Published in final edited form as:

Curr Opin Struct Biol. 2011 October ; 21(5): 597–602. doi:10.1016/j.sbi.2011.08.006.

Emerging themes in cryptococcal capsule synthesis

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Abstract

Cryptococcus neoformans, a basidiomycete yeast and opportunistic pathogen, expends significant biosynthetic effort on construction of a polysaccharide capsule with a radius that may be many times that of the cell. Beyond posing a stimulating challenge in terms of defining biosynthetic pathways, the capsule is required for this yeast to cause fatal disease. This combination has focused the attention of researchers on this system. Here we briefly review two aspects of the rapidly advancing field of capsule synthesis: the extensive variation that occurs in capsule polymers and the regulation of capsule biosynthesis.

INTRODUCTION

Biological systems offer a remarkable diversity of both form and function. One example is the capsule of *Cryptococcus neoformans*, an intricate structure of polysaccharide fibers (Fig. 1). As the flip side to its beauty, the capsule plays multiple roles in the pathogenesis of a fatal disease: *C. neoformans* is an opportunistic fungus that kills over 600,000 people annually, mainly those suffering from severe immunocompromise in the context of limited access to health care. The burden of this disease therefore falls disproportionately on HIV-positive individuals in the developing world [1].

The nineteenth century scientists who first isolated *C. neoformans* from environmental sources (fruit juice [2]) and infected tissue [3] were struck by its extensive capsule. More recently, research on this structure has accelerated, stimulated by its biological interest and pathological importance. The capsule is notable for being made by a eukaryotic microbe: although it conceptually resembles its better-studied bacterial counterparts, the details of its synthesis, assembly, and display are quite distinct. The fact that this structure is required for cryptococcal virulence adds urgency to elucidating, and potentially disrupting, the processes involved in capsule synthesis.

Significant progress has been made in the field of capsule synthesis during the last few years, from the basic enzymology of this process [4,5] to broader questions of capsule assembly. We now know that capsule polysaccharide synthesis occurs primarily in the lumen of the secretory pathway with the participation of Golgi enzymes [4], and that the polysaccharide products exit the cell via the protein secretory pathway [6,7] and are also

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found in association with vesicular structures shed from the cell [8]. These areas have been reviewed recently [9–12] and will not be revisited here, although numerous mechanistic questions about these events remain to be answered. (Readers interested in capsule function and other aspects of cryptococcal biology are also referred to an excellent new edited book on this subject [13].) In this review we focus on two related themes that have emerged from recent studies of cryptococcal capsule synthesis: the variation in structure of the component polysaccharide molecules and the dramatic regulation of capsule structure that occurs in response to environmental signals, including entry into a mammalian host.

CAPSULE POLYSACCHARIDES AND ARCHITECTURE

The capsule of *Cryptococcus neoformans* consists primarily of two polysaccharides, glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal¹), along with smaller amounts of mannoproteins. In addition to being associated with the cell, these molecules are shed as an exopolysaccharide fraction. The larger polymer, GXM (Fig. 2, top), accounts for about 90% of the capsule mass and its structure has been analyzed in detail [14]. Imaging studies suggest that GXM is associated with vesicle membranes [6,15], although the nature of this association has not been defined. GXMGal (Fig. 2, bottom) has a more complex structure [16,17] and comprises about 10% of the shed polysaccharide. Historically, a lack of biological reagents has limited studies of GXMGal, but recent work has addressed its localization and suggested a potential role in capsule formation [18]. Both polymers have been implicated in cryptococcal virulence via impairment of the host immune response (reviewed in [19]).

Glycans offer a revealing window on biological systems because they respond in structure and abundance to the environment, metabolic state, and physiological needs of the cells that produce them. The cryptococcal capsule is no exception. This dynamic structure undergoes significant changes in size in response to environmental stimuli (Fig. 3); recent studies from several groups indicate that this change is mediated at the level of synthesis of individual GXM molecules [20,21]. Characteristics other than polymer size are also highly variable. Different strains of *C. neoformans* bear GXM that varies in the amount and pattern of xylose substitution (Fig. 2) as well as in the level of 6-*O*-acetylation of backbone mannose residues [14]. These differences contribute to the distinct antigenic properties of GXM from different serotypes. To add to the complexity, switching can also occur within strains, yielding variants that differ in terms of the predominant structural reporter groups of GXM (Fig. 2) as well as in other characteristics [22]. Changes in GXM occur in a variety of physiological settings: altered growth conditions affect polysaccharide branching [23]; cell age influences GXM acetylation [25] and overall capsule density [26]; and structural modifications have been suggested to occur in the context of infection [24]. Although most of these observations derive from studies of GXM, the physical properties of GXMGal have also been shown to change with environmental conditions [27]. Determining the mechanisms of these variations and how they are regulated offers a rich area for research.

Studies of the spatial organization of the capsule have advanced significantly in the last few years, building on previous work that indicated an inner zone of higher density material, consistent with the radial arrangement of capsule fibers [28]. New methods such as optical tweezers have been applied to measuring capsule stiffness, emphasizing the elasticity of the structure and supporting the greater density of smaller capsules [29]. A recent study also indicates that GXM polymers associate by cross-linking that is dependent on the concentration of divalent cations [30], similar to a mechanism proposed for pectin assembly

¹This polymer is frequently termed GalXM in the literature, but has been renamed based on its structure as detailed in references [9,11,16].

[31]. This model suggests that Ca^{2+} forms salt bridges between the negatively charged glucuronic acid residues of neighboring GXM fibers, mediating capsule assembly through GXM-GXM aggregation. The model is supported by observations of increased GXM shedding in the absence of Ca^{2+} , decreased capsule size in the presence of EDTA or monovalent cations [30], and decreased capsule elasticity in conditions of increased solution Ca^{2+} concentration [29]. The viscosity of GXM in solution is also reduced in the presence of chelators and upon chemical reduction of the carboxyl groups of glucuronic acid [30]. Although some caution is warranted given that several of these studies were performed at Ca^{2+} levels significantly above those in mammalian plasma [29], this work still provides a consistent working model for capsule assembly. Capsule assembly is also affected by changes in the cell wall structure beneath it (Fig. 1). Cells lacking α -1,3 glucan in their cell walls do not display surface capsule, although they shed normal capsule polysaccharides (suggesting independent synthesis of these structures) [32,33]. In contrast, cells lacking cell wall β -1,3 glucan form capsules that are larger than those of wild type cells [34]. These also have increased permeability to small molecules [34], suggesting that this defect in cell wall structure affects capsule architecture.

CAPSULE REGULATION

A well-recognized feature of the cryptococcal capsule is its dramatic response to environmental conditions, easily visualized by negative staining with India ink (Fig. 3). A variety of growth conditions, notably entry into a mammalian host, 'induce' formation of a larger capsule; this results at least in part from the synthesis of longer GXM molecules (see above). *In vitro* conditions leading to this capsule response include those that induce stress and mimic aspects of host infection, such as increased carbon dioxide, reduced iron availability, and the presence of serum [9]. These observations, together with the general avirulence of acapsular cryptococci, suggest that capsule expansion is important in pathogenesis.

Colonies of cells with reduced capsule size are readily identified on solid growth medium, as they typically appear dry compared to wild type colonies. This morphological characteristic, coupled with other methods for assessing capsule phenotype (including cell sedimentation behavior [35] and antibody-based methods [36]) has allowed isolation of a spectrum of acapsular and hypocapsular mutants, although most of these have been characterized primarily with respect to capsule size or antibody reactivity rather than in structural detail. Additional mutants in capsule synthesis have been found through phenotypic analysis of the rapidly growing collection of *C. neoformans* gene deletion strains [37]. Together, these mutants have enabled the identification of numerous genes implicated in capsule synthesis [11].

Particularly intriguing in the long list of genes whose deletion results in altered capsule size are those that encode known or putative regulatory proteins, including DNA-binding factors and participants in known signaling pathways. Studies from several laboratories have implicated the cAMP pathway in capsule response [38]; recent work has similarly implicated participants in the high osmolarity glycerol (HOG) response and other signaling pathways [38–40] in this process. The demonstration of specific transcription factors affecting capsule was first reported five years ago, with the characterization of the cryptococcal Nrg1 protein, which is downstream of the cAMP pathway [41], and of Cir1, an iron-responsive transcription factor [42]. Cir1, in particular, plays a central role in gene regulation that affects capsule as well as other virulence factors of *C. neoformans*. Research in this area has accelerated recently, with the identification of seven more transcriptional regulators that affect capsule size (Gat201 [37,43], Tup1 [44], Gcn5 [45], Rim101 [46], Hap3 and Hap5 [47], and Ada2 (our unpublished work)), five of them in the last year.

Major current challenges are to understand the relationships between the cellular response pathways that influence capsule and to define how the signals they transmit are integrated to produce the final structure. Powerful genome-scale tools like chromatin immunoprecipitation and RNA sequencing are now being applied to these questions, in order to investigate both the regulators and targets of DNA-binding proteins that have been implicated in capsule regulation ([43] and unpublished work from our laboratory). Data from these approaches will enable the application of computational tools to elucidate the capsule regulatory network in *Cryptococcus* (Fig. 4), complementing more traditional avenues of research. In the near future we can expect this combination to yield greater understanding of the upstream factors that trigger changes in capsule synthesis, the interplay between the signaling pathways that they initiate, and the array of biosynthetic genes that are modulated in response to these signals to generate the final capsule structure.

LOOKING FORWARD

The cryptococcal capsule captured the imagination of microbiologists over one hundred years ago and continues to do so through the present. While this review offers a snapshot of current research in several rapidly advancing areas related to capsule synthesis, we are far from a comprehensive understanding of this fascinating and medically important topic. Continued focus on capsule synthesis during the next few years should yield additional insights into the biosynthetic pathways, spatial relationships, and gene regulation that combine to produce this unique polysaccharide structure.

Acknowledgments

We thank members of the Doering lab, Michael Brent, and colleagues in the fields of glycobiology and microbiology for stimulating discussions of capsule synthesis. Studies of capsule in the Doering lab are supported by National Institutes of Health grants GM071007 and AI087794.

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HIGHLIGHTS

- The cryptococcal capsule offers insights into glycan diversity and synthesis.
- Capsule polysaccharide size and structure change with the environment and cell age.
- Discovery of capsule regulatory factors has accelerated in the last few years.
- How regulatory pathways are integrated to change capsule structure remains to be determined.

Figure 1.

Cryptococcal surface structures. This quick-freeze deep-etch electron micrograph of the edge of a cryptococcal cell (J. Heuser and T.L. Doering, unpublished) shows a segment of the cell wall separating a region of the plasma membrane from the radiating capsule fibers. The capsule extends upwards from the cell body and only the inner portion of the structure is visible; the entire capsule would extend significantly beyond the region shown.

Figure 2.

Schematic depiction of the cryptococcal capsule polysaccharides. Single polymer repeat units are shown and all sugars are in the pyranose form unless labeled with *f* to indicate furanose. Top, the six structural reporter groups that are found in varying proportions in glucuronoxylomannan (GXM) from different strains of *C. neoformans* (as defined by Cherniak *et al* [14]). All linkages are as indicated on the M4 structure. Mannose residues, green spheres; xylose, red stars; glucuronic acid residues, half filled diamonds. Mannose acetylation is not shown. Bottom, the structure of glucuronoxylomannogalactan (GXMGal). Trisaccharide side branches that occur at every other galactose may be substituted on 0 – 3 moieties [16,17]; the two extreme cases are shown. Symbols are as above, with galactose shown as yellow spheres. Some unbranched backbone galactose residues are modified with β -1,2 and β -1,3-linked galactofuranose as shown (C. Heiss, M. Skowyra, and T. Doering, unpublished data). Shapes and colors follow the recommendations of the Consortium for Functional Glycomics. This figure is modified from [9] according to the generous policy of Annual Reviews.

Figure 3.

Growth conditions dramatically alter capsule thickness. Striking capsule induction is demonstrated by light micrographs of cells grown in an atmosphere of either room air (left) or 5% CO₂ (right). Both cultures were grown for 24 hours at 37 °C in tissue culture medium and then stained with India ink; the capsule appears as a region of ink exclusion surrounding the cell. Scale bars, 10 microns.

Figure 4.

Capsule is regulated by a complex network, as exemplified by this subset of regulatory interactions involving transcription factors that influence capsule size (red ovals; see text). Several of the interactions shown are mediated by Hog1, a MAP kinase involved in stress response and virulence factor synthesis in *C. neoformans*, which is phosphorylated by the MAPK kinase Pbs2 [39]. SAGA is a multiprotein complex that regulates transcription [48]; deletion of genes encoding two of its component proteins, Gcn5 and Ada2, yields hypocapsular cryptococci ([45] and our unpublished work, respectively). The diagram is based on our analysis of gene expression profiles generated by microarray [41–43,46] or RNA sequencing (our unpublished data on strains deleted for *NRG1*, *CIR1*, and *ADA2*). Green lines, stimulation of transcription; red lines, inhibition of transcription; solid black arrow, phosphorylation; P, phosphate; dashed arrow, reaction catalyzed by Pbs2.