The capsular dynamics of *Cryptococcus neoformans*

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***Cryptococcus neoformans* is a soil-dwelling fungus that causes life-threatening illness in immunocompromised individuals and latently infects many healthy individuals. *C. neoformans*, unlike other human pathogenic fungi, is surrounded by a polysaccharide capsule that is essential for survival and enables *C. neoformans* to thwart the mammalian immune system. The capsule is a dynamic structure that undergoes changes in size and rearranges during budding. Here, the latest information and unresolved questions regarding capsule synthesis, structure, assembly, growth and rearrangements are discussed along with the concept that self-assembly is important in capsular dynamics.**

The uniqueness and importance of the *Cryptococcus* capsule

The most distinctive feature of *Cryptococcus neoformans* is the polysaccharide capsule. *C. neoformans* is the only encapsulated human eukaryotic pathogen and the presence of a polysaccharide capsule gives this fungus some pathogenic attributes comparable to those of encapsulated bacteria. For example, like the classical encapsulated bacteria *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Neisseria meningitidis*, the polysaccharide capsule of *C. neoformans* is anti-phagocytic, poorly immunogenic and essential for virulence. However, the *C. neoformans* capsule stands alone in the microbial world as a massive structure that is synthesized by a eukaryotic microbe, with a size and complexity that dwarfs the bacterial structures. Despite occasional clinical case reports of poorly encapsulated strains, all clinical strains have some capsule material and the capsule can be considered as an invariant requirement of wild-type pathogenic strains. The capsule and polysaccharide shedding were recently identified as the virulence factor that made the largest relative contribution to the overall virulence phenotype [1].

Although the capsule is intimately associated with *C. neoformans* virulence for a variety of animal hosts, the primary habitats of this fungus are soils and trees. Consequently, the primary function of this structure must be related to environmental survival. In this regard, the capsule is essential for survival in amoebae and slime molds, which are probable predators of soil fungi [2–4].

The capsule might also provide substantial protection against desiccation and is essential for the formation of cryptococcal biofilms [5]. Hence, a full understanding of the physiological and biological role of the capsule would also require consideration of the mechanisms by which this structure increases fitness in the environment.

The *C. neoformans* capsule was last reviewed in this journal six years ago [6]. In the intervening years, there has been substantial progress in understanding the genetic, biochemical and physical processes that contribute to the assembly and maintenance of the *C. neoformans* capsule. Hence, this review focuses primarily on recent advances while placing new observations in the context of prior knowledge. Furthermore, several major unsolved areas for future investigation are identified.

Capsular polysaccharides of *C. neoformans*

The capsule of *C. neoformans* contains two major polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). GXM is a linear $\alpha(1,3)$-mannan with a $\beta(1,2)$ glucuronic acid residue attached at every third mannose, on average, and a variable amount of 6-*O*-acetylation [7,8]. Each trisaccharide of the backbone is also substituted with up to four xyloses, which are either $\beta(1,2)$- or $\beta(1,4)$-linked [7] (Figure 1). Xylosylated mannoses tend not to be acetylated [8–10]. GXM forms a complex, large molecular-weight structure that involves the self-association of molecules and the self-entanglement of fibers [11–13] (Figure 2). GXM is also released from the capsule and accumulates in tissue during infection. Effective immune responses are disrupted by GXM and this undoubtedly assists the pathogenicity of *C. neoformans* [14]. High concentrations of GXM in tissue were hypothesized to cause viscosity-dependent dysfunction of cellular processes [15]. However, recent viscosity studies suggest that this anionic polysaccharide does not increase the viscosity of ionic solutions at concentrations that are relevant *in vivo* [13]. Furthermore, the sensitivity of GXM to the surrounding ionic strength creates a capsular structure with inherent malleable properties [16].

GalXM is an $\alpha(1,6)$ galactan that contains branches of $\beta(1,3)$-galactose–$\alpha(1,4)$-mannose–$\alpha(1,3)$-mannose. In turn, the branch sugars can be linked to $\beta(1,3)$ or $\beta(1,2)$ xylose [17]. Whereas all sugars in GXM and most of the sugars in GalXM are in the pyranose configuration, GalXM does
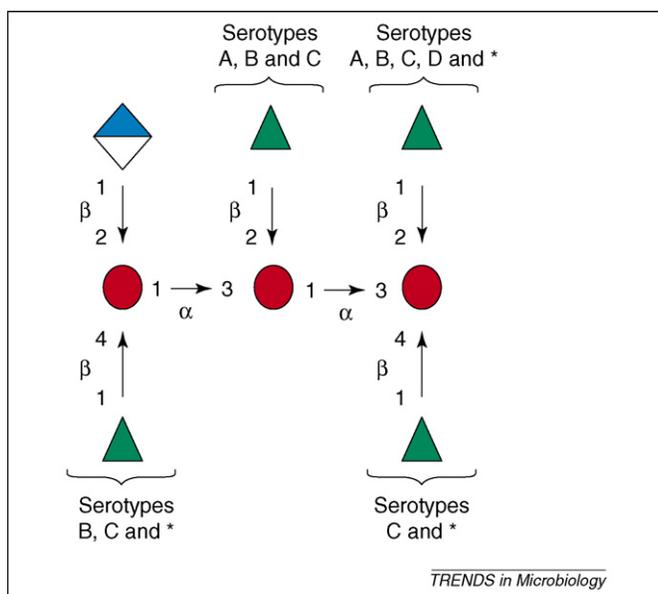
Figure 1. The six repeating units of glucuronoxylomannan found in different serotypes. Any strain of *Cryptococcus neoformans* can contain one or more of the repeating units. Each repeating unit contains three mannoses (red circles) and one glucuronic acid (half-filled diamond). The xylose side groups (green triangles) that predominate in serotypes A, B, C and D are designated with the corresponding serotype letter. A fifth repeating unit, which does not predominate in a given serotype, contains xylose side groups designated with an asterisk (*). A sixth repeating unit that does not contain any xyloses has also been identified.

contain a small amount of galactofuranose [17]. A *C. neoformans* gene has been identified that encodes a functional UDP-galactopyranose mutase, which converts galactopyranose to galactofuranose [18]. GalXM has a much smaller mass than GXM: 1.01×10^5 g mol^{-1} versus 1.7–7.4×10^6 g mol^{-1} and, consequently, more moles of
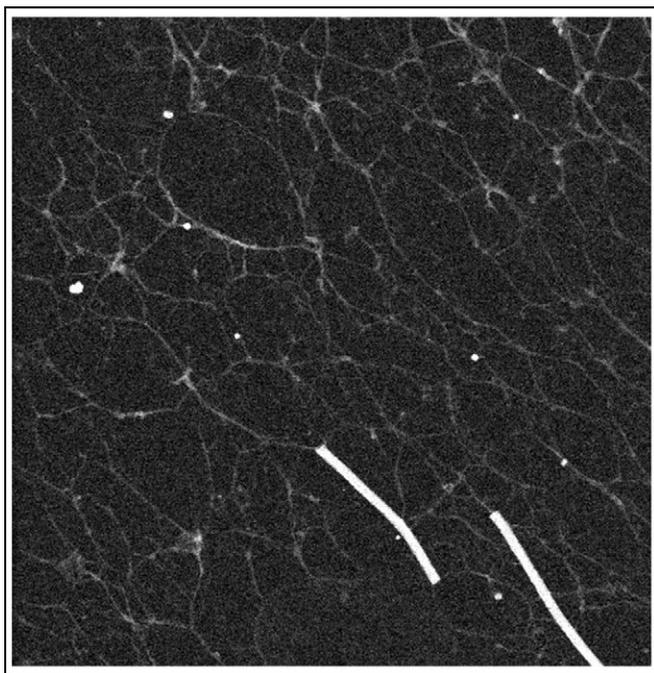
Figure 2. Scanning transmission electron microscopy image of GXM. Isolated GXM appears as thin fibers that are highly entangled. The heavy electron dense rods are tobacco mosaic virus particles that serve as an internal control for the preparation. The dark field image was digitally recorded as scans of 1.024 μm with a scanning pixel size of 20 Å².

GalXM than GXM could be present within the capsule [13]. GalXM does not substantially increase the viscosity of ionic solutions [13].

Several genes that are essential for capsule formation have been identified and previously reviewed [19]. Genes involved in, but not essential to, capsule formation have been discovered by complementation of capsular mutants that bear specific structural alterations in GXM. Two such genes, *CAS1* and *CAS3*, are involved in the acetylation of GXM. *CAS1* encodes a predicted multipass transmembrane protein that is required for the acetylation of GXM [9]. *CAS3*, which is predicted to encode a single-pass N-terminal transmembrane protein (TMHMM 2.0 program; http://www.cbs.dtu.dk/services/TMHMM), is necessary for *C. neoformans* to acetylate GXM fully in the absence of *CAS31* expression [10]. *CAS3* and five similar genes share homology to the *CAP64* gene, which is essential for capsule formation, and all contain a SGNH hydrolase conserved domain found in lipases and esterases [20]. With the availability of the *Cryptococcus* genome, other capsule-associated gene families have also been identified [19,21]. Biological analysis of acetylation-deficient capsulated strains have demonstrated the importance of the acetyl group in antibody recognition, complement activation, serum and tissue clearance and inhibition of neutrophil recruitment [22–25]. Virulence studies with the Δ*cas1* strain suggest that the acetyl group on GXM reduces the virulence of the fungus [25].

Several genes are important for the proper xylosylation of GXM. Uxs1 has UDP-glucuronic acid decarboxylase activity and converts UDP-glucuronic acid to UDP-xylose [26,27]. *UXS1* mutants produced GXM that lacked β(1,2) xylose [28]. The importance of *UXS1* to β(1,4) xylose could not be determined because the strain used only contained β(1,2) xylose. *CAS31*, *CAS32*, *CAS33*, *CAS34* and *CAS35* are homologs of *CAS3* and are involved in the addition or inhibition of xylose substitution on GXM [10]. Interestingly, Cas31 co-purified with an α(1,3) mannosyltransferase protein Cmt1 and this could hint that GXM biosynthetic enzymes exist as a complex. *CMT1* is a homolog of two capsule-related genes, *CAP59* and *CAP6* (GenBank accession number AAR84600) [10,29]. Experiments with the avirulent Δ*uxs1* strain suggest that the xyloses in the GXM structure contribute to virulence by slowing the rate of complement factor C3 deposition and altering the clearance of GXM from the spleen [25,28]. A separate screen for capsule variants isolated a variant with hypoacetylated GXM containing additional xylose sugars. In virulence studies, the variant was hypovirulent and this might suggest an interplay between the two structural alterations [30].

Structural variations in the capsule
The composition and structure of the capsule is different between strains and is clearly detailed through the extensive structural analysis of GXM from >100 isolates [7]. The six repeating units that comprise GXM are present at varying ratios in different strains, such that each strain has a potentially unique capsule composition and/or structure [7] (Figure 1). However, some strains are similar in their structures and share antigenic determinants that

enable immunological sub-grouping into serotypes. *C. neoformans* strains are divided into five serotypes: A, B, C, D and AD [31,32]. Serotypes A and D are the most common serotypes associated with human diseases; changes in their capsule structures were detected following disease relapse and are associated with virulence differences [33,34]. The GXM of serotype B and C strains are more highly xylosylated than the other serotypes [7] (Figure 1).

The serotype classification of *C. neoformans* strains is based on polyclonal rabbit sera reactivity to the capsule after differential adsorption of the sera with the non-immunizing serotypes [35]. Eight major antigenic factors in the capsule (numbered 1–8) were identified with these rabbit sera preparations. Factor 1 defines an antigenic determinant present in all strains and probably represents a complex group of epitopes [24,35]. A second pan-specific determinant was detected with monoclonal antibody (mAb) F12D2 and is independent of GXM *O*-acetylation, unlike antibody factor 1 [36]. Serotype-specific antigenic determinants are detectable with antibody factors 7, 5, 6 and 8 (serotypes A, B, C and D, respectively). mAbs CRND-8 and E1 can also be used as serotype-specific reagents and have serotype reactivities similar to antibody factors 8 and 2, respectively [37–39]. mAb 13F1 exhibits serotype-specific capsular immunofluorescence patterns and mAb F10F5 has the unusual specificity of recognizing serotypes A and B [36,40]. The various immunoglobulin reactivity patterns suggest that the variability in GXM composition aids in generating the complex antigenic structure of the capsule.

Variation in capsule structure can occur within a population derived from a single strain [39,41]. Capsular antigenic determinants can be rather heterogeneous within a population and alterations in their expression occur during infection and culturing [41,42]. Given that antigenic variations must reflect structural heterogeneity within the capsule, this phenomenon is potentially advantageous because it could enable *C. neoformans* to survive host immunity or microbial interactions in the environment, or permit dissemination and colonization within a host.

Morphological changes in the capsule

C. neoformans modulates capsule thickness in response to environmental conditions. Under standard *in vitro* conditions, the capsule thickness is often 1–2 microns (Figure 3). *In vivo*, the capsule size can be as large as 30 microns [43]. The dependence of capsule thickness on environmental conditions was described in the 1950s by Littman while studying the effects of cerebrospinal fluid components on *C. neoformans*. From his results, a synthetic medium was developed that induced an increase in capsule size [44]. This phenomenon was not investigated again until the 1980s, when the regulation of capsule size by carbon dioxide was directly related to virulence [45]. Subsequently, iron limitation was discovered to induce capsule growth [46]. Recently, other conditions that induce capsule growth have been described, such as serum or low nutrient medium that is slightly alkaline [47,48] (Figure 3). At the other extreme, conditions associated

with a small capsule include high osmotic pressure, high glucose or rich fungal growth media (e.g. Sabouraud agar) [16,49,50].

Several transduction cascades induce growth of the capsule, including the cAMP and mitogen-activated protein kinase pathways [19,47,51,52]. The fine details of the regulatory network remain to be understood but serotype-specific functions of protein kinase A isoforms, a major component of the cAMP pathway, suggest serotype differences exist [53]. Mutants in the cAMP pathway that cannot enlarge the capsule show reduced virulence, whereas mutants that overproduce capsule are hyper-virulent [52]. Although these mutants have pleiotropic phenotypes, their virulence traits are consistent with the capsule growth being important for virulence.

Induction of the capsule requires certain experimental considerations. First, the phenomenon is strain dependent, and for each strain the optimal conditions must be ascertained [47]. Additionally, some strains show substantial heterogeneity in capsule size within the population. Second, some inducing factors are effective only in certain media [47]. Third, there is a time consideration. Capsule enlargement is apparent after 4–6 h and continues for 24–48 h in inducing medium [47]. After enlargement, density differences exist within the capsule, with the most dense regions adjacent to the cell wall and then gradually decreasing outwards [16,54]. Capsule density decreases approximately sixfold in the outer layer compared with the inner layer, as shown by Fab binding to the capsule and direct polysaccharide measurements in different regions of the capsule [16]. [Fab is the immunoglobulin (antibody) fragment that contains the antigen binding site following papain digestion.] These density differences within the capsule could function as a molecular sieve that prevents large macromolecules such as antibodies from reaching the cell. Capsule size also affects the complement localization within the capsule, profoundly affecting its opsonic efficacy [55,56]. In addition, the enlarged capsule seems to have a complex spatial organization with the creation of ring-like channels that are located perpendicular to the budding pole in the high-density area [57].

Capsule size seems to be a tightly regulated process. For example, stationary phase *C. neoformans* cells grown in standard fungal media release substantial amounts of GXM in the broth, yet capsule enlargement is substantially less than under optimized conditions [58]. Continuous passage of cells with enlarged capsules in induction medium does not lead to further increases in capsule size [59]. This suggests that the initial environmental signal from the medium induces the maximal response. Factors that control cell size also seem to control capsule size because these two parameters correlate following the enlargement of the capsule [47,60,61]. Thus, an upper limit exists for the size of the capsule. *In vivo*, the capsule has been reported to be as large as 40–80 microns, which is much larger than the size found *in vitro* [62,63]. The generation of such large cells *in vitro* is not currently possible and the *in vivo* factors that induce such enormous capsules have not been identified.

Strikingly, the enlargement of the capsule is not reversible, at least in the conditions studied. Cells with
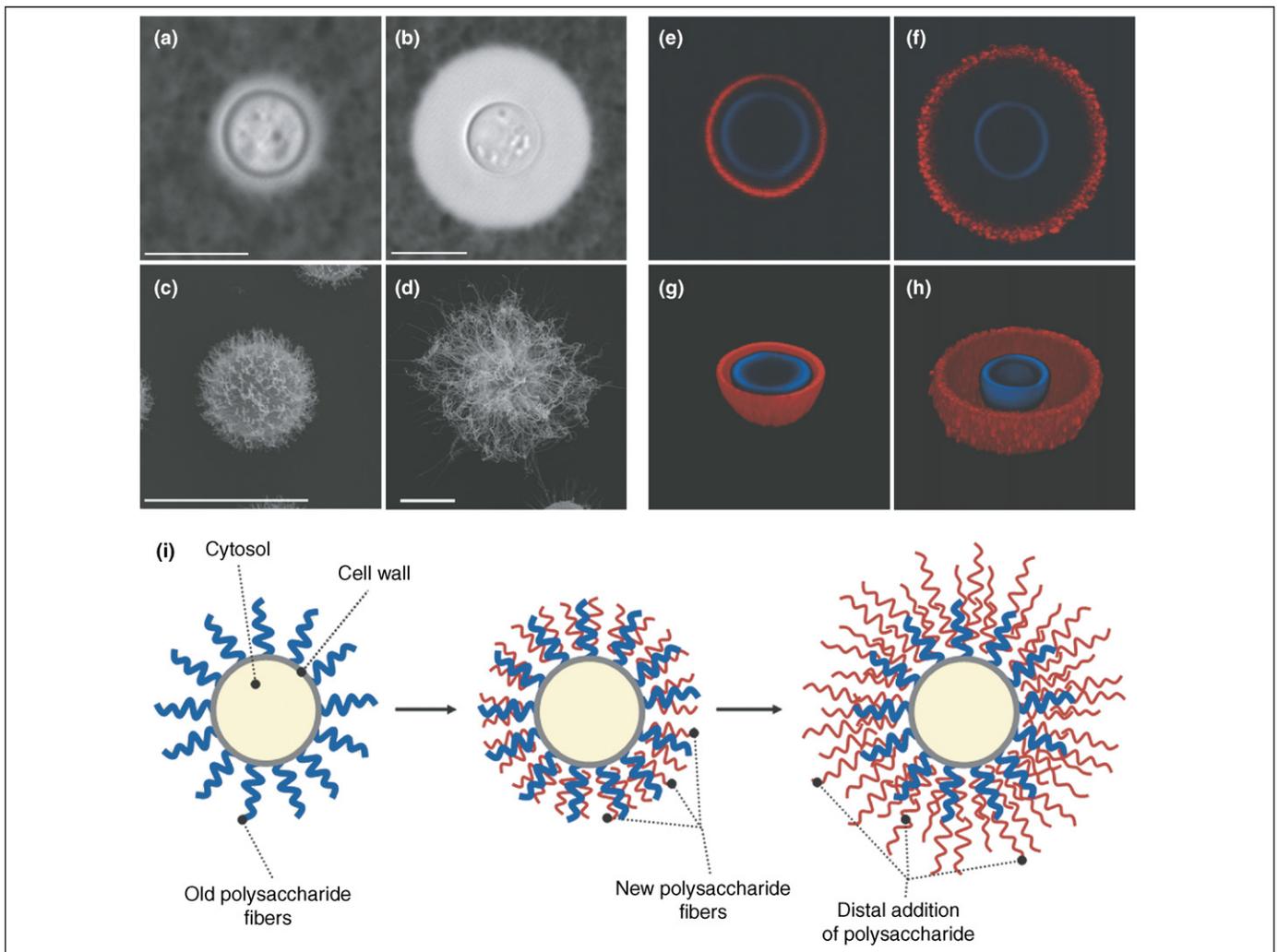
Figure 3. Micrographs of *Cryptococcus neoformans* cells with different capsule size. India-ink staining of **(a)** cells with a small capsule and **(b)** cells with an enlarged capsule. The capsule is visible as a while halo around the cell. Scanning electron microscopy of **(c)** small-capsule cells and **(d)** enlarged-capsule cells. Scale bars = 5 μm. Confocal microscopy of **(e)** small-capsule cells and **(f)** enlarged-capsule cells after coating the capsule with an IgM mAb to GXM (12A1) and detection with a goat anti-mouse IgM antibody conjugated to rhodamine (red fluorescence). The cell wall is visible by staining with calcofluor (blue fluorescence). 3D image reconstruction of **(g)** small-capsule cells shown in part (e); and **(h)** enlarged-capsule cells shown in part (f) after processing pictures taken along the *z*-axis with ImageJ software (NIH) and Voxx software (program owned by Indiana University). **(i)** Schematic showing the self-assembly and distal elongation of the capsule. New polysaccharide (red) is added to existing polysaccharide (blue).

enlarged capsules retain their capsule size after replication in rich growth medium but their daughter cells have small capsules. This suggests the absence of a capsular degradation mechanism and that the adaptation to new environmental conditions is independent of the initial capsule size [59]. Thus, the population produces offspring with capsule sizes appropriate for the new environmental conditions.

Capsule assembly and growth

The synthetic pathways for the capsular polysaccharides have not yet been delineated. A parallel approach to understanding capsule synthesis is to understand capsule assembly. Most of the knowledge in this area must be extrapolated from various independent studies. mAbs to GXM localize antigen to the cytoplasm and cytoplasmic vesicles [64,65], which suggests that GXM synthesis can occur at more than one site within the cell. Consistent with the finding of GXM in vesicles is the localization of the capsule-associated protein Cap10 in cytoplasmic

vesicles and the inference of membrane localization of the transmembrane proteins Cap59, Cas1 and Cas3 [9,10,66,67]. GXM was immunolocalized to vesicles crossing the cell wall, consistent with a vesicular-based export mechanism [64]. Interestingly, and perhaps important, is the finding that Cap59 (which is necessary to build the capsule) is involved in secretory transport [64].

The mechanism that underlies GXM attachment to the surface seems to involve non-covalent interactions because a thin capsule can be re-attached to an acapsular cell through interactions between GXM and cell wall α-1,3 glucan [68–70]. This interaction also occurs on the surface of *Histoplasma capsulatum* (which is normally not encapsulated but has α-1,3 glucan in its cell wall) but does not occur on the surface of *Saccharomyces cerevisiae* or *Candida albicans*, which lack cell wall α-1,3 glucan. Therefore, the initial attachment of GXM to *Cryptococcus* seems to use a simple mechanism that is readily available among other non-encapsulated fungi.

Despite a limited understanding of initial capsule assembly, important strides have been made in our knowledge of capsular growth, particularly given the development of efficient *in vitro* capsule enlargement protocols. Capsule growth *in vivo* is rapid and discernible differences in capsule size are apparent within five minutes of infection [65]. *In vitro*, an enlarged capsule can be detected as early as four hours after induction [47]. The first studies of capsule growth mechanics were reported in 2001 [54]. By labeling the capsule with mAb and then inducing its enlargement, the investigators observed that mAb was displaced to the capsule edge. On the basis of these studies, a model was proposed whereby new polysaccharide is added near the cell wall and old polysaccharide was displaced towards the capsule edge leading to capsule enlargement. However, the incubation time needed in these experiments and the reliance on antibody staining for tracking polysaccharide movement had the potential drawback that the antigen–antibody complex could dissociate, a concern that was recently validated [61].

By contrast, complement component C3 binds to capsular polysaccharide through a covalent bond and, thus, provides a non-reversible marker for the inner capsule. Capsule growth studies with C3 labeling revealed no migration of this protein during capsular enlargement. This result was interpreted as indicating that the new polysaccharide was added external to the C3 location [61]. As a secondary method, the capsular polysaccharide was metabolically radiolabeled during capsule growth and then the outer capsule was removed with γ-radiation [61]. Most of the radioactive label was incorporated into the outer capsule, implying growth at the capsule edge. Consistent with this mechanism was the observation that new antigenic determinants appeared at the capsule edge during *in vivo* capsular growth [71].

Most recently, the self-association of new polysaccharide fibers to pre-existing ones was suggested as a capsule assembly mechanism, which could explain the addition of new material to sites considerably distant from the cell, and the existence of two capsule layers as defined by dimethyl-sulfoxide (DMSO) solubility [13,16]. A combination of these several lines of evidence led us to propose a model of capsule growth in which new polysaccharide fibers intercalate into the existing capsule through self-association, which explains the increased density of this area. We also propose that capsule growth itself occurs by a non-enzymatic means, that is, by the self-association of fibers at the capsule edge (Figure 3i).

Capsular rearrangements during budding

Although capsular enlargement seems to be a common response of *C. neoformans* to environmental stimuli, a large capsule could be problematic for one of the most important vital functions of the cells: replication. However, daughter cell separation is not interfered with by the dense net of capsule fibers (Figure 4). Capsular rearrangements during budding initially manifests as a dimple at the capsule edge. Then, as the opening expands, capsule markers such as mAb or C3 are lost from the area [54,61]. These observations suggest that the capsule undergoes local rearrangement during budding, possibly producing
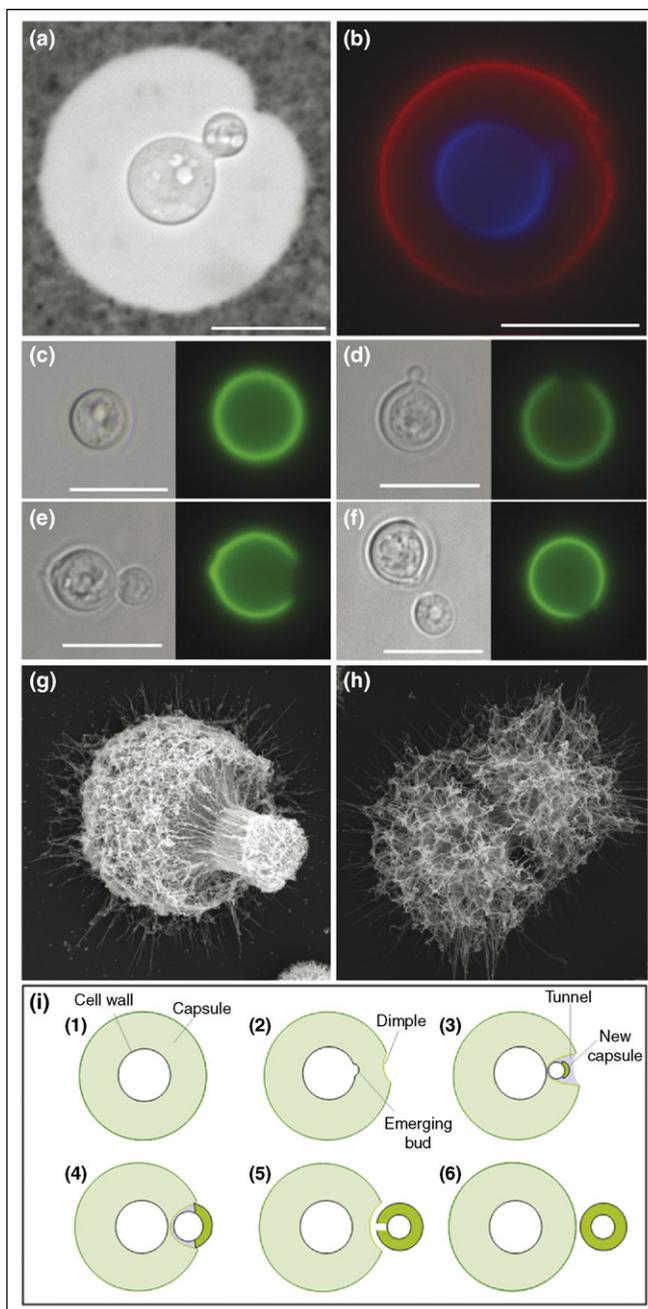
Figure 4. Capsule rearrangement in *Cryptococcus neoformans* budding cells. **(a)** Budding cell suspended in India ink. A dimple is formed in the capsule in the budding area. **(b)** Staining of a budding cell with mAb 18B7 and goat anti-mouse IgG antibody conjugated to rhodamine (red fluorescence). Disruption of uniform staining occurs in the budding area. **(c)–(f)** Cells in different budding stages after labelling the capsule with complement (green fluorescence). Bud separation produces the disappearance of complement in the budding area compared with non-budding cells [part (c)]. Scale bars = 10 μm. **(g)** and **(h)** Scanning electron micrographs of budding cells. A physical separation between the capsule of the mother cell and bud is observed. **(i)** Model of capsule growth and capsule rearrangements during budding in *C. neoformans*. (1) Newly synthesized capsule (light green) accumulates at the edge of the capsule after enlargement, with the old capsular material (light blue) close to the cell wall. When the bud begins to grow, a dimple (2) and a tunnel (3) are formed in the capsule, which enables the separation of the bud. (4) Bud growth is accompanied by capsule growth in the bud. (5) The capsule of the mother cell closes as the bud separates from the mother cell and, at the same time, the bud completes the capsule (6) without taking any polysaccharide of the mother cell. Pictures and schematic reproduced, with permission, from Ref. [58].

a tunnel for the bud to emerge. For a complete and efficient separation, the capsules of the mother and daughter cells should be distinct and this was vividly demonstrated by scanning electron microscopy [61]. How the capsules of the two cells separate without entanglement remains unsolved. GXM-degrading enzymes have not been described but capsule degradation of the mother cell might not be necessary. Separation could be a purely physical process, with force being generated by the capsule fibers of the mother cell as they move into the separation gap, or by the daughter cell as the capsule grows. This would also enable a fast separation.

Techniques for studying the capsule and capsular dynamics

For most of the 20th century, the main technique available to study the *C. neoformans* capsule was light microscopy after suspension in India ink. The capsule is not visible without India ink because its refraction index is similar to that of aqueous media; however, the capsule is easily visualized as a white halo that separates the cell from the dark background of ink particles. This simple technique has been crucial to many capsule studies. The capsule can be visualized without ink particles if coated with mAb ('quellung' or capsular reaction) because this alters the refraction index of the capsule [72].

Most of the structural information about the capsule is inferred from analysis of the capsular polysaccharides purified from *C. neoformans* culture supernatants [73]. Although biologically important, the exopolysaccharides might or might not have similar properties, such as structural composition or molecular weight, to the polysaccharides within the capsule. Until recently, treatment of the cell with DMSO was the most common means to obtain capsular material [68]. This method kills the cell and the amount and purity of the released material is often difficult to modulate because organic solvents can also remove internal polysaccharides [16,68]. Recently, γ-radiation was identified as a new method to isolate polysaccharide from the capsule. In contrast to DMSO treatment, fungal cells survive γ-radiation treatment and the degree of capsule release is a function of dose [74,75]. Radiation generates free radicals in water that are believed to react with polysaccharide molecules and mediate the release of capsular material [69,76,77]. This mechanism is supported by the protection of the capsule from γ-radiation with free-radical quenching agents, sorbitol and ascorbic acid [75]. Polysaccharide from the capsule can now be reliably obtained in sufficient amounts and with greater purity, which provides new opportunities for chemical and biological analysis.

To study the dynamics of capsule growth and rearrangements during budding, a good marker for the capsule is needed. The cell wall provides an invariant reference point and the edge of the capsule structure can be defined by India ink particle penetration. However, the identification of geographical markers within the polysaccharide capsule has been more elusive. A good capsular marker must be exclusive to the capsule and remain bound to the capsule during enlargement. Most capsule growth and rearrangement studies have used mAbs or complement to label the capsule fluorescently. mAbs to GXM are specific for the capsule and can bind to it before and after capsule induction [54]. However, the non-covalent interactions of antigen–antibody complexes are subject to the uncertainties of affinity- and concentration-dependent equilibrium considerations. These parameters can change as the capsular features change during growth, causing antibody to relocate [61]. By contrast, complement forms thioester covalent bonds and C3 irreversibly binds to the capsule.

Metabolic labeling using radioactive sugars is another approach to study capsular dynamics [54,59,78]. During incubation with ^{3}H-xylose or ^{3}H-mannose, the isotope incorporates into the capsule and can be detected by autoradiography of cells or liquid scintillation counting of capsular material released by γ-irradiation [54,59]. ^{13}C-labeling of GXM was used to investigate the synthesis of the capsular polysaccharide [78]. Liquid scintillation counting is more sensitive than autoradiography and, in conjunction with the variable exposure of cells to γ-irradiation, labeled regions of the capsule can be more easily defined.

A major problem in studying the *C. neoformans* capsule is its highly hydrated structure. Scanning or transmission electron microscopy has the inherent problem that sample preparation requires dehydration, which in turn induces capsule collapse. The capsule is better preserved for transmission electron microscopy in infected tissue samples [79]. Transmission electron microscopy on freeze-fractured cells and scanning transmission electron microscopy of isolated GXM reveals detailed images of the organization of the capsular fibers [13,54,80]. Studies on capsule porosity can take advantage of fluorescently labeled dextrans [16]. Application of these newer methods to explore *C. neoformans* capsular structure has shown that the structure apparent by India ink is, indeed, a highly dynamic structure that actively responds to external stimuli and undergoes complex rearrangements during cell growth.

Concluding remarks and future perspectives

The symmetrical growth of the *C. neoformans* capsule poses a problem in understanding the mechanism(s) of capsule growth because the geometry of spheres dictates that any increase in the capsule must result in a squaring and a cubing of the capsular surface area and volume, respectively. Hence, spherical growth necessitates that as the capsule enlarges, substantially more material must be delivered to the surface to encompass a rapidly growing volume and surface area. The radius of gyration of polysaccharide molecules shows that these are substantially smaller than the capsular thickness, which implies that assembly and growth is not the result of the growth of individual molecules. Instead, it suggests that capsular polysaccharide has self-aggregative properties, which could imply that GXM molecules contain much of the information necessary for capsular assembly. Consequently, spherical growth might require only the delivery of GXM molecules to the outer portions of the capsule. However, the mechanism to transport GXM through the capsule and the types of GXM–GXM interactions needed in the assembly of the fibrous network (which are apparent in electron micrographs) are unknown. Further complicating

any proposed capsular assembly scheme is the observation that GXM could be synthesized internally and delivered to the cellular exterior in vesicles [64,65]. Consequently, a vesicular transport mechanism to deliver capsular polysaccharide components to the capsule, and possibly to the exterior regions, could be speculated.

In addition to elucidating the mechanism of GXM delivery to the exterior space, it also remains to be determined whether GXM is synthesized in its entirety before delivery to the extracellular space or in units that are subsequently assembled. Immunohistological studies support GXM synthesis in units because mAbs that recognize different epitopes localize in different parts of the cytoplasm and cell wall [64]. If mature GXM is synthesized through the joining of units, then the cell would have considerable flexibility in varying its capsular structure. However, performing this synthesis on the outside of the cell would require enzymatic machinery in the capsule, and the different internal locations of GXM units might require different transport mechanisms with different molecular markers to deliver them to the cellular exterior and to certain destinations in the capsule.

The role of GalXM in capsular assembly, if any, remains an enigma. GalXM has been identified in the inner regions of the capsule, which have a greater density and greater resistance to removal by radiation and DMSO [17,75,81]. Whether GalXM is responsible for the greater tenacity and higher fibrous density of the capsule is unknown. The unavailability of mAbs or other reagents to study GalXM limits its study.

The relationship of shed polysaccharide to capsular polysaccharide remains largely unexplored. *C. neoformans* produces copious amounts of GXM and GalXM in culture and milligram quantities can be recovered from stationary cultures after days and weeks. Notably, polysaccharide is released *in vitro* during late stationary phase, which suggests the possibility that this effect is related to quorum-sensing [58]. In that event, shed polysaccharide could represent a different metabolic product instead of being from the extant capsule or new synthesis.

The past six years have witnessed tremendous progress in our understanding of the mechanisms involved in capsule synthesis and structure. As the detailed studies of the capsule begin in earnest, this unusual structure clearly poses a set of formidably difficult problems and questions. A fuller understanding of capsular assembly and growth dynamics will require the parallel application of genetic, biochemical and physico-chemical techniques and the development of new approaches to study fragile hydrated structures.

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