A Yeast under Cover: the Capsule of Cryptococcus neoformans

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Few fungi are pathogenic to humans. Of these, Cryptococcus neoformans has emerged as an important cause of mortality in immunocompromised patients, especially those with AIDS. As a result, extensive research efforts have addressed the pathogenesis and virulence of this organism.

C. neoformans is a basidiomycetous fungus that is ubiquitous in the environment, where it is found in soil, in association with certain trees, and in bird guano (16). Because of its ubiquity, it has been suggested that most people are exposed to C. neoformans early in life (41). The fungus is heterothallic, with mating types MATa and MATα. Asexual reproduction takes place either by budding or, in the case of MATα cells, by haploid fruiting in response to nutrient deprivation or exposure to the mating pheromone α factor (106). Sexual reproduction occurs when cells of opposite mating types come together to form a heterokaryon that ultimately leads to the production of basidia and basidiospores (64). Desiccated cells and the spores formed by haploid fruiting or sexual reproduction have all been suggested to serve as infectious particles, which must be less than 2 μm in diameter to penetrate the lung parenchyma (44, 86). Infection occurs when the fungal particles are inhaled and enter the alveolar space. In most immunocompetent individuals, this infection is either cleared or remains dormant until an immune imbalance leads to further development. In the setting of compromised immune function, however, the fungus disseminates, with particular tropism for the central nervous system. In severe cases, cryptococcal infection progresses to a meningoencephalitis that is fatal if left untreated.

C. neoformans virulence is mediated predominantly by a polysaccharide capsule that surrounds its cell wall and has multiple effects on the host immune system. This structure provides a physical barrier that interferes with normal phagocytosis and clearance by the immune system. Capsule components inhibit the production of proinflammatory cytokines, deplete complement components (by efficiently binding them), and reduce leukocyte migration to sites of inflammation (11). The capsule also constitutes the major diagnostic feature of cryptococcosis, because its components can be detected in the bloodstream and it can be visualized with light microscopy by using India ink staining. The capsule excludes the ink particles and forms characteristic halos (Fig. 1A) whose diameters are often several times that of the cell. The elaborate structure of the capsule may also be appreciated by electron microscopy (Fig. 1B and C).

Given the importance of the capsule in cryptococcal disease, tremendous effort has been applied in recent years to understanding its biology. This review focuses on the resulting advances in our understanding of the structure and synthesis of the capsular components, the incorporation of these components into the existing capsular network, the association between the capsule and the cell wall, and the regulation of capsule growth.

CAPSULE STRUCTURE

The capsule of C. neoformans is composed primarily of polysaccharides. Studies of its structure have taken advantage of the fact that capsular material is shed copiously into the external milieu. This material, called the exopolysaccharide, can easily be collected, purified, and used for structural studies. Historically, chemical degradation followed by paper, column, and/or gas-liquid chromatography and mass spectrometry was used to determine the composition of the capsular material. These techniques identified two polysaccharide components: an abundant glucuronoxylomannan (GXM) and a minor galactoxylomannan (GalXM). Their precise structures were solved by the application of nuclear magnetic resonance techniques (27) and are shown in Fig. 2.

GXM makes up about 90% of the capsule mass. Its backbone consists of mannose residues that are α-1,3 linked and decorated with xylosyl and glucuronyl side groups (Fig. 2A). Roughly two of every three mannose residues are also 6-O-acetylated (8, 26, 61), with a preference for unbranched mannose but with some acetylation of the mannose which is substituted with glucuronic acid (56). GXM differs in the degree of xylose addition and acetylation in various cryptococcal strains. These variations, differentiated by specific antibody binding, contribute to the classification of C. neoformans into four serotypes, A through D (35, 108). A fifth serotype, AD, has been proposed, as some strains exhibit characteristics of both the A and D serotypes (50). The antisera classification system, however, is not equipped to distinguish the subtle heterogeneity in GXM structure that may correlate with the range of virulence seen among C. neoformans isolates. Therefore, it has...
also been proposed that *C. neoformans* strains be classified on the basis of the minimum GXM repeating unit, as determined by proton nuclear magnetic resonance (27).

Analysis of GXM structure is complicated by variability on several levels. One instructive study involved isolates from separate episodes of cryptococcosis in individual patients (25). In several cases, the strains remained the same, as determined by analysis of restriction fragment length polymorphisms (29) and electrophoretic karyotyping (78), but alterations in GXM structure resulted in their assignment to different serotypes (25). Similar results have been observed after prolonged cultivation either in vitro or during cryptococcal infection in animal models (36). To further complicate the picture, all cells in a population may not bear identical GXM, as assessed by their binding of anticapsular monoclonal antibodies (F. Moyrand and G. Janbon, unpublished results).

GalXM constitutes about 7% of the capsular mass and has a more elaborate structure than GXM (96). It is built on an α-1,6-linked galactose polymer which bears side chains of different lengths on alternate galactose residues (Fig. 2B). As with GXM, the side chain structures, which consist in this case of galactosyl, mannosyl, and xylosyl residues, vary be-

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**FIG. 1.** Views of the cryptococcal capsule. (A) Differential interference contrast micrograph of cells that were mixed with India ink after induction of capsule formation by growth in low-iron medium (80). Scale bar, 3 μm. (B) Thin-section micrograph of a budding cell fixed in the presence of ruthenium red dye (80). Scale bar, 1 μm. (C) Quick-freeze, deep-etch image of the edge of a cell, with an arc of cell wall separating the cell interior (lower left) from the abundant capsule fibers emanating upwards. Scale bar, 0.15 μm. All images were obtained as described by Pierini and Doering (80).

**FIG. 2.** Structures of the cryptococcal capsule components. (A) GXM of serotype B (27); (B) GalXM (96). Linkages between sugars are printed next to the arrows connecting monosaccharides; arrow position is not intended to represent linkage. Blue, mannose; gray, glucuronic acid; red, xylose; green, galactose. 6-O-acetylation of mannose, which modifies the GXM backbone, is not shown.
tween serotypes. The \textit{cap67} mutant strain has been particularly useful in the elucidation of GalXM structure since it lacks GXM, thus simplifying analysis (55, 96, 97).

A third polysaccharide component identified in culture filtrates of \textit{C. neoformans} is mannoprotein, which forms a minor fraction of the exopolysaccharide (76, 83). These mannoproteins have been the focus of immunologic studies, as they appear to be involved in the induction of cell-mediated immunity (18, 28, 70, 75, 82) and cytokine production (48, 81), both critical functions for clearing the fungus. Several of these proteins have been purified from acapsular strains of \textit{Cryptococcus} by concanavalin A affinity (49, 66), and one study suggests that they move through the cell wall and are released into the external environment (99). It is not clear whether the mannoproteins are capsule components. For this review, they are not considered part of the capsule structure and will not be discussed further.

**MUTANTS IN CAPSULE SYNTHESIS**

The structures of the \textit{C. neoformans} capsule polysaccharides are complex (Fig. 2), and their biosynthesis requires many gene products (31). Both genetic and biochemical techniques have been applied to elucidate the steps in this pathway. One productive series of studies was based on analysis of acapsular cryptococcal strains (52). By complementing these mutants, Kwon-Chung and colleagues (19–22) identified four genes required for capsule production. These \textit{CAP} genes were shown to be essential for virulence in mouse models of cryptococcosis, emphasizing the importance of the capsule as a virulence factor. Although proteins encoded by two of them have been localized, Cap60p to the nuclear membrane (20) and Cap10p to the cytoplasm (21), the biochemical functions of the \textit{CAP} gene products have yet to be determined.

To investigate whether the \textit{CAP} genes are unique or members of larger gene families, we used their sequences to search the available \textit{C. neoformans} genome. Because \textit{CAP59} and \textit{CAP60} are close homologs, they were treated as part of the same gene family and only \textit{CAP10}, \textit{CAP59}, and \textit{CAP64} were used to search sequence data from The Institute for Genomic Research (www.tigr.org). The protein sequence for each gene was compared to the sequences of the assembled \textit{B3501} contig database by using TBLASTN (4), and hits were considered positive if a good match to at least half of the query sequence existed in the same contig (a good match was defined as a score greater than 50 bits, with an expect value below $10^{-10}$). There are at least two homologs of \textit{CAP10} in the genome, one of which is the recently identified \textit{CAP1} (65). Two additional homologs matched the first and last 50 amino acids of Cap10p but had weak homology to the rest of the protein. Searches with the \textit{CAP59} sequence revealed two homologous sequences in addition to \textit{CAP60}. \textit{CAP64} is also a member of a gene family, with six homologs in \textit{C. neoformans}.

To assess whether \textit{CAP} genes were specific for \textit{Cryptococcus} in the fungal world, we used the \textit{CAP10}, \textit{CAP59}, and \textit{CAP64} sequences to search all fungi with publicly available genome sequences (Table 1). The only fungus with sequences homologous to all three is another basidiomycete, the white rot fungus \textit{(Phanerochaete chrysosporium)}. Hybridization studies previously suggested that \textit{CAP59} is unique to the genus \textit{Cryptococcus} (79). However, the broader technique of sequence analysis indicates that many fungi, both pathogenic and benign, contain homologs of both \textit{CAP59} and \textit{CAP10}. This suggests that these families of genes may play roles other than capsule synthesis, as these other fungi lack capsules. Unfortunately, no genome sequencing projects are under way for other encapsulated fungi. We also looked for \textit{CAP} gene homologs beyond fungi (as assessed by BLAST analysis of all genes and gene products in the nonredundant National Center for Biotechnology Information database). Interestingly, \textit{CAP10} has matches in metazoans with sequences of unknown function in human, mouse, and \textit{Drosophila}. \textit{CAP59} also matches a hypothetical gene in the \textit{Leishmania major} genome, \textit{CAP64}, in contrast, has no detectable homologs outside the \textit{Myco}ta.

A second set of genes that were identified genetically as having a role in capsule synthesis are the \textit{CA4} genes. These were discovered when one of our laboratories screened mutagenized \textit{C. neoformans} cells for those that were unable to bind, or were defective in binding, monoclonal antibodies directed against the capsule (74). Analysis of these \textit{CA4} mutants identified six complementation groups, \textit{Cas1} through \textit{Cas6} (74), of which several members have now been examined. The \textit{CAS1} gene (56) is one of a family of genes with orthologs in human, \textit{Drosophila}, and plants. Analysis of the \textit{cas1} mutant capsule showed defects in O acetylation, and interestingly, \textit{cas1Δ} strains were more virulent than the parental wild type in a murine model of cryptococcosis (56). The \textit{CAS2} gene (74) was found to correspond to the previously identified \textit{UXS1} gene. \textit{UXS1} encodes a UDP-glucuronate decarboxylase which forms UDP-xylene from UDP-glucose (5). This gene is necessary for both capsule xylosylation and fungal virulence (74).

**STEPS REQUIRED FOR CAPSULE SYNTHESIS**

Genetic screens have clearly proven valuable in identifying genes whose products are required for capsule synthesis. Biochemical studies have tremendous power to complement this approach and help elucidate the functions of these gene products. These experiments have generally focused on the specific events in capsule biosynthesis that are outlined in Fig. 3. The
FIG. 3. Steps in GXM synthesis. Proteins with a role in biosynthesis are indicated by red letters and are discussed further in the text. (A) Phosphomannose isomerase; (B) UDP-glucose dehydrogenase; (C) UDP-glucuronic acid decarboxylase; (D) nucleotide sugar transporters specific for GDP-mannose (D1), UDP-glucuronic acid (D2), and UDP-xylene (D3); (E) mannosyltransferase; (F) xylosyltransferase; (G) glucuronosyltransferase; and (H) acetyltransferase. The color coding is the same as described in the legend to Fig. 2, with the addition of purple to represent glucose.

first to be considered here is the production of the activated precursors that serve as donors for capsule components. GXM synthesis requires GDP-mannose, UDP-xylene, and UDP-glucuronic acid as biosynthetic precursors; GalXM additionally requires UDP-galactose. The acetyl donor to GXM has not been identified. Since GXM is the major component of the capsule and has a mannan backbone, biosynthesis of GDP-mannose is a critical early step. This requires the activity of three enzymes that are highly conserved in the fungal world, phosphomannose isomerase, phosphomannomutase, and GDP-mannose pyrophosphorylase (or GDP-mannose synthase) (Fig. 3, element A). The gene encoding phosphomannose isomerase in C. neoformans (MAN1) has been identified and cloned (107). man1 mutants are defective in capsule production and GXM secretion, have morphological abnormalities, and are avirulent.

The nucleotide sugars UDP-glucuronic acid (which donates a major component of GXM) and UDP-xylene (required for both GXM and GalXM) can be made from UDP-glucose by the sequential actions of two enzymes. UDP-glucose dehydrogenase leads to the formation of UDP-glucuronic acid (Fig. 3, element B), while UDP-glucuronic decarboxylase converts UDP-glucuronic acid to UDP-xylene (Fig. 3, element C). These activities were detected in C. neoformans by Jacobson and Payne (51, 53), and subsequent work in one of our laboratories has identified the genes encoding both of these enzymes. One of these genes (UXS1), encoding UDP-glucuronic decarboxylase, was found through its homology to a bacterial gene that had been hypothesized to have a similar function (5). Although this decarboxylase is important for synthetic processes across biological kingdoms, the sequence for this enzyme had not previously been obtained in any system.

Once biosynthetic precursors are made, the next probable step in capsule synthesis is their localization to the site of polysaccharide construction. This is likely to require several specific transporters (Fig. 3, element D), since the charged precursors are generally formed in the cytosol, while most eukaryotic glycan synthesis occurs in membrane-bound compartments. Several genes encoding putative nucleotide sugar transporters have been identified in C. neoformans by sequence homology performed in our laboratories. Functional assays of these putative transporters should clarify their potential role in capsule formation and cryptococcal biology.

After appropriate precursors are transported into the compartment of capsule biosynthesis, the monosaccharides must be assembled into polymers. As previously noted (31), there are at least 11 linkages present in the various configurations of GXM and GalXM. Each linkage probably reflects the function of a different sugar transferase, because these enzymes are usually specific for the substrate, the moiety transferred, and the bond formed. Constructing GXM and GalXM would therefore require an array of sugar transferases (for examples, see Fig. 3, elements E to G) and an acetyltransferase (Fig. 3, element H).

Several distinct mannosyl transferase activities have been identified in C. neoformans by using crude membrane preparations. Two of these, identified in efforts to isolate enzymes involved in capsule synthesis, are in fact α-1,2-mannosyltransferases and therefore lack the requisite activity (32, 104). A third, however, can transfer mannose from GDP-mannose to α-1,3-linked mannobioside and would therefore be capable of building the GXM backbone (32). This enzyme has been purified, and its peptide sequence has been used to obtain the corresponding gene sequence. Inhibition of its expression shows that it is required for normal capsule synthesis (U. Sommer, H. Liu, and T. L. Doering, unpublished data). In addition, xylosyltransferase (Fig. 3, element G) and glucuronyltransferase (Fig. 3, element F) activities have been detected in crude membrane preparations of C. neoformans (103). Study of these activities led to the proposal that the order of addition of side groups to the mannan backbone is first, acetyl; second, glucuronyl; and third, xylosyl (103). However, subsequent investigation of GXM structures in a mutant lacking xylose suggests that acetylation follows the addition of xylose (74).

CAPSULE ASSEMBLY AND DEGRADATION

Once individual capsule components are generated by the biosynthetic pathways discussed above, they must be assembled into the elaborate meshwork of the mature capsule. How are these new materials incorporated to generate and maintain this crucial virulence factor? Capsule synthesis has been investigated in two physiologically important situations: bud formation, which occurs with concomitant encapsulation of the daughter cell, and capsule synthesis on mature cells, which is induced by altering growth conditions (80). In budding cells, new-capsule synthesis is directed into the bud, with no incorporation of the maternal capsule into the nascent polysaccharide structure overlying the daughter cell (Fig. 4). In mature cells, new capsule material is incorporated just outside the cell wall, at the inner aspect of the existing capsule structure (80). This displaces the existing material outwards, although there is also some mixing of old and new polysaccharide and an overall increase in density of the polysaccharide structure with time. Material displaced by new synthesis could conceivably be shed.
from the cell into the environment or it could remain associated with the capsule, perhaps fixed in place by cross-linking processes such as those suggested to occur during cell wall maturation (67). To fully understand these processes, the mechanism by which the capsule is initially associated with the cell must first be defined.

Early studies of how capsule material associates with the cell were based on the observation that capsular exopolysaccharide could bind to acapsular cells. Capsule material purified from growth medium of wild-type cryptococcal cells was first shown to bind to acapsular mutants and to protect them from phagocytosis (12, 13, 91). This interaction was specific for cryptococcal cells and cryptococcal capsule material (62) and was shown to be saturable and reversible (63). Chemical treatments of acapsular cells and purified capsule material were subsequently investigated to test their effects on binding (89). These studies led to a model in which polysaccharide capsule material binds to a specific receptor on the surface of acapsular mutants.

To pursue the question of how capsule polysaccharides interact with the cell, an in vivo assay for the binding of capsule material to acapsular cells has been employed. In this assay, conditioned medium from wild-type cells serves as a capsule source and indirect immunofluorescence with a fluorophore-tagged anti-capsule antibody is used to evaluate the transfer of polysaccharides to acapsular acceptor cells. Studies using this model strongly implicate cell wall glycan in capsule binding (A. J. Reese and T. L. Doering, submitted for publication).

The relationship of the capsule to the cell wall necessitates an examination of C. neoformans cell wall composition. While much is known about the cell wall structures of Saccharomyces cerevisiae and Candida albicans, considerably less is known about those of other fungal organisms (Table 2). Common cell wall structural building blocks include β-glucans and chitin (14, 15). Other possible cell wall components in fungi include glycoproteins (particularly mannoproteins), α-glucans (14), and melanin (17). In S. cerevisiae (90) and Candida albicans (60), the main cell wall network is formed by branched β-1,3-linked glucans, with chitin and several types of mannoproteins linked directly to it. Other fungi, including C. neoformans, have a high α-1,3-glucan content, and their cell wall structures might be considerably different. Binding assays indicate that α-1,3-glucan at the cryptococcal surface is required for acapsular cells to bind exogenous capsule material. Posttranscriptional silencing of a cryptococcal α-glucan synthase gene yields cells with poor ability to assemble capsules, although the components are still made (Reese and Doering, submitted). It is interesting that in the pathogenic fungi Blastomyces dermatitidis and Histoplasma capsulatum, virulence is associated with α-1,3-glucan levels (47, 59), although these fungi are not encapsulated.

While studies of capsule synthesis have progressed, capsule degradation remains poorly understood. Although enzymes from soil microbes can degrade capsules (37), no cryptococcal or human enzymes with this activity have been found. Nonetheless, capsule material must be degraded or remodeled by the cell to allow bud emergence, since buds are coated with newly synthesized capsule material (Fig. 4) (80). The mammalian host may also have mechanisms for capsule breakdown, but further studies in this area are needed to clarify these events.

### TABLE 2. Major cell wall components of selected fungal organisms

| Organism            | β-Glucan | α-Glucan | Mannoproteins | Chitin | Able to melanize |
|---------------------|----------|----------|---------------|--------|------------------|
| Cryptococcus neoformans | 15%β (mostly β-1,6; some β-1,3) | 35%β (mostly α-1,3; some α-1,4) | Present (99) | Present (16) | Yes (102) |
| Saccharomyces cerevisiae | 50% β-1,3; 10%β-1,6 (67) | None (67) | 40% (67) | 1–3% (67) | No |
| Candida albicans | 40%β-1,3; 20%β-1,6 (60) | None (60) | 35–40% (60) | 1–2% (60) | No |
| Schizosaccharomyces pombe | 55% β-1,3; 6% β-1,6 (46) | 28% α-1,3 (46) | Likely present | 0.5% (46) | ? |
| Aspergillus fumigatus (mycelial form) | 70% β-1,3; 4%β-1,6; 10%β-1,3,1,4 (7) | Present (7) | 3.5% (7) | Present (7) | Yes (7) |
| Paracoccidioides brasiliensis | Yeast form, 5%β-1,3 (87); mycelial form, mostly β-1,3 (9) | Yeast form, 95%α-1,3 (87); mycelial form, little α-1,3 (9) | Likely present | Present (yeast, mycelial form) (9) | Yes (42) |
| Blastomyces dermatitidis (yeast form) | Present (58) | 95% (58) | Likely present | Present (10) | Likely* |
| Histoplasma capsulatum (yeast form) | Present | Present | Likely present | Likely present | Yes (77) |

* The ratio of cell wall components is highly dependent on whether the organism is byphal or yeast form (15, 87, 90), so these are listed separately. Equivalent analyses have not been performed for all organisms listed.

β Percentage of cell wall glucan.

* Percentage of total cell wall mass.

* J. D. Nosanchuk, personal communication.


**REGULATION OF THE CAPSULE**

The *C. neoformans* capsule varies tremendously in size and morphology under different growth conditions. As early as 1958, Littman studied the effects of various nutrients on capsule formation and formulated a capsule-inducing medium containing thiamine, sodium glutamate, mineral salts, maltose, and sucrose (68). Low-iron medium (98), alterations in pH, and a high ratio of CO2 to HCO3 also induce capsule formation, while other medium additives, such as 1 M sodium chloride, suppress capsule formation (54). Suppression of capsule synthesis by sodium chloride is specific, as other hypertonic solutions do not have this effect. Capsule size also increases dramatically during infection (6, 40). The extent of capsule formation depends on the tissue location, with cells in the brain having larger capsules than those in the lungs (84). This is probably due to differences in the CO2/HCO3 ratio or other capsule-modulating factors in those sites, again stressing the highly regulated nature of this virulence factor.

Efforts to understand capsule regulation have focused attention on second messengers, which mediate aspects of growth and development in eukaryotic cells. In particular, studies have addressed the second messenger cyclic AMP (cAMP), which is produced in cells by the enzyme adenylyl cyclase. The activity of adenylyl cyclase in mammalian cells is regulated by heterotrimeric G proteins, which are in turn activated by seven transmembrane receptors of the β-adrenergic receptor family. Binding of an appropriate ligand to the receptor leads the heterotrimeric G protein to exchange GDP for GTP, causing dissociation of the α subunit from the βγ dimer (Fig. 5). Depending on the organism, either one of these products may activate an adenylyl cyclase. The subsequent production of cAMP has diverse effects, one of the best known of which is the activation of cAMP-dependent protein kinase A (PKA). PKA in turn regulates downstream targets, including transcription factors, leading to broad changes in cellular physiology (92).

cAMP has been shown to regulate capsule production in *C. neoformans* (2, 3). Implicated in the upstream pathway are the GPA1 and CAC1 gene products, which have been shown to control intracellular cAMP production. The GPA1 product is homologous to the *S. cerevisiae* Gα subunit Gpa2p (94), while the CAC1 product is the adenylyl cyclase (3). Both gpa1 and cac1 mutants are unable to induce capsule production and are correspondingly avirulent, but overproduction of CAC1 can suppress the gpa1 mutant defects (3). These defects can also be corrected by the addition of cAMP to the medium, thereby bypassing the requirement for Gpa1p and Cac1p and supporting the crucial role that signaling via cAMP plays in capsule formation.

Among the potential targets of cAMP, the *C. neoformans* PKA1 gene product has a clear role in capsule production (33), since a pka1 strain is acapsular and avirulent. In this case, capsule production is not restored by exogenous cAMP, suggesting that PKA acts downstream of cAMP in the signaling pathway (Fig. 5). Further investigations of PKA in *C. neoformans* are based on its subunit structure. Inactive PKA is a tetramer of two catalytic subunits (encoded by PKA1) and two regulatory subunits (encoded by PKR1). Binding of cAMP to the regulatory subunits causes them to undergo a conformational change and to dissociate from the catalytic subunits, releasing the latter from inhibition (Fig. 5). Loss-of-function mutations of PKR1 should, therefore, lead to constitutive activation of PKA1. This is indeed the case, as pkr1 mutants produce larger capsules than wild-type cells do and are hypervirulent in animal models. pkr1 mutants can also suppress the capsule production defect of gpa1 cells (33). However, the capsules produced by the pkr1 gpa1 double mutant are larger than those produced by the pkr1 single mutant. This suggests that GPA1 may have additional targets, yet to be identified, that modulate capsule production (34). Once active, Pka1p kinase can cause changes in gene expression and physiology by phosphorylation of effector proteins. One candidate target of PKA, which is involved in positive regulation of capsule production, is the transcription factor encoded by STE12, which is found in two mating-type-specific forms (105, 110). This transcription factor is probably only one of several targets (Fig. 5), because loss of function of STE12α or STE12α leads only to a decrease in capsule size (23, 24, 110) and overexpression of STE12α does not overcome the capsule production defect of a pka1 strain (33).

In many other systems, the Ras signaling protein plays a role in the activation of adenylyl cyclase. However, RAS1 does not appear to be involved in capsule production in *C. neoformans* (1, 2). Other major signaling pathways in *C. neoformans* that have been investigated include the mitogen-activated protein kinase pathway and the signaling pathway of cyclophilins. Although both of these pathways have been shown to regulate virulence and mating (100, 101), they are not clearly implicated in capsule production.

**OTHER CAPSULES**

*C. neoformans* is unique in being the only fungus that is both pathogenic to humans and in possession of a true polysaccharide capsule outside its cell wall. *Tremella mesenterica*, a close phylogenetic relative, possesses a similar capsule but does not cause disease (30). Several other fungi have capsule-like structures, but they have not been well characterized. These fungi include *Malassezia furfur* (73), *Rhinosporidium seeberi* (93), *Trichosporon beigelli* (72), *Blastocystis hominis* (71), and *Sporothrix schenckii* (38).

Among other microbes, many gram-negative and gram-pos-
ative bacteria also bear polysaccharide capsules. The numerous examples among pathogens include Streptococcus pneumoniae, Neisseria meningitidis, and Pseudomonas aeruginosa (109). Capsules protect bacterial cells from desiccation, mediate their adherence to host tissue, and assist in evasion of the host immune response (85). Some bacteria also shed exopolysaccharides into their surroundings as a method of avoiding immune system clearance. There is a tremendous variety of polysaccharide structures present in bacterial capsules (85, 109). All of them, however, are quite distinct from those of the C. neoformans capsule and, therefore, require an independent set of en-
zymes for their synthesis and assembly.

The value of capsules has also been appreciated beyond the microbial world with the use of microencapsulation techniques in the food industry. In this application, alginate or other polysaccharides are used to microencapsulate bacteria (57). This process makes use of the protective nature of polysaccharide capsules to assist in the survival of probiotic bacteria.

FUTURE DIRECTIONS

The biogenesis of the capsule in Cryptococcus is a complicated process, and rapid advances in understanding its structure, synthesis, and regulation have been seen in the past few years. However, there are vast areas that are still unexplored and numerous questions that remain unanswered. Filling these gaps will require concerted effort in all fields of biology—biophysical, biochemical, genetic, and cell biological.

The structure of the major capsular polysaccharides has been established and suggests the synthetic precursors required, but how is the synthesis of these polysaccharides initiated? Similarly, the question of whether the addition of new material to the capsule occurs in units of single monosaccharides, short oligosaccharides, or extensive polysaccharide chains remains unanswered.

Our present understanding of the biochemical reactions in capsule synthesis is patchy. The participants in some of the predicted reactions, such as GXM acetylation, have not yet been defined. Other capsular synthetic activities have been biochemically identified only in lysates and have yet to be studied in detail or correlated with a corresponding gene. Only a few have been purified and cloned. Without pure enzyme preparations or strains deficient in specific activities, it becomes extremely difficult to determine the order of the reactions that add side chain subunits to the polysaccharide backbone and, therefore, to delineate the entire biosynthetic pathway.

There are several approaches that can identify additional genes with potential roles in capsule synthesis. These include identifying gene families within Cryptococcus, finding cryptococcal genes with homology to those encoding relevant proteins in other organisms, and examining mutants with altered capsules. Once important enzymes are identified and functionally validated, their localization will be crucial in determining the sites and organelles involved in capsule synthesis. Determination of the transport pathways that carry capsule components to the cell surface is another area that is important but largely unexplored.

In the search for the molecular basis of capsule regulation, a cAMP-signaling pathway that modulates capsule production has been identified. Genetics has been used to define many of the members of this pathway. However, unanswered questions remain. What is the nature of the signal that activates the pathway? What are the identities of the transmembrane receptor and of downstream effectors? A variety of nutrients and environmental conditions also affect capsule production, but what are the regulatory pathways by which they do so? The regulation of capsule structure presents fascinating questions in addition to those about how much capsule is made. Capsule structure depends on environment (95), and studies with anti-capsule antibodies suggest that structure may vary among cells in a population (Moyrand and Janbon, unpublished results), but the mechanisms controlling these differences are yet to be defined.

The capsule polysaccharides that are shed into the external milieu enable C. neoformans to resist the host immune response. Little is known, however, about how this occurs. Are the old parts of the capsule sloughed off as new material is added, or is the release of capsular material a regulated process under specific cellular control? Also, how does degradation of the capsule take place? The molecular mechanisms of these processes remain obscure.

Finally, although the structures of the main components of the capsule are now known, how do these components combine to form the three-dimensional structure of the capsule? Electron micrographs show an elaborate network of fibers that extend out from the cell wall. However, these images do not address whether these strands are composed of one or multiple polysaccharide components or how they group and branch. A better understanding of the biochemical and biophysical properties of the capsule is needed to answer these questions.

For many years, the study of the cryptococcal capsule was hindered by the absence of appropriate tools. This picture has been completely redrawn by the recruitment and adaptation of new technologies to the cause of cryptococcal research, from genome sequencing (45, 88) and RNA interference (69) to structural studies. These tools, coupled with the facts that gene disruptions are now routinely performed and animal models are in place to study in vivo effects, have considerably advanced our understanding of the Cryptococcus capsule. The coming years will be an exciting time for studies of cryptococcal biology.

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