformans* wall morphology differs between serotypes (122) and changes upon mutation (122), during infection (49), and with culture age, nutrient status, and temperature (A. Yoneda & T.L. Doering, unpublished). These dramatic and coordinated changes must be exquisitely regulated, an area of active interest (38,61,87,114,140).

The composition and synthesis of spore walls is a related but relatively unexplored area of cryptococcal research. The surfaces of these potentially infectious particles react with antibody to a capsule polysaccharide and with lectins that bind polymers of mannose and N-acetylglucosamine (16), suggesting that they share structural motifs with cell walls and capsule. Further analysis should illuminate the synthetic relationships between these entities.

It has been assumed that cell wall components and organization in *C. neoformans* resemble those of model yeasts. However, whenever these processes have been examined in detail, intriguing differences have come to light. Given the fundamental importance of cell walls, the validation of β -glucan synthesis as a drug target, and the immunological role of cell wall mannoproteins, it is crucial to pursue the many unanswered questions in this area.

CRYPTOCOCCAL CAPSULE

In a clinical setting, the distinct halo appearance of the cryptococcal capsule (Figure 2*b*) is diagnostic for cryptococcal disease. In the research world the halo immediately stimulates questions about the nature of this striking material, its arrangement, and its biosynthesis. Beautiful scanning and thin-section EM (34, 136) and dramatic quick-freeze deep-etch images (119, 155) provide some hints, such as the intertwined fibers of capsular material emanating radially from close association with the cell wall (Figure 1), but many compelling questions remain.

Almost all investigations of capsule polysaccharides have been based on material that is shed from the cell, most often using polysaccharides precipitated from culture supernatant fluids. This readily obtained material has been used for immunological studies (46,103,118) and structural analysis (33,145) and to generate monoclonal antibodies (mAbs) that have been key to the study of capsule (11,44,109). But does it represent the capsule as displayed upon cells? A recent report (53) addressed this question by physically comparing polysaccharides that were actively released from cells with dimethyl sulfoxide (DMSO) or by radiolysis with shed polysaccharides recovered from culture medium by filtration or precipitation. Although the samples differed in multiple properties, the relationship between shed material and cell-associated polysaccharides remains enigmatic. For example, cell-associated material released by irradiation was most similar to shed material recovered by filtration in terms of average molecular mass, radius of gyration, rigidity, and zeta potential. These two populations differed in mAb reactivity, however. By that criterion the radiation-released material was most similar to shed polysaccharide precipitated from culture medium. Notably, the composition of all four samples was similar when normalized to the mannose backbone, although the actively released populations included large quantities of glucose, presumably derived from the cell wall (53). Shed material somewhat resembles cell-associated polysaccharide microscopically (99), but defining their relationship may need to await mechanistic understanding of capsule assembly and release. Until then we must be careful to interpret experimental results in the context of the polysaccharide origin.

Capsule Components

Early studies of shed capsule indicated that it consisted of two large polysaccharides along with mannoproteins (31,144). Concerted efforts by Cherniak and others defined the major capsule polysaccharide as a linear mannan substituted with glucuronic acid and xylose, descriptively named glucuronoxylomannan (GXM) (Figure 3*a*) (13,32,71). The degree of xylose substitution is strain dependent (33) (Figure 3*a*); acetylation of mannose in the GXM backbone also varies between strains and can exceed 60% (13,79). GXM as shed from the cell is a large molecule (1–7 MDa) (32,99) that comprises about 90% of the capsule mass (32). Mutant strains lacking surface GXM are avirulent (23), a central observation in the characterization of this polymer as a major virulence factor of *C. neoformans*. Immunological studies support the importance of GXM in the pathogenesis of cryptococcal infection (46, 102,103).

The second capsule polysaccharide is also a repeating polymer (Figure 3*b*), with a molecular weight of $\sim 10^5$ Da (32, 99). This polysaccharide was termed galactoxylomannan (GalXM) based on early composition analysis; we propose glucuronoxylomannogalactan (GXMGal) as

a new name that reflects the recently revised structure of a galactose backbone with side chains containing mannose, xylose, and glucuronic acid (69) (see sidebar, A New Structure for a Familiar Polysaccharide) (Figure 3). Studies of GXMGal function have been limited because it is harder to purify than GXM (153), and mutants that produce altered GXMGal became available only recently (83, 105). Nonetheless, multiple reports suggest that this polymer also interferes significantly with the host immune response (21, 43, 118).

Models of Capsule Polymer Synthesis

A central challenge in deciphering cryptococcal capsule construction is that we do not yet know the overall scheme of polymer synthesis. The repeating structures of GXM and GXMGal raise the possibility that individual subunits are generated and then linked together (Figure 4a), reminiscent of bacterial peptidoglycan synthesis. Alternatively, extended segments of the polymer might be generated, either in mature form (as with the processive synthesis of some bacterial capsule polysaccharides) or as an extended precursor that is then sequentially modified (Figure 4b), analogous to biosynthesis of chitosan or mammalian heparan sulfate. Numerous variations on or combinations of these schemes can also be envisioned, although there is a dearth of definitive evidence for any synthetic model.

One pertinent question is the precision of the polysaccharide repeats. NMR analysis of shed GXM has defined six structural reporter groups (Figure 3a), which occur in reproducible combinations in various strains (33). Importantly, such analysis cannot distinguish whether the observed structural heterogeneity results from the coexistence of multiple reporter groups within individual GXM molecules, from distinct molecules on the same cells, or from different cells within a population. This question was addressed by using mass spectroscopy to assess GXM fragments produced by acid hydrolysis. The data indicated that some repeats were underxylosylated, leading the authors to conclude that individual GXM molecules were created by linkage of different subunits and to suggest a model similar to Figure 4a (100). This study is certainly thought-provoking, although the observed microheterogeneity of the GXM structure could also arise during either processive synthesis or sequential modification of a glycan (72).

Fully defining capsule synthesis presents a formidable challenge, due partly to the tremendous variability of GXM structures between strains (33). Capsules on cells within clonal populations may also differ, as evidenced by anti-GXM antibody reactivity and polysaccharide analysis (27,56). Adding complexity, *C.neoformans* readily undergoes microevolution (30,51,70), potentially enabling environmental pressure to select specific capsule variants during infection (56). Reversible phenotypic switching has also been observed in multiple strains, with differences between switch variants including structural changes in GXM (76). A final twist is offered by the suggestion that cellular mechanisms may edit residues aberrantly incorporated into GXM (93). It is likely that these multiple levels of variability reflect differential regulation of the capsule synthetic machinery, a fascinating prospect that is largely unexplored.

Synthesis of Capsule Polysaccharide Donors

On the basis of the known composition of the capsule polysaccharides (Figure 3), we anticipate that their synthesis would require GDP-mannose, UDP-galactose, UDP-glucuronic acid, and UDP-xylose donors. UDP-glucuronic acid is generated from UDP-glucose by dehydrogenation; it is then either consumed in synthetic reactions or decarboxylated to UDP-xylose. Early experiments in *C. neoformans* and *C. laurentii* detected both activities (2,3,74); the *C. neoformans* enzymes responsible were subsequently cloned and expressed for biochemical studies (7,8). Deletion of the gene encoding UDP-glucose dehydrogenase (*UGDI*) yields cells with no UDP-glucuronic acid or UDP-xylose (66), demonstrating that no other pathways exist for their synthesis. The mutants appear acapsular, although trace surface

material is observed by EM, and they are stress- and temperature-sensitive, indicating poor cell wall integrity. They are also misshapen and are avirulent in animal models of cryptococcosis (66,106).

Identification of the cryptococcal UDP-glucuronic acid decarboxylase (Uxs1p) by expression studies allowed independent examination of UDP-xylose formation (7). Deletion of *UXS1* yielded cells lacking UDP-xylose, which accordingly synthesized GXM with no xylose residues. These cells bore capsules of reduced size with deformed fibers and were avirulent in animal models. However, they did not display the cell integrity problems of *ugd1Δ* cells, which lack both UDP-glucuronic acid and UDP-xylose (66,107). Those defects presumably result from alterations in other glycoconjugates that require UDP-glucuronic acid for synthesis.

As part of a systematic mutagenesis and virulence study, Janbon and colleagues (108) deleted *UGE1*, which encodes a putative epimerase that generates UDP-galactose from UDP-glucose. Capsule polysaccharides of these mutants lacked galactose, suggesting the absence of GXMGal. Staining with anti-GXM mAbs indicated the capsules were enlarged, raising the possibility that GXMGal helps organize capsule structure. The mutants also demonstrated a defect in cell wall integrity, suggesting an independent role for galactose addition in wall construction (105), perhaps via protein modification (143). Finally, the authors noted aberrant organ colonization and proposed a role for GXMGal in this process (105).

Synthesis of GDP mannose has not been studied directly, but one study addressed phosphomannose isomerase, a critical enzyme in mannose metabolism (152). This protein generates a precursor for the synthesis of GDP mannose; the latter is required for all forms of protein glycosylation as well as for GXM and GXMGal synthesis. Deletion of the corresponding gene yielded cells that were abnormally shaped, hypocapsular, and avirulent in animal models.

Terminal galactofuranose is a minor component of GXMGal (77,145). The cryptococcal protein responsible for synthesis of the corresponding donor molecule, UDP-galactofuranose, has been identified (12), but deletion of the corresponding gene produced no obvious phenotypic alterations besides the expected change in GXMGal components (H. Liu & T.L. Doering, unpublished data).

Enzymology of Capsule Synthesis

Carbohydrate structures result from a complex interplay between glycosyltransferase activities, the levels of sugar donors, and acceptor availability. Further, glycosyltransferases are typically specific for both the reactants and the linkages created. Examination of capsule polysaccharide structures (Figure 3) suggests that a variety of glycosyltransferases are required for their synthesis (42), but to date only one has been demonstrated to act in this process (83). This sharp contrast with our more detailed understanding of cell wall synthesis is partly due to lack of precedent. *S. cerevisiae*, for example, does not use xylose or glucuronic acid and performs little galactosylation. It is also currently impossible to predict glycosylation activity from sequence data.

The only enzyme proven to directly mediate capsule synthetic reactions in *C. neoformans* is a xylosyltransferase called Cxt1p, which was biochemically purified from cryptococcal membrane extracts (84). In vitro Cxt1p catalyzes UDP-xylose-dependent addition of xylose in β 1,2-linkage to the reducing sugar of a mannose- α -1,3-mannose dimer (84). Dramatically, *cxt1Δ* mutants lacked all β 1,2-linked xylose on GXMGal (Figure 3b), indicating that Cxt1p is solely responsible for this modification. The β 1,3-linked xylose on GXMGal was also absent; although Cxt1p may catalyze linkage of this residue directly, it is more likely that the enzyme mediating β 1,3-linked xylose addition requires the prior addition of the more proximal β 1,2-

linked xylose (Figure 3*b*), suggesting the temporal order of these two reactions. Beyond the striking lack of xylose in GXMGal, *cxt1Δ* lacked ~30% of the β1,2-linked xylose on its GXM (dominated in this strain by the M1 structural reporter group) (Figure 3*a*) (83). The partial loss suggests that multiple enzymes act in this synthetic step, a frequent theme in fungal glycan synthesis (85). The mutant also lacked all xylose on glycoinositolphosphoceramides, revealing Cxt1p as a fascinating trifunctional enzyme (20).

The GXM backbone is highly 6-O-acetylated, a feature important for polysaccharide antigenicity (13,31,86). A mutant screen based on capsule reactivity with mAbs implicated *CAS1* in GXM acetylation; this gene encodes a multimembrane-spanning domain protein with homology to glycosyltransferases. Although the specific function of Cas1p is not known, GXM in *cas1Δ* cells was not acetylated (79). An analysis of GXM structures in this work further suggested that acetylation follows side chain addition and occurs preferentially on unsubstituted mannose residues, with acetylation of glucuronylated mannose as a second choice. [GXMGal, which is also acetylated (145), was not examined.] Curiously, *cas1Δ* was hypervirulent compared with isogenic wild-type strains (79).

Several additional enzyme activities identified in *C. neoformans* might participate in capsule synthesis. Deletion of the gene encoding an α1,3-mannosyltransferase (Cmt1p) did not yield a morphologic capsule defect, although structural analyses were not performed (136). Cmt1p may act in GXMGal synthesis, may be one of several functionally redundant enzymes, or may play an unrelated role in the cell. Another potential participant is Cps1p, a hyaluronic acid synthase that is required for virulence and whose product may act in capsule formation (22, 80). Additional glycosyltransferase activities detected in *C. neoformans* and *C. laurentii* (133,148,149) have not been purified or studied at the molecular level.

The products of four *CAP* genes [*CAP10*, *CAP59*, *CAP60*, and *CAP64* (23–26)], identified by complementation of acapsular mutants (75), are strong candidates for proteins with enzymatic roles in capsule synthesis. *CAP64* and five homologs were implicated in GXM xylosylation and acetylation (104); one of these homologs copurified with Cmt1p. *CAP60* and *CAP59* are homologous to *CMT1*. *CAP10* (along with four other cryptococcal genes) is highly homologous to *CXT1* (84); one of the other homologs also encodes a functional xylosyltransferase (J.S. Klutts & T.L. Doering, unpublished data). Thus, although specific functions of the original *CAP* gene products have not been demonstrated, these data and their predicted structures suggest they participate directly in capsule construction, perhaps within multienzyme complexes.

The Site of Capsule Synthesis

A critical question for understanding capsule construction is the site of synthesis. One potential location is within intracellular organelles, analogous to synthesis of cell wall mannan or plant pectin (4). Alternatively, capsule polymers could be synthesized at the cell surface, similar to bacterial polysaccharides, plant cellulose, or mammalian hyaluronan. Finally, steps in synthesis could be spatially separated, perhaps depending on polymer type, stage of synthesis, or regulatory signals. Indirect evidence that capsule synthesis occurs within membrane-bound organelles comes from studies of nucleotide sugar transport. Most nucleotide sugar donors are generated in the cytosol, while the eukaryotic enzymes that use them frequently act within membrane-bound organelles. Cells solve this topological problem with specific transporters to move sugar donors into appropriate compartments. A UDP-galactose transporter that was identified in *C. neoformans* by homology (105) has the expected transport activity (A. Ashikov, R. Gerardy-Schahn, & T.L. Doering, unpublished) and is required for GXMGal production and normal virulence in mice (105). Two GDP-mannose transporters were similarly identified in *C. neoformans* and shown to be functional; deletion of one of these produced hypocapsular cells with a defect in capsule induction (36). The requirement for these transporters to achieve

normal capsule synthesis suggests that at least the back bones of GXM and GXMGal are made within membrane-bound organelles, although it remains formally possible that this effect is indirect (e.g., via glycosylated enzymes). UDP-glucuronic acid and UDP-xylose transporters have not been studied, although the genome includes candidate genes (15).

A direct route to identifying the site of capsule synthesis is to localize proteins involved in this process. However, many of the enzymes required act upstream of multiple synthetic processes and would not serve as specific markers for capsule formation; Cxt1p, the only molecularly confirmed capsule glycosyltransferase, has not yet been localized. As an alternative approach, previous investigations have sought to localize the capsule polymer itself, proposing a variety of locations and structures for synthesis (reviewed in Reference 157). We recently took a cell biological approach to this question and generated a cryptococcal mutant that is conditionally defective in classical secretion. Reducing secretory vesicle fusion with the plasma membrane (Figure 5a arrow) led to vesicle accumulation; immuno-EM indicated that these vesicles contain cargo that reacts with anti-GXM mAbs (Figure 6), suggesting that significant steps in capsule synthesis occur intracellularly (157).

Intracellular synthesis of even the largest GXM polymers is conceptually possible, as their calculated volume can be accommodated in typical post-Golgi vesicles (T.L. Doering, unpublished calculations). Measured radii of gyration of shed or actively released GXM (53) support this assessment, and several studies hint that it occurs. Thin-section EM images show material that appears similar to capsule fibers present in secretory vesicles fusing with the plasma membrane (Figure 7a). Consistent with this image, the pattern of deposition of colloidal gold-labeled mAbs to GXM suggests binding to linear fibers, both on the cell surface and in secretory vesicles within the cytosol (Figure 7b). These images do not definitively establish this model, but they provide intriguing visual evidence that capsule fibers are generated internally.

Capsule Architecture

Imaginative approaches have been used to examine the elaborate capsule architecture, with several themes emerging from the data. First, the capsule varies in porosity, as demonstrated by diffusion into the capsule of sized populations of beads (59). This physiologic heterogeneity is complex, as indicated by variable porosity at the site of emerging buds (119) and growth-dependent differences in zones of bead or antibody exclusion (59). Related to this theme is the emerging concept that concentric regions of the capsule differ in physical properties. This is supported by variable patterns of allowed permeation and binding of anti-GXM mAbs and Fab fragments (59,97) and by density measurements (59). These results are echoed by studies of complement binding to capsule, which is also restricted to specific zones of the structure, depending on the serum source (58,161).

Further support for the existence of distinct capsule regions is offered by DMSO release of capsule from cells; such studies consistently show an internal DMSO-resistant region that remains cell associated (18,59). More recently, consecutive bursts of γ -irradiation have been used to sequentially release populations of capsule, suggesting the existence of multiple capsule regions of similar composition but different density (18,97). These studies assume that the capsule layer released by each burst is the most superficial one; it is conceivable that more susceptible regions are internal but this has not been directly addressed.

Capsule architecture is dynamic, like that of the cell wall. For example, capsule growth may be observed in vitro in capsule-inducing media (160) or as dramatic enlargement in animal tissues (125,160). This expansion is likely relevant to pathogenesis, as large capsules inhibit host phagocytosis (41) and cells unable to induce capsule formation exhibit reduced virulence (36). The capsules of cells that have been grown in vitro under inducing conditions are more

dense than those from cells with small uninduced capsules (59). Capsule density also increases with time of induction, as assessed by quick-freeze deep-etch imaging and susceptibility to irradiation (97,119). The capsules on cells isolated from animals are similar in size to those on in vitro-induced cells, but the in vivo-grown capsules are even more dense and have an extended DMSO-resistant zone (59). This finding must be considered when in vitro induction is used to model in vivo events.

Most studies of capsule arrangement have focused on GXM because it is the more abundant polymer and because mAbs to it are readily available. mAbs to GXMGal were reported in 1990 but are no longer available (146); an exciting recent development is the production of polyclonal antibodies to this polymer (40). Immunolocalization with these antibodies placed GXMGal at the cell wall in acapsular cells and the outer capsule in wild-type cells (40).

Our understanding of capsule architecture is hampered by the absence of information about the physical interactions of the two capsule polymers and whether either is branched or otherwise modified. Nonetheless, creative and elegant studies in this area have given us an excellent foundation on which to build with newly available methods and reagents.

Capsule Transport and Assembly

Evidence reviewed above suggests that at least some capsule components are manufactured within the cell, although the structure itself is extracellular. This raises the related questions of how capsule material exits the cell, and how it is incorporated as the structure grows. Studies of *C. neoformans* mutants impaired in classical secretion show that secretory vesicle contents react with anti-GXM mAbs (157). The membranes of such vesicles, by definition, fuse with the plasma membrane; this would release the vesicle contents, which were produced in secretory organelles, outside the cell, where they would be available for incorporation into growing capsule (Figure 5a).

Nonclassical secretory pathways could also export capsule components from an intracellular site of synthesis. Heterogeneous membrane-bound vesicles in the supernatant fraction of cultured cryptococcal cells have been reported; these fractions contain anti-GXM mAb-reactive material as well as membrane lipids and proteins of both cytosolic and membrane origin (128,129). There are two ways that cells can release intact vesicles. In one, the plasma membrane blebs outward, capturing cytosolic material to form extracellular vesicles (Figure 5b) (110). In the other, vesicles may be generated within another intracellular membranous structure, as with formation of mammalian multivesicular bodies (141). When the outer compartment fuses with the surface, the inner vesicles are released (Figure 5c). Multivesicular structures in *C. neoformans* have been observed (128,138,139; A. Yoneda & T.L. Doering, unpublished data), but their contents have not been probed for capsule components; they could be related to other processes observed in *C. neoformans* such as autophagy (47,113). Vesicles released intact from cells, whether produced via blebbing or multivesicular body formation (Figure 5b,c), would have contents that are derived from the cytosol (47). In contrast, classical secretory vesicles (Figure 5a) bear luminal components. The anti-GXM mAb-reactive cargo in these classes of vesicles must therefore originate in distinct compartments and may include different populations of capsule-related molecules.

When interpreting all these studies we must be aware of the limits of mAb localization techniques. These Abs have been powerful tools for the study of *C. neoformans*, and several have been analyzed in detail (44,86,109,159), but the specific epitopes they recognize are not known and may not be exclusive to capsule components. Thus, although antibody binding is consistent with the presence of capsule material, it might also indicate the presence of other cellular glycoconjugates with shared structural motifs (20). Further, antibody binding does not indicate the size or composition of the polymers present. Direct analysis of vesicle contents

will ultimately be needed to illuminate the location of capsule biogenesis and the export of capsule polysaccharides.

Once capsule materials exit the cell, they must be incorporated into the existing structure. One circumstance in which this must occur is new bud formation. Capsule around buds may differ in arrangement from that of mature cells, but it is clearly present on the earliest identifiable budding structures (34,119) (Figure 1). In pulse-chase studies of fluorescently tagged *C. neoformans* we demonstrated de novo formation of cell wall and capsule around nascent buds (119), studies later reproduced by others (162). These experiments demonstrate the polarized delivery of polysaccharide components to the bud site, consistent with observations on cell wall synthesis in *S. cerevisiae*.

Another occasion for capsule assembly is the growth of existing capsule on mature cells. In a study based on antibody labeling, pulse-chase EM-autoradiography, and quick-freeze deep-etch imaging, we suggested that new capsule material is incorporated close to the cell wall, displacing existing material outward while intercalating with existing fibers to increase the overall density of the structure (119). Other investigators noted that complement used to covalently label proximal capsule regions was not displaced distally over time; they concluded that addition of new material to the capsule instead occurs at the distal edge, while noting that intercalation throughout the structure is likely (162). In this study radiolabeling indicated that newly incorporated xylose was most susceptible to capsule release by irradiation (162), supporting a model of progressive capsule cross-linking (119), but the capsular site of radiolabel incorporation was not assessed.

The reagents potentially complicate interpretation of both studies of capsule expansion. For example, even low amounts of antibody might alter the conformation and arrangement of capsule fibers; antibody may also redistribute, although this was not observed in budding studies (119,162). Complement binds the cell wall in addition to the capsule (162) and could label an inner capsule layer that is not displaced while antibody-binding layers translocate outward; this model is supported by the observation of concentric rings of complement and antibody binding (162). Finally, both investigations used capsule induced in vitro, although this differs substantially from capsule expanded in vivo (59). One fascinating study that was performed in vivo suggests a rapid shift in capsule antigenicity during infection, with a later epitope present in an extracellular cloud suggestive of released exopolysaccharide surrounding an earlier epitope (27); this observation certainly warrants further investigation.

Multiple mechanisms likely act in concert to coordinate capsule expansion and the concomitant density increase, potentially regulating both polysaccharide arrangement and the characteristics of the polymers themselves. We have examined the gel migration of anti-GXM mAb-reactive material that is shed as cells are cultured in inducing conditions. The electromobility of this polysaccharide is inversely related to capsule radius (158). The electromobility change upon induction is independent of the presence of cations, suggesting it is not due to the charge-based aggregation that occurs in concentrated GXM preparations and is hypothesized to influence capsule assembly (98,111). The simplest interpretation of these results is that cells synthesize GXM molecules of greater length in response to conditions that stimulate capsule expansion. However, this behavior could also reflect other modifications such as branching, cross-linking, or substitution. These results offer an intriguing model for capsule enlargement, which will be interesting to test in material actively released from cells.

CONCLUDING THOUGHTS

Why is study of the cryptococcal cell wall and capsule so compelling? Is it their key roles in cell integrity and pathogenesis? Is it because each is a fungal-specific structure needed for *C. neoformans* to succeed in overcoming the host? Is it the hope that investigating their synthetic

pathways might elucidate other important areas of biology? Whatever the appropriate combination of reasons, researchers in multiple areas will benefit from these investigations. Even while the wall and capsule differ in detail from other extracellular polymers, they are similar in concept and share fundamental questions of topology and synthesis. This has allowed research in *Cryptococcus* to benefit from groundbreaking work in other systems; this fungus has now begun to return the favor by inspiring new methods and experimental approaches that may ultimately be broadly applicable.

A NEW STRUCTURE FOR A FAMILIAR POLYSACCHARIDE

Structural analysis of the galactose-containing capsule polysaccharide is difficult owing to its complex structure and the nonstoichiometric glycosylation of some residues. Despite these challenges, Cherniak and colleagues (145) used NMR to define this structure, based on a composition of xylose, mannose, and galactose. While studying capsule synthesis, we (83) generated a mutant (*cxt1*Δ) that produces a form of this polysaccharide without xylose, greatly simplifying the NMR spectra (69). During analyses of these spectra we discovered inconsistencies between our data and the published structure. All our studies (compositional and methylation analysis, NMR, and gas chromatography-mass spectrometry) were consistent with the unexpected presence of d-glucuronic acid and yielded a revised structure in which the galactose backbone is modified with side branches composed of galactose, mannose, xylose, and glucuronic acid. (The original structure had xylose in place of the glucuronic acid residue, but was otherwise identical.) We propose that this polymer be renamed glucuronoxylomannogalactan (GXMGal) instead of galactoxylomannan (GalXM) (69). GXMGal better represents the polysaccharide in terms of its overall structure (a galactan, not a mannan) and its substituents (now shown to include glucuronic acid), making it both accurate and consistent with the nomenclature used for GXM.

SUMMARY POINTS

1. The cell wall and capsule of *C. neoformans* are crucial for the success of this pathogen. Understanding their synthesis may help identify targets for greatly needed new therapies.
2. Cryptococcal cell wall glucans include a higher proportion of α1,3-linked structures than is found in *S. cerevisiae* and *Candida albicans*; these polymers are required for capsule association with the cell.
3. Chitosan, the deacetylated form of chitin, is abundant in *C. neoformans* cell walls and is required for normal cell integrity and separation. Formation of chitin and chitosan may be physically linked, adding a layer to the already complex synthesis of fungal chitin.
4. The physical properties of GXM, the major capsule component, depend on whether it has been shed or actively released and on the method of isolation employed. This must be kept in mind during interpretation of studies involving this material.
5. Application of both biochemical and molecular approaches has advanced our understanding of capsule synthesis. In a convergence of these methods, sequence homology to enzymes that have been biochemically identified suggests that the proteins encoded by the original *CAP* genes identified by Kwon-Chung and colleagues, along with many of their homologs, likely act directly in glycan synthesis.
6. mAb localization indicates that GXM-related material is associated with both intracellular and extracellular vesicles, suggesting various models of capsule

formation. These exciting observations must be tempered with the recognition that structural motifs found in capsule polysaccharides may occur in other glycoconjugates, as has already been demonstrated by a shared synthetic motif that directs xylose transfer.

FUTURE ISSUES

1. What are the structures of the N-linked and O-linked glycans in *C. neoformans*, and which are present in the highly immunogenic cell wall proteins? How do these structures relate to those of capsule polysaccharides, and can they provide synthetic clues to the synthesis of those polymers?
2. What and where is the machinery for capsule glycan synthesis? Do the relevant enzymes participate in other glycan synthetic pathways? Do they occur in multienzyme complexes? How are they regulated?
3. Are GXM and GXMGal formed on primer molecules or de novo, and how does elongation occur? Are these polymers branched? Do they cross-link with other molecules of the same type, to each other, or to other cellular components? Does the cell degrade capsule polymers? What is the mechanism of cell association and release? How are these processes controlled?
4. Capsule architecture is complex and dynamic, with properties of this extraordinary structure varying spatially, temporally, and with growth conditions. What is the detailed arrangement of this structure and the mechanism for its construction? How is synthesis of the various zones of the capsule regulated in response to physiological conditions?
5. Why are cryptococcal cells resistant to the echinocandin antifungal compounds? What can be done to enable use of these effective drugs to help patients with cryptococcal disease? What other avenues of cell wall and capsule synthesis can be explored to improve current therapy?

Glossary

EM	electron microscopy
GPI	glycosylphosphatidylinositol
ER	endoplasmic reticulum
mAb	monoclonal antibody
Glucuronoxylomannan (GXM)	the major capsule polysaccharide in terms of mass
Galactoxylomannan (GalXM)	historical term for the galactan-based capsule polysaccharide
Glucuronoxylomannogalactan (GXMGal)	revised term for the galactan-based capsule polysaccharide

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RELATED RESOURCES

Casadevall, A.; Perfect, JR. *Cryptococcus neoformans*. Washington, DC: ASM; 1998.

Serotype A genome sequence.

http://www.broad.mit.edu/annotation/genome/cryptococcus_neoformans/MultiHome.html

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Figure 1. Quick-freeze deep-etch image of the edge of a budding cryptococcal cell. The plasma membrane (*lower left*) is separated by the double-layered cell wall from the capsule meshwork (*upper right*). The wall and capsule surround both the parent cell and the newly emerging bud. Reprinted, with permission, from the cover image associated with Reference ¹²².

Figure 2.

(a) Cryptococcosis is contracted by inhalation of the infectious particle, which initiates a pulmonary infection. The most common site of dissemination is the central nervous system. (b) Images (top to bottom): infected lung tissue, infected brain tissue, and an India Ink stain of cryptococcal cells showing the characteristic halo appearance where capsule material excludes the ink particles. Lung histology image adapted from Reference ¹¹⁷; brain histology image courtesy of Dimitri Agamonolis.

Figure 3.

Capsule polysaccharide structures. (a) The six structural reporter groups of glucuronoxylomannan (GXM) defined by Cherniak et al. (33). Mannose residues are α 1,3 linked; xylose and glucuronic acid residues shown above the mannose backbone are β 1,2 linked; xylose shown below the backbone is β 1,4 linked. Mannose acetylation (see text) is not shown. (b) The structure of glucuronoxylomannogalactan (GXMGal), formerly termed GalXM (69) (see sidebar). Trisaccharide side branches occur at every other galactose and may be substituted on 0–3 moieties (69); the two extreme cases are shown. Linkages are indicated on the diagram. In both panels all sugars are in pyranose form, and single polymer repeat units

are shown. Shapes and colors follow the recommendations of the editors of *Essentials of Glycobiology* (see Related Resources).

Figure 4.
Two possible models of capsule polymer synthesis. See text for details.

Figure 5.

Models of *Cryptococcus neoformans* vesicular traffic. (a) Vesicles formed via the classical secretory pathway fuse with the plasma membrane to release their contents outside the cell. (b) Vesicles formed by plasma membrane blebbing are released from the cell surface. (c) Vesicles formed within intracellular membrane-bound structures are released intact at the cell surface. Drawing is not to scale. See text for details.

Figure 6.

A *Cryptococcus neoformans* secretion mutant accumulates vesicles that contain capsular material. A section through a new bud was labeled with anti-GXM monoclonal antibody and gold-conjugated antimouse antibody. Similar sections of wild-type buds (not shown) demonstrate only occasional vesicles with antibody labeling (157). Reprinted, with permission, from Reference 157.

Figure 7.

Images suggesting intracellular synthesis of capsule fibers. (a) Transmission EM shows intracellular vesicles (*arrowheads*) fusing with the cell surface; the vesicles contain fibers that are morphologically similar to the extracellular capsule fibers. (b) Image prepared as in Figure 6, showing a linear labeling pattern (*arrowheads*) on both vesicle contents and external capsule fibers. Panel *a* is reprinted, with permission, from Reference ⁶⁶.