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The capsule of the fungal pathogen *Cryptococcus neoformans*

Oscar Zaragoza¹, Marcio L. Rodrigues², Magdia De Jesus³, Susana Frases³, Ekaterina Dadachova^{4,3}, and Arturo Casadevall^{3,5,*}

¹ Servicio de Micología. Centro Nacional de Microbiología. Instituto de Salud Carlos III. Crta Majadahonda-Pozuelo, Km2. Majadahonda 28220. Madrid. Spain

² Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, RJ, 21941-902 Brasil

³ Microbiology and Immunology Department. Albert Einstein College of Medicine. 1300 Morris Park Avenue. Bronx, NY 10461

⁴ Nuclear Medicine Department, Albert Einstein College of Medicine. 1695A Eastchester Rd. Bronx, NY 10461

⁵ Medicine Department. Albert Einstein College of Medicine. 1300 Morris Park Avenue. Bronx, NY 10461

Abstract

The capsule of the fungal pathogen *Cryptococcus neoformans* has been studied extensively in recent decades, and a large body of information is now available to the scientific community. Well-known aspects of the capsule include its structure, antigenic properties and its function as a virulence factor. The capsule is composed primarily of two polysaccharides, glucuronoxylomannan (GXM) and galactoxylomannan (GalXM), in addition to a smaller proportion of mannoproteins (MP). Most of the studies on the composition of the capsule have focused on GXM, which comprises more than 90% of the capsule's polysaccharide mass. It is GalXM, however, that is of particular scientific interest because of its immunological properties. The molecular structure of these polysaccharides is very complex and has not yet been fully elucidated. Both GXM and GalXM are high molecular mass polymers with the mass of GXM equaling roughly 10 times that of GalXM. Recent findings suggest, however, that the actual Mw might be different to what it has traditionally been thought to be. In addition to their structural roles in the polysaccharide capsule, these molecules have been associated with many deleterious effects on the immune response. Capsular components are therefore considered key virulence determinants in *Cryptococcus neoformans*, which has motivated their use in vaccines and made them targets for monoclonal antibody treatments. In this review we will provide an update on the current knowledge of the *C. neoformans* capsule, covering aspects related to its structure, synthesis, and particularly, its role as a virulence factor.

Introduction

The adaptation of microorganisms to their environment is often associated with the acquisition of certain attributes that help improve survival in specific ecologic niches. Such adaptations include signal transduction pathways that optimize metabolism to respond to the nutritional environment, stress conditions and interaction with other biological systems, such as other microbes, environmental predators, and symbiotic hosts. In addition, it is common to find morphological changes and the development of specialized structures that provide the microbe with survival benefits during its life cycle. Among these structures, many microbes possess

(*) Corresponding author. Arturo Casadevall. Departments of Microbiology and Immunology and Medicine. Albert Einstein College of Medicine. 1300 Morris Park Avenue. Bronx, New York 10461. Phone + 718 430 2811. casadeva@aecom.yu.edu.

capsules surrounding their cell body. Microbial capsules are usually composed of polysaccharides although some organisms, like *Bacillus anthracis*, have capsules composed of polymerized D-glutamic acids. Microbial capsules play important roles in the lives of these microorganisms, providing resistance to stressful conditions (such as dehydration), and playing a key role in the interaction with the environment.

Although capsules are commonly found among bacteria, there are a few encapsulated fungal species. The best characterized fungal capsule belongs to *Cryptococcus neoformans*. The capsule of this microorganism has been extensively studied because it is the main virulence factor of this pathogenic organism (McClelland et al., 2006). In the environment, the capsule plays a role in the protection of the organism against some stress conditions, such as dehydration (Aksenov et al., 1973). The *C. neoformans* capsule has some functional similarities to those of encapsulated bacteria such as, *Streptococcus pneumoniae* and *Haemophilus influenzae* (De Jesus et al., 2008; Kang et al., 2004). In fact, the cryptococcal polysaccharide is known to share some antigenic determinants with certain pneumococcal polysaccharides (Maitta et al., 2004b; Pirofski and Casadevall, 1996). The capsule is important for virulence, since acapsular mutants do not produce disease in murine models (Fromtling et al., 1982). The definitive experiment establishing the capsule as a virulence factor was accomplished when acapsular mutants were created and shown to be significantly less virulent than wild-type or capsule-reconstituted strains (Chang and Kwon-Chung, 1994). These mutants can survive and replicate in normal laboratory conditions but exhibit a markedly reduced virulence during infection in murine models. Interestingly, acapsular strains can be pathogenic for severely immunocompromised hosts implying a residual pathogenic potential for non-encapsulated yeast cells (Salkowski and Balish, 1991). These studies established that the capsule plays a predominant role in the interaction with the host. Consequently, this structure has been the main focus of attention in many experimental studies. Furthermore, studies have also shown that the capsular polysaccharide has strong immunomodulatory properties and promotes immune evasion and survival within the host (Monari et al., 2006a; Vecchiarelli, 2000). Besides mammalian hosts, studies focused on the capsule have also been extended to include environmental predators such as amoebae, since *C. neoformans* is both a pathogen and an environmental yeast and therefore interacts with multiple types of hosts.

A vast amount of knowledge has been accumulated on the biology, structure and role of the capsule during infection. The purpose of this manuscript is to give an overview of the main aspects of the capsule, including its structure, synthesis, and in particular, its role as a virulence factor.

Section A Capsule components and structure

The most characteristic feature of *C. neoformans* is a polysaccharide capsule that surrounds the cell body. The capsule is not visible by regular microscopy because it is highly hydrophilic, and due to its high water content it has the same refraction index as the medium. However, it can be easily made visible by several techniques. The classic image of the capsule is that of a halo surrounding the cell made visible by suspending the yeast in India ink preparations. The halo effect is a consequence of the fact that the capsule does not stain with India ink, visible only by a translucent area. It can also be nicely observed by other microscopic techniques, such as scanning electron microscopy and fluorescence. In figure 1 we have collected a series of images in which the capsule is made visible by means of these techniques. The cryptococcal capsule is composed of polysaccharide, causing it to be highly hydrophilic with an extremely high water content of 99% of the total weight of the capsule (Maxson et al., 2007a). The high hydration of the capsule makes it difficult to study. The polysaccharide capsule confers a strong negative charge by virtue of the glucuronic acid residues on its main polysaccharide component (Nosanchuk and Casadevall, 1997). The polysaccharides that constitute the capsule are found

in two different locations. The first location is attached to the cell wall, forming the physical structure defined as the capsule. These polysaccharides are also constitutively released by the cell into the surrounding medium and environment, and they can be isolated as exopolysaccharides after certain purification protocols. It is not known whether the capsule's release into the medium is an active phenomenon regulated by the cell, or if it is just an unspecific capsule shedding. It is noteworthy that practically all our information about *C. neoformans* capsular polysaccharides originates from studies of exopolysaccharide components released from cells and recovered from culture supernatants. The field has operated under the notion that exopolysaccharide material is shed capsular polysaccharide and has extrapolated results obtained with this material to infer capsular characteristics. Recent evidence seems to suggest, however, that this assumption may be incorrect and that capsular and exopolysaccharide materials originate from different pools (see below, (Frasés et al., 2008)). The exopolysaccharide material contains two major types of polysaccharides (PS), glucuronoxylomannan (GXM) and galactoxylomannan (GalXM). GXM comprises around 90-95% of the mass, and GalXM around 5-8%. In addition, a small proportion of mannoproteins (MP) have been identified (<1%), but this component has not been studied in detail and its role in capsule architecture, if indeed it has any, remains unknown. The polysaccharides have a complex structure that has not been completely characterized yet. An intriguing finding is that capsule structure is very variable depending on the strain (and on its the environment). It also changes during the course of an infection (Cherniak et al., 1995). Depending on these criteria, five different serotypes (A, B, C, D and AD) have been defined to classify *C. neoformans* strains, depending on the reactivity of the capsule with different rabbit polyclonal sera. Some of these classified serotypes include *C. neoformans* var. *grubii* (serotype A), *C. neoformans* var. *gattii* (serotypes B and C) and *C. neoformans* var. *neoformans* (serotypes D and AD). Recently, *Cryptococcus gattii* serotypes B and C have been proposed as new species because they present significant genetic and biological differences with serotypes A and D (Kwon-Chung and Varma, 2006). The capsular structures of the different serotypes have been studied using a variety of techniques, providing insights on the composition and organization of the capsular polysaccharides. In the next sections we will briefly review the main features of the main capsular components.

A1. Structure of Capsular Components

1) GXM structure—Based on the analysis of GXM purified from culture supernatants, this component has a high molecular mass with a complex structure. The average M_w can range from 1700 to 7000 kDa, depending on the strain (McFadden et al., 2006), and it comprises about 90% of the capsule's mass. There is evidence that the size distribution of the GXM molecules is more complex than initially observed (McFadden et al., 2006; McFadden et al., 2007). Structurally, GXM consists of a linear α -(1,3)-mannan main chain with β -(1,2)-glucuronic acid residues attached to every first mannose forming the basic core, with this unit repeated for all serotypes (Figure 2). Mannosyl residues can also be 6-O-acetylated and substituted with xylosyl units in β -(1,2)- or β -(1,4)-linkages depending on the serotype (Cherniak et al., 1988; Cherniak and Sundstrom, 1994; McFadden et al., 2006; McFadden et al., 2007). Molar ratios of xylose, mannose, and glucuronic acid residues vary depending on the serotype with ratios 1:3:1, 2:3:1, 3:3:1, and 4:3:1 for serotypes D, A, B and C, respectively (Cherniak and Sundstrom, 1994).

In contrast to bacterial polysaccharides that have a single oligosaccharide repeating unit, GXM has at least six different structure reported groups (SRGs) identified to date (Cherniak et al., 1998), based on the shift of the anomeric protons and mannosyl residues by $^1\text{H-NMR}$ (Cherniak et al., 1998). These SRGs, classified as motifs 1 to 6 (M1-M6) of GXM, are shown in Figure 2. $^1\text{H-NMR}$ analysis from GXM of 106 *C. neoformans* isolates showed six different chemotypes (Cherniak et al., 1998). A seventh GXM repeating unit was recently described by

Nimrichter and co-workers (Nimrichter et al., 2007), who characterized a substituted triad in GXM from encapsulated cells that had only been described in polysaccharide fractions from a hypocapsular mutant (Bacon et al., 1996). This structure, called hexasaccharide 1 by Bacon and colleagues (Bacon et al., 1996), is also shown in Figure 2. Later, McFadden et al. found evidence of co-polymerization of different GXM repeating units in one polysaccharide using mass spectrometry (McFadden et al., 2007). In some *C. neoformans* strains GXM is composed of a single repeating unit whereas in other strains the polysaccharide contains multiple units. Furthermore, those strains that utilize the same set of repeating units often differ from one another by the ratio of those units within the GXM molecule. These differences in ratios result in GXM's compositional similarity while allowing for slight differences in structures that translate into antigenic differences (McFadden et al., 2007).

All the structural studies available to date use exopolysaccharides extracted by cetyl trimethylammonium bromide (CTAB) precipitation as described by Cherniak et al (Cherniak et al., 1998). More recently, a new method of exopolysaccharide extraction based on filtration and the tendency of polysaccharide to self-aggregate has been described (Nimrichter et al., 2007). Using this method, concentration of *C. neoformans* supernatants resulted in the formation of a jellified polysaccharide film providing a one-step procedure for its isolation (Nimrichter et al., 2007). Polysaccharides obtained from the ultrafiltration display differences in the reactivity with GXM-specific monoclonal antibodies when compared with polysaccharides obtained by differential precipitation with CTAB. Viscosity analysis associated with inductively coupled plasma mass spectrometry and measurements of zeta potential in the presence of different ions, strongly suggested that polysaccharide aggregation was a consequence of the interaction between the carboxyl groups of glucuronic acid (GlcA) and divalent cations. The length of a GXM fiber from solution supernatants is shorter than the capsule diameter (McFadden et al., 2006). All these results suggest that capsular assembly in *C. neoformans* results from divalent cation-mediated self-aggregation of GXM molecules (Nimrichter et al., 2007).

Polysaccharides recovered from culture supernatants have historically provided an ample and convenient source of material for structural and immunological studies. A major assumption in such studies is that the structural features of the exopolysaccharide material faithfully mirror those of the capsular polysaccharide. This remains a mere assumption, and the exact reasons why the polysaccharide is released are still unknown. In fact, it is conceivable that exopolysaccharide differs from capsular polysaccharide in that the former has structural features intended for extracellular release, and thus, the two materials differ in fundamental ways. Suspension of acapsular cells in soluble capsular polysaccharide resulted in the presence of a capsule forming around the cell as was visualized by fluorescence, but the degree of encapsulation was much lower than is commonly seen in regular encapsulated strains. The binding was dependent on the polysaccharide concentration, with high concentrations resulting in non-specific polysaccharide binding to the cell (Kozel, 1977; Kozel and Hermerath, 1984). These findings suggest that a proportion of exopolysaccharides retain their ability to form a capsule. The finding that this capsule is much smaller than what is generally found in encapsulated strains, suggests that a significant proportion of exopolysaccharides have different structural features than the polysaccharide that make up the normal capsule in wild-type strains. Structural analysis of the capsular polysaccharides (not exopolysaccharide) has been limited for a long time because of a lack of efficient protocols for separating the capsule from the cell. However, in the last years, different groups have described the exposure of the cells to γ -radiation or suspension in DMSO resulting in the release of the capsule from the cells (Bryan et al., 2005; Dembitzer et al., 1972; Gates et al., 2004). This has led to the possibility of studying the structural features of the polysaccharide that remain attached to the cell, forming a visible capsule. This approach allowed for the detailed study of different regions of the capsule (inner capsule, medium capsule and outer edge). An additional advantage of stripping

the capsule with γ -radiation versus DMSO is that the cells remain viable after radiation treatment (Bryan et al., 2005).

Comparison of exopolysaccharide obtained by two isolation techniques with capsular polysaccharide (PS) stripped from cells exposed to gamma radiation or treated to DMSO, revealed significant differences in glycosyl composition, mass, size, charge, viscosity, circular dichroism spectra and reactivity with monoclonal antibodies (mAbs). This implies that exopolysaccharides and capsular PS are structurally different and originate from different synthetic pools. Hence, the method used to isolate PS can significantly influence the structural and antigenic properties of the product (Frases et al., 2008).

Structural information provided by antibodies to GXM—A large number of monoclonal antibodies (mAbs) that bind to the *C. neoformans* capsule have been obtained (Casadevall et al., 1994; Casadevall et al., 1992; Dromer et al., 1987b; Eckert and Kozel, 1987; Pirofski et al., 1995; Todaro-Luck et al., 1989; van de Moer et al., 1990). Most of these antibodies were obtained after mouse immunization with purified GXM. At present, antibodies differing in affinity, specificity, and isotype are available for the study of the capsule. In addition to immunological studies examining their protective effect during infection, these antibodies have proven to be a useful tool to study the structure and properties of the *C. neoformans* capsule. Studies with mAbs have revealed that the capsule has a very heterogeneous structure, and that several different epitopes are likely to be present in the same GXM molecule (Belay et al., 1997; Todaro-Luck et al., 1989). They have shown that this heterogeneity can be found between different strains, as well as within a population of the same strain (McFadden et al., 2007). Several factors determine the specificity and affinity of the mAbs to GXM, such as acetylation and xylosylation (Kozel et al., 2003; Todaro-Luck et al., 1989). Several genetic screenings have been performed with the aim of obtaining mutants with altered binding to specific mAbs. These mutants were not acapsular, but deficient in acetylation and xylosylation (Kozel et al., 2003), demonstrating the importance of this process for some of the functions associated with GXM. Monoclonal antibodies to the capsule have also been used in immunoblotting experiments, which have shown that the size distribution of the GXM molecules is very complex (McFadden et al., 2006). It has also been shown that the binding affinity of these antibodies varies according to the different capsule regions (Maxson et al., 2007b). This confirms that capsule structure is very heterogeneous, not only at the level of a single GXM molecule, but also in terms of the spatial and structural organization of the capsule.

2) GalXM structure and localization in the capsule—GalXM constitutes about 8% of the capsular mass (Bose et al., 2003; Vaishnav et al., 1998) and has an α -(1 \rightarrow 6)-galactan backbone containing four potential short oligosaccharide branch structures (Figure 3). The branches are 3-O-linked to the backbone and consist of an α (1 \rightarrow 3)-Man, α (1 \rightarrow 4)-Man, β - galactosidase trisaccharide with variable amounts of β (1 \rightarrow 2)- or β (1 \rightarrow 3)-xylose side groups (Bose et al., 2003; McFadden et al., 2006; Vaishnav et al., 1998). The GalXM backbone consists of galactopyranose and a small amount of galactofuranose (Vaishnav et al., 1998), unlike GXM, which contains only mannopyranose (Bose et al., 2003). Compositional analysis of GalXM was confirmed by gas chromatography/mass spectrometry of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the sample by acidic methanolysis. The analysis revealed that the molar percentage for GalXM were as follows: xylose, 22%; mannose, 29%; and galactose, 50% (De Jesus et al., 2008; Vaishnav et al., 1998). GalXM is very sensitive to oxidation with sodium periodate, which destroys the integrity of most of the carbohydrates. Xylose residues are completely eliminated and two-thirds of galactose and mannose residues oxidized (Cherniak, 1982). To elucidate the proposed structure of GalXM repeats, proton nuclear magnetic resonance spectrum analysis was used to determine the anomeric region of the galactan building unit. The results showed the anomeric region to be between 5.4 and 4.3 ppm in a one-dimensional ^1H spectrum recorded at 600 MHz

and 56°C (De Jesus et al., 2008; Vaishnav et al., 1998). Although the GalXM from serotypes A, C and D all contained galactose, mannose and xylose, it has been shown that the molar ratios are not identical, suggesting that there may be more than one GalXM entity. GalXMs are thought to be a group of complex closely related polysaccharides (James and Cherniak, 1992; Vaishnav et al., 1998).

A recent study showed that GalXM is significantly smaller than GXM (1.7×10^6 Da), with an average mass of 1×10^5 Da (McFadden et al., 2006). Additionally, the radius of gyration (R_g), defined by the average distance between the center to the outer edge of the molecule, was measured for both GXM and GalXM. The results revealed that for GXM different strains showed differences of 68-208 nm while GalXM isolated from the acapsular strain cap67 was 1.4-fold smaller than GXM with an R_g of 95 nm (McFadden et al., 2006). Attempts to image GalXM by scanning transmission electron microscopy (STEM) were unsuccessful, possibly because the molecule was too narrow to be detected (McFadden et al., 2006). GalXM is the most abundant polysaccharide in the capsule based on its molar mass, since GalXM has a comparatively smaller molecular weight, with 2 to 3.5 mol of GalXM for each mole of GXM. The widespread assumption that GXM was the most abundant molecule was based on the total amount of material that was recovered from the culture supernatant (McFadden et al., 2006). It is noteworthy that all studies of GalXM used material obtained from non-encapsulated strains that failed to secrete GXM. This is due to an absence of adequate methods available that are able to separate GalXM from GXM in exopolysaccharide preparations to yield GalXM of sufficient purity for structural studies. However, given that non-encapsulated strains often have secretion defects (Garcia-Rivera et al., 2004), this introduces the conundrum that the GalXM so obtained may not be in its native state.

While GXM seems to be distributed throughout the capsule, the exact location of GalXM is not known as there is no visualization method available to help determine this (such as an antibody to GalXM). Experiments aimed at purifying GalXM revealed that supernatant fractions following homogenization of cell walls derived from the acapsular strain *cap67* contained both GalXM and mannoproteins. It was demonstrated that GalXM and MP do not form a part of the cell wall matrix since they are not covalently bound to glucans within the cell wall (James and Cherniak, 1992). GalXM and MP can be separated using Concanavalin A affinity chromatography which binds MP, with the eluate containing GalXM (James and Cherniak, 1992; Vaishnav et al., 1998).

Capsular studies by Bryan *et al.* noted the absence of galactose in the fraction removed by radiation alone, which represents the outer most section of the capsule (Bryan et al., 2005). This was interpreted to indicate an absence of GalXM in the outer layers of the capsule. The compositions of the outer layer of the capsule removed by either DMSO or radiation alone were similar. This result was consistent with the observation that both DMSO and radiation removes the same part of the capsule. Neither radiation nor DMSO removed the portion of the capsule in the region closest to the cell wall. This can be explained by the fact that the inner radiation- and DMSO-resistant capsule and the outer radiation- and DMSO-susceptible regions have differences in terms of their chemical compositions, branching or the density of molecules packed. In these studies it was also noted that GXM protects against radiation with a possibility that GalXM in the inner capsule also contributed to the effect of radioresistance observed by that layer (Bryan et al., 2005).

Another study utilized low doses of gamma radiation to investigate the structure of the *C. neoformans* capsule (Maxson et al., 2007b). The authors prepared two different fractions of the capsule by irradiating *C. neoformans* H99 strain for 20 minutes, enough time to allow for the lower density part of the capsule to be stripped. The second part of the experiment included washing and irradiating for another 20 minutes, which stripped away the higher density region

of the capsule. The results revealed no differences in terms of the carbohydrate composition. However, the occurrence of galactose was found with both strippings, which was interpreted to signify that GalXM is present throughout the capsule (Maxson et al., 2007b).

Structural information provided by antibodies to GalXM—In the early 1990's, van de Moer *et al.* generated six mAbs against spheroplast lysate of *C. neoformans* serotype A. MAb CN6 of the IgM isotype was reactive with GalXM. The CN6 antibody binding was blocked by the presence of GXM but the acapsular mutants were able to bind the antibody (James and Cherniak, 1992; van de Moer et al., 1990). Immunogold transmission electron microscopy and a microagglutination assay demonstrated through epitope recognition by the monoclonal antibody CN6 that GalXM was located within the cytoplasm and the cell wall in acapsular strains (Vaishnav et al., 1998). Unfortunately, the antibodies were not used again for any subsequent studies and are apparently no longer available.

Mannoproteins—Classically, mannoproteins (MP) have been considered as minor components of the capsule, but their role in capsule architecture has never been established. Their presence was identified in culture filtrates by ¹³C-NMR analysis of the GXM-free portion of the GalXM-enriched fraction (Murphy, 1988; Reiss et al., 1985). Very little is known, however, about their function and the role they play in the capsule. Some articles have investigated the location of MP on cryptococcal cells and concluded that they are mainly found in the inner cell wall, and not associated with GXM or GalXM (Vartivarian et al., 1989). It has been shown that mannoproteins are highly immunogenic during infection, which will be discussed in a different section.

A.2.- Capsule dynamics

In this section, we will review an important aspect of the capsule that has important implications for the virulence of the organism, namely the ability of *Cryptococcus neoformans* to modify the size and structure of its capsule in response to various stimuli. Since the capsule is presumably the first fungal structure that interacts with host cells, the capability for multiple combinations of capsule size and structure makes this a very versatile structure. Changes in capsule size are typical features among *C. neoformans* strains during the interaction with the host, and are considered an early morphologic response during infection. Capsule enlargement occurs after a few hours of infection in murine models (Feldmesser et al., 2001). Moreover, it is also observed to occur during *C. neoformans* intracellular parasitism. This phenomenon has been elegantly illustrated using live imaging techniques (see supplemental movie 1 in (Ma et al., 2006)). In addition, during the course of infection, the immunogenic properties of the capsule can change, a finding that is also considered as an adaptation phenomenon to survive in the host.

Capsular enlargement—The size of the capsule is very variable, not only among strains, but also between individual cells of the same strain, to such an extent that a given strain can exhibit large differences in capsule size depending on the specific environmental conditions. There are many different conditions that lead to capsule enlargement in natural environments. In laboratory conditions there are several factors that are known to influence capsule size.

In the late 1950s, it was Littman who described for the first time that capsule size in *C. neoformans* could be modulated in vitro by placing the cells in different growth conditions. He described several minimal media in which he observed a significant increase in capsule size (Littman, 1958). The inducing factors in that media still remain unknown. In 1985, carbon dioxide was described as a factor that had an influence on capsule size. In that work, Granger et al. found that when the cells were placed in a CO₂ enriched atmosphere (5%) the capsule manifested a significant enlargement (Granger et al., 1985). These authors also isolated mutants

defective in capsule growth in the presence of CO₂, and demonstrated that it had reduced virulence in murine models, which provided the first evidence that the phenomenon of capsular enlargement played a role in the pathogenesis of the microorganism. Consistent with these findings it was found that strains which differed in terms of their capsule size also differed in their virulence and in their effects on macrophages. The strain with reduced virulence was not able to increase capsule size in the presence of CO₂. During infection, the strain unable to induce capsule size elicited a rapid inflammatory response in the host and was efficiently cleared from the lungs (Blackstock et al., 1999; Blackstock and Murphy, 1997). These reports provided strong evidence that capsule enlargement is a process that plays an important role during infection. Several studies have found, however, that virulence is not correlated with in vitro capsule size (Clancy et al., 2006; Dykstra et al., 1977; Littman and Tsubura, 1959). This lack of correlation is most probably due to the fact that in vitro capsule size does not predict the degree of encapsulation of a strain during infection seen in vivo. Furthermore, *C. neoformans* has multiple virulence factors that contribute to the overall virulence phenotypes and it is possible that differences in non-capsular virulence factor expression affect the overall virulence potential to obscure a correlation with capsule size (McClelland et al., 2006). In 1993, iron limitation was shown to result in capsule enlargement (Vartivarian et al., 1993). The fact that both CO₂ and iron limitation influenced capsule size is particularly interesting because these conditions mimic some situations that the microorganism encounters during lung colonization. In addition, capsule size during infection varies according to the organ infected, the lung being the place where higher capsule size is found, which indicates that microenvironments during infections have a great impact on capsule size (Rivera et al., 1998). Iron is an essential nutrient for the life of the yeast whose concentration in the host is normally very low, so the yeast must compete with iron binding proteins from the host such as transferrin. It is not known why *C. neoformans* responds to iron limitation by enlarging its capsule, but it has been suggested that the capsule might contribute to iron uptake (Vartivarian et al., 1993). Therefore, capsule growth could increase the iron concentration available to the yeast.

More recently other media that induce capsule growth have been described. In the context of media that mimic infection situations, mammalian serum was found to induce in vitro capsule growth in the absence of CO₂ (Zaragoza et al., 2003a). Those studies also established that the incubation media had a major determinant effect on the efficacy of inducing factors, such as serum or CO₂. Furthermore, results showed that the behavior of different strains varies and that the optimal conditions to obtain capsule growth should be standardized for every strain.

The signals that induce capsule growth are very heterogeneous (iron concentration, CO₂, serum), and it is not clear if they induce capsule enlargement through different pathways and whether there is a common element to all of these factors that the cell senses as a capsule-inducing factor. In all the media described that are known to induce capsule growth a significant decrease in the growth rate of the yeast has been observed. It is therefore believed that capsule growth occurs under certain conditions where yeast grows very poorly. Accordingly, media which contain low nutrient concentrations, such as diluted Sabouraud buffered at a neutral pH, induced a significant increase in capsule size (Zaragoza and Casadevall, 2004), supporting the hypothesis that capsule growth is probably inversely related to the growth rate of yeast. While a slow as opposed to a rapid growth rate of yeast appears to be a requirement for induction of capsule growth, this is apparently not sufficient, since conditions have been observed in which the yeast grows slowly that do not lead to capsule enlargement.

Physical factors that affect capsule size—Capsule size also changes in the presence of certain factors that affect its structure or its packing without affecting the amount of polysaccharide accumulated in the capsule. One such factor is the effect of the osmotic pressure of the medium. Addition of a high NaCl concentration produces a reduction in capsule size

(Dykstra et al., 1977). The same effect is produced by glucose concentrations over 10%, a finding that has been correlated to an increase in osmotic pressure of the medium. There are several explanations for these findings. It is possible that a high osmotic pressure produces an effect whereby a significant part of the water content of the capsule is lost in these conditions, which in turn, results in an increase in the packing of the polysaccharide fibers and thereby a decrease in capsule size. Concerning glucose effect, it has also been described that growth of the cells in high glucose concentration produces a higher polysaccharide release to the growth medium (Cleare and Casadevall, 1999), which suggests that carbon metabolism might be an important factor in capsule synthesis. Another element that has been reported to influence capsule size is the binding of monoclonal antibodies. Cleare and Casadevall reported that appearance of the cells by scanning electron microscopy was affected by the presence of mAb 2H1 (Cleare and Casadevall, 1999). Later on, it has been described that binding of a high amount of mAb 18B7 to the capsule produces a drastic decrease in capsule size (Zaragoza and Casadevall, 2006). Monoclonal antibodies tend to bind to each other through interactions of the constant regions, which could feasibly produce a force within the capsule resulting in a compaction of the polysaccharide fibers toward the cell body. Moreover, capsule size decreases in the presence of monoclonal antibodies can result not only in a diminished capsular size, but also in one with irregular edges, in which the morphology changes and the capsule loses its characteristic spherical shape (Zaragoza and Casadevall, 2006).

However, recent findings suggest new explanations for the different packing of the polysaccharide fibers in different conditions. Capsule enlargement is facilitated under alkaline conditions (Zaragoza and Casadevall, 2004), which is consistent with the view that capsule growth requires the acidic groups of glucuronic acid (GlcA) residues to be ionized, possibly so that they can react with divalent cations for capsule assembly (see below). It is noteworthy that mammalian physiological fluids are usually slightly alkaline. In addition, since the finding that high NaCl concentration blocks capsule growth does not generally apply to other solutes, it has been proposed that capsule growth could be inhibited in the presence of Na⁺ by univalent neutralization of negatively charged GlcA residues (Nimrichter et al., 2007).

In plant cell walls, galacturonans can be cross-linked by insertion of Ca²⁺ ions between the unesterified carboxyl groups of the uronosyl residues (Vincken et al., 2003). The same property was recently described for GXM which self aggregates through a divalent metal-mediated process (Frasés et al., 2008; McFadden et al., 2006; Nimrichter et al., 2007). The efficacy of this association depended on water removal and increased polysaccharide concentration (Nimrichter et al., 2007), which allowed an efficient interaction between GXM fibers and divalent ions at neutral and alkaline pH (pK of glucuronides, ≥ 3). Accordingly, carboxyl-reduction of GXM, as well as its treatment with EDTA, monovalent ions and excessive concentrations of Ca²⁺ impaired self-aggregation (Nimrichter et al., 2007). These observations suggested that divalent metals could regulate the aggregation of GXM fibers.

The cultivation of cryptococci in the absence of divalent ions was associated with smaller capsules and a concomitant increase in the concentration of GXM in supernatants, presumably as a result of incomplete assembly (Nimrichter et al., 2007). In contrast, the addition of increasing concentrations of Ca²⁺ to the culture medium resulted in augmented capsule expression that was associated with decreased levels of soluble GXM in fungal cultures. Finally, high concentrations of Ca²⁺ caused a decrease in capsule sizes (Nimrichter et al., 2007). Interestingly, dead *C. neoformans* cells incorporated exogenous GXM in the presence of varying Ca²⁺ concentrations. A possible explanation for these findings is based on the stoichiometric ratio between divalent cations and glucuronic acid residues. According to this model, divalent ions would be expected to positively modulate capsule assembly by forming ionic bridges between glucuronic acid residues in different GXM fibers. In this context, metal ion concentrations compatible with a 1:2 metal ion-GlcA ratio in cryptococcal cultures would

promote capsule growth, while the capsule would be reduced when the metals were provided in excess. These results are consistent with and support the notion that divalent cation-mediated aggregation of GXM is involved in capsule enlargement.

Recent findings suggest that capsule size and packing depends on the interaction of its basic components, GXM and GalXM. Mutants that cannot synthesize GalXM manifest larger capsules than the isogenic wild-type strains, suggesting that GalXM plays an important structural role in maintaining a certain capsule size, and in turn, structure and density. It could also suggest that in the absence of GalXM the cell responds by overproducing GXM molecules, resulting in a higher capsule size (Moyrand et al., 2007).

Physical changes associated with capsule growth—Capsule growth is achieved by addition of polysaccharide to the capsule, which implies that capsule enlargement is a high energy consuming process. Addition of new polysaccharide to the capsule leads to an increase in the density of the polysaccharide, especially in the regions closer to the cell wall. This has been proven by different groups using different approaches, based on microscopy, immunogenic properties and direct polysaccharide measurement in different capsule regions (Gates et al., 2004; Maxson et al., 2007b; Pierini and Doering, 2001). A characteristic feature of the encapsulated cells is a very high negative zeta potential value due to the high number of glucuronic acid residues in the capsular polysaccharide of cryptococcal cells. Different studies suggest that capsule enlargement is associated with a decrease in the zeta potential value consistent with larger negative surface charges (Maxson et al., 2007b; Nosanchuk and Casadevall, 1997). It has been reported that the binding of mAbs to the capsule changes its permeability, a phenomenon that is correlated with a phenomenon known as quellung reaction (MacGill et al., 2000; Mukherjee et al., 1995a). Yoneda and Doering (2007) observed in immunoblotting experiments that the size distribution of exopolysaccharide GXM varied when the capsule enlargement was induced (Yoneda and Doering, 2008), which suggests that in these conditions, the GXM fibers that are produced and incorporated in the capsule are different, at least in size.

Capsule growth model—The mode of polysaccharide accumulation on the capsule has been the subject of many different studies. For this purpose, different methods have been used to label the capsule prior to induction and trace the label once the cell triggers the signals to increase capsule size. The first study used a monoclonal antibody and radioactive xylose (Pierini and Doering, 2001). This approach revealed that the marker moved to the edge of the capsule after induction, an observation that was interpreted as indicating that the capsule grows by accumulation in the inner part of the capsule, displacing the old capsule to the edge. However, since antibody binding is not covalent and antibody-antigen interactions are prone to dissociation and association, there was some concern that the antibody was not a stable capsule geographical marker. More recently a similar approach was employed, using a marker that covalently binds to the capsule (Zaragoza et al., 2006b). This marker was provided by proteins from the complement systems, which bind to the polysaccharide capsule fibers through covalent bonds. Using this approach, it was shown that the old polysaccharide fibers remained in a position close to the cell wall after capsule induction, suggesting that capsule growth was achieved by addition of the polysaccharide fibers at the capsule edge (Figure 4A). The authors confirmed this result by giving a pulse of radioactive mannose prior capsule induction, and then establishing the localization of the radioactive label in different regions of the capsule obtained after gamma radiation treatment of the cells. Consistent with this last model, Charlier et al. found that during infection, there is a difference in the binding pattern of specific antibodies to the capsule after capsular enlargement during infection (Charlier et al., 2005). These investigators infected mice with a *C. neoformans* strain that reacted to mAb E1 which is specific for serotype A. After 6 hours of infection, they observed that a new area surrounding the E1-binding region appeared which was not reactive to this antibody anymore, but to another

mAb named CRND-8, raised against serotype D strains. These findings suggest that new polysaccharide is added at the capsule edge such that capsular enlargement proceeds by apical growth.

Capsule rearrangements during budding—Capsule growth poses a problem for the cell because during budding, the new cell has to traverse the width of the parental capsule to separate from the mother cell. Theoretically, the presence of a dense net of polysaccharide fibers around the cell wall could interfere with bud migration and separation. However, the fact that a cell buds successfully implies the existence of mechanisms to ensure bud separation. Several lines of evidence have suggested the existence of a mechanism for this phenomenon. 1) When a *C. neoformans* cell is coated with a mAb and then placed in a medium that induces budding, the mAb does not migrate to the bud (Pierini and Doering, 2001; Zaragoza et al., 2006b); 2) the binding of mAbs to the capsule over the bud is different from the binding to the capsule of the mother cell, which suggests that the capsules does not mix during budding (Zaragoza et al., 2006b); 3) when budding cells with large capsule are incubated with complement, immunofluorescence staining for complement proteins is not observed in the regions that separate the bud and the mother cell (Zaragoza et al., 2006b); 4) when cells with large capsule start budding, it is possible to observe some rearrangement at the regions of the capsule of the mother cell where the bud is forming. Furthermore, it is possible to observe a dimple on encapsulated budding cells which suggests that the capsule disappears at the region where the bud will appear. In cells coated with complement it is also possible to observe some rearrangements in the capsular region of bud emergence. This suggests that during budding, the capsule of the mother cell opens, leaving an empty space for the bud to migrate through; 5) the region of the capsule where the bud is going to emerge becomes highly permeable to India Ink (Pierini and Doering, 2001); 6) when budding cells with large capsule are placed in the presence of serum for a very short time period, thus allowing complement deposition, complement proteins accumulate in the region between the bud and the mother cell, suggesting that in the capsule region where the capsule is going to pass there are not fibers, which would facilitate the migration of the bud; 7) scanning electron micrographs have shown a physical separation between the capsules of the mother cells and the bud (Zaragoza et al., 2006b).

All these observations led us to propose a model to explain how the bud crosses the capsule of the mother cell without being retained, or entangled, within the polysaccharide fibers of the mother cell (see Figure 4C). We hypothesized that when the bud emerged from the mother cell, there are some rearrangements in the region where the bud has to pass, which produce the formation of a tunnel in the capsule, creating a physical space that allows the bud to migrate through the capsule. The description of bud scars in the capsule supports this idea (Cleare and Casadevall, 1999). The presence of chitin-like structures in the cell wall which are projected into the capsule have been recently described (Rodrigues et al., 2008a). Staining for this structure is particularly abundant in the neck around the bud, which suggests that these structures could be involved in the migration of the bud through the mother's cell capsule.

Capsule degradation—An interesting feature that has hardly been addressed is how the capsule is degraded and how the cells diminish their capsule size in adaptation to the environment. For example, after passage through hosts, the capsule of the *C. neoformans* cells reverts to a small size. During budding, the creation of the tunnel described above could imply the degradation of the polysaccharide fibers in the budding area of the capsule. However, despite these situations in which a reduction or degradation of capsule size seems to be required, no capsule degrading enzymes have been described so far in *C. neoformans*. Moreover, preliminary findings indicate that when cells with a large capsule are shifted to a medium where the capsule is small, the cells do not degrade the capsule, and they retain a considerable capsule size (Zaragoza et al., 2006b). Although the cells initially placed in the new medium remain with an enlarged capsule, the buds that emerge in the new medium have a small capsule, which

suggests that when *C. neoformans* is shifted to a medium which does not require a large capsule, the adaptation mechanism cause the cells to invest their cellular energy in producing buds with the proper capsule size, and not in degrading the capsule (figure 4B). It also seems to suggest that during budding, a mechanism of capsule release occurs in the budding area without actual capsule degradation. Since budding implies cell wall degradation, and the capsule is linked to the cell wall, it is believed that the capsule fibers are released by rearrangements in cell wall, rather than through direct capsular polysaccharide degradation. This issue remains a key point in the cryptococcal biology that needs to be further examined.

Capsule structural changes *in vitro* and during infection—In addition to capsule growth, several other phenomena indicate that the capsule is dynamic, which is related to an inherent ability of *C. neoformans* cells to alter their capsular structure. As mentioned above, not only are physical changes observed in terms of density, but also alterations with regards to the structure of the polysaccharide fibers. Turner et al. found that capsule structure from different strains from within the same serotype (serotype A) differed in their structure as shown by chemical analysis and ¹³C NMR spectroscopy (Turner et al., 1992). Different groups have found that even within the same population, there are significant changes in the immunoreactivity of *C. neoformans* cells to specific mAbs (Garcia-Hermoso et al., 2004; McFadden et al., 2007). This heterogeneity is also a characteristic feature of these cells seen during infection (Garcia-Hermoso et al., 2004).

Structural changes are relevant during the course of infection, especially during the interaction with phagocytic cells. Addition of purified capsular polysaccharide isolated from different serotype A strains to acapsular mutants produces different effects on phagocytosis, for example (Small and Mitchell, 1989). Furthermore, *C. neoformans* phagocytosis by amoeba is influenced by the incubation medium, a phenomenon that has been related to differences in capsule structure in accordance with the surrounding environment (Zaragoza et al., 2008b). Finally, capsule structure changes have been implicated in dissemination (Charlier et al., 2005). These implications will be discussed in more detail in the coming sections.

Capsule and phenotypic switching—As is seen in other fungi (such as *Candida albicans*), *C. neoformans* undergoes macroscopic changes, determining different phenotypic morphologies, through a process known as phenotypic switching (Goldman et al., 1998). Two different variants have been defined, smooth and mucoid. These variants manifested different virulence, and it was later shown that they differed in terms of their capsular structure (Fries et al., 1999; McFadden et al., 2007). Similar findings have recently been reported for *Cryptococcus gattii* (Jain et al., 2006). Although it has not been demonstrated that capsule changes are responsible for phenotypic switching, this phenomenon and its correlation with capsule variation is a clear example of how *C. neoformans* can produce dynamic capsule changes in correlation to variations in phenotypes and related to virulence.

Section B Capsule synthesis in *Cryptococcus*

B.1) Genes, enzymes and signaling pathways

The CAP genes—During the last decade, different genes involved in capsule biosynthesis have been identified. The first one, *CAP59*, was cloned by complementation of an acapsular mutant isolated by uv random mutagenesis (Chang and Kwon-Chung, 1994). The gene was deleted by homologous recombination and resulted in an acapsular phenotype associated with loss of virulence in an animal model of cryptococcal infection. The restoration of virulence by complementation of the acapsular phenotype represented the first molecular evidence for the capsule as a virulence factor of *C. neoformans*. At the time of the study by Chang and Kwon-Chung (Chang and Kwon-Chung, 1994), no sequences similar to that found in the *CAP59* gene had been reported. More recently, *CAP59* sequence presents homology with a cryptococcal

α -1,3-mannosyltransferase (Sommer et al., 2003). Cap59p has a putative transmembrane domain, and it was further suggested to play a role in capsule secretion (Garcia-Rivera et al., 2004). Interestingly, *CAP59* was considered to be related to the *L27* gene (Chang and Kwon-Chung, 1994), which encodes for a ribosomal protein that is present in secretory vesicles carrying capsular polysaccharides in *C. neoformans* (Rodrigues et al., 2008b; Rodrigues et al., 2007).

Following the molecular characterization of *CAP59*, three other *CAP* genes were identified. *CAP64* was the second capsule-related gene described in *C. neoformans* (Chang et al., 1996). The gene demonstrated similarity to the gene encoding the yeast proteasome subunit, *PRE1*. Deletion of *CAP64* resulted in an acapsular strain that was avirulent in mice. As with *CAP59*, complementation of the *cap64* mutation restored both capsule expression and virulence. Interestingly, the *CAP64* gene from a serotype D isolate complemented the acapsular phenotype of a serotype A strain, indicating that the gene product of *CAP64* did not contribute to serotype specificity of *C. neoformans* polysaccharides (Chang et al., 1996).

The third gene required for capsule formation was *CAP60* (Chang and Kwon-Chung, 1998), which is closely linked to a cellulose growth-specific gene from *Agaricus bisporus*, *CEL1*. *CAP60* is located on the same chromosome as *CAP59* but, although similarity exists between Cap59p and Cap60p, the gene products cannot functionally substitute each other by direct complementation or by domain swap experiments. Immunogold labeling localized Cap60p at the nuclear membrane, although antibody reactivity was also detected in both the plasma membrane and the cell wall (Chang and Kwon-Chung, 1998). Deletion of *CAP60* resulted in acapsular cells that were moderately virulent in mice and complementation of the mutation restored capsule expression and virulence (Chang and Kwon-Chung, 1998).

CAP10 was the fourth characterized gene required for capsule formation and virulence in *C. neoformans* (Chang and Kwon-Chung, 1999). The gene sequence shows homology with a gene encoding a cryptococcal xylosyltransferase (Klutts et al., 2007). *Cap10* mutants were unable to produce fatal infection in mice, as demonstrated with other acapsular strains such as *cap59*, *cap60*, and *cap64* (Chang and Kwon-Chung, 1994, 1998, 1999; Chang et al., 1996). Complementation of the *cap10* mutation restored capsule and virulence (Chang and Kwon-Chung, 1999). The construction of Cap10p as a green fluorescence protein-conjugate revealed that the product of *CAP10* is distributed in patches within the cytoplasm of yeast cells, in cluster formations resembling vesicles.

Cryptococcal enzymes involved in GXM biosynthesis—The connections between the building units of GXM require the activity of different glycosyl transferases. The activity of glycosyltransferases involves the transfer of a monosaccharide from an activated sugar phosphate to an acceptor molecule. Most commonly, sugar nucleotide derivatives are used as glycosyl donors and, in the case of GXM, these basic building units consist of UDP-glucuronic acid, UDP-xylose and GDP-mannose. Some of the genes encoding for the enzymes responsible for the synthesis of these activated sugar donors have been characterized in *C. neoformans* and shown to have key roles in capsule biosynthesis and function, as detailed below.

Mannose is the most abundant sugar unit in GXM, and its incorporation into the polysaccharide depends on the synthesis of GDP-mannose. The GDP-mannose biosynthesis pathway is initiated by the cytosolic conversion of fructose-6-phosphate into mannose-6-phosphate by phosphomannose isomerase (PMI). Mannose-6-phosphate, the product of the reaction catalyzed by PMI, is then sequentially converted into mannose-1-phosphate and GDP-mannose by the enzymes phosphomannomutase and GDP-mannose pyrophosphorylase, respectively. The *C. neoformans* gene encoding for PMI, the enzyme responsible for the first step of GDP-mannose biosynthesis, was characterized (Wills et al., 2001). This gene, denominated

MANI, was disrupted and the resulting mutant had a poor ability to form a capsule. The mutant also produced reduced levels of exopolysaccharide and, in comparison with wild-type and reconstituted cells, had attenuated virulence in both rabbit and mouse models of cryptococcosis (Wills et al., 2001).

GDP-mannose is used as a mannose donor for polysaccharides, but also for glycolipids and glycoproteins. As mentioned above, this activated sugar is produced in the cytosol, although the process of mannosylation generally occurs within different organelles. GXM synthesis, for example, is thought to occur in Golgi-related structures (Yoneda and Doering, 2006). The activity of GDP-mannose transporters is therefore required for the transfer of nucleotide-activated mannose from the cytosol to the organelles in which mannosyl transfer to acceptor molecules will occur. The occurrence of two functional GDP-mannose transporters (*Gmt1* and *Gmt2*) has been recently reported in *C. neoformans* (Cottrell et al., 2007). Interestingly, this study demonstrates that deletion of *GMT1* generates poorly encapsulated cells that are not responsive to capsule induction conditions. In contrast, deletion of *GMT2* does not affect capsule expression. This observation clearly indicates that the transporters have distinct roles, some of them apparently relevant for capsule biosynthesis, while others are probably related to mannosylation of other glycoconjugates.

Glucuronic acid is the second sugar unit of GXM. Its addition to the polysaccharide requires the synthesis of UDP-glucuronic acid, which occurs via oxidation of UDP-glucose by UDP-glucose dehydrogenase in the cytoplasm. The gene encoding for the enzyme (*UGDI*) was cloned and expressed in *Escherichia coli* (Bar-Peled et al., 2004), revealing that the cryptococcal enzyme is a dimer whose activity is regulated by its NAD^+ - and UDP-glucose-binding sites. *Igd1p* showed membrane localization. The result was unexpected since there is no signal in the polypeptide sequence typical of membrane association or anchorage, which suggested the indirect association with the membrane via interactions with other proteins. Two independent studies revealed that disruption of *UGDI* results in acapsular cells with alterations in cell integrity, morphological defects at the bud neck, lack of growth in an animal model of cryptococcosis, and enhanced sensitivity to temperature, detergent, NaCl, and sorbitol (Griffith et al., 2004; Moyrand et al., 2004).

Xylose is the third monosaccharide component of GXM. It is used by fungal glycosyltransferases in the form of UDP-xylose, which is synthesized from the decarboxylation of UDP-glucuronic acid by UDP-glucuronic acid decarboxylase. The subcellular location of UDP-glucuronic acid decarboxylase is uncertain. In plants, UDP-xylose synthesis occurs both in the cytosol and in membranes (Reiter, 2002), and it is currently not known which source of UDP-xylose the different Golgi-localized xylosyltransferases (described later in this section) are utilizing. UDP-xylose and UDP-glucuronic acid transporters, supposedly present at the Golgi apparatus membrane, are also probably required for GXM synthesis in *C. neoformans*. As expected, mutant cells that had *UGDI* disrupted and, consequently, lacked UDP-glucuronic acid synthesis, failed to produce UDP-xylose (Griffith et al., 2004; Moyrand et al., 2004). The cryptococcal gene encoding for UDP-xylose synthase (*UXSI*) was also characterized and deleted, but *uxs1* Δ strains were encapsulated and grew at 37°C (Kozel et al., 2003). These results suggest that xylose incorporation into GXM is not essential for capsule formation, although it is clearly relevant for biological functions (Klutts and Doering, 2008).

Nucleotide-activated sugar donors are used by cryptococcal glycosyltransferases to form GXM. Considering that the polysaccharide consists of an α -1,3 mannan backbone containing *O*-acetyl, β -1,2/ β -1,4 xylosyl- and β -1,2 glucuronyl substitutions, proteins like α 1,3 mannosyltransferase, *O*-acetyltransferase, β -1,2 glucuronyltransferase, and β -1,2 and β -1,4 xylosyltransferase are expected to occur in *C. neoformans*. In fact, both early and more recent studies describe characterization of some of these enzymatic activities (Jacobson, 1987; Klutts

and Doering, 2008; Klutts et al., 2007; Klutts et al., 2006; Moyrand et al., 2004; Sommer et al., 2003; White et al., 1990; White and Jacobson, 1993). In several cases, the genes encoding these enzymes were characterized, as described below.

An α -1,3-mannosyltransferase that catalyses the transfer of mannose from GDP-mannose to α -1,3-linked mannose disaccharides has been described (Sommer et al., 2003). The related gene, *CMT1*, was shown to be homologous to *CAP59*, a cryptococcal gene involved in capsule synthesis by participating in the process of GXM export (Garcia-Rivera et al., 2004). *C. neoformans* cells in which *CMT1* expression was affected by RNA interference or genetic deletion had no 1,3-mannosyltransferase activity (Sommer et al., 2003). Interestingly, the product of *CMT1*, Cmt1p, co-purifies with a protein (Cas31p) that is homologous to the *CAP64* gene. Cas31p is required for xylosylation and *O*-acetylation of GXM (Moyrand et al., 2004). As discussed by Sommer and co-workers (Sommer et al., 2003), the co-purification of two *CAP* protein homologues suggests that these polypeptides might normally be associated. In addition, the hypothesis that Cmt1p is a mannosyltransferase could suggest the product of *CAP59* also acts in the synthesis of different glycans, since mannosylation is a common feature of different cryptococcal glycoconjugates (Gutierrez et al., 2007; Heise et al., 2002).

The analysis of capsular phenotypes revealed that approximately 50% of the cells in which expression of the putative mannosyltransferase (Cmt1p) was blocked were acapsular (Sommer et al., 2003). The remaining population, however, presented modified capsules, with lower density and shorter fibers than wild-type cells. Therefore, paradoxically, at least some of the *cmt1* mutant cells had no mannosyltransferase, but were encapsulated. A hypothesis raised by the authors is that, since fungi have a propensity to have multiple enzymes of overlapping specificity involved in glycan synthesis, other enzymes could compensate for the loss of Cmt1p. However, the hypothesis that Cmt1p is not directly related to GXM synthesis cannot be ruled out.

Once the α -1,3-mannan is synthesized, *O*-acetyl, glucuronyl and xylosyl residues are added to the growing polysaccharide. Cryptococcal protein fractions containing xylosyltransferase and glucuronyltransferase activities were described by White and co-workers (White et al., 1990). Potential acceptor molecules were investigated and, interestingly, *O*-acetylated mannan served as an acceptor for glucuronyl residues but xylomannan did not. In addition, glucuronomannan served as an acceptor for xylosyl residues, although acetylated mannan did not. These observations led to the conclusion that, during GXM synthesis, mannan is first assembled and then *O*-acetylated. Glucuronate is then added to the polysaccharide, followed by xylosyl residues.

Janbon and co-workers described for the first time a capsule synthesis gene, *CAS1*, coding for an enzyme that is apparently required for GXM *O*-acetylation (Janbon et al., 2001). The gene product, Cas1p, had no homology with any known protein putatively involved in protein or polysaccharide *O*-acetylation, but sequence analysis using a program designed to find putative protein families when querying a new sequence that has failed identification using alignment methods classified Cas1p as a putative glycosyltransferase. Mutant cells in which *CAS1* had been deleted synthesized regular GXM, except that it lacked *O*-acetyl groups. Strikingly, *cas1* mutant cells were more virulent than the parental strain (Janbon et al., 2001). Further studies revealed that *O*-acetylation is important for recognition of GXM by monoclonal antibodies, complement activation, tissue accumulation of GXM and interference with neutrophil migration, although it had no relevance on phagocytosis by neutrophils (Kozel et al., 2003). The relationship between the hypervirulent phenotype of *cas1* mutants and the properties described above remain unclear. It is noteworthy that antibody selection was used to isolate a spontaneous *O*-acetylation deficient variant that was hypovirulent (Cleare et al., 1999b). Another gene (*CAS3*) necessary to complete *O*-acetylation of the capsule was

described by Moyrand et al (Moyrand et al., 2004). The product of *CAS3*, Cas3p, shares homology with five other putative proteins, named Cas31p, Cas32p, Cas33p, Cas34p, and Cas35p. Mutational analysis of the related genes demonstrated that a *cas31* mutant produces GXM with the same *O*-acetylation as that of the parental strain, while the polysaccharide from a *cas3* mutant strain had a 70% loss of *O*-acetylation. Interestingly, GXM from a *cas3-cas31* double mutant was completely de-*O*-acetylated. Cas31p and Cas3p were demonstrated to have very similar sequences, suggesting that they correspond to proteins that have similar functions and different regulatory mechanisms (Moyrand et al., 2004).

Klutts and co-workers (Klutts et al., 2007) have recently described a β 1,2-xylosyltransferase activity that transfers xylose to an α 1,3-mannose dimer. The corresponding gene, *CXT1*, was cloned and successfully expressed in *Saccharomyces cerevisiae*, which normally lacks endogenous xylosyltransferase activity. Interestingly, the product of *CXT1*, Cxt1p, is homologous to Cap10p, a product of the capsule-related gene *CAP10*. The characterization of Cxt1p represented the first *CAP* gene homolog whose product had a defined biochemical function, and this homology suggests that *CAP10* and its homologs (*CAP1*, -2, -4, and -5) encode glycosyltransferases that are likely to be involved in capsule synthesis. The cryptococcal xylosyltransferase is not homologous to any related enzyme in mammalian or plant cells, so its characterization defined a novel family of glycosyltransferases. This family includes *C. neoformans* Cap10p and its homologs and is exclusive to fungi. Cas31p, described above to be involved in capsule *O*-acetylation, is also required for xylosylation (Moyrand et al., 2004), suggesting that at least some of the proteins involved in capsule assembly are multifunctional.

CXT1 deletion did not affect *in vitro* growth of the mutant cells or the general morphology of their capsules, as concluded from India ink staining and immunofluorescence with mAbs to GXM (Klutts and Doering, 2008). This result was in agreement with findings by Moyrand and Janbon (Moyrand et al., 2004), showing that mutant strains that lost the ability to form UDP-xylose are still encapsulated. Structural analysis of GXM and GalXM measured by NMR from *cxt1* mutants prepared on encapsulated or acapsular backgrounds revealed that the enzyme is involved in the synthesis of both polysaccharides (Klutts and Doering, 2008). However, NMR structural analysis of GXM and GalXM, revealed that both polysaccharides were missing β -1,2-xylose residues. A 30% reduction of β -1,2-xylose was noted in the abundance of this residue in GXM as compared to wild-type strains. In the mutant strains GalXM was almost completely devoid of β -1,2-linked xylose and β -1,3-linked xylose residues. Interestingly, fungal burden in the lungs of mice infected with the *cxt1* strain was significantly reduced compared to wild-type strains, indicating that deletion of *CXT1* results in an attenuated virulence (Klutts and Doering, 2008). Recently, Cxt1p has been involved in the addition of xylose residues to glycosphingolipids (Castle et al., 2008), a fact which could contribute to the reduced virulence observed.

According to Doering and colleagues, at least twelve glycosyltransferases would be required for GXM synthesis (Doering, 2000; Klutts et al., 2006). It is clear, therefore, that several of these enzymes remain yet to be characterized, including key molecules such as xylosyltransferases forming β -1,4 linkages with mannose, as well as glucuronyltransferase.

Cryptococcal enzymes involved in GalXM synthesis—Recently, GalXM mutant strains were generated for a putative UDP-glucose epimerase (*Uge1*) and a putative UDP-galactose transporter (*Ugt1p*). The study revealed that galactose metabolism plays a central role on the virulence of *C. neoformans* (Moyrand et al., 2007). The *UGE1* gene encodes a putative UDP-glucose epimerase, an enzyme that converts UDP-glucose and UDP galactose. The *UGT1* gene encodes a putative Golgi UDP-galactose transporter. In infection studies, *uge1* mutants are cleared from the brain and lungs. The *ugt1* mutant is found in a significantly

lower burden in the brain, lung and spleen. Over time *C. neoformans* is completely eliminated in the brain and lung with diminished levels in the spleen. Carbohydrate composition analysis revealed that the mutants do not secrete GalXM although their capsule size is larger than the isogenic wild-type strain. Both mutants had a growth defect at 37°C that was suppressed by 1% galactose addition to the medium, which suggested that absence of these enzymes also produced a cell wall defect. To date, the reason why GalXM deficiency results in diminished brain dissemination is unknown, and it is unclear if the effect is due to a reduction in fungal replication in the brain, or because of an impairment in crossing through the blood- brain barrier. Further studies are required to clarify the role of GalXM in dissemination.

Signaling pathways involved in capsule synthesis—The particular cellular distribution of the *CAP* genes in *C. neoformans* suggests that capsule formation is a complex and finely coordinated biosynthetic pathway. In fact, different compounds and environmental conditions have been shown to regulate capsule size by affecting the expression of *CAP* and other genes. Analyses of capsule size and quantitative real-time PCR indicated that, under capsule-inducing conditions, the expression of *CAP* genes is increased (Okabayashi et al., 2005). In contrast, capsule-repressing conditions, such as supplementation of culture media with high glucose concentrations, results in the concomitant decrease of capsule size and *CAP* gene expression (Okabayashi et al., 2005). The functions of these genes have not been clearly described, however. Since homologs of the *CAP* genes have been identified in fungal species that do not produce capsules (Janbon, 2004), their roles are probably not exclusively related to capsule production. Components of the cyclic AMP signal transduction pathway, as well as mechanisms that regulate pH and CO₂ sensing, utilization of carbon, nitrogen, serum components and iron, are also relevant to capsule expression in *C. neoformans*, as summarized in Table 1 and discussed below.

Iron sensing and capsule synthesis in *C. neoformans*—As stated above, iron concentration is one of the main environmental factors that influence capsule synthesis. Recently, genes encoding high affinity iron permeases (CFT1 and CFT2) were shown not to be involved in capsule size regulation. SAGE and Northern blot analyses demonstrated that iron limitation results in elevated transcripts of the *CAP60* gene (Lian et al., 2005). The iron-responsive transcription factor Cir1 was also demonstrated to be a requirement for elaboration of the capsule, since *cir1* mutants of both the serotype D and serotype A strains were acapsular (Jung et al., 2006). The *cir1* mutants also failed to form polysaccharide capsules in the presence of elevated CO₂ concentrations. Microarray analysis allowed the evaluation of the potential targets of Cir1 regulation resulting in the loss of capsule (Jung et al., 2006). The only gene that had its regulation altered in the *cir1* mutants was *CAS32*, which belongs to a family (CAS) of genes that are homologs of *CAP64* (Moyrand et al., 2004). The CAS genes are involved in insertion of xyloxyyl substitutions and/or *O*-acetylation of GXM (Moyrand et al., 2004). Therefore, it was not clear whether transcriptional changes in capsule-related genes caused the capsule defect in the *cir1* mutants. It has therefore been speculated that Cir1 could be necessary for capsule formation through the regulation of genes needed for sensing iron and CO₂ levels (Lian et al., 2005).

Signaling cascades involving cAMP regulation control capsule synthesis in *C. neoformans*—Elements of the cyclic AMP (cAMP) dependent pathway control melanin and capsule production in *C. neoformans* (D'Souza and Heitman, 2001). Strains in which the cryptococcal gene encoding for adenylyl cyclase (*CAC1*) was disrupted lost the ability to synthesize capsule and melanin (Alspaugh et al., 2002). Consequently, the mutants were unable to cause disease in animal models of cryptococcosis. Exogenously added cAMP suppressed the capsule defect of adenylyl cyclase mutant strains (Alspaugh et al., 2002). The reintroduction of the *CAC1* gene in the mutant strains reverted the *cac1* phenotype to wild-type phenotypes.

An upstream regulatory element of adenylyl cyclase, Aca1 (for adenylyl cyclase-associated protein 1) was further demonstrated to regulate capsule production via the Cac1-cAMP-protein kinase A pathway (Bahn et al., 2004). In addition, the activity of Cac1 is stimulated by HCO_3^- , produced through hydration of CO_2 by carbonic anhydrase 2 (Can2) (Mogensen et al., 2006), which is consistent with the well-described ability of *C. neoformans* to increase capsule size in response to CO_2 (Granger et al., 1985; Zaragoza et al., 2003a).

The gene encoding the major cAMP-dependent protein kinase catalytic subunit in *C. neoformans* (*PKA1*) was identified and disrupted (D'Souza et al., 2001). *Pka1* strains failed to produce melanin or capsule and were avirulent as a result. Interestingly, disruption of the gene encoding for the protein kinase A regulatory subunit (*PKR1*) in mutant strains which have a constitutively activated pathway resulted in overproduction of capsule. These mutant strains were hypervirulent in animal models (D'Souza et al., 2001). *Pka1* catalytic subunit acted downstream of the *Pkr1* regulatory subunit, since the phenotype of a *pkr1-pka1* double mutant was similar to that observed in single *pka1* mutants (D'Souza et al., 2001).

The cAMP pathway is highly regulated by the cell, and genes involved in this regulation have been identified. An early study (Alspaugh et al., 1997) revealed that the α -subunit 1 of the cryptococcal G protein (*Gpa1*), which in other species is necessary to activate adenylyl cyclase, regulates capsule expression in *C. neoformans* in response to environmental signals. The impaired ability of mutant cells lacking *Gpa1* to produce capsule and melanin led to an attenuated virulence in animal models of cryptococcosis. Exogenous cAMP restored mating, melanin, and capsule production in *gpa1* mutant strains (Alspaugh et al., 1997). This observation supported the hypothesis that cAMP-dependent signaling proteins were involved in capsule formation. Indeed, it has been further demonstrated that *Gpa1* transcriptionally regulates at least nine genes that are known or presumed to function in capsule synthesis or assembly, including *CAP10*, *CAP59*, and *CAP64* and genes encoding for *O*-acetyltransferase and UDP-xylose synthase (Pukkila-Worley et al., 2005). *Gpa1* is apparently under the control of the G protein-coupled receptor *Gpr4*, which engages amino acids in the cAMP-PKA pathway involved in the regulation of capsule formation (Xue et al., 2006). Recently it has been shown that disruption of *CRG2*, encoding a G-protein, results in increased cAMP levels and capsule enlargement, which suggests that *Crg2* negatively regulates the *Gpa1*-cAMP pathway (Shen et al., 2008).

Using a gene microarray approach, Cramer and co-workers (Cramer et al., 2006) demonstrated that the expression of *NRG1*, a gene encoding for a homolog of the *Candida albicans* *Nrg1* and *Saccharomyces cerevisiae* *Nrg1/Nrg2* family of transcription factors regulated in response to carbohydrate availability, is altered in a *gpa1* mutant of *C. neoformans*. *Nrg1* is under transcriptional control of the cAMP pathway (Liu, 2001). In fact, glucose and intact cAMP are required for wild-type levels of transcription of the cryptococcal *NRG1* gene (Cramer et al., 2006). Disruption of the *C. neoformans* *NRG1* gene by homologous recombination resulted in hypocapsular mutants with attenuated virulence. The reduced ability of *nrg1* mutants to form a capsule was probably associated with an effect on the expression of *UGD1*, which encodes a UDP-glucose dehydrogenase that is required for capsule formation (Moyrand et al., 2004). Interestingly, the *Nrg1* protein contains a consensus sequence for phosphorylation by the cAMP-dependent protein kinase (PKA) that is required for its full function (Cramer et al., 2006).

Intracellular cAMP concentrations depend on the activity of the low- and high-affinity phosphodiesterases (PDEases), *Pde1* and *Pde2*, respectively. In *C. neoformans*, mutational analysis revealed that *Pde2* has no apparent role in regulating intracellular cAMP levels. In contrast, *Pde1* is clearly relevant to cAMP-dependent signaling pathways regulating capsule formation (Hicks et al., 2005). Intracellular cAMP levels are elevated in a *pde1* mutant. In

addition, deletion of the *PDE1* gene restored the ability of *gpa1* mutants to form capsule (Hicks et al., 2005). Measurements of cAMP intracellular content revealed that the levels of this second messenger in a double *pde1-pka1* mutant strain is approximately 15-fold higher than the cAMP content in a *pka1* single mutant strain. In addition, a mutation in a consensus Pka phosphorylation site reduces Pde1 function. These results clearly indicate the occurrence of a feedback regulation system controlling cAMP levels, via its degradation by Pde1 (Hicks et al., 2005).

B.2.- GXM traffic in *C. neoformans*

Microbial polysaccharides are usually polymerized at the outer layer of the plasma membrane or even at the extracellular space. Bacterial capsular polysaccharides, for instance, are synthesized by plasma membrane glycosyltransferases whose active sites face the extracytoplasmic environment (Raetz and Whitfield, 2002; Whitfield, 2006; Whitfield et al., 2003). In fungal cells, glucan and chitin synthases, enzymes responsible for the synthesis of cell wall components, also localize to the plasma membrane (Leal-Morales et al., 1994; Ortiz and Novick, 2006; Valdivia and Schekman, 2003), which supports the idea that chitin and glucan are polymerized at extracellular sites. In contrast to other microbial polysaccharides, GXM is synthesized intracellularly, (Feldmesser et al., 2001; Garcia-Rivera et al., 2004), more specifically inside the Golgi apparatus (Yoneda and Doering, 2006). GXM traffic to the cell surface apparently involves transport by secretion of polysaccharide-containing vesicles (Feldmesser et al., 2001; Garcia-Rivera et al., 2004; Rodrigues et al., 2007), which somehow cross the cell wall, releasing their content into the extracellular space. The released polysaccharide is then connected to the cell wall or incorporated into the growing capsule by putative mechanisms that will be discussed below.

The first evidence that GXM is synthesized intracellularly was presented in a study by Feldmesser et al. (Feldmesser et al., 2001), who noted that monoclonal antibodies to the polysaccharide reacted with cytoplasmic structures of about 100-200 nm in diameter consistent with vesicles. These results were further supported by subsequent studies using immunogold labeling. Four different monoclonal antibodies to GXM reacted with cytoplasmic vesicles in four strains of *C. neoformans* (Garcia-Rivera et al., 2004), suggesting that the capsular polysaccharide is synthesized in the cytoplasm and exported to the exterior of the cell in secretory vesicles.

Different genes related to secretion mechanisms in yeast have been described in the last three decades (Lyman and Schekman, 1996). In yeast cells, highly conserved small GTPases are fundamental to exocytic processes (Spang and Schekman, 1998). One of the GTPases regulating exocytosis is Sec4p, a highly conserved molecule that belongs to the Sec4/Rab8 subfamily of Rab GTPases (Lipschutz and Mostov, 2002). This protein has homologs in several eukaryotic systems and, in fact, a Sec4p cryptococcal homolog was recently described (Yoneda and Doering, 2006). A gene encoding for a Sec4p homolog in *C. neoformans*, *SAVI*, has been cloned, and disruption of the gene resulted in decreased acid phosphatase secretion, suggesting a role in protein secretion. The most prominent cellular feature of the *sav1* mutant, however, was a marked accumulation of post-Golgi exocytic vesicles in the cytoplasm (Yoneda and Doering, 2006). These vesicles were recognized by an antibody to GXM, confirming that the synthesis of the polysaccharide occurs at intracellular sites. This study also supported the hypothesis that GXM traffic involves secretory vesicles derived from the Golgi apparatus.

The fact that treatment of *C. neoformans* with brefeldin A, an inhibitor of the Golgi-derived transport of molecules, resulted in a significant inhibition of capsule formation (Hu et al., 2007) supports the notion of capsular polysaccharide synthesis in golgi-derived vesicles. However, Golgi-derived vesicles, in the classic secretory pathway, are expected to fuse with the plasma membrane and release their internal content to the extracellular space

(Mallabiabarrena and Malhotra, 1995). Therefore, such a secretion mechanism could explain how GXM reaches the periplasmic space, but not how the polysaccharide reaches the outer layer of the cell wall to be incorporated into the growing capsule. Late endosomes, the cellular precursors of MVBs, can be connected with structures from the Golgi and the *trans*-Golgi network (Fevrier and Raposo, 2004). Therefore, one possibility is that the GXM-containing, post-Golgi vesicles of *C. neoformans* connect to the endosomal pathway, resulting in the release of exosome-like structures. In this case, the production of GXM-containing extracellular vesicles by *C. neoformans* would be expected. In fact, different studies supported the hypothesis that *C. neoformans* produce exosome-like vesicles containing the polysaccharide (Rodrigues et al., 2008b; Rodrigues et al., 2007; Rodrigues et al., 2000).

Membrane vesicles that migrate from the plasma membrane to the cell wall of *C. neoformans*, as well as in the region between the cell wall and the capsule, have been described in different studies (Rodrigues et al., 2000; Takeo et al., 1973a, 1973b). The occurrence of vesicles within the cell wall of cryptococci or in its adjacent extracellular microenvironment was also evidenced after *in vitro* growth, and vesicle-like structures near the edge of the capsule or in the cryptococcal cell wall were also observed in lung sections of mice infected with *C. neoformans* (Rodrigues et al., 2007). These vesicles were isolated from *C. neoformans* cultures by differential centrifugation and analyzed by transmission electron microscopy (Rodrigues et al., 2007). They corresponded to spheres within the diameter range of 20 to 400 nm presenting as bilayered membranes. Using morphological criteria, four main vesicle groups were identified, including i) electron dense and ii) electron lucid vesicles, iii) vesicular structures with membrane-associated electron dense regions and iv) vesicles containing hyperdense structures resembling a dark pigment. Vesicle production required cell viability and mass spectrometry analysis of vesicular components revealed the presence of different lipids (Rodrigues et al., 2007) and proteins (Rodrigues et al., 2008b) associated with virulence mechanisms of *C. neoformans*.

Vesicle secretion is apparently not exclusive to encapsulated cells. Although immunogold labeling of vesicles purified from encapsulated cells with an antibody to GXM followed by transmission electron microscopy revealed that the polysaccharide is present in the vesicular matrix, extracellular vesicles are also detectable in culture supernatants of acapsular mutants (Rodrigues et al., 2008b; Rodrigues et al., 2007). The mechanism of vesicular secretion of GXM, however, is putatively relevant for capsule assembly, since induction of capsule expression is accompanied by an enhanced detection of GXM in vesicular fractions (Rodrigues et al., 2007). Acapsular cells were able to extract GXM from vesicular fractions and incorporate the polysaccharide into their cell surfaces, indicating that the fungus has the metabolic apparatus to extract GXM from membrane vesicles. The mechanisms potentially involved in GXM release from vesicles and incorporation into the growing capsule are still not clear. According to Siafakas and co-workers (Siafakas et al., 2007), the cell wall-associated enzyme phospholipase B could be involved in the release of the molecules transported across cell walls inside membrane-bound vesicles.

The biogenesis of the *C. neoformans* secretory vesicles is still obscure, but several different lines of evidence lead to the hypothesis that they are exosome-like structures. Early studies by Takeo and co-workers described in *C. neoformans* unusual plasma membrane invaginations containing small vesicles, which would be associated with capsule synthesis (Takeo et al., 1973a, 1973b). Multivesicular compartments in close association with the cell wall were also recently described in *C. neoformans* (Rodrigues et al., 2008b). Finally, proteomic analysis of the *C. neoformans* vesicles revealed a complex protein composition that included several proteins described in mammalian exosomes (Rodrigues et al., 2008b). These observations, together with morphological data, support the idea that extracellular vesicles produced by *C.*

neoformans are exosome-like structures. A scheme comprising vesicle formation and GXM traffic in *C. neoformans* is proposed in Figure 5.

2. Polysaccharide connections at the *C. neoformans* surface—The cell wall of *C. neoformans* is a complex molecular network that includes, chitin and mannoproteins, lipids, pigments, and bioactive enzymes besides well-characterized structural components such as glucans (Nimrichter et al., 2005). This heterogeneous structure is surrounded by multiple layers of capsular polysaccharides, which implies that capsule assembly in *C. neoformans* involves the connection of capsular components to cell wall elements, as well as molecular interactions within the capsule.

Capsule anchoring to the cryptococcal cell wall—Proteins are apparently not involved in the connection of cell wall components to the capsule (Reese and Doering, 2003; Rodrigues et al., 2008a). In contrast, polysaccharides play a key role in capsule attachment to the cell wall. It has been demonstrated that GXM anchoring to the cryptococcal wall requires α -1,3-glucan (Reese and Doering, 2003; Reese et al., 2007). Cryptococcal cells with a disrupted α -1,3-glucan synthase gene lost the ability to synthesize α -1,3-glucan, (Reese et al., 2007). These cells had the ability to secrete capsular material, although they lacked surface capsule (Reese and Doering, 2003). Interestingly, acapsular mutants of *C. neoformans* treated with glucanase, but not protease, still bound to GXM, but in a defective manner (Reese and Doering, 2003). This result indicated that cell wall molecules other than glucan are required for capsule attachment to the cell wall.

The presence of cell wall chitin-derived molecules in *C. neoformans* has been demonstrated by microscopic and molecular approaches (Baker et al., 2007; Banks et al., 2005; Rodrigues et al., 2008a). Chitosan, the de-*O*-acetylated form of chitin, was demonstrated to be necessary for the correct architecture of the cell wall, melanin and capsule complex (Baker et al., 2007). In addition, chitin-derived oligomers, whose presence at the surface of *C. neoformans* had been suggested a few years ago (Woyke et al., 2002), have been recently shown to be involved in capsule anchoring to the cell wall (Rodrigues et al., 2008a).

The wheat germ agglutinin (WGA) is a lectin that binds to sialic acids and β -1,4-*N*-acetylglucosamine (GlcNAc) oligomers. In *C. neoformans*, different techniques revealed that chitin-bound oligomers of GlcNAc, but not sialic acids or intact chitin, are the molecules recognized by the lectin (Rodrigues et al., 2008a). Confocal microscopy demonstrated that the chitin-like oligomers recognized by WGA form projections linking the capsule to the cell wall (Figure 6). Interestingly, some of the molecules recognized by WGA formed unique ring-like structures connecting the capsule to the cell wall during yeast budding (Rodrigues et al., 2008a). The projection of chitin-like oligomers into the capsule, combined with the study of Zaragoza and colleagues showing the occurrence of capsule channels (Zaragoza et al., 2006a) strongly supports the concept that the capsule contains heterogeneous microenvironments despite a relatively homogenous appearance when visualized by India ink suspension and light microscopy. The mechanisms by which this singular architecture is maintained are still not understood.

Treatment of encapsulated *C. neoformans* cells with chitinase released GXM from the cell surface and reduced capsule size (Rodrigues et al., 2008a). The fact that chitinase treatment did not fully remove the cryptococcal capsule may be explained by the ability of cell wall glucans to anchor capsular polysaccharides (Reese and Doering, 2003; Reese et al., 2007). Based on the well documented ability of acapsular mutants to bind GXM to the cell surface (Kozel, 1977), it was demonstrated that chitinase-treated acapsular cells still bound soluble GXM (Rodrigues et al., 2008a). However, in contrast to untreated cells, yeast cells that were pre-incubated with the enzyme bound GXM in a manner that formed a loose polysaccharide

coat at the surface of *C. neoformans*, as previously described for glucanase-treated acapsular mutants (Reese and Doering, 2003). In combination, these results indicate an association of chitin-derived structures and α -1,3-glucan with GXM. However, the hypothesis that other components connect glucans and chitin-derived structures to capsular components cannot be discarded, since loss of cell wall polysaccharides would disturb cell wall assembly and, consequently, capsule anchoring.

Section C Capsule functions in *C. neoformans*. The capsule as a virulence factor. Function during the interaction with the host

C. neoformans virulence is a complex process and there is evidence that both fungal components and the immune response to infection combine to damage the host (Casadevall and Pirofski, 2003). Concerning fungal factors, the capsule is the major virulence factor involved in the pathogenesis. As stated previously, the polysaccharide from the capsule can be found attached to the cell forming the physical structure, and released to the medium, as exopolysaccharide. Multiple studies indicate that both types of polysaccharide confer beneficial effects for the fungus during its interaction with the host. In general, the capsule virulent function is conveyed by mediation of a protective effect for cryptococcal cells, such as interfering with phagocytosis by macrophages and ameboid environmental predators. However, the capsular polysaccharide also contributes to virulence through multiple deleterious effects on the immune response, which in turn facilitates fungal propagation in tissues and ultimately kills the host.

C.1 Role of the capsule during interaction with the host

The presence of the capsule confers beneficial effects to *C. neoformans* during infection, especially during its interaction with phagocytic cells and environmental predators.

Phagocytosis—Phagocytosis is an important process required for the clearance of a large number of pathogens. From the first studies in the early 1970s, it was shown that the capsule had strong antiphagocytic properties, which was the first evidence of its role as virulence factor. As a consequence, the capsule is involved not only in evading killing by phagocytic cells, but also by means of inhibiting a subset of several phenomena which are dependent of phagocytosis. For example, a reduced phagocytosis translates into reduced T-cell proliferation (Syme et al., 1999), and antigen processing and presentation by macrophages, all resulting in an interference with the T-cell response (Vecchiarelli et al., 1994a; Vecchiarelli et al., 1994b). Despite the inhibitory role of the capsule, phagocytosis does occur during infection. During the first hours of infection, a significant proportion of the yeast cells injected in the lungs are found inside phagocytic cells (Feldmesser et al., 2000). This result indicates that during infection, there are mechanisms that overcome the antiphagocytic effect of the capsule. So far two mechanisms for this have been described: 1) the presence of opsonins, 2) direct interaction of the polysaccharide fibers with phagocytic receptors that occur after capsule structure rearrangements.

Opsonins required for phagocytosis—Classically, there have been two types of opsonins that induce *C. neoformans* phagocytosis, which are antibodies and proteins from the complement system. These opsonins bind to the capsular polysaccharide. We will briefly review the interaction that is formed between these opsonins and the polysaccharide fibers.

a) Antibody mediated phagocytosis: A large number of mAbs that recognize GXM have been obtained (Casadevall et al., 1992; Dromer et al., 1987b; Eckert and Kozel, 1987; Kozel et al., 1998; Pirofski et al., 1995; Todaro-Luck et al., 1989). In addition to the protective effect of these antibodies, their binding to the capsule and function as phagocytic opsonins have also

been tested. IgG antibodies bind the *C. neoformans* capsule, and they induce phagocytosis through binding to the Fc receptors (FcRI and FcRIII). Interestingly, IgM to capsular polysaccharide is opsonic in the absence of complement. IgM opsonic efficacy correlates with the binding pattern of the Abs to the capsule. IgM to GXM can bind in two different patterns to the capsule as shown by immunofluorescence, punctuate and annular (Cleare et al., 1999a; Cleare and Casadevall, 1998; Nussbaum et al., 1997). Annular pattern correlates with a protective effect in mice and with a good opsonic efficacy. In contrast, mAbs that bind to the capsule in a punctuate pattern are not protective and do not induce phagocytosis. Since no FcR has been identified for IgM antibodies, the mechanism by which this isotype induces *C. neoformans* phagocytosis posed a challenging question. Analysis of this phenomenon revealed that phagocytosis of *C. neoformans* in the presence of IgM antibodies occurred through binding of the polysaccharide fibers to the complement receptors, and specifically to CD18 (see below). It was hypothesized that the binding of the Ab to the capsule produced some structural rearrangements that resulted in the exposure of CD18 binding sites in the polysaccharide, which in turn produced phagocytosis through the complement receptors (CR) (Taborda and Casadevall, 2002). A similar effect was demonstrated for IgG antibodies and F(ab)₂ fragments (Netski and Kozel, 2002; Zaragoza et al., 2003b). In addition to mAbs to the capsule, other opsonic antibodies have been described. Other examples of this phenomenon may exist, such as the report by Merkel and Scofield who described the isolation of an opsonic mAb whose epitope was abundantly found, not in the capsule, but at the level of the cell wall (Merkel and Scofield, 1999).

b) Complement mediated phagocytosis: The other opsonin whose function has been extensively studied in *C. neoformans* is complement. The capsule of *C. neoformans* is a strong inducer of complement system activation through the alternative pathway, resulting in a large deposition of iC3b on the capsule (Kozel and Pfrommer, 1986; Kozel et al., 1989). Encapsulated cells and purified GXM induced C3 release from peritoneal cells, and the amount of C3 released correlated with the degree of encapsulation of the cell (Blackstock and Murphy, 1997). However, binding of C3 to the capsule is not sufficient to induce phagocytosis, because there are many *C. neoformans* strains that are not phagocytosed in the presence of complement. It was later found that there was a negative correlation between the phagocytic index of the strains and the size of the capsule (Yasuoka et al., 1994), suggesting that complement-mediated phagocytosis inhibition correlated with the molecular size of the GXM molecules produced by different strains. Earlier studies had suggested that cells with a large capsule bound a lower amount of C3, also suggesting a mechanism by which cells with a larger capsule were less efficiently phagocytosed (Kozel et al., 1996). More recently, studies using confocal microscopy revealed that C3 deposition was different in cells with small and large capsules. In cells with large capsule, C3 was located close to the cell wall, away from the capsule edge. This differential localization suggested a mechanism by which capsule enlargement interfered with the interaction of C3 in the capsule with its corresponding phagocytic receptor (Zaragoza et al., 2003b). Interestingly, C3 proteins from different sources (human, mouse, rat) bound differentially to the capsule, suggesting that the complement pathway from different hosts may have different functions in the defense against *C. neoformans* (Gates and Kozel, 2006), which could explain the differences in susceptibility of the host to *C. neoformans* infection (see comment in (Pirofski, 2006)).

c) Effect of the interaction between the different opsonins with the capsule: During the course of an infection, both complement and antibodies are available, and it has been shown that the presence of both opsonins can result in different outcomes. For example, the presence of Abs to the capsule produces strong complement activation through the classical pathway, leading to a fast deposition of complement on the capsule (Kozel et al., 1998). This effect is largely dependent on the isotype of the Ab. While IgG mAbs have a strong effect, IgM produces a moderate activation. In addition, it has also been shown that addition of mAbs to the capsule

not only produces C activation through the classical pathway, but, when added in a sufficient amount, allows complement deposition at the capsule edge, as opposed to deep inside the capsule, resulting in an increase in C-mediated phagocytosis (Zaragoza and Casadevall, 2006).

2) GXM Receptors—Several receptors have been described that interact with the capsular polysaccharide, triggering different responses in phagocytic cells (Ellerbroek et al., 2004c; Levitz, 2002; Monari et al., 2005a; Yauch et al., 2004). These receptors are involved in phagocytosis, either because they directly induce or prevent pathogen engulfment, or because they belong to pattern recognition receptors, which are involved in phagocytosis regulation, such as Toll-like receptors. In the first group, GXM can interact with CD18, which forms part of the complement receptor, and this interaction can trigger CR mediated phagocytosis in the absence of opsonin. GXM can also directly interact with Fc RIIB (Monari et al., 2005a), which has been involved in *C. neoformans* uptake by phagocytic cells (Monari et al., 2005a; Syme et al., 2002). However, FcR γ II engagement produces inhibitory signals that contribute to immune unresponsiveness, as will be reviewed later on. In the second group, it has been described that GXM can interact with CD14 and Toll-like receptors 2 and 4. These receptors are involved in macrophage stimulation, and their functions have been characterized.

The role of other receptors that are known to bind mannose and other polysaccharides has been studied, since the capsular polysaccharide is particularly rich in mannose. These receptors are the mannose receptor and SIGN-R1. In the case of the mannose receptor, it has been shown that it plays a role in *C. neoformans* uptake by dendritic cells (Syme et al., 2002). In addition, *C. neoformans* produces immunomodulation through secretion of mannoproteins which interact with the mannose receptor (Levitz and Specht, 2006). The SIGN-R1 receptor binds bacterial polysaccharides, but in the case of *C. neoformans*, it has been shown that it does not bind GXM (De Jesus et al., 2008).

The role of various cellular receptors in phagocytosis and GXM localization has been studied using knock-out mouse strains. None of the receptors were completely required for serum clearance or hepatosplenic polysaccharide degradation (Yauch et al., 2005). In another study, Yauch *et al.* (Yauch et al., 2004) sought to study the role of the CD14, TLR2, and TLR4 receptors and the function of MyD88, in cryptococcal infection *in vitro*. The results revealed that MyD88 plays a critical role during cryptococcosis while the TLR2 and CD14 receptors play minor roles. That study demonstrated that MyD88^{-/-} mice succumbed to *C. neoformans* infection significantly earlier than wild-type mice when infected either i.n. or i.v. MyD88^{-/-} mice infected intranasally had increased numbers of CFU in the lungs and elevated GXM levels in the lungs and sera, which correlated with the reduced survival of these mice. Additionally, mice lacking TLR2 had significantly reduced survival only after pulmonary challenge, while CD14^{-/-} mice displayed a trend towards reduced survival after intravenous infection. Since the MyD88^{-/-} mice were more susceptible to cryptococcosis than the TLR2 and TLR4 mutant mice, the authors suggested that MyD88 besides serving as an adaptor protein for TLR SIGN-R1 there are other yet-to-be identified receptors for GXM on red pulp macrophages (De Jesus et al., 2008). They also suggested that it does not appear to be involved in macrophage uptake of GXM.

Capsule role during macrophage intracellular parasitism and intracellular GXM accumulation—A key feature of cryptococcal pathogenesis is its ability to evade killing inside phagocytic cells. *C. neoformans* is considered a facultative intracellular pathogen. This finding was first observed in the early 70s (Diamond and Bennett, 1973) and it has been more carefully studied in the last years in macrophages, microglial cells, and endothelial cells (Coenjaerts et al., 2006; Chretien et al., 2002; Feldmesser et al., 2000; Lee et al., 1995b). *C. neoformans* can survive inside macrophages, and exploits the intracellular macrophage

environment for its replication. Furthermore, there are several mechanisms described that allow *C. neoformans* extrusion from infected macrophages (Alvarez and Casadevall, 2006; Ma et al., 2006) or even cell-to-cell spread (Alvarez and Casadevall, 2007; Ma et al., 2007). Concerning the role of the capsule in this process, it has been shown that acapsular mutants cannot replicate inside phagocytic cells (Feldmesser et al., 2000), which indicates that the capsule plays a key role in intracellular parasitism. Along these lines, acapsular mutants are not extruded by macrophages, and this phenotype is reverted by pre-coating of acapsular cells with GXM (Alvarez and Casadevall, 2006). A typical feature after *C. neoformans* engulfment is that there is an important accumulation of GXM-containing vesicles (Lee et al., 1995b; Tucker and Casadevall, 2002). These vesicles became permeable during the course of intracellular infection, which suggested a new mechanism by which *C. neoformans* interfered with macrophage functioning (Tucker and Casadevall, 2002). Since phagocytic cells have several receptors for GXM, they can directly engulf this capsular polysaccharide component (Monari et al., 2003). However, binding and internalization differ between phagocytic cells. GXM accumulated constitutively in macrophages, in contrast to neutrophils, in which it was rapidly expelled (Monari et al., 2003). Accumulation of GXM by macrophages was dependent on the cytoskeleton and several macrophage intracellular pathways (Chang et al., 2006b). The presence of intracellular polysaccharide altered the function of the macrophages at multiple levels. For example, once phagocytosed, encapsulated cells do not induce nitric oxide synthase, a phenomenon that does occur when acapsular mutants are ingested (Naslund et al., 1995). Other mechanisms by which capsular components can interfere with the activity of immune cells will be reviewed below. Finally, it has recently been shown that the capsule confers protection against reactive oxygen species, which are important antifungal molecules produced by macrophages during phagocytosis. In addition, capsule enlargement also increased the survival of the cells in the presence of these radicals and antifungal molecules, such as defensins and Amphotericin B (Zaragoza et al., 2008b). These results suggest mechanisms for how *C. neoformans* evades killing inside the phago-lysosome, and are in agreement with published data in *Klebsiella pneumoniae* (Campos et al., 2004), which demonstrate that the bacterial capsule confers protection against defensins..

Capsule and dissemination—Extrapulmonary dissemination is a key process for disease causation during fungal pathogenesis (reviewed in (Eisenman et al., 2007; Filler and Sheppard, 2006)). Several microbial factors, such as laccase, phospholipase and the capsule are involved in the dissemination process. The capsule plays an important role in this process, since acapsular mutants have impaired dissemination to the brain (Wilder et al., 2002). Although this effect could be due to the lack of virulence of the strain and rapid clearance by the immune system, there are reports that the polysaccharide capsule plays an active role in this process. For example, a seminal report indicates that during dissemination through the organism, *C. neoformans* manifests important capsule structure changes that may be involved in tissue invasion (Charlier et al., 2005). These authors found that a serotype A strain (H99) that was exclusively reactive with a serotype A-specific mAb in vitro, became reactive to a serotype D-specific mAb during endothelial crossing of the blood-brain-barrier. These findings suggest that *C. neoformans* is able to modulate its capsule structure during dissemination, allowing for adaptation to different environments, thereby enhancing dissemination.

During the course of infection and dissemination, *C. neoformans* must cross epithelial and endothelial barriers. *C. neoformans* can bind to epithelial cells (Merkel and Cunningham, 1992). In this way the capsule contributes to the binding of *C. neoformans* cells to human alveolar epithelial cells (Barbosa et al., 2006). This interaction resulted in a decreased viability of epithelial cells. In a different report the same authors demonstrated that epithelial cells did bind GXM through interaction with CD14, and that this binding resulted in an increased IL-8 production (Barbosa et al., 2007). Other reports, however also suggest that acapsular mutants can efficiently bind to human lung epithelial cell lines (Merkel and Scofield, 1997), implying

that binding to epithelia can also occur through selective binding of yeast adhesins present in the cell wall.

Concerning endothelial cells, capsular and acapsular *C. neoformans* cells cross brain microvascular endothelial cells through transcytosis (Chen et al., 2003). Transcytosis was accompanied by disruption of tight junctions and alterations in the cytoskeleton. The role of the capsule in binding endothelial cells is not clear. Binding to endothelial cells occurs for both encapsulated and acapsular strains, although binding, and transcytosis, was more efficient for encapsulated strains (Chen et al., 2003). In agreement with this idea, a mutant defective in CPS1 (encoding a putative glycosyltransferase) manifested a slight decrease in capsule size, and presented a marked reduced adherence to brain endothelial cells (Chang et al., 2006a). However, although this mutant has a smaller capsule size, the structure is still present, which indicates that the capsule is not a complete requirement for *C. neoformans* to bind to endothelial cells. In this sense, Ibrahim et al found that acapsular strains produced damage in umbilical vein endothelial cells and were more efficiently phagocytosed than encapsulated strains, suggesting that the escape from the vasculature and dissemination occurred through acapsular or poorly encapsulated cells (Ibrahim et al., 1995), and that the invasive mechanisms in the BBB are different from other blood vessels. Accordingly, acapsular mutants were recently shown to manifest *in vitro* invasive growth, a phenomenon not observed in encapsulated strains (Zaragoza et al., 2008a). Mutants defective in inositol phosphosphingolipid-phospholipase C1 are hyperencapsulated and do not disseminate to the brain (Shea et al., 2006). Similar findings have been found with mutants that cannot synthesize GalXM, which are hyperencapsulated, and fail to disseminate to the brain during infection (Moyrand et al., 2007). Such mutants presumably have a capsule with a different structure and lack GalXM, which might play an independent role in dissemination. Thus, the attribution that larger capsules inhibit dissemination must be considered tentative until validated by subsequent studies.

Once the fungal cell has been internalized by the endothelial cell, transcytosis to the abluminal side of the cell occurs. During this process it seems that endothelial cells have antimicrobial mechanisms based on the production of free radicals. Coenjaerts et al. found that endothelial cells have the ability to kill *skn7 C. neoformans* mutant cells (lacking a transcription factor that regulates oxidative stress response) through production of free radicals (Coenjaerts et al., 2006). The fact that wild-type *C. neoformans* cells can evade this antimicrobial activity suggests that there are mechanisms that protect the cell from killing, enhancing survival, transcytosis and dissemination. We recently described that the capsule and capsular enlargement plays a key role in the protection against free radicals (Zaragoza et al., 2008b), and it is reasonable to think that encapsulated strains are more likely to survive and disseminate than acapsular strains.

C.2. Role of the exopolysaccharides during infection

The capsular components are released into the surrounding environments, and are found in serum and tissues during infection. In fact, non-*Cryptococcus* cell associated exopolysaccharides have been found freely in various types of tissues (De Jesus et al., 2008; Goldman et al., 1995; Lee et al., 1996; Lendvai et al., 1998; Lee and Casadevall, 1996). Various approaches have been carried out to study the role of capsular polysaccharides on the immune response. They included investigation of the role of encapsulated and acapsular strains, as well as *in vitro* or *in vivo* studies using purified capsular fractions, the latter based on exposure in host models. These approaches have shown that these exopolysaccharides exert a large number of effects inside the host, which will be reviewed in the following sections.

GXM unresponsiveness—Early studies using total polysaccharide from *C. neoformans* revealed that this antigen had a propensity for inducing immunological paralysis (Blackstock

and Hall, 1984; Gadebusch, 1958a, 1958b; Robinson et al., 1982). Several reports indicate that the capsular polysaccharide plays an important role in *C. neoformans*-induced immunological unresponsiveness. Murphy *et al.* showed that the polysaccharide caused a dose-dependent primary immunological response in mice using the hemolytic plaque assay as indicated by the increase in antibody producing cells. However, after a subsequent challenge with capsular polysaccharide, a state of immunological unresponsiveness was induced. There was a diminution of antibody response and the abolition of spleen plaque forming cells rather than neutralization of antibody by excess antigen (Murphy and Cozad, 1972). The results supported the view that tolerance was due to terminal differentiation without proliferation of immunocompetent cells. A second study showed that the induction of unresponsiveness in the host appears to be dependent upon the total amount of polysaccharide to which the host is exposed rather than the duration of exposure. The study suggested that cryptococcal polysaccharide is sequestered until a sufficient amount of polysaccharide is accumulated for the induction of paralysis (Kozel et al., 1977).

In addition to inhibition of Ab production, *C. neoformans* and capsular polysaccharides interfere with proper inflammatory responses. Mody and Syme found that *C. neoformans* GXM diminished human lymphocyte proliferation (Mody and Syme, 1993). This process was mainly due to the antiphagocytic effect elicited by the capsular polysaccharides (Syme et al., 1999). Blackstock et al. compared the immune response elicited by two different *C. neoformans* strains, which differed in their ability to induce in vitro capsule enlargement and virulence (Blackstock et al., 1999). The authors found that after infection the strain was unable to induce capsule size, was not associated with GXM accumulation in serum, and was cleared from infected lungs after an early inflammatory response. In contrast, the virulent strain, which was heavily encapsulated, produced a delayed DTH response (after 15 days of infection) and a significant accumulation of polysaccharide in serum. This delayed response correlated with a difference in the cytokine expression pattern. Mice infected with heavily encapsulated strain failed to upregulate Th1 cytokines, such as IL-2 and IFN γ , and induced a significant accumulation of IL-10, which was not found in mice infected with the hypocapsular mutant. These results suggested that *C. neoformans* and its capsular components, produced anergy through IL10 upregulation and Th1 cytokines downregulation (Blackstock et al., 1999).

Older studies suggest that *C. neoformans* GXM polysaccharide produces some of its immunosuppressive effect by activation of a specific subset of T-suppressive cells which secrete T-suppressive factors (TsF) (Blackstock, 1996). This factor induced macrophage unresponsiveness and in turn, inhibited phagocytosis (Blackstock et al., 1987; Morgan et al., 1983; Rubinstein et al., 1989). GXM is a type 2 T-cell independent antigen (Sundstrom and Cherniak, 1992). GXM tolerance was T-cell mediated at a low dose, but T-cell independent at high doses. Moreover, the effect seemed to be mediated through CD4+ T-cells. In rat experimental models, it was also shown that *C. neoformans* produced an immunosuppressive state after two and three weeks of infection, which was associated with function of accessory cells (such as macrophages) and inhibition of spleen cell proliferative response to mitogens (Rossi et al., 1998). This unresponsiveness was also induced by purified GXM, and was associated with a decrease in the number of viable spleen mononuclear cells, increased production of IL-10 and IL-4, reduced levels of IL-2, and decreased CD11a surface expression (Chiapello et al., 2001). Addition of GXM to dendritic cells can inhibit T-cell proliferation. Recently, Yauch et al. described that GXM inhibited T-cell proliferation when exposed to dendritic cells. This inhibition occurred not only in the presence of dendritic cells, but also when T-cells were activated by anti-CD3 antibody, concanavalin A, or ionomycin (Yauch et al., 2006).

In addition to modulation of acquired immune system (B and T cells), capsular polysaccharide also interferes with some elements of the innate immune system. For example, the capsule

inhibits aggregation of *C. neoformans* cells by surfactant protein D, which is an important component of the lung's innate defense system (van de Wetering et al., 2004). Recently, it has been demonstrated that direct engagement of GXM to FcR γ II expressed in monocytes, macrophages and dendritic cells, leads to an inhibitory signal that contributes to immune suppression (Monari et al., 2006b).

The immune unresponsiveness resulting from GXM does convey some beneficial effects on the host during other types of inflammatory pathologies. For example, GXM acts as a potent immunosuppressor during induced arthritis (Mirshafiey et al., 2004).

GXM immunomodulation—During *C. neoformans* infection, the expression of a large number of immune mediators are modulated by GXM (reviewed in (Monari et al., 2006a; Vecchiarelli, 2000)). In addition, these immunomodulatory effects vary depending on cell type.

The outcome of the interaction between *C. neoformans* and monocytes differs depending on whether the yeast cell has a capsule. Acapsular strains elicited higher levels of some pro-inflammatory cytokines from monocytes, such as TNF- α , IL-1- β and IFN- γ (Vecchiarelli et al., 1995; Walenkamp et al., 1999). This effect of the capsular polysaccharide is largely mediated through GXM, which can influence the expression of many molecules with important functions in the immune response. GXM also induces IL-10 and IL-8 secretion by human monocytes (Walenkamp et al., 1999). Since IL-10 is a potent downregulator of pro-inflammatory cytokines (such as TNF- α and IL1- β), this finding suggests a mechanism by which *C. neoformans* polysaccharide interferes with cell mediated immunity (Vecchiarelli et al., 1996). *C. neoformans* encapsulated cells induced IL-12 poorly when compared to acapsular mutants. It was later described that GXM reduced IL-12 through the increase in IL-10 expression, since neutralization of IL-10 with antibody reversed the GXM suppressive effect (Retini et al., 2001). It has recently been shown in murine macrophage cell lines that GXM induces TNF-beta accumulation (Villena et al., 2008). Cytokine regulation by *C. neoformans* and GXM is also mediated through the complement pathway (Chaka et al., 1997b). GXM also induces IL-6 in monocytes (Delfino et al., 1997). The same effect was found in rat alveolar macrophages. In this case, the effect was mediated after phagocytosis of encapsulated cells opsonized with specific Abs (Li and Mitchell, 1997). The expression of CD4 on monocytes was different when challenged with encapsulated and acapsular strains. Acapsular strains induced higher CD4 levels, and this effect was downregulated by addition of exogenous GXM (Pietrella et al., 1998)

In contrast, the capsule and purified GXM can have different effects on PMN cells. As mentioned above, GXM causes L-selectin shedding from neutrophils, a phenomenon which is accompanied with reduced TNF- α receptor expression, but increased CD15 and CD11b expression in neutrophils (Dong and Murphy, 1996). Furthermore, *C. neoformans* and purified GXM induce the expression of pro-inflammatory cytokines (TNF- α , IL-1 β , IL-6, and IL-8) in neutrophils. Interestingly, in the case of encapsulated cells, the effect was dependent on the size of the capsule (Retini et al., 1996). However, this effect was not directly mediated through GXM, but through the complement activation pathway by capsule components, especially C3a y C5a, which stimulate PMN to produce pro-inflammatory molecules (Vecchiarelli et al., 1998). Some studies showed higher IL-6 expression in peripheral blood mononuclear cells after prolonged stimulation with acapsular strains (Siddiqui et al., 2006). The capsule also modulates the expression of C5aR (Monari et al., 2002). Exposure of PMN cells to encapsulated cells or acapsular mutants treated with GXM resulted in a drastic decrease of C5aR on the PMN cells membrane, suggesting a mechanism by which GXM interferes with neutrophil function.

The reason why GXM produces different effects on macrophages and neutrophils is not known, but these cell types interact differently with GXM (see above, (Monari et al., 2003)). While GXM is expelled from the cells in neutrophils, macrophages show a continuous intracellular accumulation of GXM. This difference may, in turn, produce different effects in cytokine production.

Consistent with the idea of GXM producing different effects in different cell types are findings that GXM from certain serotypes induces IL-8 production in human fetal microglial cells, but not in astrocyte cultures (Lipovsky et al., 1998a). Barluzzi et al. studied the effect of encapsulated and acapsular strains in microglial cell cultures. These authors did not find any difference in the expression of several cytokines, such as TNF- α , IL-1 β , IL-6, IL-12p40 and granulocyte macrophage colony stimulating factor. They did find, however, that lipopolysaccharide-induced TNF α expression was blocked by encapsulated or acapsular yeasts or exposure to purified GXM or GalXM (Barluzzi et al., 1998).

GXM also regulates cytokine expression by CD4+ T cells (Mariano Andrade et al., 2003). GXM does not induce CD4 cell proliferation, but interfered with the immune system, resulting in increased fungal replication through a mechanism that involved enhancement of IL-10 and IL-4 production. These findings were interpreted as indicative that GXM contributes to virulence by interfering with the development of an effective cell-mediated immune response that was unable to inhibit fungal replication.

GalXM immunomodulation—Although most of the immunomodulatory effects of the capsular polysaccharide studies have focused on GXM, GalXM can also contribute to these effects. GXM, GalXM and MP can each elicit an increase in TNF- α in whole blood cultures (Delfino et al., 1996). Another study demonstrated that GXM-induced TNF- α accumulation was dependent on the presence of serum. In the case of GalXM, however, TNF- α was serum independent (Chaka et al., 1997b). GalXM induces IL-6 in monocytes (Delfino et al., 1997). In another study, the cytokine expression profile of human monocytes in the presence of different capsular components was investigated, demonstrating that GalXM and GXM exerted similar effects (Walenkamp et al., 1999). Recent findings in murine macrophages (RAW 264.7 cell line) showed that GalXM and GXM exert different effects on cytokine production, with GalXM having a profound effect on TNF-alpha induction in addition to NO production through ad iNOS expression (Villena et al., 2008). In microglial cells neither the addition of GalXM or GXM was associated with an increase in TNF- α accumulation, but both GXM and GalXM blocked this induction in the presence of LPS (Barluzzi et al., 1998). These results might suggest that GXM and GalXM share immunomodulatory properties.

Effect of GXM on antigen presentation—*C. neoformans* encapsulation is associated with reduced antigen presentation by macrophages (Collins and Bancroft, 1991). Purified GXM inhibited antigen presentation in monocytes exerted by acapsular cells (Retini et al., 1998). This effect was dependent on phagocytosis and was mediated through an increase in IL-10, resulting in a significant reduction in MHCII. In addition, the capsule was a poor stimulator of co-stimulatory molecule expression required for antigen presentation, such as B7-1 (CD80) and B7-2 (CD86), although encapsulated cells increased expression of ligand CTLA-4 when compared to acapsular cells (Pietrella et al., 2001b). This result is relevant for the host's defense since B7 co-stimulatory molecules are necessary to elicit an efficient T-cell response against *C. neoformans* (Monari et al., 1999b). Despite the negative effects of GXM on the expression of co-stimulatory and MHC molecules, injection into mice of antigen-presenting cells previously exposed in vitro to GXM inhibited T-suppressive cell responses, a phenomenon that led to enhanced survival of infected mice (Blackstock and Casadevall, 1997). This protective effect of GXM-APC stimulated cells was associated with an early DTH response development, and was mediated through increased production of some cytokines, such as IL-2

and IFN- γ (Blackstock et al., 2000). The authors concluded that during this process, GXM-dependent and GXM-independent effects occurred. GXM-specific effects were dependent on MHC class II, while GXM independent effects required B7-1 and B7-2 molecules (Blackstock, 2003).

The capsular polysaccharide also interferes with the normal function of dendritic cells, which are professional antigen presenting cells necessary to initiate T-cell mediated responses. Acapsular *C. neoformans* mutants are rapidly ingested by dendritic cells, which produces upregulation of maturation markers, such as MHCI and II, CD40, and CD83 (Monari et al., 2002). This maturation was not observed when dendritic cells were exposed to encapsulated cells or purified GXM, and suggested another mechanism by which the capsule interfered with T-cell mediated responses.

GXM induced apoptosis—Several groups have demonstrated that soluble GXM can induce apoptosis in different systems (see figure 7). This phenomenon was first described in rat lymphocytes (Chiapello et al., 2003). GXM-mediated apoptosis was first observed *in vitro*, and the results were corroborated in animal models, which proved that there was a high proportion of apoptotic cells in rat lungs and spleens during *C. neoformans* infection. A similar finding was observed when rats were treated with purified GXM (Chiapello et al., 2004). This process correlated with the modulation of some cytokines by GXM, such as IL-10, IL-2, IFN- γ and TNF- α . GXM was also shown to induce expression of the Fas ligand on murine cells. This induction required GXM binding to TLR4 and correlated with GXM accumulation in the macrophages. Moreover, these macrophages induced apoptosis of T-cells or jurkat cells that expressed Fas (Monari et al., 2005b). Recently the mechanism of GXM-induced apoptosis of T-cells was further elucidated. It was found that both *in vitro* and *in vivo* systems, the binding of FasL produced by antigen presenting cells, by Fas on T-cells triggers caspase cascades that cause T-cell death (Monari et al., 2008). Caspase 8 was identified to play a central role in apoptosis since it cleaves caspase 3 via the extrinsic and intrinsic apoptotic pathways. It was also found that caspase 9 cleavage can also activate caspase 3 independent of caspase 8. Therefore, the caspase 8 and caspase 9 pathways co-operate in an amplification loop for efficient cell death. The authors provide evidence that both caspase 8 and 9 are activated in one single cell. Collectively, the data indicate that GXM-induced apoptosis involves, a cross-talk between pathways within a single cell. Recently, GXM was also shown to induce apoptosis in macrophages through a mechanism that involves an increase in both Fas and FasL in the phagocytic cells (Villena et al., 2008).

GalXM induced apoptosis—GalXM inhibited peripheral blood mononuclear cells and T-cell proliferation, increased IFN- γ and IL-10 production and induced apoptosis of T-lymphocytes by DNA fragmentation through the activation of caspase 8 (Pericolini et al., 2006). GalXM induces death receptors such as Fas and through subsequent activation of caspase-8, it is most likely that the type I apoptotic pathway is involved in this process. These results suggested the possibility of a putative receptor for GalXM on T-cells that may be involved in triggering apoptotic signals. GXM- and GalXM-mediated apoptosis appear to have fundamentally different mechanisms. GXM does not interact directly with T- cells, but rather mediates apoptosis by inducing FasL on antigen-producing cells such as macrophages (Monari et al., 2005b). In contrast, GalXM appears to directly interact with galectins on T-cells to induce apoptosis. However this mechanism needs to be further elucidated (Pericolini et al., 2006). GalXM is also more potent than GXM at the induction of Fas/FasL expression and apoptosis on macrophages (Villena et al., 2008).

Regulation of chemotaxis and cellular migration by capsular components—Leukocyte migration to infected tissues is a complex process that involves chemotaxis of immune cells, binding to endothelial surfaces and movement across the endothelium. The

capsular components can influence chemotaxis (see review in (Ellerbroek et al., 2004d)). The first evidence suggesting that the capsule interfered with cell migration was provided by the finding that capsular components induced PMN migration through the activation of the complement system by the alternative pathway (Diamond and Erickson, 1982; Laxalt and Kozel, 1979). Consistent with this finding, CSF from rabbits with chronic cryptococcal meningitis contained a host-derived factor with chemotactic activity toward PMN and monocytes (Perfect and Durack, 1985). In addition, encapsulated cells induced chemotaxis of human neutrophils through a direct mechanism (Dong and Murphy, 1993). Acapsular strains also exerted this effect, but in an indirect manner. The direct effect was mediated by GXM, while the other components of the capsule (GalXM and MP) played an indirect role through complement pathway modulation. Interestingly, capsular components from *C. gattii* did not have any effect on neutrophil migration (Dong and Murphy, 1995a) which suggests a mechanism by which *C. gattii* evades the interaction with this type of phagocytic cells.

The interplay of these chemotactic activities *in vivo* is very complex. Despite the chemotactic properties of GXM, there is a characteristic inhibition of leukocytes infiltration into infected tissues in cryptococcosis (Baker and Haugen, 1955). Dong and Murphy also showed that elements from the capsule inhibited leukocyte migration (neutrophils, lymphocytes, and monocytes) to infected areas (Dong and Murphy, 1995b). Furthermore, GXM induced shedding of L-selectins from neutrophil membranes as well as reducing TNF receptor expression, but increased CD15 and CD11b (Dong and Murphy, 1996). L-selectins are surface molecules necessary for neutrophil migration into tissues. GalXM and MP did not affect L-selectin shedding by comparison. These findings suggest a mechanism by which GXM may reduce neutrophil migration into infected tissues by preventing neutrophil adhesion to endothelial surfaces. A similar effect was found on T-cells (CD4 and CD8) through a mechanism that required tyrosine kinase (Dong et al., 1999). Some reports have suggested that L-selectin shedding was not the determinant factor that inhibits migration, and the same authors involved E-selectin in the migration process (Ellerbroek et al., 2002). The same authors reported that GXM interfered with rolling and fixed binding of neutrophils on the endothelium using a flow model (not static), and that this interference was mediated through TLR4, CD14 and E-selectin (Ellerbroek et al., 2004c). The effect was dependent on the 6-O-acetylation of mannose residues in GXM (Ellerbroek et al., 2004a). In addition, GXM can bind to CD18, interfering with its role in neutrophil migration (Dong and Murphy, 1997). GXM also interfered with PMN migration in response to IL-8 (Lipovsky et al., 1998a) and in agreement with this finding, it was found that the CSF leukocyte cell count was inversely correlated with the amount of GXM present in the serum (expressed as the GXM proportion in serum related to the amount present in CSF) (Lipovsky et al., 1998b). GXM also inhibited leukocyte migration into CSF in rabbit experimental models of bacterial meningitis (Lipovsky et al., 2000), as well as in some models which induce non-related neutrophil migration, such as rat model of myocardial ischaemia (Ellerbroek et al., 2004b).

Mannoproteins (MPs)—Although it has not been demonstrated that these immunogenic proteins are in fact capsular components, we will briefly review the effect of mannoproteins on the host.

C. neoformans MPs are highly immunogenic (Levitz and Specht, 2006)). Most immunological responses to mannoproteins in *C. neoformans* are pro-inflammatory (Murphy, 1988), and MPs in general elicit stronger effects than GXM or GalXM. The mannoprotein fraction CneF stimulated delayed-type hypersensitivity whereas the fraction MP4 can desensitize neutrophils toward chemoattractant challenges. MPs produce a strong TNF α accumulation (Delfino et al., 1996), dependent on serum-labile components (Chaka et al., 1997b). MPs also regulated the expression of other cytokines, such as IL-12, IL-6, IL-10, IFN γ , and IL-8 in monocytes (Delfino et al., 1997; Pitzurra et al., 2000; Walenkamp et al., 1999). The effect of MPs has also been

tested *in vivo*, and these studies have shown that treatment of mice with MP induced IL-12 and IFN- γ expression, which was associated with a protective response to *C. neoformans* that included enhanced antifungal activity, early inflammatory responses, and clearance of fungal burden from the brain (Pietrella et al., 2001a). *In vivo* impairment of IL-12 induction interfered with the protective effect elicited by MPs (Pietrella et al., 2004). Furthermore, these mannoproteins elicit a protective response, not only against *C. neoformans*, but also against other fungal pathogens such as *Candida albicans* (Pietrella et al., 2002). In addition, MPs induced proliferation of human peripheral blood mononuclear cells (PBMC) (Orendi et al., 1997), a phenomenon not observed with GalXM. This induction resulted in an enhancement of HIV replication in infected PBMC, which suggested a mechanism by which *C. neoformans* enhanced the viral infection. Later, a specific MP of a molecular size of 105 kDa was demonstrated to be involved in monocyte proliferation induction (Pitzurra et al., 1997).

Mannoproteins also promote maturation and activation of dendritic cells (Pietrella et al., 2005). MP-induced expression of mature DC markers and IL-12, and MP-activated DC efficiently activated both CD4 and CD8 T-cells. MPs also exert a cooperative stimulation of DC with CpG-oligodeoxynucleotides, which induces pro-inflammatory cytokines. (Dan et al., 2008b)

Mannosylation is required for MP-mediated T-cell stimulation (Specht et al., 2007), consistent with the finding that MP stimulatory effect are the result of interactions with human mannose binding protein (Chaka et al., 1997a) or the mannose receptor (Dan et al., 2008b; Mansour et al., 2002). More recently, MP uptake was shown to be similar by dendritic cells from wild-type and mannose receptor KO mice, although MPs only induced CD4 lymphoproliferation in cells from wild-type mice, but not in KO mice (Dan et al., 2008a). This finding indicated that the mannose receptor is required for some of the effects mediated by MPs. There are other mechanisms by which DC can bind MPs, although these other mechanisms were not effective in inducing a protective T-cell response.

Several *C. neoformans* mannoproteins have been isolated. Mansour *et al.* identified a specific 98 kDa mannoprotein (MP98) involved in the stimulation of T-cell responses (Levitz et al., 2001). The gene for MP98 was cloned (CDA2), and was found to contain a domain with similarity to chitin deacetylase. The role of chitin deacetylases is to convert chitin to chitosan, the deacetylated form of chitin. Using a collection of putative chitin deacetylase deletion strains *cda1*, *cda2*, *cda3* and a polysaccharide deacetylase *fpd1*, Baker *et al.* characterized the deacetylases and their biological roles (Baker et al., 2007). The study revealed that a single chitin deacetylase does not adversely affect the ability of *C. neoformans* to produce chitosan. Strains with a *cda1* Δ *cda2* Δ mutation had increased capsular diameters. The results imply that chitosan produced by Cda1 and Cda2 is sufficient to retain normal capsule integrity. However, a quadruple mutation *cda1cda2cda3fpd1* abolished chitosan production. Strains lacking the chitosan had incomplete mother daughter cell separation and were sensitive to cell wall inhibitors. Additionally, mutants lacking chitosan were unable to completely retain melanin, another major virulence factor in *C. neoformans* (Baker et al., 2007).

In 2002, the same group identified a new MP involved in T-cell activation. It had an apparent molecular weight of 88 kDa, and was therefore named MP88 (Huang et al., 2002). This protein contained a putative glycosyl phosphatidyl inositol anchor site, and had homology to eleven other *C. neoformans* proteins, including MP98 (Huang et al., 2002).

Recently new immunogenic MPs have been identified, with molecular weights of 250, 125, 115, and 84 kDa. The genes encoding MP84 and MP115 were cloned and had homology with polysaccharide deacetylases and carboxylesterases, respectively (Biondo et al., 2005).

C.3. Origin of the capsule as virulence factor

An intriguing issue in the *C. neoformans* capsule field is how this structure has evolved as a virulence factor in mammals. It is difficult to understand how an environmental yeast that produces infection after inhalation can possess such a specialized structure for virulence in animals while not really requiring an animal host for any aspect of its life cycle.

To properly answer this question, it is important to emphasize that although *C. neoformans* is an environmental organism, it frequently interacts with a large number of non-conventional hosts widely found throughout nature (Casadevall and Perfect, 1998). For example, *C. neoformans* is known to interact with amoebas, such as *Acanthamoeba castellanii* and *A. polyphaga* and the slime mould *Dictyostelium discoideum* (Bunting et al., 1979; Castellani, 1955; Steenbergen et al., 2003). Some amoebas can phagocytose and kill *C. neoformans* while this fungus can survive ingestion by others (Steenbergen et al., 2001). In addition, *C. neoformans* has developed some mechanisms that allow phagocytotic inhibition, intracellular fungal replication, and killing evasion (Steenbergen et al., 2001; Zaragoza et al., 2008b).

C. neoformans intracellular pathogenesis in mammalian cells is associated with GXM accumulation in specific vesicles (Tucker and Casadevall, 2002). Remarkably, the interaction of *C. neoformans* with ameboid protozoal cells results in cytopathic changes in the host cell that are strikingly similar to those observed with mammalian cells. In both amoeba and macrophages, the capsular polysaccharide appears to function as an intracellular aggressin whereby polysaccharide-filled vacuoles from phagosomal membrane blebbing and accumulate in the cytoplasm (Steenbergen et al., 2001). In addition, *C. neoformans* can infect nematodes (Mylonakis et al., 2002), insects (Apidianakis et al., 2004; Mylonakis et al., 2005), toads (Seixas et al., 2008) and goats (Chapman et al., 1990).

In most of these interactions, the capsule plays a critical protective role for *C. neoformans*. Acapsular mutants are more efficiently engulfed by amoebas, and killed to a higher degree than encapsulated fungal cells (Steenbergen et al., 2001). Moreover, capsule enlargement and structural changes induced by starvation can also inhibit *C. neoformans* phagocytosis by amoebas (Zaragoza et al., 2008b). In the case of *Caenorhabditis elegans*, the capsule is also required for virulence and killing of the nematode, since acapsular mutants were cleared by the worm (Mylonakis et al., 2002). A similar situation was found with the caterpillar *Galleria mellonella* (the greater wax moth), a model in which acapsular mutants showed a markedly reduced virulence (Mylonakis et al., 2005). However, the role of the capsule in the virulence in *Drosophila melanogaster* was different. In this model, acapsular mutants showed a slightly reduced virulence in comparison to encapsulated cells, but were still able to kill the insect (Apidianakis et al., 2004), indicating that the capsule is dispensible for virulence during *D. melanogaster* infection.

The fact that the capsule plays a protective role during interaction of *C. neoformans* with environmental factors (such as dehydration (Aksenov et al., 1973)) and in the interaction with environmental predators suggests a mechanism by which the *C. neoformans* capsule has adapted to become a virulence factor in multiple hosts. It is hypothesized that during evolution, *C. neoformans* has adapted the capsule from a protective structure to a feature that confers selective advantage during the interaction with the different hosts that it interacts with. As a consequence, it acquired the ability to produce a large number of deleterious effects on mammalian hosts (Casadevall et al., 2003). This hypothesis provides an explanation for the origin of virulence factors in environmental microbes that are pathogenic for animals, and allows us to propose how virulence is a feature specifically acquired after numerous exposures and interactions with different types of hosts by natural selection (Casadevall and Pirofski, 2007).

Section D Use of capsular components as antifungal targets and vaccine

Given the critical role of the capsule in virulence considerable efforts have been undertaken to develop antibodies and vaccines that target this structure. Two general approaches have been taken. First, many monoclonal antibodies have been developed with the purpose of targeting the capsular components, as a passive immunization. Studies with these mAbs have shown that antibody function against *C. neoformans* is extremely complex in the sense that some immunoglobulins confer protection, while others do not, and still others may even result in enhancement of disease. In a second approach the use of the capsular polysaccharide or capsule-mimetic structures as a protective active vaccine has been proposed. In the following section, we will briefly review the main advances in these two fields.

D.1 Capsule as an antifungal target. Monoclonal antibodies to the capsule as therapeutic alternative

Although the protective role of the humoral response during *C. neoformans* infection has historically been questioned and remains uncertain, there have been two main reasons for developing these mAbs: 1) the capsule is the main virulence factor of this fungal pathogen, so blocking it with mAb theoretically could produce some protective effects; 2) it was observed that although the capsule is poorly immunogenic, there are some epitopes that can elicit protective Abs (Cleare et al., 1994).

The binding interactions between mAbs and the capsule and the interactions between *C. neoformans* and the host have been extensively studied. Although the capsule is dispensable for the life of the yeast, binding of mAbs to this structure produces a large number of effects in *in vitro* and *in vivo* models. The protective effect of antibodies binding to GXM has been extensively tested in murine models (Dromer et al., 1987a; Feldmesser and Casadevall, 1997; Fleuridor et al., 1998; Mukherjee et al., 1995c; Mukherjee et al., 1992), and the ability to change the outcome of disease has been observed in various infection models (Mukherjee et al., 1993; Mukherjee et al., 1994b). Numerous studies have been performed and a specific mAb (18B7) was evaluated in a Phase I/II trial in patients with HIV-associated cryptococcosis as an adjunct to antifungal therapy for cryptococcal meningitis (Larsen et al., 2005). To improve the potential efficacy of antibody therapy in humans, mouse-human chimeric and human Abs have been created. These Abs prolong mouse survival and this effect is dependent on the characteristics of the binding of the mAbs, such as specificity and isotype (Fleuridor et al., 1998; Maitta et al., 2004a; McLean et al., 2002). Moreover, mAbs to GXM enhance antimicrobial effects of some antifungal drugs, such as amphotericin B, fluconazole and flucytosine (Dromer and Charreire, 1991; Feldmesser et al., 1996; Mukherjee et al., 1995b; Mukherjee et al., 1994a).

Passive Ab administration is not always associated with a protective effect. For example, mAbs vary in efficacy depending on the infecting *C. neoformans* strain (Mukherjee et al., 1995d), which suggests that strain differences could be a key determinant in the protective effect of the Abs. Another key factor that determines the effect of mAbs to *C. neoformans* is the variable region of the mAb. Several studies have demonstrated that Ab efficacy depends on the isotype (Mukherjee et al., 1992; Sanford et al., 1990). IgG1 isotype is generally considered protective, whereas IgM is either protective or neutral, and IgG3 is found to be disease-enhancing in some strains of mice. There are numerous explanations for the different effects according to the Ab isotype, including differences in distribution in the body, half-life, opsonic capacity and complement activation ability. However, antibodies expressing the same variable regions can differ in terms of their affinity, specificity, and protective efficacy (Torres et al., 2007; Torres et al., 2005). For some antibodies, differences in the binding to the capsule correlate with different *in vivo* effects (Beenhouwer et al., 2007). There is evidence that the binding pattern of the Abs to the capsule influences the final effect of the Abs. For example, as stated above, IgM Abs,

which bind to the capsule in a punctuate pattern, are not protective, while IgM mAbs which bind in an annular pattern prolong survival in mice (Cleare and Casadevall, 1998; Nussbaum et al., 1997).

In a recent study, computer modeling to predict pore-hindered diffusion and binding of the GXM-specific antibody within the *C. neoformans* capsule was modeled (Rakesh et al., 2008). Using the finite element method (FEM), they created a model which represents the *in vivo* binding of GXM-specific antibody to a *C. neoformans* cell taking into account the intravenous infusion time of antibody, antibody diffusion through capsular pores, and Michaelis-Menten kinetics of antibody binding to capsular GXM (see figure 8). The model predicted rapid diffusion of antibody to all regions of the capsule where pore size was greater than the Stokes diameter of the antibody. Binding occurred primarily at intermediate regions of the capsule. The GXM concentration in each capsular region was the principal determinant of the steady-state antibody-GXM complex concentration, while the forward binding rate constant influenced the rate of complex formation in each region. The concentration profiles predicted by the model closely matched experimental immunofluorescence data (Fig. 8). Inclusion of different antibody isotypes (IgG, IgA and IgM) into the modeling algorithm resulted in similar complex formation in outer capsular regions, but different depth of binding at inner regions. These results have implications for the development of new antibody-based therapies (Rakesh et al., 2008).

Several studies have established that dose is a critical parameter in determining the efficacy of passive antibody administration. A single mAb can behave as protective, not protective or disease-enhancing depending on the Ab dose and / or the number of infecting pathogens (Taborda and Casadevall, 2001; Taborda et al., 2003). At high antibody doses, some mAbs lose protective efficacy. This feature, the so-called prozone effect, is an important issue that must be considered when interpreting passive Ab administration results.

MAbs to the capsule play an important role as opsonins during phagocytosis (see section above), and also in regulating complement activation (Kozel et al., 1998). In addition, mAbs to the capsule can enhance the antifungal activity of macrophages and microglia (Lee et al., 1995a; Mukherjee et al., 1996; Mukherjee et al., 1995e) and also of neutrophils from HIV patients (Monari et al., 1999a). However, the function of mAbs is critically dependent on the host's cell-mediated immunity (Yuan et al., 1997), B-cells (Rivera et al., 2005) and Th1 and Th2 cytokines (Beenhouwer et al., 2001). These studies indicate that mAbs to the capsule exert their *in vitro* action by modulating the inflammatory response, rather than through a direct antifungal effect. The mechanism by which mAbs binding to the capsule exert their action is therefore very complex, and it seems that binding to the epitope is not sufficient to achieve protection. mAbs to the capsule can regulate the inflammatory response elicited during infection, possibly by altering the release of mediators of inflammation such as cytokines (Feldmesser et al., 1998; Feldmesser et al., 2002). Engagement of Fc receptors appears to be a requirement for efficacy, since mAbs are not protective in FcRI^{-/-} or FcRIII^{-/-} KO mice (Yuan et al., 1998). Interestingly, mAbs can be protective in some cases of immunodeficiency, such as the absence of complement component 3 (Shapiro et al., 2002). In addition, the mouse genetic background is an important determinant for the effect of the Abs, which confirms that host elements are important to determine whether a mAb binding to the capsule will have a protective effect or not (Rivera and Casadevall, 2005).

In addition to passive mAb administration, new strategies based on enhancing the activity of mAbs to the capsule have been developed to improve their efficacy. For example, radiolabeling of these mAbs converted an antibody into a microbicidal molecule that causes reduction in capsule size, changes in cellular metabolism and an apoptosis-like death in fungal cells (Dadachova et al., 2006). This effect is highly dependent on the antibody isotype and the

affinity constant of the mAb binding to the polysaccharide capsule (Dadachova et al., 2007). The same dependence of the fungicidal ability of radiolabeled antibodies on the isotype was observed for *C. neoformans* biofilms which are characterized by massive deposits of exopolysaccharide (Martinez et al., 2006). Administration of radiolabeled GXM-specific mAb 18B7 prolonged survival of AJ/Cr mice lethally infected with *C. neoformans* (Dadachova et al., 2003). The success of these studies has led to the emergence of the whole new field of radioimmunotherapy (RIT) of infectious diseases which has been subsequently expanded to treatment of bacterial and viral infections (reviewed (Dadachova and Casadevall, 2008)).

D.2 Use of capsular components as vaccine

GXM synthesis is different from that of other capsular polysaccharides in that it is a heteropolymer that, in theory, could display an almost infinite number of structural combinations, which could translate into antigenic changes and enhance its structural complexity (McFadden et al., 2007). Fortunately, mAbs that recognize all strains have been identified, suggesting that certain immunodominant epitopes may be conserved across strains. The rationale for GXM-based conjugate vaccines is that they elicit antibodies that are opsonic and bind soluble polysaccharide, promoting its clearance.

The first immunization studies with GXM were done by injecting the polysaccharide with an ion exchange resin or in an emulsion with Freund's adjuvant. This strategy, however, only elicited a weak antibody response mostly of the IgM isotype (Cauley and Murphy, 1979; Gadebusch, 1958a; Kozel et al., 1977). In the late 1960's Goren and Middlebrook generated the first GXM (or cryptococcal polysaccharide)-based glycoconjugate vaccine by coupling unfractionated capsular polysaccharide (which most likely contained all three capsular components) to a bovine gamma globulin by nitrocarbanilation and diazotization (Goren and Middlebrook, 1967). This vaccine was highly immunogenic but did not elicit a protective antibody response. In the 1990's, a second glycoconjugate vaccine was developed based on fractionating the GXM component and linking it to tetanus-toxoid (TT) (Devi et al., 1991). Boosting immunization yielded Ab's of the IgG isotype. mAb 18B7 was generated using the GXM-TT conjugate.

An alternative approach in using *C. neoformans* as a vaccine antigen that is conceptually related to glycoconjugate vaccines in terms of the type of immunity that is elicited, is the development of a peptide that mimics a GXM epitope. A mimetic is a peptide epitope that inhibits the binding of an antibody to its native antigen (Pirofski, 2001). The peptide can elicit a protective antibody response by stimulating the production of protective antibodies that bind to polysaccharide (Pirofski, 2001). The approach used to develop the peptide mimetope vaccine employed a decapeptide phage display library in which peptide epitopes were expressed on the surface protein of the M13 bacteriophage which were then screened by using human IgM monoclonal antibody to *C. neoformans* GXM, 2E9. This mAb was produced from immortalized lymphocytes from a volunteer who received the GXM-TT vaccine (Valadon et al., 1996). A group of these phage peptide epitopes were isolated and were considered GXM mimetics.

The peptide mimetic P13 was conjugated to TT or BSA and used to vaccinate mice. The vaccine elicited both IgM and IgG antibodies to both GXM and P13, suggesting that P13 is a GXM mimetope. The P13-TT conjugate induced protection in mice that is probably antibody mediated. Despite promising results, mimetope mediated protection and vaccine efficacy is not fully understood.

There have been several attempts to synthesize oligosaccharides that reproduce the structural repeat of GXM (Garegg et al., 1996; Oscarson et al., 2005; Vesely et al., 2008), including a heptasaccharide representing the putative immunodominant motif of serotype A GXM (Oscarson et al., 2005). The heptasaccharide consists of two M2 repeats (see Figure 2 for

structural interpretation) and one mannose residue. The M2 repeat was selected because it is commonly found in GXM of serotype A strains, although this repeat is also found in other serotypes. The synthetic heptasaccharide was strongly recognized by two different IgMs (Oscarson et al., 2005). Conjugation of the heptasaccharide to human serum albumin resulted in an immunogenic compound that elicited high-titer IgG responses in mice when given with complete Freund's adjuvant. The antibody response elicited by the oligosaccharide conjugate vaccine had characteristics of a T-cell-dependent response (Oscarson et al., 2005). The immunogenic oligosaccharide with the structural motif of GXM therefore represents a potential synthetic vaccine against this fungal pathogen.

Future perspectives

After 50 years of intensive study that has included serological, biochemical, immunological, microscopic, genetic and physical investigations, much is known about the *C. neoformans* capsule. The capsule is widely acknowledged to be an indispensable virulence factor and continues to occupy much of the investigative attentions of the cryptococcal field. However, despite hundreds of publications detailing capsule-related studies, the capsule remains an enigmatic structure that continues to defy complete understanding. At this time there is probably a reasonably good understanding of the various mechanisms by which the capsule contributes to virulence. While the chemical composition of capsular polysaccharide has been well described, we still do not know the answer to some basic questions concerning capsular biochemistry such as whether some fibrils include branches. There is no current evidence for branched polysaccharides but the biochemical methods do not rule out occasional branched molecules. The areas in which the least is known include the architecture, organization, and synthesis of the capsule. No comprehensive model has yet been proposed for molecular mechanisms responsible for capsular enlargement.

The major problem with regards to studying the architecture of the capsule is a dearth of techniques that yield structural information for a highly hydrated, fragile, and variable polysaccharide fibrillar mesh. Therefore much of what we know about the capsule has been inferred from studies of soluble polysaccharide. A better understanding of how the capsule is assembled may require the development of new approaches that combine approaches from various disciplines.

On the immunological front several important open questions remain. The role of the capsule during intracellular pathogenesis provides the paradoxical finding that a structure that inhibits phagocytosis also functions to undermine the host cell after being ingested. The origin of polysaccharide-containing vesicles that fill the cytoplasm of infected macrophages has not been solved. Similarly, the mechanism by which the capsule contributes to the phenomenon of cryptococcal exocytosis is unknown. Capsular polysaccharides are powerful immunomodulators but their cellular receptors have not been fully characterized.

C. neoformans has the largest, most distinctive and best characterized capsule in the field of medical microbiology and studies of capsular structure, synthesis, and function will continue to occupy the field for many years to come.

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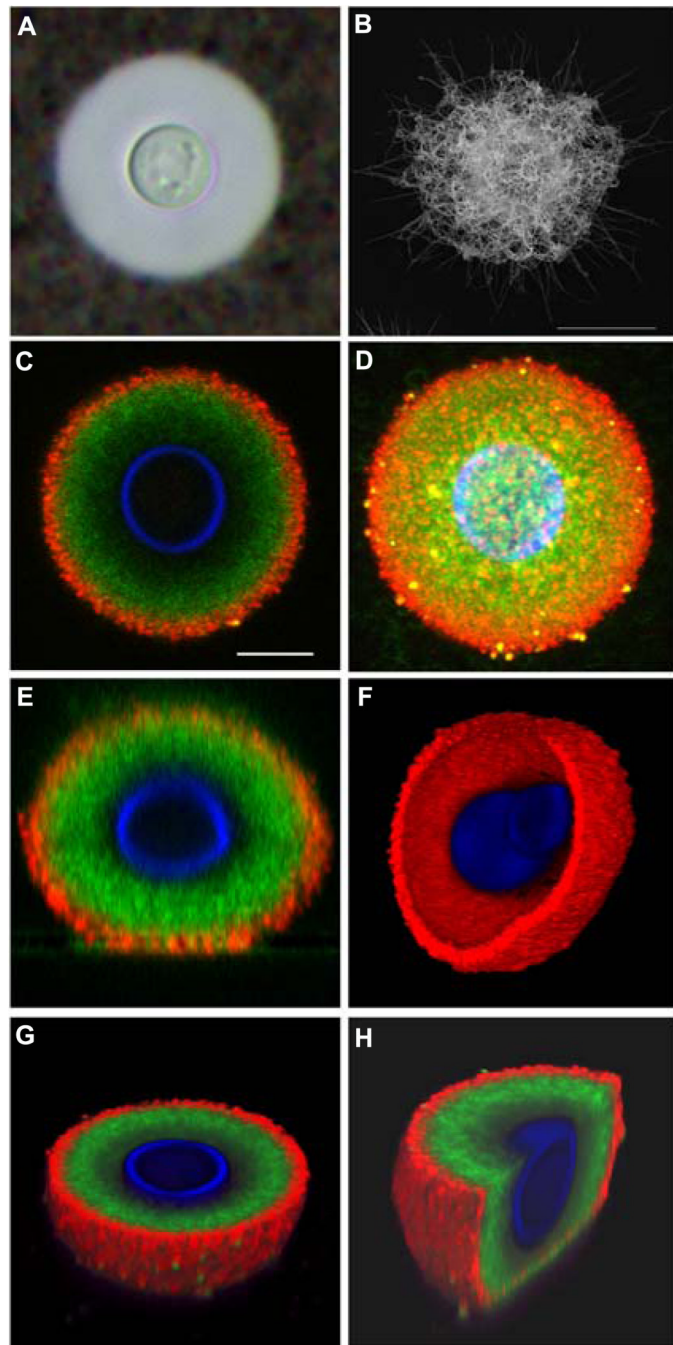


Figure 1. Different micrographs and compositions showing the polysaccharide capsule of *C. neoformans*. A) Suspension of the cells in India Ink; B) Scanning electron microscopy; C-H) Immunofluorescence using specific mAbs to the capsule (green and red fluorescence) showing also the cell wall localization (blue fluorescence). D) 3D image composition of a *C. neoformans* cell labeled with two different mAbs to the capsule. In blue, the cell wall. E) Side view of a section of cell shown in D. F-H) Sections showing the 3 dimension of the capsule, visualized after staining with mAbs (green and red). Pictures by Oscar Zaragoza, and from (Maxson et al, 2007b).

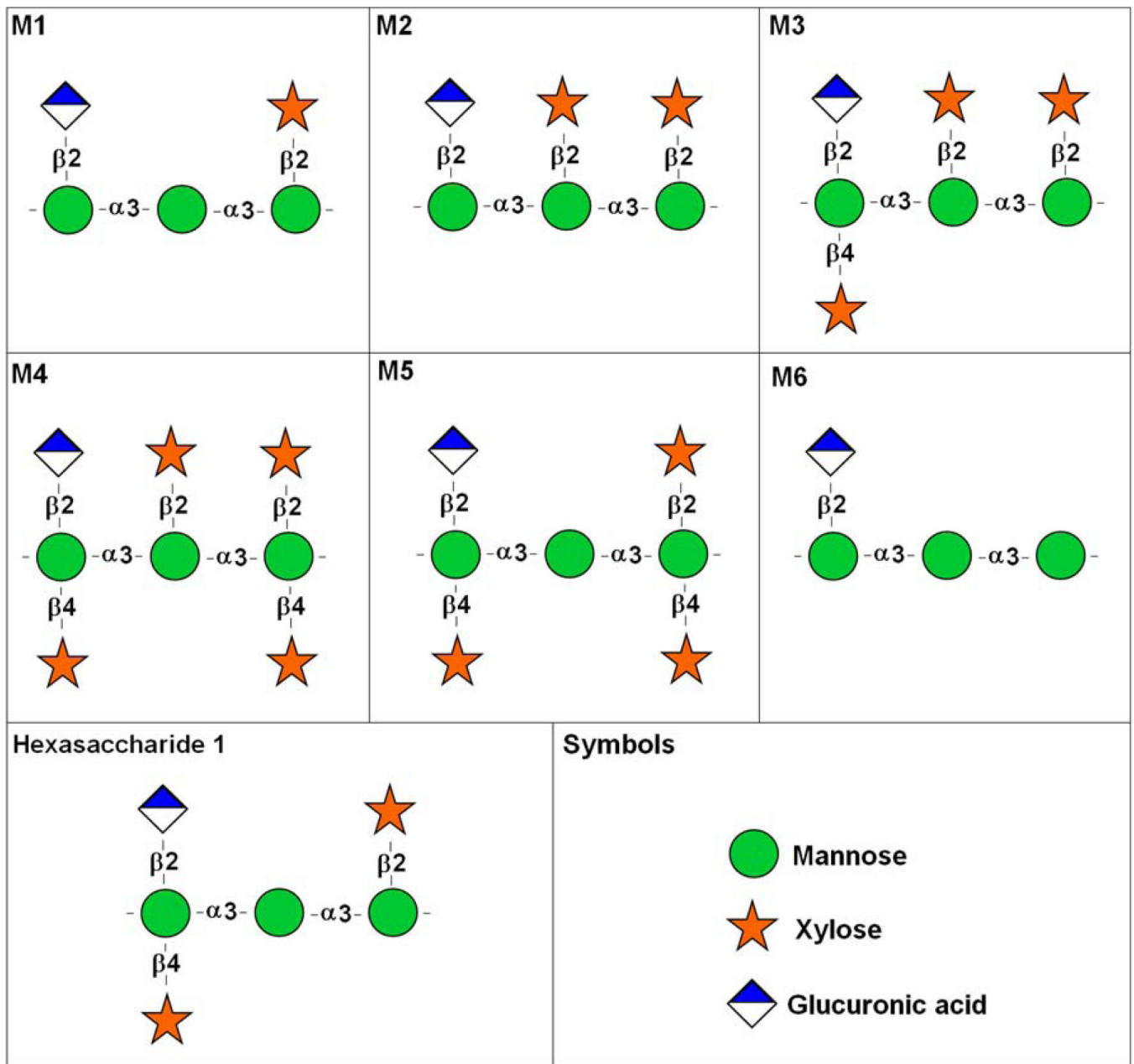


Figure 2. Repeating polysaccharide motifs of GXM

Motifs M1 to M6, which were described by Cherniak and colleagues (1998) as the major structure reporter groups (SRG) of GXM, are shown. Hexasaccharide 1, an additional GXM substituted triad, is also presented. Symbol nomenclature for glycans followed the format available in <http://grtc.ucsd.edu/symbol.html>.

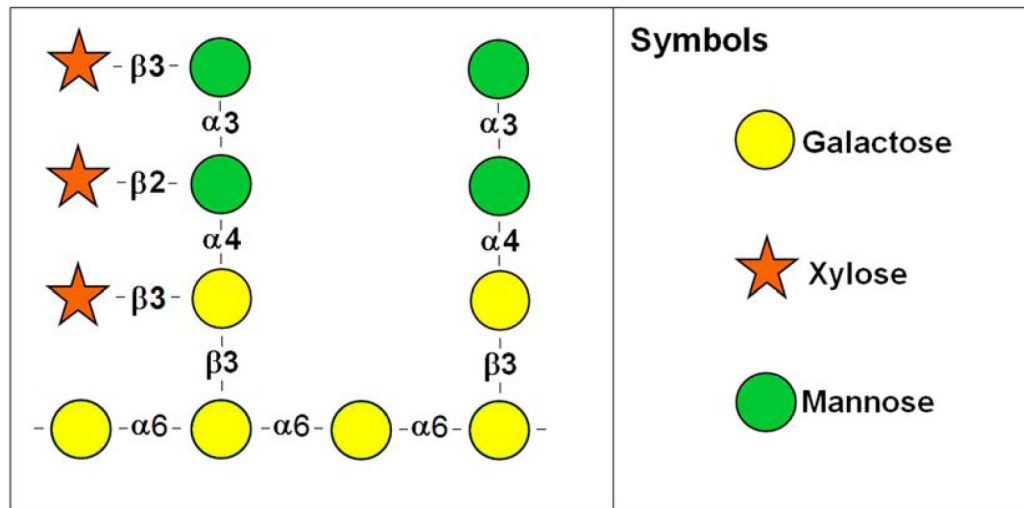


Figure 3. Basic GalXM structure

A GalXM motif has a $\alpha(1\rightarrow6)$ galactan backbone. The branches are $\beta(1\rightarrow3)$ -O-linked to the backbone and consist of an $\alpha(1\rightarrow3)$ -Man, $\alpha(1\rightarrow4)$ -Man, β -galactosidase trisaccharide with variable amounts of $\beta(1\rightarrow2)$ - or $\beta(1\rightarrow3)$ -xylose side groups. Symbol nomenclature for glycans followed the format available in <http://grtc.ucsd.edu/symbol.html>.

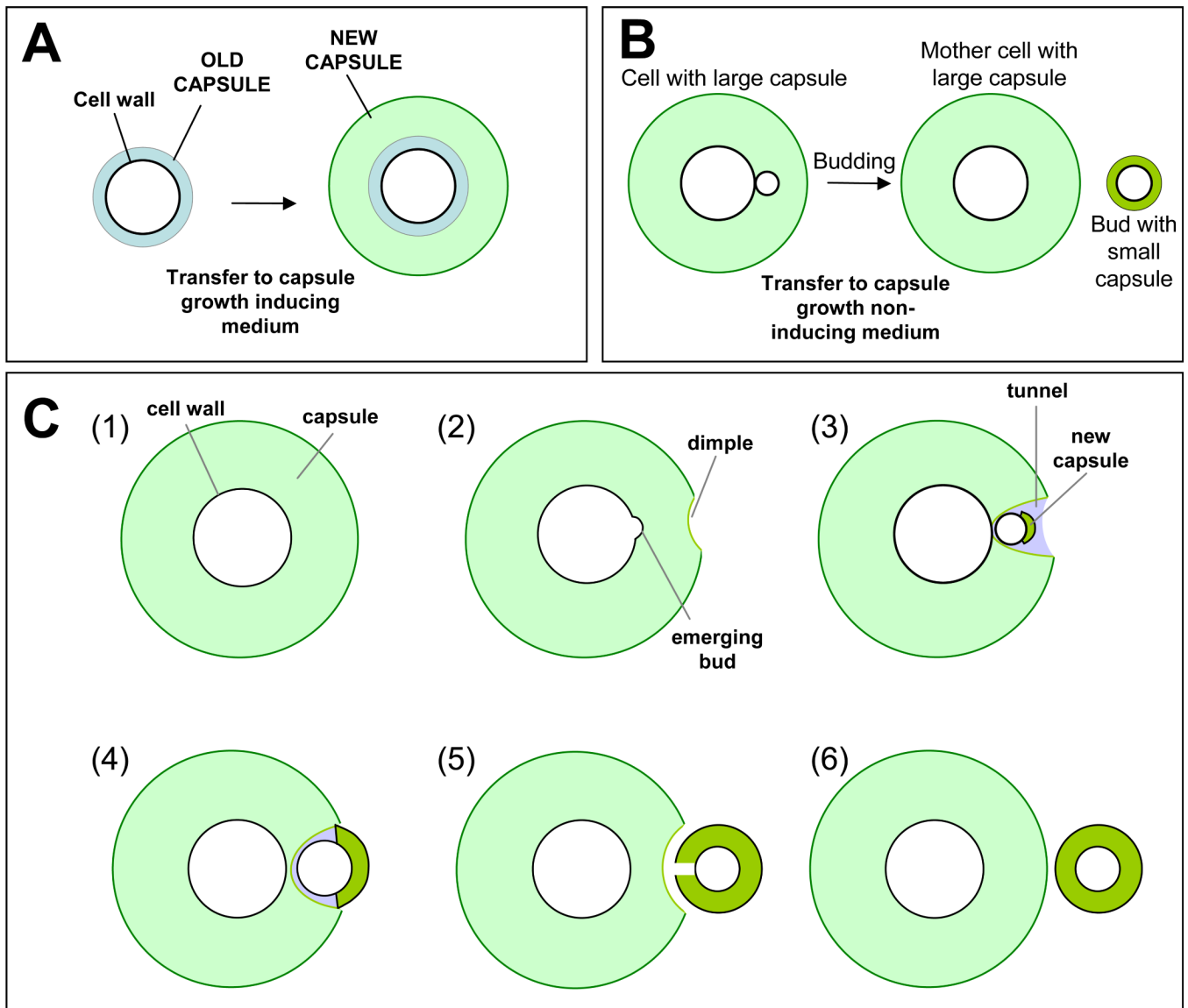


Figure 4. Model of capsule growth and capsule rearrangements during budding in *C. neoformans* (From Zaragoza et al, 2006b). A. Model of capsule growth. After capsule enlarges a newly synthesized capsule (light green) accumulates at the edge of the capsule, with the old capsule (light blue) remaining close to the cell wall. B. Model of adaptation to non-capsule growth inducing conditions. Cells with large capsules, when transferred to a medium that does not induce capsule growth, cannot degrade the capsule, but the new emerging buds have a small capsule. C. Rearrangements of the capsule during budding. Panels 1–6 illustrate schematically different stages of budding. When bud arises, a dimple and a tunnel are formed (2, 3), which allows the separation of the bud. Bud growth is accompanied by capsule growth in the bud (3, 4). Capsule of the mother cell closes as the bud separates from the mother cell, at the same time that the bud completes the capsule without taking any polysaccharide of the mother cell (5), which will allow for the complete separation of the bud (6).

☼ Capsular polysaccharide

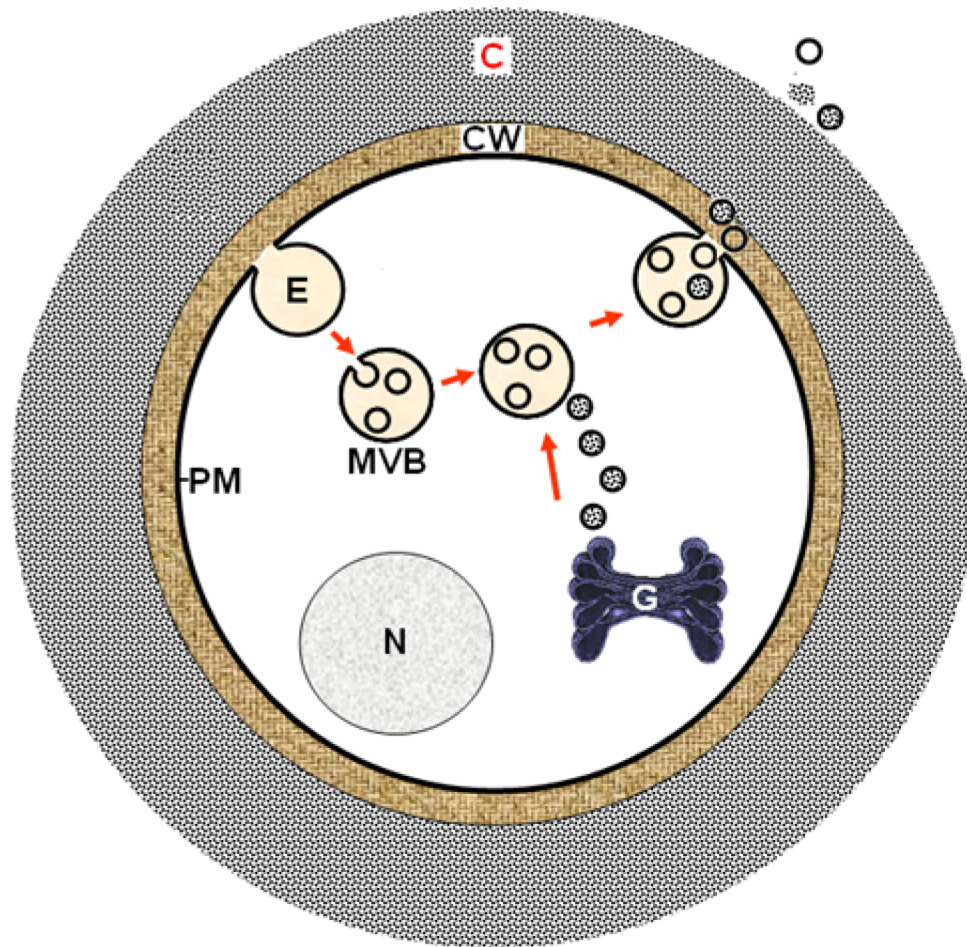


Figure 5. Proposed model for GXM traffic in *C. neoformans*

GXM is synthesized in the Golgi apparatus and released in post-Golgi vesicles. These vesicles would be integrated into endocytic compartments / multivesicular bodies that are further addressed to the plasma membrane. After fusion of endosome-derived structures with the plasma membrane, small vesicles are released into the periplasmic space to be translocated to the extracellular space. Extracellular vesicles would then release their content, which includes capsular polysaccharides. Abbreviations: N, nucleus; G, Golgi apparatus; PM, plasma membrane; E, endosome; MVB, multivesicular body; CW, cell wall; C, capsule.

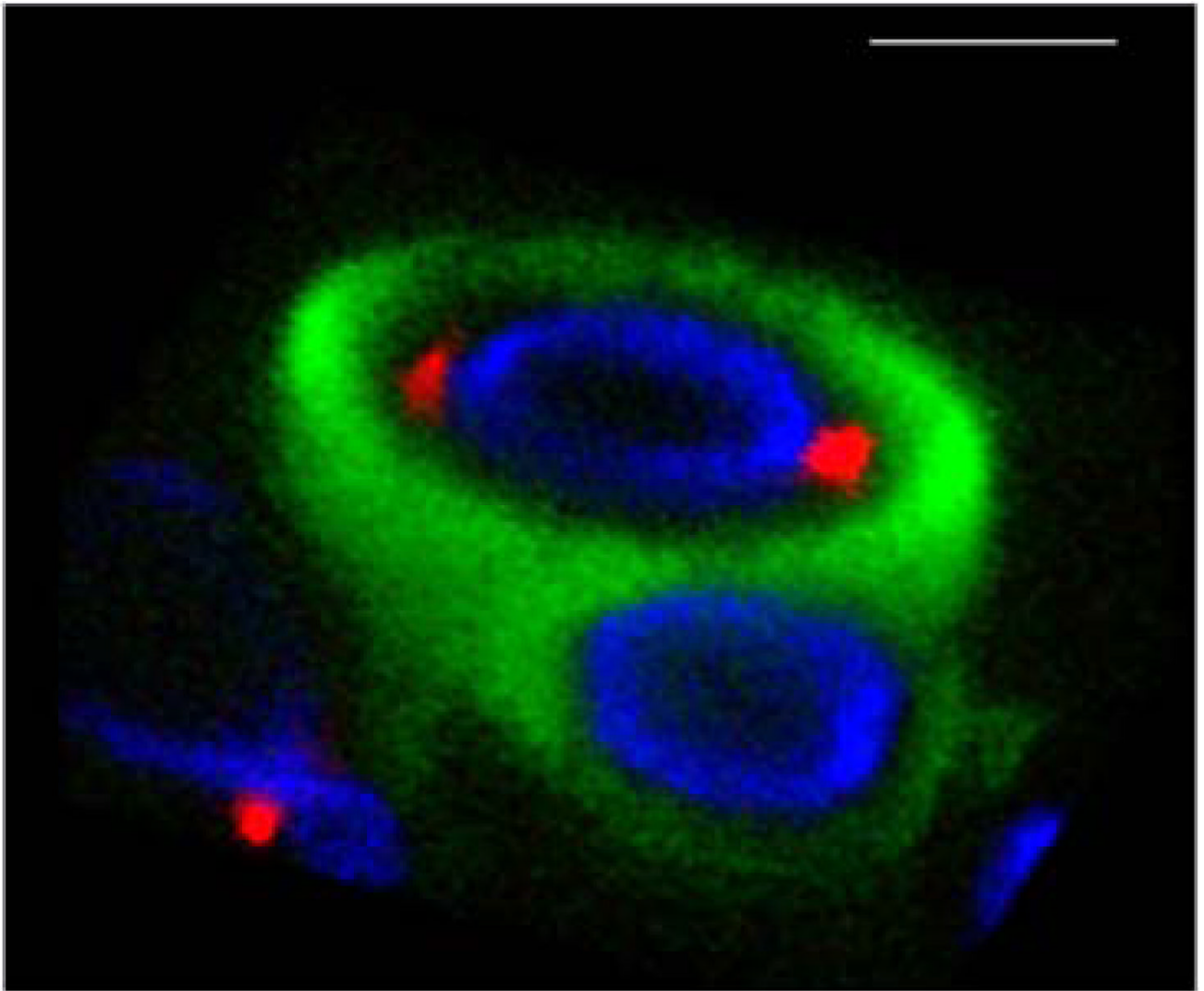


Figure 6. Cell wall-capsule connections in *C. neoformans*

Staining of capsular structures with a monoclonal antibody to GXM (green), cell wall chitin with calcofluor white (blue), and chitin-derived oligomers with WGA (red) followed by confocal analysis reveals that projected structures recognized by WGA connect the cell wall and the capsule. For experimental details, see (Rodrigues et al., 2008a).

Capsular Polysaccharide Antigens Activate Cell Death.

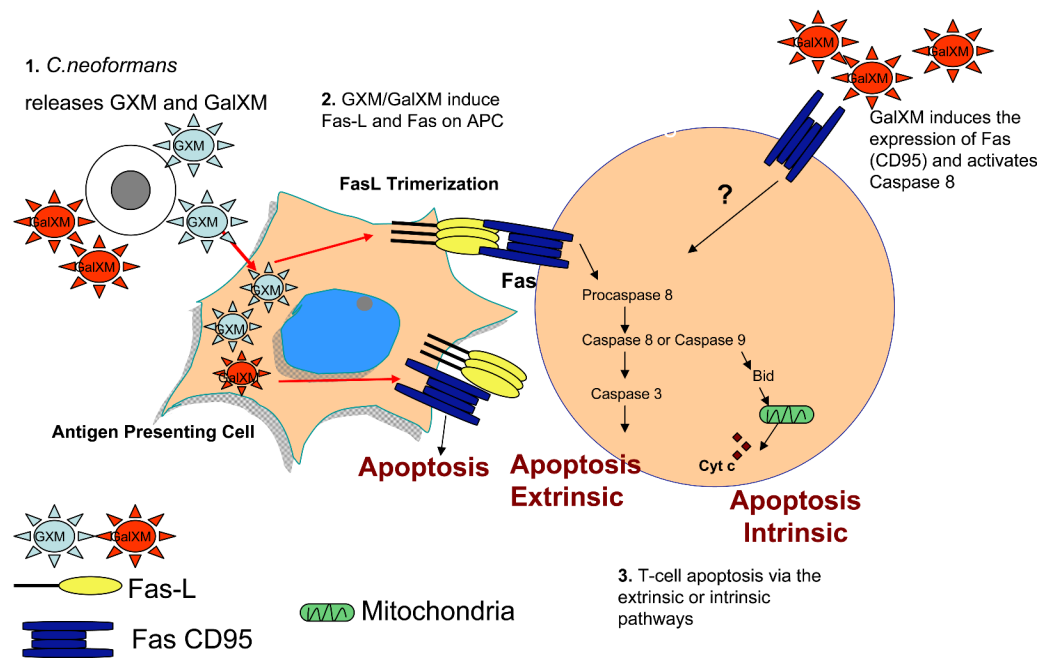


Figure 7. Scheme summarizing all the levels at which capsular polysaccharides induce apoptosis on antigen presenting cells and T-cells. GXM and GalXM induce apoptosis on antigen presenting cells, such as macrophages, and T-cells. In macrophages, GXM and GalXM induce accumulation of molecules involved in apoptosis induction, such as FasL and Fas. These molecules induce apoptosis in both macrophages and T-cells. In addition, in T-cells, GalXM can directly induce apoptosis through Fas molecules. Apoptosis occurs through caspase pathway.

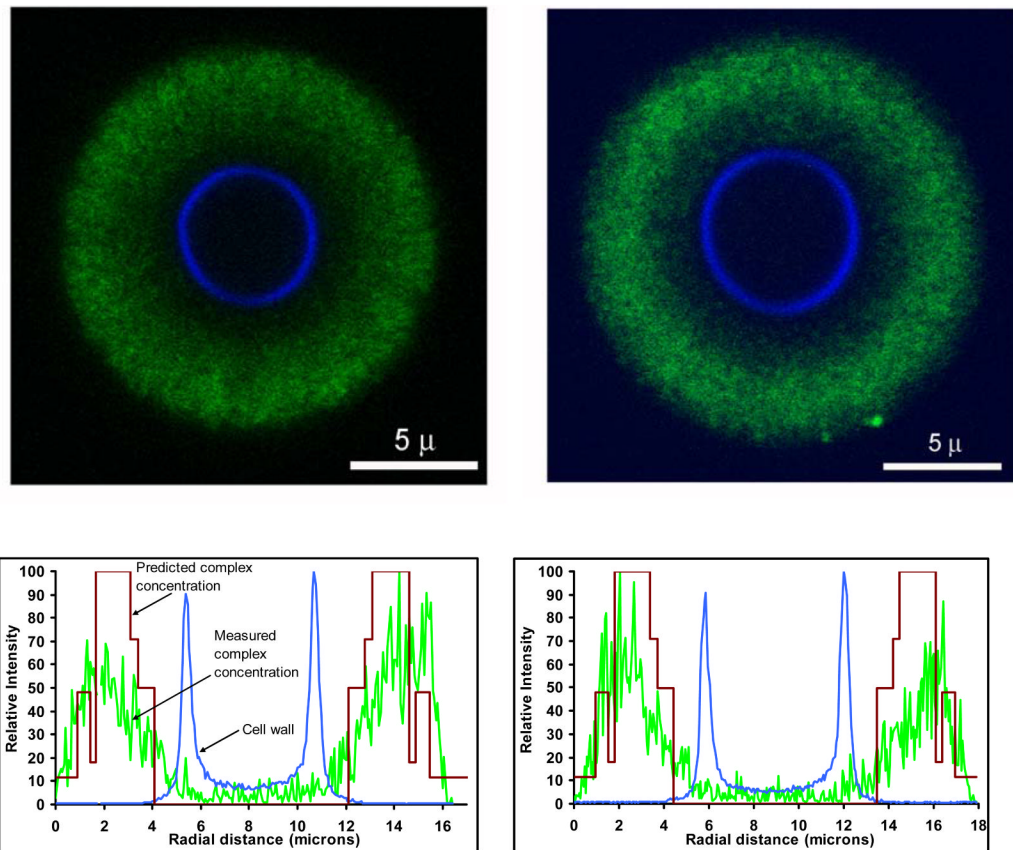


Figure 8. Comparison of model prediction with experimental results obtained for *C. neoformans* cells using immunofluorescence. Green shows the concentration of complex. The boundaries of the cell wall are shown in blue, detected using calcofluor. Shown are data for two cell sizes (adapted from Rakesh et.al, 2008).

Table 1Genes involved in capsule synthesis in *C. neoformans*.

Gene	Putative biochemical functions	Reference
<i>CAP59</i>	Similar to <i>CMT1</i> , a gene encoding for mannosyltransferase; regulation of polysaccharide secretion	(Chang and Kwon-Chung, 1994; Garcia-Rivera et al., 2004; Sommer et al., 2003)
<i>CAP64</i>	Displays similarity to <i>PRE1</i> , a gene encoding the yeast proteasome subunit	(Chang et al., 1996)
<i>CAP60</i>	Displays similarity to similarity to <i>CEL1</i> , a cellulose growth-specific gene of <i>A. bisporus</i> ,	(Chang and Kwon-Chung, 1998)
<i>CAP10</i>	Similar to <i>CXT1</i> , a gene encoding xylosyltransferase	(Chang and Kwon-Chung, 1999; Klutts et al., 2007)
<i>CIR1</i>	Iron sensing	(Lian et al., 2005)
<i>GPA1</i>	Protein G alpha subunit 1, signal transduction pathways	(Alspaugh et al., 1997)
<i>GPR4</i>	G protein-coupled receptor, signal transduction pathways	(Xue et al., 2006)
<i>PKA1</i>	cAMP-dependent protein kinase catalytic subunit, signal transduction pathways	(D'Souza et al., 2001)
<i>PKR1</i>	Protein kinase regulatory subunit, signal transduction pathways	(D'Souza et al., 2001)
<i>PDE1</i>	Low-affinity phosphodiesterases; regulation of cAMP levels	(Hicks et al., 2005)
<i>CAC1</i>	Adenylyl cyclase, signal transduction pathways	(Alspaugh et al., 2002)
<i>ACA1</i>	Adenylyl cyclase-associated protein 1, signal transduction pathways	(Bahn et al., 2004)
<i>CAN2</i>	Carbonic anhydrase 2, CO ₂ sensing	(Mogensen et al., 2006)
<i>NRG1</i>	Carbohydrate-regulated transcription factor	(Cramer et al., 2006)
<i>MAN1</i>	GDP-mannose biosynthesis	(Wills et al., 2001)
<i>GMT1</i>	Mannose transporter	(Cottrell et al., 2007)
<i>UGD1</i>	UDP-glucuronic acid biosynthesis	(Bar-Peled et al., 2004; Griffith et al., 2004; Jacobson, 1987; Moyrand and Janbon, 2004)
<i>UXS1</i>	UDP-xylose biosynthesis	(Bar-Peled et al., 2001; Moyrand et al., 2004)
<i>CMT1</i>	Mannosyltransferase	(Sommer et al., 2003)
<i>CAS1</i>	GXM O-acetylation	(Janbon et al., 2001)
<i>CAS3</i>	GXM O-acetylation	(Moyrand et al., 2004)
<i>CAS31</i>	GXM O-acetylation	(Moyrand et al., 2004)
<i>CXT1</i>	Xylosyltransferase	(Klutts and Doering, 2008; Klutts et al., 2007)