

# Chitin-Like Molecules Associate with *Cryptococcus neoformans* Glucuronoxylomannan To Form a Glycan Complex with Previously Unknown Properties

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In prior studies, we demonstrated that glucuronoxylomannan (GXM), the major capsular polysaccharide of the fungal pathogen *Cryptococcus neoformans*, interacts with chitin oligomers at the cell wall-capsule interface. The structural determinants regulating these carbohydrate-carbohydrate interactions, as well as the functions of these structures, have remained unknown. In this study, we demonstrate that glycan complexes composed of chito oligomers and GXM are formed during fungal growth and macrophage infection by *C. neoformans*. To investigate the required determinants for the assembly of chitin-GXM complexes, we developed a quantitative scanning electron microscopy-based method using different polysaccharide samples as inhibitors of the interaction of chitin with GXM. This assay revealed that chitin-GXM association involves noncovalent bonds and large GXM fibers and depends on the *N*-acetyl amino group of chitin. Carboxyl and *O*-acetyl groups of GXM are not required for polysaccharide-polysaccharide interactions. Glycan complex structures composed of cryptococcal GXM and chitin-derived oligomers were tested for their ability to induce pulmonary cytokines in mice. They were significantly more efficient than either GXM or chitin oligomers alone in inducing the production of lung interleukin 10 (IL-10), IL-17, and tumor necrosis factor alpha (TNF- $\alpha$ ). These results indicate that association of chitin-derived structures with GXM through their *N*-acetyl amino groups generates glycan complexes with previously unknown properties.

**C**ryptococcosis is a life-threatening disease caused by the yeast-like pathogens *Cryptococcus neoformans* and *C. gattii*. This systemic mycosis is rampant in Africa, where mortality indices of cryptococcosis exceed those of tuberculosis (37). Currently available anticryptococcal therapies are unsatisfactory such that high mortality and morbidity, drug resistance, and recurrence of treated infection are relatively common (4).

Cryptococcal cells are coated by a polysaccharide capsule that contributes to virulence through multiple mechanisms (46). Although it is accepted that capsule formation and its assembly are necessary for pathogenesis (38), many structural and functional aspects of capsular components remain unknown (42). The cryptococcal capsule contains the polysaccharides glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (previously called galactoxylomannan) (46). GXM is the major capsular component, accounting for approximately 85% of the capsular mass. This polysaccharide is the main virulence factor of *C. neoformans* (31), and its neutralization by passively administered monoclonal antibodies is associated with disease control (6). GXM is also a prototype of vaccine development (8).

Capsule assembly in *C. neoformans* is a complex event, and the mechanisms by which polysaccharide components are organized to form this structure are only now beginning to be elucidated (24). Carbohydrate-carbohydrate interactions are presumably required for capsule organization and, in fact, GXM interacts with other polysaccharide molecules at the cell surface (46). Self-aggregation (GXM-GXM interactions) is required for capsule enlargement (24, 35), but the polysaccharide is also likely to interact with

cell wall glucans, which are essential for capsule anchoring and cell wall architecture (14, 25, 39, 40). Microscopic examination revealed that GXM is also associated with GXMGal at the capsular network (17). Therefore, assuming that GXM is linked to other components of the capsule, glycan complexes are presumably formed, but the function of these complexes is still unknown.

Our recent studies demonstrated that GXM interacts with chitin-like structures within the capsular matrix by combining light scattering analysis, fluorescence microscopy, and chromatographic methodology (21, 41). Chitin and oligomeric subunits (chito oligomers) consist of  $\beta$ -1,4-linked units of *N*-acetylglucosamine (GlcNAc). In *C. neoformans*, chitin is synthesized through the activities of eight different chitin synthases, and the de-*N*-acetylated form, called chitosan, is also found (1, 2). Inhibition of chitin-related molecule synthesis through the use of a synthetic inhibitor resulted in the formation of faulty capsules (21), suggesting that chitin-GXM association contributes to correct capsule assembly. In fact, complexes formed by chitin-derived oligomers and GXM are formed in association with the capsule or in soluble

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**TABLE 1** Analytical models for analysis of the interaction of GXM with chitin

Structural aspect assessed	Approach used
Molecular specificity of <i>C. neoformans</i> -chitin interaction	Pretreatment of the chitin particles with WGA or with soluble GXM; pretreatment of yeasts with anti-GXM
Involvement of noncovalent bonds	Treatment of chitin- <i>C. neoformans</i> complexes with urea, NaCl, and EDTA
Requirement of <i>O</i> -acetyl groups of GXM	Pretreatment of the chitin particles with de- <i>O</i> -acetylated GXM and comparison with results obtained with samples of native GXM; treatment of yeast cells with de- <i>O</i> -acetylating agent, followed by incubation with chitin particles
Requirement of carboxyl groups of GXM	Pretreatment of the chitin particles with carboxyl-reduced GXM, followed by comparison with results obtained with samples of native GXM
Influence of GXM molecular mass	Treatment of chitin- <i>C. neoformans</i> complexes with EDTA; pretreatment of chitin particles with fractions containing GXM with different molecular masses
Requirement of chitin amino groups	Replacement of chitin by cellulose in SEM tests
Requirement of <i>N</i> -acetyl groups from chitin	Pretreatment of yeast cells with chitosan

form during regular growth of *C. neoformans* (21). Thus, assuming that the association of chitin-like molecules with GXM is in fact physiological, a glycan complex is putatively formed during the regular metabolism of *C. neoformans*. However, despite the potential relevance of these structures for capsule assembly and pathogenesis (21), it is still unknown whether they affect the interaction of *C. neoformans* with the host. The structural determinants regulating the interaction of chitin with GXM were also unknown and have been investigated in the present work.

In this study, we investigated whether glycan complexes formed by GXM and chitin-like molecules would be formed during macrophage infection by *C. neoformans* and if they would differ in function from each polysaccharide/oligosaccharide component tested separately. We detected glycan complexes during infection of the phagocytes by encapsulated fungi and observed that these complexes were significantly more efficient than either GXM or oligomeric chitin structures alone to induce the production of lung cytokines in mice. Formation of glycan complexes depended on the mass of GXM fibers, noncovalent bonds, and the *N*-acetyl groups of chitin. These observations suggest the occurrence of cryptococcal hybrid molecules with previously unknown functions and with the potential to influence the pathogenesis of *C. neoformans*.

## MATERIALS AND METHODS

**Microorganism and growth conditions.** The *C. neoformans* strain used in most experiments described in this study was the standard serotype A isolate H99. The only exception was the assay that sought to detect glycan complexes after fungal growth or macrophage infection, where the heavily encapsulated ATCC 24067 isolate (serotype D) and the acapsular mutant strain Cap67 were also included. Yeast cells were inoculated into 100-ml Erlenmeyer flasks containing 50 ml of minimal medium composed of 15 mM glucose, 10 mM MgSO<sub>4</sub>, 29.4 mM KH<sub>2</sub>PO<sub>4</sub>, 13 mM glycine, and 3 μM thiamine-HCl (pH 5.5). Fungal cells were cultivated for 2 days at 30°C, with shaking. Yeast cells were obtained by centrifugation, washed in phosphate-buffered saline (PBS), and counted in a Neubauer chamber. All media were prepared with apyrogenic water, and glassware was rendered sterile and free of pyrogen by heating at 190°C for 4 h.

**GXM fractionation and chemical modifications.** GXM was isolated as described previously (35) by sequential filtration of fungal supernatants in Amicon ultrafiltration cells (cutoffs, 1, 10, 100, and 300 kDa; Millipore, Danvers, MA). After concentration of the supernatant, the viscous GXM-containing film layer was collected with a cell scraper and was transferred to plastic tubes. GXM was chemically modified using standard techniques. Carboxyl-reduced GXM was prepared as described by Taylor and Conrad (43), with conversion of approximately 60% of the glucuronyl

residues into glucose (not shown). Removal of *O*-acetyl groups was performed by dissolving 5 mg of GXM in 1 ml of H<sub>2</sub>O adjusted to pH 11.25 with NH<sub>4</sub>OH. The resulting solution was stirred for 24 h at 23°C and dialyzed against water. For polysaccharide quantification, capture enzyme-linked immunosorbent assay (ELISA) (7), carbazole reaction for hexuronic acid (18), and the method of Dubois et al. for hexose detection (19) were used. In all experiments, lipopolysaccharide (LPS)-free water and plastic material were used.

### Quantitative assay of polysaccharide-polysaccharide interactions.

The interaction of chitin with GXM was determined based on the following: (i) the cells of *C. neoformans* contain abundant amounts of surface GXM (46) and (ii) particles of commercial chitin (isolated from shrimp; Sigma-Aldrich) are insoluble in water and therefore can be handled and separated by regular techniques for cell fractionation and visualization. Thus, considering the previously described affinity of chitin for GXM (21), we used chitin particles for interaction with GXM fibers on the surface of *C. neoformans*. In this case, the complex formed by chitin and *C. neoformans* could be visualized and quantified by scanning electron microscopy (SEM). This method was validated in initial tests developed in our laboratory, and optimized protocols are described. The specificity of GXM binding to chitin and the structural determinants involved in this interaction were assessed by inhibition of complex formation as summarized in Table 1.

*C. neoformans* cells (10<sup>7</sup> cells) were washed three times with PBS by centrifugation and incubated in the presence of 2 mg of chitin for 12 h at room temperature with stirring in minimal medium. Suspensions containing yeast cells or chitin alone were prepared following the same protocol. In some experiments, the insoluble particles of chitin were replaced by cellulose, a polymer composed of repeating units of β-1,4-linked glucose. Chitin-*C. neoformans* complexes were washed extensively in PBS by centrifugation and fixed in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde for 1 h. The complexes were then washed in a buffer containing 0.1 M sodium cacodylate, 0.2 M sucrose, and 2 mM MgCl<sub>2</sub>. The samples were fixed on coverslips coated with poly-L-lysine for 20 min. Preparations were then serially subjected to dehydration in alcohol (30%, 50%, and 70% for 5 min and 95% and 100% for 10 min), critical point drying, and metallization. The complexes were observed in a scanning electron microscope (JEOL JSM-5310). Quantification of the formation of chitin-*C. neoformans* complexes by SEM was achieved by counting the number of yeast cells adhered per polysaccharide particle per microscopic field (minimum of 10 fields). In this analysis, only chitin particles within the range of 40 to 60 μm were considered.

The structural determinants involved in the interaction of cryptococcal GXM with chitin were examined by the inclusion of several blocking or competing agents in the experimental system described above. Specificity was first evaluated by the incubation of chitin-free particles with wheat germ agglutinin (WGA) at 100 μg/ml for 1 h at room temperature before adding *C. neoformans* cells. WGA recognizes oligomeric sequences of

$\beta$ -1,4-*N*-acetylglucosamine. Alternatively, chitin particles were incubated with purified GXM samples using either the native polysaccharide or samples that were de-*O*-acetylated, carboxyl reduced, or fractionated according to molecular mass. All these preincubations were performed with samples of GXM at 100  $\mu$ g/ml in PBS. After extensive washing by centrifugation, the suspensions containing particles of chitin were incubated with *C. neoformans* cells as described above, followed by preparation for SEM. Additional systems included preincubation of fungi with the monoclonal antibody (MAb) to GXM MAb 18B7, chitosan, *N*-acetylglucosamine, or chitotriose [(GlcNAc)<sub>3</sub>], all at 100  $\mu$ g/ml. Owing to chitin insolubility, the chitoooligomer (GlcNAc)<sub>3</sub> was used in assays that required GlcNAc-containing structures in their water-soluble form. After washing, yeast cells were incubated with chitin as described above. To assess whether noncovalent interactions were involved in GXM-chitin interaction, the complexes formed as described above were washed in PBS, suspended in EDTA (100 mM), NaCl (1 M), or urea (8 M), and stirred for 1 h at room temperature for subsequent SEM analysis.

**Preparation of glycan complexes.** GXM and chitotriose (GlcNAc)<sub>3</sub> were dissolved in minimal medium to form a solution in which each molecule reached the final concentration of 3 mg/ml. This solution was incubated overnight at 25°C and extensively washed with minimal medium in a Centricon ultrafiltration system (10 kDa cutoff) to remove unbound oligomers. GXM-(GlcNAc)<sub>3</sub> association was monitored by light scattering analysis as described recently (21) (data not shown). GXM alone was subjected to the same conditions.

**Mice immunization and cytokine determination.** Female BALB/c mice (4 to 8 weeks old;  $n = 5$ ) were anesthetized intraperitoneally with ketamine (0.125 mg/g) and xylazine (0.01 mg/g). Animals were then given 50  $\mu$ l of a PBS solution of GXM, chitotriose (GlcNAc)<sub>3</sub>, or a combination of both, all at 500  $\mu$ g/ml intranasally. Mice were sacrificed 24 h after immunization by cervical hyperextension. Lungs were then excised, weighed, and macerated in PBS with a cell strainer for cytokine determination using the DuoSet ELISA development system kit (R&D System). Interleukins 17 and 10 (IL-17 and IL-10) and tumor necrosis factor alpha (TNF- $\alpha$ ) were chosen as the prototypes for cytokine tests based on previous literature on the anticryptococcal immunity (11, 32) and chitin-induced cellular responses (15, 16). Cytokine contents were normalized to the mass of lungs removed from each animal. For all experiments, LPS-free water and glassware were used.

**Preparation of antibody-coated polystyrene beads.** Polystyrene spheres (10<sup>8</sup>, 3- $\mu$ m diameter) were incubated for 1 h under gentle stirring in a 10  $\mu$ g/ml solution of MAb 18B7 at room temperature. The spheres were washed in PBS by centrifugation and blocked for 1 h at room temperature in PBS-bovine serum albumin (BSA). After washing with PBS, the spheres were suspended in Dulbecco's modified Eagle's medium (DMEM) and used for precipitation of polysaccharides as described below.

**Precipitation of glycan complexes from supernatants of fungal cultures and infected phagocytes.** Fungal cells (strain Cap67 or ATCC 24067) were incubated in culture medium as described above for times varying from 15 min to 12 h. Supernatants (1-ml samples) were collected and centrifuged at 10,000  $\times g$  for the removal of cells and debris, and the MAb 18B7-coated spheres (final density, 10<sup>8</sup> spheres/ml) were then added. The suspension was stirred for 1 h at room temperature to precipitate GXM-related molecules. To evaluate whether glycan complexes were precipitated by this methodology, the beads were incubated with tetramethyl rhodamine isocyanate (TRITC)-labeled WGA (30 min, 5  $\mu$ g/ml). After washing, the spheres were analyzed in a FACSCalibur flow cytometer as described previously (3). The possible participation of fungal chitinases on the formation of the hybrid glycan complexes was evaluated in experimental systems where the medium was supplemented with 3-methylxanthine, an inhibitor of fungal chitinases (44). Methylxanthine was used at final concentrations corresponding to 0.05 and 0.5 mM, at which no antifungal activity was observed (44). Control preparations for all systems were developed as described above by incubating antibody-coated

spheres with sterile medium or with supernatants obtained at time zero of incubation. Additional controls included experiments where encapsulated *C. neoformans* was replaced by the acapsular strain Cap67. For detection of the complexes during macrophage infection, the murine macrophage-like cell line RAW 264.7 (ATCC) was cultivated under LPS-free conditions in complete DMEM supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mg/ml gentamicin, minimal essential medium (MEM) nonessential amino acid solution (catalog no. 11360; Gibco-Invitrogen), 10 mM HEPES, and 50 mM 2- $\beta$ -mercaptoethanol at 37°C under a 7.5% CO<sub>2</sub> atmosphere as described previously (36). For interaction with the phagocytes, fungal suspensions (strain Cap67 or ATCC 24067) were prepared in DMEM at a ratio of 10 yeasts per macrophage (host cell). Interactions between fungi and host cells were studied at 37°C with 5% CO<sub>2</sub> for periods varying from 15 min to 12 h. Supernatants were collected and incubated with antibody-coated spheres as described above. Treatment with TRITC-WGA and flow cytometry analysis of the spheres were performed under the conditions described above.

**Statistical analysis.** Student's *t* test was used for comparison of two different groups, and analysis of variance was used for comparison of several groups. Statistical tests were performed with GraphPad Prism (version 5.0).

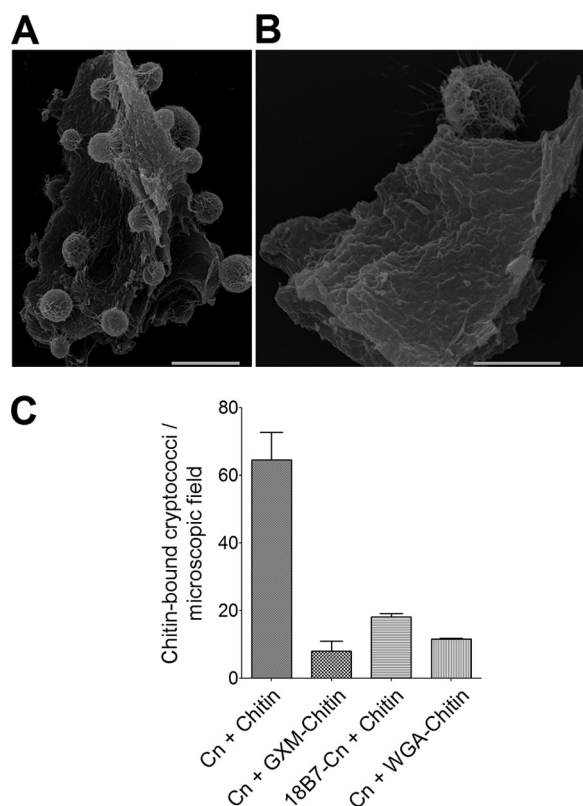
## RESULTS

### Evaluation of the interaction between chitin and GXM by SEM.

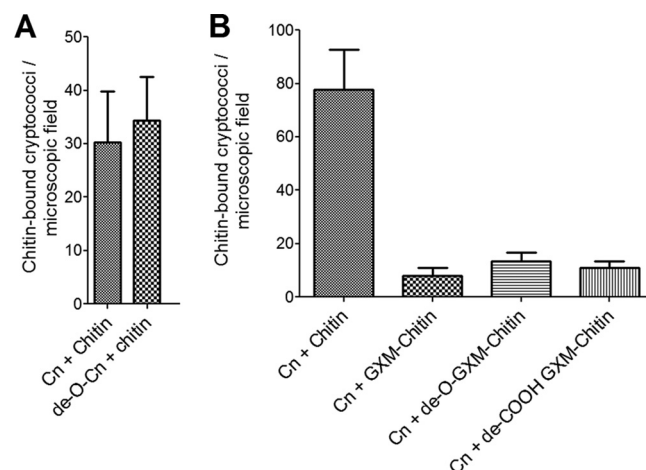
Cryptococcal GXM has affinity for chitin (21). Based on this observation, we developed a quantitative scanning electron microscopy (SEM) method to investigate the structural determinants required to form chitin-GXM complexes. Since GXM is abundant at the surface of *C. neoformans*, the addition of exogenous insoluble chitin particles formed aggregates containing encapsulated yeast cells and chitin, which could then be visualized microscopically (Fig. 1A). Capsular fibers were seen as projections onto the surface of chitin particles, suggesting that components of the capsule were required for the association of *C. neoformans* with the  $\beta$ -1,4-GlcNAc polymer. In fact, treatment of chitin particles with GXM before incubation with *C. neoformans* resulted in a marked decrease in the number of yeast cells that adhered per particle of chitin (Fig. 1B). Quantification of the number of yeast cells adhered to chitin particles showed that the interaction of *C. neoformans* with the insoluble polysaccharide was inhibited by pretreatment with GXM (87% inhibition,  $P < 0.001$ ) or the chitin-binding lectin WGA (72% inhibition,  $P < 0.001$ ), confirming the specificity of this interaction. Treatment of *C. neoformans* with MAb 18B7 before exposure to chitin produced similar results (82% inhibition,  $P < 0.001$ ), which allowed us to validate the model.

**Chemical nature of chitin binding to GXM.** Formation of aggregates containing *C. neoformans* cells and chitin were sensitive to treatment with chaotropic agents (NaCl and urea) (Fig. 2A;  $P < 0.001$  in comparison to untreated systems), suggesting that the polysaccharide-polysaccharide interactions between GXM and chitin involved noncovalent bonds. EDTA, a cation chelating agent, produced an even stronger effect in comparison with both urea and NaCl (Fig. 2A;  $P < 0.05$ ). Formation of the *C. neoformans*-chitin aggregates in the presence of EDTA was also greatly reduced ( $P < 0.001$ ).

Although chitin does not bind to divalent metals (5), GXM associates strongly with Ca<sup>2+</sup> and Mg<sup>2+</sup> (35), which promotes enlargement of polysaccharide fibers. EDTA could impair formation of chitin-*C. neoformans* aggregates by sequestering divalent metals, which make salt bridges connecting GXM polymers, and



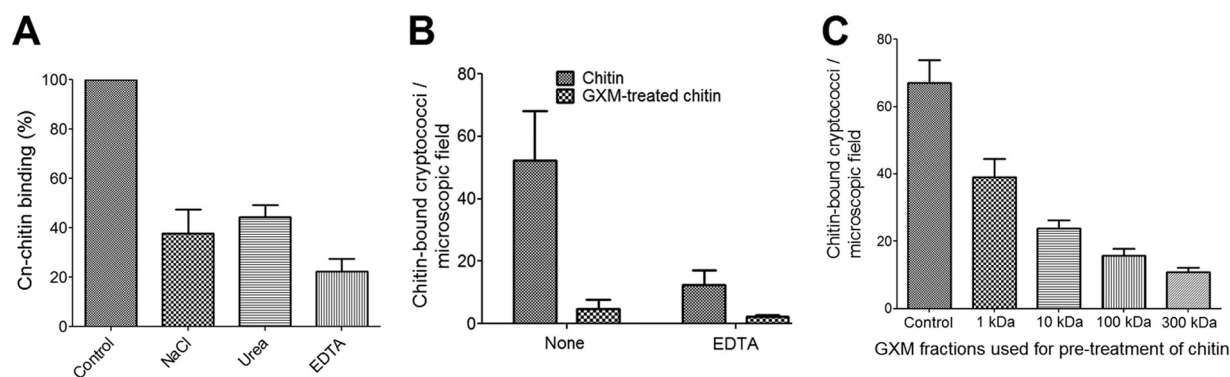
**FIG 1** Interaction of cryptococcal GXM with chitin as assayed by SEM. (A) SEM analysis of aggregates formed after incubation of yeast cells with chitin in minimal medium. Yeast capsular fibers (spherical forms) are apparently in close contact with the surface of a chitin particle (larger and amorphous particle). At least 10 microscopic fields were examined in each system. Scale bar, 10  $\mu$ m. (B) Treatment of chitin particles with soluble GXM before incubation with *C. neoformans* resulted in decreased number of yeast cells per chitin particle. Scale bar, 2  $\mu$ m. (C) Quantification of the results of the systems illustrated in panels A (Cn + Chitin [where Cn is *C. neoformans*]) and B (Cn + GXM-Chitin) and also the effects of pretreatments of yeast cells with a monoclonal antibody to GXM (18B7-Cn + Chitin) and of chitin particles with a chitin-binding lectin (Cn + WGA-Chitin). Results are expressed as means  $\pm$  standard deviations.



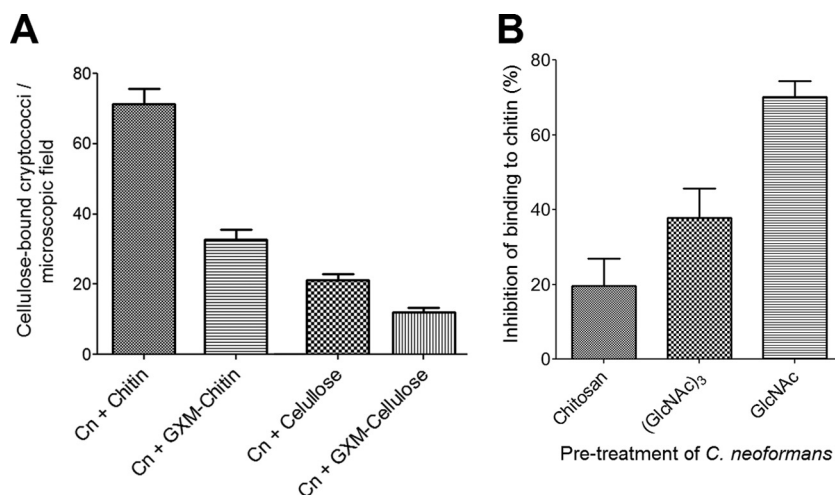
**FIG 3** GXM O-acetylation and glucuronic acid-associated negative charges are not involved in interaction with chitin. (A) Incubation of standard (Cn) or de-O-acetylated *C. neoformans* cells (de-O-Cn) with chitin results in similar levels of association. (B) The use of native, de-O-acetylated (de-O-GXM) or carboxyl-reduced (de-COOH) GXM fractions to treat chitin before exposure to *C. neoformans* cells reveals that all samples had the same efficacy as inhibitors of formation of cell-polysaccharide aggregates. Results are expressed as means  $\pm$  standard deviations.

consequently decreasing the mass of GXM fibers. In fact, the ability of GXM to block the interaction of chitin with *C. neoformans* cells depended on its molecular mass (Fig. 2C). This observation suggests that chitin interacts more efficiently with larger GXM fibers.

We then investigated some structural features in GXM required for interaction with chitin. Mannose O-acetylation and the carboxyl groups of this polysaccharide are known to regulate some biological properties of the polysaccharide (29, 35). We observed that interaction of control cryptococci or de-O-acetylated yeast cells with chitin produced similar levels of binding (Fig. 3A;  $P = 0.7455$ ), suggesting that O-acetyl groups are not required for the carbohydrate-carbohydrate interaction studied in our model. In fact, native GXM and its de-O-acetylated form were equally efficient in blocking the binding of yeast cells to chitin (Fig. 3B;  $P = 0.2602$ ). Also, the use of GXM with reduced carboxyl groups to



**FIG 2** Chitin-GXM interactions involve noncovalent bonds and large GXM fibers. (A) Incubation of *C. neoformans* cells with chitin followed by exposure to chaotropic molecules (NaCl and urea) or a chelating agent (EDTA) inhibits aggregate formation in comparison with systems that were exposed to PBS. (B) Formation of the mixed aggregates is impaired in the presence EDTA. (C) The use of GXM fractions of different molecular masses to treat chitin before exposure to *C. neoformans* cells reveals that samples of higher molecular masses are more effective in inhibiting the formation of cell-polysaccharide aggregates. Results are expressed as means  $\pm$  standard deviations.



**FIG 4** Requirement of amino groups and *N*-acetylation for the interaction of chitin with GXM. (A) Replacement of chitin by cellulose results in decreased levels of attachment of *C. neoformans* (Cn) cells to chitin and reduced sensitivity to inhibition by previous exposure to GXM. (B) A chitooligomer [(GlcNAc)<sub>3</sub>] and GlcNAc are more efficient than chitosan as inhibitors of the interaction of *C. neoformans* with chitin, as determined by comparison of systems in which fungal cells were treated with PBS or the amino sugars before incubation with chitin with systems in which cryptococci were treated with minimal medium alone. Results are expressed as means  $\pm$  standard deviations.

bind chitin before the cryptococci produced inhibitory effects that were similar to those observed with native GXM ( $P = 0.4609$ ). This result suggests that the negative charges associated to the glucuronyl residues of GXM are not involved in the binding of this polysaccharide to chitin.

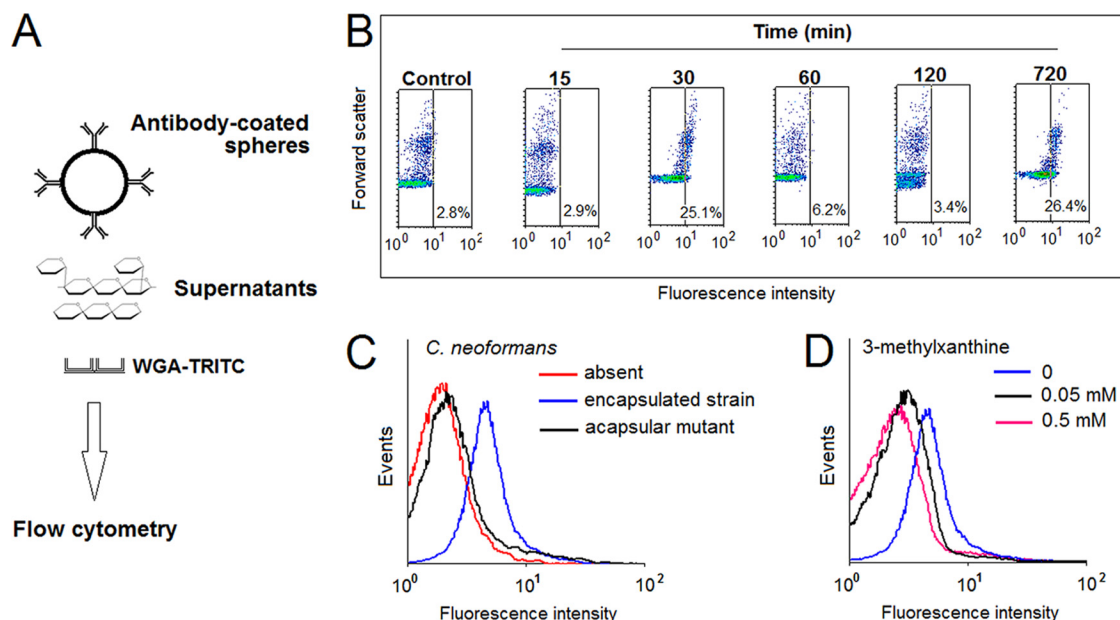
We then evaluated the structural features of chitin that were required for interaction with GXM. GlcNAc, the building unit of chitin, is an amino sugar. The amine group in chitin confers polarity and is required for nucleophilic interactions. When chitin was replaced by cellulose that has  $\beta$ -1,4-linked glucose and lacks the *N*-acetyl amino group of chitin, we found that although *C. neoformans* adhered to cellulose and this interaction was partially inhibited by pretreatment with GXM, this association was weaker than that observed when chitin was used ( $P < 0.0001$ ), indicating that the *N*-acetyl amino groups of the GlcNAc polymer are likely involved in chitin-GXM interactions (Fig. 4A). To specifically evaluate whether the *N*-acetyl moiety of this group was involved in binding to GXM, the inhibitory potential of chitosan, the de-*N*-acetylated form of chitin, was compared to those observed with a chitin oligomer and the GlcNAc monosaccharide. Chitosan was significantly less efficient in inhibiting the interaction of *C. neoformans* with chitin than GlcNAc and chitotriose (GlcNAc)<sub>3</sub> (Fig. 4B;  $P < 0.0002$  and  $P = 0.017$ , respectively), suggesting that *N*-acetylation is an important feature in chitin-GXM interactions. The higher efficacy of GlcNAc as an inhibitor is likely related to its molar concentration (0.45 mM for GlcNAc versus 0.15 mM for chitotriose, both corresponding to 100  $\mu$ g/ml). In summary, our SEM-based model revealed that binding of GXM to chitin is non-covalent, requiring large GXM fibers and the *N*-acetyl amino group of chitin.

**Formation of glycan complexes composed of GXM and chitooligomers during *C. neoformans* growth.** Glycan complexes composed of GXM and chitooligomers were detected in *C. neoformans* cultures in a previous study (21). However, the kinetics of formation of the hybrid glycan and the elements regulating this process remain unknown. To address these questions, *C. neoformans*

was cultivated in minimal medium for periods varying from 15 min to 12 h for supernatant collection using the experimental model described in Fig. 5A. Based on initial data aiming at experimental optimization, strain H99, which was used in all experiments in this study, was replaced by strain ATCC 24067 of *C. neoformans*, a heavily encapsulated isolate with a high efficiency in producing extracellular GXM (12, 35). Our approach was to precipitate soluble fractions of GXM released during fungal growth and then test whether they came out associated with chitin-derived molecules and, consequently, reacted with WGA. Sequential incubation of supernatants with antibody-coated spheres and TRITC-WGA did not give positive reactions (control). Similar results were obtained at time zero (data not shown) and 15 min of incubation (Fig. 5B). After a 30-min incubation of *C. neoformans* in the medium, however, glycan complexes were detected in approximately 25% of the antibody-coated spheres. This value progressively decreased to basal (control) levels after 120 min of incubation of the fungus in minimal medium. After 12 h (720 min) of growth, another peak of hybrid glycan detection was observed.

Based on the results described in Fig. 5B, the 30-min period of incubation of the fungus in minimal medium was selected for analysis of the specificity of glycan detection, as well as of possible biological factors influencing its formation. Specificity was confirmed by experiments where encapsulated cells were replaced by the Cap67 mutant of *C. neoformans*. In contrast to what was observed for supernatants of encapsulated cells, detection of the complexes containing GXM and chitooligomers was similar when sterile culture medium and supernatants from the acapsular mutant were tested (Fig. 5C).

To evaluate the role of fungal chitinases on the formation of complexes containing GXM and chitooligosaccharides, incubation of the fungus for 30 min in culture medium followed by glycan detection was performed in the presence of methylxanthine, an inhibitor of fungal chitinases (44). Detection of the complexes containing GXM and chitooligomers was at the background level when the chitinase inhibitor was added to the



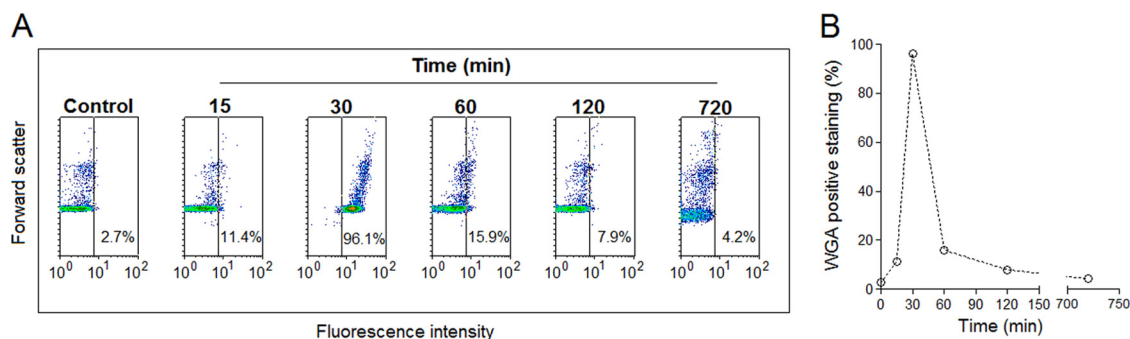
**FIG 5** Formation of glycan complexes composed of GXM and chitin-derived structures during incubation of *C. neoformans* in culture medium. (A) Flow cytometry-based experimental model designed for detection of glycan complexes. (B) Reactivity of 18B7-coated spheres with TRITC-WGA after incubation with culture supernatants. (C) Analysis of the reactivity of antibody-coated spheres with TRITC-WGA after incubation with sterile medium or after a 30-min incubation of encapsulated or acapsular cells in the medium. (D) Effect of a chitinase inhibitor (3-methylxanthine) on the formation of glycan complexes in cultures of *C. neoformans* incubated for 30 min at room temperature. Images are representative of three different experiments.

medium (Fig. 5D), supporting the supposition that formation of the hybrid molecule requires chitinase-mediated release of chitin oligomers.

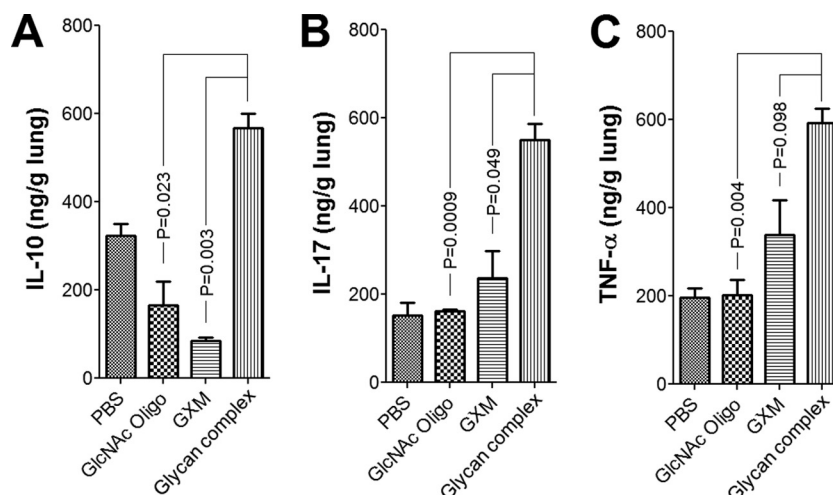
**Detection of glycan complexes composed of GXM and chitooligomers after macrophage infection by *C. neoformans*.** Glycan complexes containing GXM and chitin-derived structures could possibly exert differential biological functions compared to GXM alone. We initially asked whether glycan complexes would be formed spontaneously in soluble form during the interaction of *C. neoformans* with host cells. Yeast (strain ATCC 24067) and host cells were coincubated for periods varying from 15 min to 12 h for supernatant collection, followed by precipitation of the glycan complexes as illustrated in Fig. 5A. During interaction of macrophages with *C. neoformans*, a peak of detection of the hybrid glycans was observed after a 30-min incubation (Fig. 6). At this point, 96% of the antibody-coated beads were positive for the presence

of hybrid glycans. This number progressively decreased to approximately 7% after 2 h of incubation and reached less than 5% after 12 h in contrast to what was observed for assays in the absence of host cells. Sequential incubation of supernatants of uninfected macrophages with antibody-coated spheres and TRITC-WGA did not give positive reactions (control). Experiments using acapsular cells instead of encapsulated fungi also produced negative results (data not shown). We concluded that glycan complexes containing GXM and chitin-derived structures could be formed during infection of host cells by *C. neoformans*.

**Glycan complexes induce a differential cytokine response in infected mice compared with GXM and chitooligomers.** Glycan complexes were prepared with chitotriose and GXM and tested for their ability to induce selected lung cytokines in mice (Fig. 7). IL-10 determinations after 24 h of intranasal immunization with each polysaccharide preparation revealed that both chitotriose



**FIG 6** Formation of glycan complexes composed of GXM and chitin-derived structures during infection of macrophages by *C. neoformans*. (A) Reactivity of 18B7-coated spheres with TRITC-WGA after incubation with macrophage supernatants, incubated with *C. neoformans* for the periods indicated at the top of each panel. (B) Kinetics of formation of the hybrid complexes based on the values obtained in panel A. Images are representative of three different experiments.



**FIG 7** Cytokine determination in the lungs of mice exposed to PBS (control), chitooligomer [(GlcNAc)<sub>3</sub>; GlcNAc Oligo], GXM, or glycan complex structures. Analysis of the production of IL-10 (A), IL-17 (B), and TNF-α (C) revealed that the glycan complex structures are much more efficient than chitin-like structures or GXM alone in their ability to induce pulmonary cytokine responses.

and GXM tended to cause a decrease in the basal levels (mock immunization) of the cytokine ( $P = 0.05$  for chitotriose and  $P = 0.0008$  for GXM). Immunization of mice with the glycan complexes, however, induced a significantly higher IL-10 production in comparison with controls ( $P = 0.0091$ ) and with (GlcNAc)<sub>3</sub> or GXM ( $P = 0.023$  and  $0.003$ , respectively). The levels of IL-17 in the lungs of control mice and of animals immunized with chitotriose were similar ( $P = 0.7359$ ). Immunization with GXM tended to cause an increase in lung IL-17 production, although the difference was not statistically significant ( $P = 0.2477$ ). Exposure of mice to the glycan complexes, however, significantly enhanced IL-17 production in comparison to immunization with chitotriose and GXM separately ( $P = 0.009$  and  $0.049$ , respectively). A similar profile was observed for TNF-α production. Altogether, these results demonstrate that glycan complex structures produced enhanced immunosuppressive and proinflammatory cytokine responses compared to a chitin oligomer and GXM alone.

## DISCUSSION

In the current study, we examined some functional and structural aspects of the interaction of GXM with chitin-like structures, based on previous reports showing that both components interacted within the capsular network (41) and in culture supernatants (21). GXM, the major capsular polysaccharide produced by *C. neoformans*, is abundantly secreted *in vitro* and during animal and human infections (46). The polysaccharide is extremely hydrophilic and has numerous motifs that can interact with neighboring structures sharing H bonds (10) such as other polysaccharides. In addition, GXM may function as a chelating agent (35), sequestering divalent metals in different environments and establishing electrostatic bonds. Therefore, it is reasonable to assume that, during infection, GXM may exist either in its free form or in association with other molecules potentially exhibiting still unknown biological activities.

Studies on the bioactivity of GXM have been classically performed with its free, soluble form. These studies may not reflect the complete set of activities of this major capsular polysaccharide, since a comparison between the properties of capsular-associated

and extracellular polysaccharide samples revealed differential biophysical properties, biological functions, and carbohydrate compositions (23). For instance, capsular extracts of *C. neoformans* contained the building units of GXM but also large amounts of glucose (23). It remains unknown, however, whether the modified sugar compositions of capsular and extracellular extracts were due to contamination with noncapsular molecules or whether GXM was in fact associated with other polysaccharides in the capsule. In this regard, it is clear that carbohydrate-carbohydrate interactions are multiple within the capsule. Mutant *C. neoformans* cells lacking the ability to synthesize α-1,3-glucan also lost their ability to anchor GXM to the cell wall (39, 40). GXM-GXM interactions also occur in the capsule, and formation of these aggregates depends on divalent metal bridges (35). Microscopic evidence suggests that GXM and GXMGal are in close association in some regions of the capsule (17, 28), and in fact, the basic components of the Gal-containing polysaccharide are frequently detected in samples prepared according to protocols aiming at GXM purification (23). Apparently, mannoproteins also interact with capsular polysaccharides (28). Finally, confocal microscopy in combination with biochemical and biophysical methods revealed that GXM associates with chitin-derived structures in the capsular matrix and in culture supernatants (21, 41). The possibility that GXM takes part in complex glycan structures during the regular metabolism of *C. neoformans* led us to explore its ability to interact with chitin-like structures, aiming at investigating whether such complexes would manifest novel biological properties.

Glycan complexes composed of chitooligomers and GXM were isolated from both fungal supernatants and cultures of infected macrophages, supporting the hypothesis that these structures can be formed during both regular growth and infection. The profile of formation of complex glycans as a function of time was different when the fungus was cultivated in culture medium alone or in the presence of macrophages. In fungal cultures, the usually high rate of replication of *C. neoformans* may support a dynamic process where the hybrid glycans are formed and degraded in regular cycles, depending on the availability of GXM, chitooligomers, and possibly hydrolases with affinity for these molecules. In the pres-

ence of macrophages, however, fungal viability is expected to vary considerably (34), which may have a direct impact on the availability of GXM and chitin-derived structures in their soluble forms. In this context, different kinetics of formation of the hybrid polysaccharides would indeed be expected in these two experimental models.

Chitoooligomers are the products of enzymatic hydrolysis of chitin. In this sense, it was described recently that chitinase expression is induced during animal pulmonary cryptococcosis (45) and in the bronchoalveolar lavage fluid of asthmatic children (26), a finding that has led to the proposal that this fungal product is involved in the pathogenesis of some forms of asthma (27). The surface expression of chitoooligomers in *C. neoformans* is in fact increased in the lung of infected rats (21). It is also likely that chitoooligomers produced through the activity of chitinase are released to the extracellular space, considering their high hydrophilicity and consequent solubility in water. GXM, on the other hand, is constitutively released to the extracellular space (46). We therefore raised the possibility that the chitoooligomers formed in our experimental model were originated through the activity of fungal chitinases, yielding molecules that could associate with GXM to form glycan complexes. In fact, the hybrid glycans were detected at background levels when *C. neoformans* was incubated in the presence of methylxanthine, an inhibitor of fungal chitinases (44). The effects of methylxanthines on cryptococcal molecules other than chitinases are still unknown, which implies that the possibility of interference with additional biological events in *C. neoformans* cannot be ruled out. The reduced formation of hybrid glycan complexes as a consequence of chitinase inhibition, however, is in accordance with *in vivo* observations, demonstrating that chitoooligomer detection and capsule enlargement are more evident in host tissues manifesting higher activity of this enzyme (21). In this sense, a putatively synergistic activity of host and fungal chitinases (26, 44, 45) could provide an explanation for the higher detection of glycan complexes in supernatants of infected macrophages in comparison with the values observed for fungal cultures.

Our observations indicate that the key structural determinants mediating the interaction between chitin-like structures and GXM are the *N*-acetyl groups present in the former. In *C. neoformans*, chitin exists as polysaccharide and chitoooligomeric forms (21, 41). The de-*N*-acetylated form, or chitosan, is also present (1, 2). This polymer is required for cell wall integrity in *C. neoformans* (1). Exposure of *C. neoformans* cells to chitosan induces capsule reduction (30), suggesting that this chitin derivative and GXM may also interact. The present study and previous work (20, 21, 41), however, suggest that chitin and chitoooligomers have higher affinity for GXM. Chitin had higher affinity for GXM fractions of higher molecular mass. All polysaccharide fractions tested in the present work had the same concentration, thus it is unlikely that this observation would simply reflect a higher number of chitin-binding sites. GXM tridimensional structure is highly complex (13), and recent observations indicate that polysaccharide samples of different molecular masses manifest functional differences (22). Thus, we believe that the superior efficacy of high molecular mass fractions to interact with chitin may reflect still unknown structural properties that would foster stable interactions between both polysaccharides.

The ability of GXM to stimulate/inhibit neutrophil and monocyte/macrophage cytokines has been extensively explored in the

literature (33). Chitin, on the other hand, is considered an immunologically inert polysaccharide, although chitin particles of reduced dimensions contain pathogen-associated molecular patterns that stimulate TLR2, dectin-1, and the mannose receptor (15). The effects of the association of both polysaccharides, however, are unknown. In our model, complex glycan structures containing GXM and chitoooligomers were more efficient in inducing the production of IL-10, IL-17, and TNF- $\alpha$  than each molecule alone in the lungs of mice. This observation indicates that glycan complexes are functionally different from each molecule tested individually and that they may influence the pathogenesis of *C. neoformans* if produced during infection as they are *in vitro* (21).

Our results further highlight molecular complexity of the cryptococcal capsule and its constituents (13, 46). The attributes of the capsular components have classically been examined with isolated polysaccharide fractions (9), although a number of studies strongly suggest that these molecules are associated to other components of the cell surface of *C. neoformans* (14, 17, 20, 21, 28, 35, 39–41). Our results suggest that chitin-derived structures associate with GXM through their *N*-acetyl amino groups generating complex glycans with previously unknown functions. We believe that the present results will open new venues of investigation on the functions of capsular components in *C. neoformans*, including tests on the influence of other glycan complexes (e.g., GXM-GXMGal) on the interaction of this pathogen with elements of the host immune response.

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