The Overlooked Glycan Components of the *Cryptococcus* Capsule

Fernanda L. Fonseca, Flavia C. G. Reis, Bianca A. G. Sena, Luísa J. Jozefowicz, Livia Kmetzsch and Marcio L. Rodrigues

Contents

Abstract Pathogenic species of *Cryptococcus* kill approximately 200,000 people each year. The most important virulence mechanism of *C. neoformans* and *C. gattii*, the causative agents of human and animal cryptococcosis, is the ability to form a

F. L. Fonseca
Centro de Desenvolvimento Tecnológico Em Saúde (CDTS), Fundação Oswaldo Cruz, Rio de Janeiro, Brazil

F. C. G. Reis · B. A. G. Sena · L. J. Jozefowicz · M. L. Rodrigues
Instituto Carlos Chagas, Fundação Oswaldo Cruz (Fiocruz), Curitiba, Brazil

L. Kmetzsch
Centro de Biotecnologia, Universidade Federal Do Rio Grande Do Sul, Porto Alegre, Brazil

L. Kmetzsch
Departamento de Biologia Molecular E Biotecnologia, Universidade Federal Do Rio Grande Do Sul, Porto Alegre, Brazil

M. L. Rodrigues
Instituto de Microbiologia Paulo de Góes (IMPG), Universidade Federal Do Rio de Janeiro, Rio de Janeiro, Brazil

M. L. Rodrigues (✉)
Cidade Industrial de Curitiba, Rua Professor Algacyr Munhoz Mader, 2135-2261, Curitiba, PR 81310-020, Brazil
e-mail: marcio.rodrigues@fiocruz.br

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polysaccharide capsule. Acapsular mutants of *C. neoformans* are avirulent in mice models of infection, and extracellularly released capsular polysaccharides are deleterious to the immune system. The principal capsular component in the *Cryptococcus* genus is a complex mannan substituted with xylosyl and glucuronyl units, namely glucuronoxylomannan (GXM). The second most abundant component of the cryptococcal capsule is a galactan with multiple glucuronyl, xylosyl, and mannosyl substitutions, namely glucuronoxylomannogalactan (GXMGal). The literature about the structure and functions of these two polysaccharides is rich, and a number of comprehensive reviews on this topic are available. Here, we focus our discussion on the less explored glycan components associated with the cryptococcal capsule, including mannoproteins and chitin-derived molecules. These glycans were selected for discussion on the basis that i) they have been consistently detected not only in the cell wall but also within the cryptococcal capsular network and ii) they have functions that impact immunological and/or pathogenic mechanisms in the *Cryptococcus* genus. The reported functions of these molecules strongly indicate that the biological roles of the cryptococcal capsule go far beyond the well-known properties of GXM and GXMGal.

1 Introduction: The Cryptococcal Capsule

Capsular structures are common surface components of bacterial pathogens including *Streptococcus pneumoniae*, *Haemophilus influenzae,* and *Neisseria meningitidis* (Roberts 1996). *C. neoformans* and *C. gattii* are the only known eukaryotic pathogens that harbor capsules. The capsular network of *Cryptococcus* is the outermost layer surrounding the cell, concealing the cell wall and the plasma membrane (Fig. 1). The capsular barrier imposes additional difficulties for the design of new antifungal chemotherapies against cryptococcosis.

The capsule of *Cryptococcus* has been extensively studied, which is justified by its major role as a virulence factor (Bose et al. 2003; Zaragoza et al. 2009; Doering 2010; Wang et al. 2018). Structurally, the cryptococcal capsule is mainly composed by two polysaccharides. Glucuronoxylomannan (GXM) is the most abundant polysaccharide and consists of a chain of α1,3-linked mannose units with xylosyl and glucuronyl substitutions (Cherniak et al. 1998). GXM, which comprises around 90% of the total mass of the capsule, is also abundantly found in the extracellular milieu in the form of a heterodisperse, high molecular mass polysaccharide (1700–7000 kDa) (McFadden et al. 2006). The remainder 10% of the total mass of the capsule is mostly composed of glucuronoxylomannogalactan (GXMGal). GXMGal is formed by α1,6-linked galactose units with mannosyl, xylosyl, and glucuronyl substitutions (Heiss et al. 2009). The molecular mass of GXMGal corresponds to approximately 100 kDa (Zaragoza et al. 2009).

GXM and GXMGal are key players in physiological and immunopathogenic events in the *Cryptococcus* genus. Their biological roles, however, have been discussed in detail in several reviews (Doering 2010; Agustinho et al. 2018).

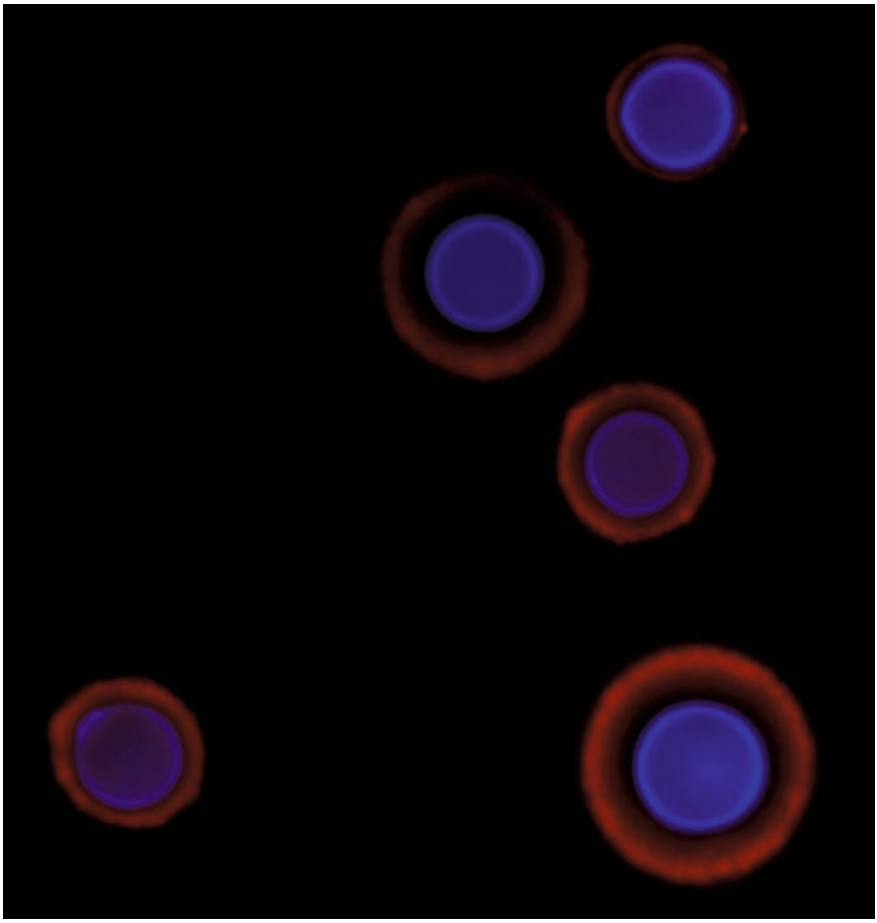

Fig. 1 Cell surface of *Cryptococcus*. Major surface components of *Cryptococcus* can be evidenced by fluorescence microscopy. Capsular structures are stained in red, while the cell wall is stained in blue. Capsule size is usually a variable within cryptococcal populations, which likely impacts pathogenic mechanisms (Fonseca et al. 2010; Albuquerque et al. 2014). Experimental details about cell surface staining of the cryptococcal surface are available in Rodrigues et al. (2008), Fonseca et al. (2009)

Other components associated with the capsule have been overlooked. Mannoproteins (MPs) are considered by a number of authors as the third glycan component of the capsule (Zaragoza et al. 2009), but the connections of these glycoproteins with the major capsular structures have not been established. Additional glycans have also been observed within the capsular network of *Cryptococcus*, including chitin-derived structures. Although these glycans are not covalently linked to the major capsule components (Ramos et al. 2012), they have been consistently detected in different layers of the cryptococcal capsule (Rodrigues et al. 2008, 2015; Fonseca et al. 2009,

2013). Both MPs and chitin-derived structures participate in the interaction of *Cryptococcus* with the host (Voelz and May 2010; Fonseca et al. 2013; Teixeira et al. 2014; Rodrigues et al. 2015). Here, we will discuss the roles of these two classes of glycans as components of the cryptococcal capsule.

2 Chitin and Capsular Architecture of *Cryptococcus*

Chitin metabolism in fungi involves a number of key structures, including its de-acetylated form (chitosan), the products of chitin hydrolysis (chitooligomers), and the enzymes involved in the balance between chitin synthesis (chitin synthases) and hydrolysis (chitinases). Each of them participates in the capsular architecture of *Cryptococcus*, as detailed in the following topics.

2.1 Chitin and Chitin Synthases

Chitin is a structural component of the fungal cell wall, accounting for approximately 2% of the wall mass (Camacho et al. 2017; Agustinho et al. 2018). This ancestral polysaccharide is a linear homopolymer composed of ß-1,4-linked units of *N*-acetylglucosamine (GlcNAc). Microfibrils of stacked chitin chains are stabilized by hydrogen bonds, assuring insolubility and mechanical strength (Doering 2010; Gonzalez et al. 2010). However, the view that chitin is an exclusive structural component providing protection from the internal hydrostatic pressure exerted on the wall by the cytoplasm and/or by environmental stresses (Gow et al. 2017) is now considered minimalist. In *Cryptococcus*, chitin is in fact a scaffold structure of the cell wall (Agustinho et al. 2018), but the polysaccharide also participates in capsular architecture (Rodrigues et al. 2008, 2018; Fonseca et al. 2009; Ramos et al. 2012) and assembly of melanin into the cell wall (Banks et al. 2005; Baker et al. 2007; Camacho et al. 2017). In addition, chitin has been demonstrated to modulate the host's immune response (Da Silva et al. 2008, 2009; Wagener et al. 2014; Wiesner et al. 2015; Ost et al. 2017). Therefore, this molecule is crucial for both cryptococcal physiology and pathogenesis.

Chitin is polymerized by membrane-associated chitin synthases, which use cytosolic UDP-*N*-acetyl-D-glucosamine as a sugar donor (Doering 2010). Eight putative cryptococcal chitin synthase genes (*CHS*1–*CHS*8) and three regulator proteins (Csr1–Csr3) have been identified in *C. neoformans* (Banks et al. 2005). It is therefore assumed that chitin synthesis is highly redundant in this fungus, which is likely related to its low susceptibility to nikkomycin, an inhibitor of chitin synthesis (Rinaldi 1999; Banks et al. 2005). Importantly, none of the chitin synthase or chitin synthase regulator genes are essential for cryptococcal viability (Banks et al. 2005; Rodrigues et al. 2018). Recent evidence, however, indicated that chitin synthesis is required for the correct capsular architecture of *C. neoformans*.

Deletion of seven out of the eight *CHS* genes resulted in aberrant capsular morphologies and altered polysaccharide dimensions (Rodrigues et al. 2018). Five of the eight *CHS* genes were required for the serological reactivity of capsular GXM, while the functionality of all eight genes was required for full polysaccharide secretion, in comparison with parental cells regularly expressing the *CHS* genes. (Rodrigues et al. 2018). These results clearly demonstrate that chitin synthesis and capsular formation are connected in *C. neoformans*. Remarkably, deletion of *CHS* genes also affected extracellular vesicle formation and chitinase activity (Rodrigues et al. 2018), which are events that also participate in capsular architecture (Rodrigues et al. 2007; Fonseca et al. 2009).

2.2 Chitooligomers

Chitooligomers or chitooligosaccharides are chitin-derived structures composed of 3–20 residues of β1,4-linked *N*-acetylglucosamine, produced enzymatically by chitinase-mediated chitin hydrolysis (Fonseca et al. 2013). These structures efficiently react with the wheat germ lectin (WGA) (Foster et al. 2004).

The demonstration that chitin-related structures are part of the capsular network in *C. neoformans* was generated by a combination of microscopic, biochemical, and pharmacological approaches. The first suggestion that chitin-derived structures and capsular components were associated was originated from the microscopic detection of chitin oligomers within the capsular network of both *C. neoformans* and *C. gattii* (Rodrigues et al. 2008). In *Cryptococcus*, WGA recognized chitooligomers with high affinity (Rodrigues et al. 2008; Fonseca et al. 2009, 2013). The chitooligosaccharides were detected at the cell wall but, unexpectedly, they were also found as projections emerging from the cell wall into the cryptococcal capsule, apparently connecting these two layers of the fungal surface. Chitooligomers formed round and hook-like structures detected within the capsule, as well as ringlike structures around the bud neck (Rodrigues et al. 2008).

The apparent association between capsular components and chitooligosaccharides was confirmed through different approaches. Chromatographic analysis revealed that hybrid glycans containing GXM and chitooligomers were found in their soluble form in culture supernatants, suggesting that formation of the complexes is a common event in the cryptococcal physiology (Fonseca et al. 2009). In the presence of the oligomers, capsular fibers increased in size, reinforcing the suggestion of intermolecular interactions between GXM and chitin-derived structures (Fonseca et al. 2009). Pharmacological inhibition of the synthesis of glucosamine 6-phosphate, a precursor of UDP-GlcNAc synthesis, resulted in decreased chitin detection and faulty capsules with clearly reduced dimensions (Fonseca et al. 2009). Structural analysis of the complexes formed by chitin and GXM revealed the requirement of chitin's *N*-acetyl groups for an efficient, non-covalent interglycan interaction (Ramos et al. 2012). The association of chitin-related structures and capsular components had a dramatic impact on the physiology of *C. neoformans*. Blocking of chitooligomers

with WGA resulted in reduced concentrations of extracellular GXM and decreased capsular dimensions (Fonseca et al. 2013). In addition, the transcription levels of genes involved in the synthesis, cellular traffic, and signaling pathways controlling capsule formation were also reduced (Fonseca et al. 2013).

The functional impact of the GXM–chitin association was not limited to the cryptococcal physiology. The interaction between chitooligomers and GXM resulted in stable, hybrid glycans with immunological functions that differed from each molecule alone (Ramos et al. 2012). Hybrid molecules were efficient inducers of lung tumor necrosis factor alpha (TNF-α), interleukin 10 (IL-10) and IL-17 in mice, suggesting that the association of GXM with chitooligomers produced molecules with unique immunological functions (Ramos et al. 2012). This observation is also in agreement with the notion that molecular interactions within the capsule can generate a number of complex structures with still unknown immunological functions.

Chitin-derived molecules were detected in outer layers of the capsule of *C. neoformans* and *C. gattii* (Rodrigues et al. 2008), suggesting that they could participate in the interaction of each pathogen with host cells. In fact, chitin-derived surface components affected the interaction of *C. neoformans* with the host at multiple levels (Fonseca et al. 2013). Toll-like receptor (TLR) 2, Dectin-1, and mannose receptor have been associated with immune responses to chitin resulting in the production of TNF-α and IL-10 (Da Silva et al. 2008, 2009; Bueter et al. 2013). Both murine and human macrophages produced IL-10 in response to cryptococcal chitin, in processes that likely required activation of NOD2 and TLR-9 (Heung 2017). WGA-treated *C. neoformans* were attenuated in virulence and had a poor capacity of dissemination to the central nervous system (Fonseca et al. 2013). Treatment of *C. neoformans* with WGA also resulted in reduced levels of interaction with host macrophages through mechanisms that required TLR-2 (Fonseca et al. 2013), suggesting that chitooligomer recognition is part of a Trojan horse mechanism of dissemination to the brain (Casadevall 2010). Accordingly, brain infection with *C. gattii* was further associated with increased chitooligomer distribution at the surface of fungal cells in mice (Rodrigues et al. 2015). Moreover, chitin stimulated Th2 responses during *C. neoformans* infection of mice through mechanisms that required polysaccharide cleavage by chitotriosidase, a mammalian chitinase (Wiesner et al. 2015). Increased chitooligomer distribution also correlated with peaks of chitinase activity in the lungs of infected mice (Rodrigues et al. 2015), suggesting that chitin hydrolysis, chitooligomer formation, capsule assembly, and pathogenesis are linked in *Cryptococcus*, as discussed below.

2.3 Chitinases

Chitooligomers are produced through chitin hydrolysis by both fungal and host chitinases (Goldman and Vicencio 2012; Ramos et al. 2012). Fungal chitinases have an important role in cell wall remodeling during growth, morphogenesis, and cell division (Adams 2004). In experimental models of cryptococcosis, expression

of chitinases varied according to the infected anatomic site (Fonseca et al. 2009). For instance, chitinase activity was induced in mice lungs infected with *C. neoformans*, but not in the brain (Overdijk et al. 1999; Vicencio et al. 2008). Intratracheal infection with *C. neoformans* also resulted in increased detection of chitinase activity in bronchoalveolar lavage fluids and lung homogenates of rats (Vicencio et al. 2008). Importantly, surface detection of chitooligomers in both *C. neoformans* and *C. gattii* was increased in lung tissues manifesting higher activity of chitinase (Fonseca et al. 2009, 2013; Rodrigues et al. 2015). Chitinase activity was also responsible for producing soluble, extracellular oligomeric structures of chitin that formed hybrid glycans with GXM during regular growth and macrophage infection, as concluded from the reduced formation of the hybrid structures in the presence of a chitinase inhibitor (Ramos et al. 2012; Rodrigues and Nimrichter 2012). Chitinase activity in *C. neoformans* under stress conditions (Rodrigues et al. 2018), which are known to induce capsule formation (Zaragoza and Casadevall 2004), was enhanced in both intracellular and extracellular fractions from *C. neoformans* (Rodrigues et al. 2018), suggesting that enzyme activity and chitooligomer formation are stimulated during capsule enlargement.

2.4 Chitosan

Differently from other fungi, chitin in *C. neoformans* is mostly de-acetylated by chitin deacetylases to form chitosan (Banks et al. 2005). The *CDA1*, *CDA2,* and *CDA3* genes are required for chitin deacetylation, but the presence of only one of these three chitin deacetylases is sufficient for chitosan production, suggesting metabolic redundancy (Baker et al. 2007). Cryptococcal chitosan levels may exceed the cellular amount of chitin by up to tenfold (Banks et al. 2005). The de-acetylated polysaccharide confers flexibility to the cell wall (Wang et al. 2018), which is essential for the molecular traffic across this cellular layer (Rodrigues and Casadevall 2018). Chitosan also contributes to the maintenance of cell wall integrity and bud separation (Banks et al. 2005; Wang et al. 2018). Importantly, chitosan is essential for melanin deposition on the cell wall of *C. neoformans* (Banks et al. 2005; Baker et al. 2007, 2011). Melanin is a cell wall pigment implicated in fungal pathogenicity (Nosanchuk and Casadevall 2006), resistance to environmental stress (Wang and Casadevall 1994; Nosanchuk and Casadevall 2006), and decreased susceptibility to antifungal drugs (Wang and Casadevall 1994; Martinez and Casadevall 2006).

Lack of chitosan critically impacts *C. neoformans* virulence (Baker et al. 2011). Fungal cells lacking chitosan synthesis manifest unstable cell walls, slow growth at 37 °C, and increased vulnerability to host defense mechanisms, with consequent inability to kill mice (Baker et al. 2011). Furthermore, chitosan-deficient *cda1cda2cda3*Δ mutants were not pathogenic and induced robust inflammatory, protective responses (Baker et al. 2011; Upadhya et al. 2016). In this context, heat-killed *cda1cda2cda3*Δ cells have been suggested as prototypes for vaccine development in the *Cryptococcus* model (Upadhya et al. 2016).

3 Mannoproteins

The cryptococcal surface contains highly mannosylated glycoproteins, namely mannoproteins (MPs). The main structural features of MPs include a signal sequence for post-Golgi secretion, a site for attachment of glycosylphosphatidylinositol (GPI) anchors, and a serine-/threonine-rich region, which bears extensive *O*-mannosylation (Levitz and Specht 2006). MPs are in close association with the cryptococcal capsule, and early estimates suggest they account for less than 1% of the capsular mass (Bose et al. 2003; Zaragoza et al. 2009). MPs are predominantly located in the inner region of the capsule, close to the cell wall (Jesus et al. 2010), but the fact that they contain secretory tags might suggest that they are transitory capsular components being transported to the extracellular space (Biondo et al. 2006; Eigenheer et al. 2007; Jesus et al. 2010; Agustinho et al. 2018).

Cryptococcal MPs are highly immunogenic and immunostimulatory (Chaka et al. 1997; Levitz and Specht 2006). MPs stimulate a massive production of IL-12 by human monocytes (Pitzurra et al. 2000). Binding of MPs to the mannose receptor of dendritic cells led to activation of T cells and protective immunity against *C. neoformans* (Specht et al. 2007; Dan et al. 2008a, b). Inhibition of mannose receptors or MP deglycosylation strongly prevented activation of T cell responses, indicating the essential contribution of mannosylation to immunogenicity (Levitz and Specht 2006). On the basis of these observations, MPs have been proposed as potential vaccine candidates against cryptococcosis (reviewed by Van Dyke and Wormley (2018)).

Bioinformatic analysis using the proteomes of *C. neoformans* and *C. gattii* revealed the putative occurrence of 43 and 36 predicted MPs, respectively (Reuwsaat et al. 2018). However, most of them remain to be characterized at the functional and structural levels. Levitz and colleagues explored the functions of *C. neoformans* MP98 in stimulating T cell responses using murine hybridomas (Levitz et al. 2001). MP98 is encoded by chitin deacetylase 2 gene (*CDA2*), which is responsible for converting chitin to chitosan. This protein, which is GPI-anchored to the plasma membrane, is associated with the cell wall. However, MP98 association with the cell wall is independent of both the GPI anchor and β-1,6-glucan (Gilbert et al. 2012). Similarly, MP88 is involved in T cell activation, sharing several structural features with MP98, which include a serine-/threonine-rich C-terminal region and a GPI anchor motif (Huang et al. 2002). Nevertheless, a supposed function for MP88 based on sequence similarity analysis has not been assigned (Levitz and Specht 2006). Other cryptococcal MPs that have been studied at the functional level include MP84 and MP115, which are recognized by serum antibodies from AIDS patients with cryptococcosis (Biondo et al. 2005). MP84 and MP115 have homology to polysaccharide deacetylase and carboxylesterase proteins, respectively. Furthermore, MP84 mediates the adhesion of *C. neoformans* to lung epithelial cells (Teixeira et al. 2014). Lastly, the *C. neoformans CIG1* gene encodes a secreted mannoprotein (Cig1) involved in a heme uptake system. Cig1 influences *C. neoformans* virulence in a mouse model of cryptococcosis but only in a strain that also lacked the high-affinity iron uptake system (Cadieux et al. 2013).

The roles of cryptococcal MPs in capsule structure and assembly have not been fully addressed. Recently, Reuwsaat et al. characterized a *C. gattii* putative MP, namely Kpr1, and suggested that it participates in capsular structure and GXM release (Reuwsaat et al. 2018). The *kpr1* null mutant cells were more sensitive to congo red, a classical cell wall stressor that disrupts beta-glucan synthesis. Gene knockout affected cell-associated cryptococcal polysaccharide thickness and phagocytosis by J774.A1 macrophages. Furthermore, recombinant Krp1 was selectively recognized by serum from patients with cryptococcosis (Reuwsaat et al. 2018). The in vivo pathogenic potential of *C. gattii*, however, was not affected by *KPR1* deletion. These data suggest a role of Kpr1 in capsule assembly in *C. gattii*.

The studies showing key biological functions of MPs contrast with the fact that only a minor fraction of cryptococcal MPs has been functionally characterized. In addition, the glycan moieties of cryptococcal glycoproteins have never been structurally determined. This scenario stimulates studies on the roles of MPs in the pathogenesis of *C. neoformans* and *C. gattii*.

4 Conclusions

It is now clear that the capsule of *Cryptococcus* is much more complex in composition and functions than initially thought. GXM and GXMGal are unquestionably major capsular components with fundamental biological functions, but the capsular network assuredly includes less abundant and even transitory components that might impact biological functions and pathogenic potential. The roles of these overlooked capsular components remain largely unexplored. Finally, intermolecular interactions within the capsular network are expected, as well as the formation of multimolecular structures with completely unknown roles in physiopathogenesis. Although the progress in the understanding on how the capsular components of *Cryptococcus* impact physiology and pathogenesis is incontestable, it seems clear that there is still much to learn about the functional multiplicity of the cryptococcal capsule.

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