



## A *Paracoccidioides brasiliensis* glycan shares serologic and functional properties with cryptococcal glucuronoxylomannan

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### ABSTRACT

The cell wall of the yeast form of the dimorphic fungus *Paracoccidioides brasiliensis* is enriched with  $\alpha$ 1,3-glucans. In *Cryptococcus neoformans*,  $\alpha$ 1,3-glucans interact with glucuronoxylomannan (GXM), a heteropolysaccharide that is essential for fungal virulence. In this study, we investigated the occurrence of *P. brasiliensis* glycans sharing properties with cryptococcal GXM. Protein database searches in *P. brasiliensis* revealed the presence of sequences homologous to those coding for enzymes involved in the synthesis of GXM and capsular architecture in *C. neoformans*. In addition, monoclonal antibodies (mAbs) raised to cryptococcal GXM bound to *P. brasiliensis* cells. Using protocols that were previously established for extraction and analysis of *C. neoformans* GXM, we recovered a *P. brasiliensis* glycan fraction composed of mannose and galactose, in addition to small amounts of glucose, xylose and rhamnose. In comparison with the *C. neoformans* GXM, the *P. brasiliensis* glycan fraction components had smaller molecular dimensions. The *P. brasiliensis* components, nevertheless, reacted with different GXM-binding mAbs. Extracellular vesicle fractions of *P. brasiliensis* also reacted with a GXM-binding mAb, suggesting that the polysaccharide-like molecule is exported to the extracellular space in secretory vesicles. An acapsular mutant of *C. neoformans* incorporated molecules from the *P. brasiliensis* extract onto the cell wall, resulting in the formation of surface networks that resembled the cryptococcal capsule. Coating the *C. neoformans* acapsular mutant with the *P. brasiliensis* glycan fraction resulted in protection against phagocytosis by murine macrophages. These results suggest that *P. brasiliensis* and *C. neoformans* share metabolic pathways required for the synthesis of similar polysaccharides and that *P. brasiliensis* yeast cell walls have molecules that mimic certain aspects of *C. neoformans* GXM. These findings are important because they provide additional evidence for the sharing of antigenically similar components across phylogenetically distant fungal species. Since GXM has been shown to be important for the pathogenesis of *C. neoformans* and to elicit protective antibodies, the finding of similar molecules in *P. brasiliensis* raises the possibility that these glycans play similar functions in paracoccidioidomycosis.

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### 1. Introduction

Paracoccidioidomycosis (PCM) is the most prevalent mycosis in Latin America (Queiroz-Telles and Escuissato, 2011). The importance of PCM, however, has been long underestimated, probably because of the geographic distribution of its etiologic agent, *Para-*

*coccidioides brasiliensis* (Teles and Martins, 2011), which limits it to South America. In Brazil, about 50% of the deaths caused by systemic mycoses between 1996 and 2006 were due to *P. brasiliensis* (Prado et al., 2009). Although there have been advances in methods of diagnosis and disease prevention, there is no consensus on the best diagnostic and preventative approaches (reviewed in Teles and Martins (2011)). Moreover, treatment of PCM requires months to years of antimicrobial therapy and relapses are common (Morejon et al., 2009).

Polysaccharides influence physiology and pathogenesis of fungal species through multiple mechanisms. In fungi, these mole-

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cules are required for cell wall architecture, interaction with host cells, modulation of immunological responses, and virulence (reviewed in Fukazawa et al. (1995); Pirofski (2001); Rodrigues et al. (2011a); San-Blas et al. (2000); Taylor and Roberts (2005); Zaragoza et al. (2009)). Because of their structural and functional particularities, fungal polysaccharides are often utilized in diagnostic tests, including therapeutic monitoring, in mycoses patients (Fukazawa et al., 1995; Lopes et al., 2011). Furthermore, fungal polysaccharides have been shown to elicit protective antibody responses when incorporated into conjugate vaccines (Torosantucci et al., 2005). However, the current literature clearly indicates that many aspects related to structure and functions of polysaccharides remain unknown (Rodrigues et al., 2011a).

Glucuronoxylomannan (GXM) is a heteropolysaccharide produced by fungal pathogens belonging to the *Cryptococcus* and *Trichosporon* genera (Fonseca et al., 2009a; Zaragoza et al., 2009). This  $\alpha$ 1,3-mannan with  $\beta$ -xylosyl and -glucuronyl substitutions is an active immunomodulator that is essential to the pathogenesis of *C. neoformans* and *C. gattii*, the causative agents of human cryptococcosis (Zaragoza et al., 2009). Antibodies to GXM have been shown to alter the course of experimental animal cryptococcosis to the benefit of the host (Feldmesser and Casadevall, 1998; Pirofski, 2001), supporting the notion that GXM-binding antibodies might be useful in preventing and treating human cryptococcosis (Larsen et al., 2005). In addition, mimetic peptides sharing immunological properties with GXM are protective in animal models of vaccination (Pirofski, 2001). In this context, GXM represents an attractive target for antifungals and vaccine design in the *Cryptococcus* and *Trichosporon* models. Currently, it is not known if other pathogens can synthesize GXM-like molecules.

In *C. neoformans* and *C. gattii*, GXM is both cell-wall associated and released to the extracellular space (Zaragoza et al., 2009). GXM association to the cell wall in *C. neoformans* requires  $\alpha$ 1,3-glucans (Reese and Doering, 2003; Reese et al., 2007). In fact, a *C. neoformans* mutant lacking expression of the gene coding for  $\alpha$ 1,3-glucan synthase displayed an acapsular phenotype despite ongoing synthesis of capsular components (Reese and Doering, 2003). Disruption of the  $\alpha$ 1,3-glucan synthase gene also resulted in increased sensitivity to temperature and in reduced levels of cell division (Reese et al., 2007). Loss of  $\alpha$ 1,3-glucan was accompanied by a compensatory increase in chitin/chitosan and a redistribution of  $\beta$  glucan between cell wall fractions (Reese et al., 2007). These observations clearly indicated that metabolism and cellular distribution of polysaccharides are integrated and required for fungal virulence. They also established a consistent connection between glucan synthesis and GXM anchoring to the cell wall in *C. neoformans* and, possibly, other fungi. In fact, capsular material from *C. neoformans* binds to isolates of *Histoplasma capsulatum* containing cell wall-associated  $\alpha$ 1,3-glucan, but not to *H. capsulatum* strains lacking synthesis of this glucan (Reese and Doering, 2003).

Synthesis of  $\alpha$ 1,3-glucan by *P. brasiliensis* yeast cells is associated with fungal virulence (San-Blas and San-Blas, 1977; San-Blas and Vernet, 1977). The presence of a polysaccharide with the ability to anchor *C. neoformans* GXM in the parasitic forms of *P. brasiliensis* led us to investigate whether these two pathogenic species would share metabolic events related to the synthesis of GXM. By combining serologic, chromatographic, and microscopic approaches with a phagocytosis model of polysaccharide-coated yeast cells, we observed that *P. brasiliensis* produces glycans that share structural, serologic and functional properties with cryptococcal GXM. These results suggest that some of the pathogenic mechanisms used by *C. neoformans* to damage the host and/or avoid host responses may be similar to those used by *P. brasiliensis*, and these observations open new avenues for the identification of therapeutic and diagnostic targets in *P. brasiliensis*.

## 2. Materials and methods

### 2.1. Fungal strains and growth conditions

The *P. brasiliensis* strain used in this study was the reference strain Pb 18, provided by Dr. Rosely Zancope-Oliveira (Fiocruz, Rio de Janeiro, Brazil). Yeast forms were cultivated in Fava Netto's medium (proteose peptone 3 g, peptone 10 g, beef extract 5 g, sodium chloride 5 g, yeast extract 5 g, dextrose 40 g, H<sub>2</sub>O 1 L) (Fava-Netto, 1955) for 7 days at 37 °C. A number of experiments in this study included *C. neoformans* cells; strains used were the standard serotype A isolate H99 and the acapsular mutant Cap67. *C. neoformans* yeast were cultivated in a 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  $\mu$ M thiamine-HCl (pH 5.5) for 2 days at 30 °C, with shaking. *P. brasiliensis* and *C. neoformans* yeast cells were obtained by centrifugation, washed in phosphate-buffered saline (PBS) and counted in a Neubauer chamber. Some of the serologic tests developed in this study required mycelial cells of *P. brasiliensis* (Pb 18), in addition to control systems using *Candida albicans* (strain IBEX 11, Tavares et al., 2008) and *Saccharomyces cerevisiae* (strain BY4741, provided by Dr. Marcos Pereira, Federal University of Rio de Janeiro) yeast cells. *P. brasiliensis* mycelia were cultivated in Sabouraud broth with shaking for 7 days at 25 °C. Filamentous cells were collected and washed by filtration as previously described (Soares et al., 1993). *S. cerevisiae* and *C. albicans* yeast cells were cultivated for 48 h in Sabouraud broth with shaking, followed by centrifugation and washing.

### 2.2. Protein database searches

Numerous genes related to GXM synthesis and export have been characterized in the last two decades (Chang et al., 1997, 1996, 1995; Chang and Kwon-Chung, 1998, 1999; Cottrell et al., 2007; Garcia-Rivera et al., 2004; Janbon et al., 2001; Klutts and Doering, 2008; Kmetzsch et al., 2011; Kumar et al., 2011; Moyrand et al., 2002; Panepinto et al., 2009; Sommer et al., 2003; Yoneda and Doering, 2006). Sequences of the proteins related to these processes were obtained from the Uniprot database ([www.uniprot.org](http://www.uniprot.org)) and used in Blast searches (protein query/protein database) to find homologous in the *P. brasiliensis* proteomic database. Hits showing the highest scores of similarity were selected and are presented in Table 1.

### 2.3. Preparation of *C. neoformans* GXM

*C. neoformans* GXM was isolated as previously described by filtration of fungal supernatants in Amicon (Millipore, Danvers, MA) ultrafiltration cells (cutoff 100 kDa) (Nimrichter et al., 2007). After concentration of the supernatant, the viscous GXM-containing film layer was collected with a cell scraper and was transferred to plastic tubes for carbohydrate quantification. Carbohydrate contents were determined as per Dubois et al. (1951).

### 2.4. Preparation of fungal glycan fractions

Cellular fractions were used to search for *P. brasiliensis* glycans using a protocol that was previously established for extraction of *C. neoformans* GXM (Maxson et al., 2007b). *P. brasiliensis* yeast cells ( $2 \times 10^9$ ) were suspended in DMSO (15 ml) and incubated for 15 min with shaking at room temperature. Supernatants containing released glycans were collected by centrifugation and the pellet was again suspended in 15 ml DMSO for a second extraction under the same conditions. Supernatants were combined and extensively dialyzed against water for subsequent lyophilization and dry

**Table 1**Proteins required for capsule formation, GXM synthesis and polysaccharide export in *C. neoformans* and their *P. brasiliensis* Pb18 homologs.

Gene (references)	<i>C. neoformans</i> protein (UniProt accession number)	<i>P. brasiliensis</i> protein (UniProt accession number)	Identity	E-value	Protein family
<i>Cellular events: capsule formation and/or polysaccharide synthesis</i>					
CAP10 (Chang and Kwon-Chung, 1999)	Capsule-associated protein 10 (Q9HGG9)	Conserved hypothetical protein (C1G0T3)	33%	$7 \times 10^{-16}$	Glycosyltransferase
CAP64 (Chang et al., 1997, 1996)	Capsular-associated protein 64 (Q9HGG3)	Not found	–	–	–
CAP60 (Chang and Kwon-Chung, 1998)	Capsular-associated protein 60 (Q9HGG6)	Not found	–	–	–
GMT2 (Cottrell et al., 2007)	GDP-mannose transporter (P0CS04)	GDP-mannose transporter (C1GF36)	57%	$1 \times 10^{-104}$	Nucleotide-activated monosaccharide transporter
GMT1 (Cottrell et al., 2007)	GDP-mannose transporter (P0CS02)	GDP-mannose transporter (C1GF36)	59%	$1 \times 10^{-104}$	Nucleotide-activated monosaccharide transporter
CXT1 (Klutts and Doering, 2008)	Xylosyltransferase (Q5K8R6)	Conserved hypothetical protein (C1G0T3)	28%	$3 \times 10^{-45}$	Glycosyltransferase
CMT1 (Sommer et al., 2003)	$\alpha$ 1,3-Mannosyltransferase (A5D938)	Conserved hypothetical protein (C1GGS1)	24%	$7 \times 10^{-6}$	Glycosyltransferase
CAS1 (Janbon et al., 2001; Moyrand et al., 2002)	O-acetyltransferase (Q8X226)	Not found	–	–	–
<i>Cellular event: GXM export</i>					
SAV1 (Yoneda and Doering, 2009)	Rab/GTPase (E2IF16)	GTP-binding protein (C1G092)	73%	$2 \times 10^{-77}$	Ras-like proteins
SEC6 (Panepinto et al., 2009)	Vesicle fusion protein (Q5K9B3)	Conserved hypothetical protein (C1G3V8)	26%	$3 \times 10^{-78}$	Exocyst complex component
CAP59 (Chang et al., 1995; Garcia-Rivera et al., 2004)	Capsular-associated protein 59 (O93823)	Conserved hypothetical protein (C1GGS1)	29%	$8 \times 10^{-5}$	Glycosyltransferase
GRASP (Kmetzsch et al., 2011)	Golgi reassembly and stacking (A6ZZA4)	Conserved hypothetical protein (C1G8Y3)	26%	$5 \times 10^{-20}$	GRASP55/65 PDZ-like domain

weight determination. Negative controls consisted of similar preparations obtained from *C. albicans* and *S. cerevisiae*. Glycan fractions from *P. brasiliensis* mycelial cells (2.5 g, wet weight) were similarly prepared for serologic tests. For light scattering analysis, the same protocol was used to extract GXM from *C. neoformans* cells.

### 2.5. Alkali treatment of fungal glycans

Removal of O-acetyl groups of GXM and similar groups potentially present in the *P. brasiliensis* molecule was performed by dissolving 5 mg of the glycans 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.

### 2.6. Fluorescence microscopy

The different components tested in this study included *P. brasiliensis* yeast forms, in addition to glycan-coated Cap67 cells. Yeast cells ( $10^6$ ) were suspended in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) and incubated for 30 min at room temperature. Fixed yeast cells were washed twice in PBS and incubated in 1% bovine serum albumin in PBS (PBS-BSA) for 1 h. For glycan staining, blocked cells were incubated with different monoclonal antibodies (mAbs) to cryptococcal GXM. MABs to GXM that were available at our laboratory were generated in previous studies and included IgMs (mAbs 13F1 and 2D10) (Cleare and Casadevall, 1998; Garcia-Rivera et al., 2004) and IgG1 (mAb 18B7) (Casadevall et al., 1998). These mAbs differ in fine specificity and protective efficacy. MAb 13F1 is not protective and produces punctate immunofluorescence (Mukherjee et al., 1995, 1992). MAb 2D10 (IgM) reacts with cell wall and capsular epitopes of *C. neoformans* and is protective in a murine model of cryptococcosis (Garcia-Rivera et al., 2004). MAb 18B7 is a protective IgG1 that has been tested as a therapeutic tool in animals and humans (Casadevall et al., 1998; Larsen et al., 2005). This antibody reacts with all GXM serotypes. After incubation in primary antibodies, yeast cells were washed and then incubated with Alexa Fluor 488-labeled goat anti-mouse (IgG or IgM) antibodies (Invitrogen). In some

conditions, yeast cells were incubated with the mAbs in the presence of the *P. brasiliensis* glycan fraction (1–100 µg/ml). After washing, yeast cells were applied to slides and observed using an Axioplan 2 fluorescence microscope (Zeiss, Germany). Images were acquired using a Color View SX digital camera and were processed with the analysis software system (Soft Imaging System) prior to processing with ImageJ software (provided by NIH, <http://rsb.info.nih.gov/ij/>). For controls, mAbs were replaced by irrelevant isotype-matched antibodies. Yeast grown in minimal medium alone were used as a control for the experiments examining glycan-coated acapsular cells. Exposure times were similar for all conditions.

### 2.7. Serologic reactivity of fungal glycans with mAbs to GXM

The reactivity of the *P. brasiliensis* glycan fractions with mAbs to cryptococcal GXM was determined by enzyme-linked immunosorbent assays (ELISAs), using modifications of a previously described protocol for GXM detection (Casadevall et al., 1992). Polystyrene 96-well plates were coated with 0.5 µg/ml solutions of the *P. brasiliensis* glycan fraction and incubated for 1 h at 37 °C. Alternatively, the plates were coated with DMSO extracts from *S. cerevisiae* or *C. albicans* (negative controls) at the same concentration. After washing to remove unbound polysaccharide, the plates were blocked with 1% bovine serum albumin, followed by addition of the mAbs to GXM. After incubation for 1 h at 37 °C, the plates were washed five times with tris-buffered saline (TBS) supplemented with 0.1% Tween 20, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse secondary antibodies for 1 h. Color reaction developed after the addition of *p*-nitrophenyl phosphate disodium hexahydrate and the absorbance was measured at 405 nm with a microplate reader (Biotek EL808 reader). Primary antibodies and alkaline phosphatase-conjugated antibodies corresponded to 1 µg/ml. A comparison between the serologic reactivity of the native glycan fraction obtained from *P. brasiliensis* yeast cells with alkali-treated (de-O-acetylated) and mycelial glycans was also included in antibody-binding assays. Since these tests involved quantitative comparisons between chemically



modified molecules with still undetermined ability to coat polystyrene plates, glycan antigens (0.03–1 µg in 5 µl PBS) in these assays were loaded onto nitrocellulose membranes for dot blot analysis (Nimrichter et al., 2007). The membranes were allowed to dry for 1 h at 37 °C and then were blocked with PBS containing 1% bovine serum albumin. Blocked membranes were incubated with mAb 18B7 at 1 µg/ml. After being washed extensively, membranes were sequentially incubated with alkaline phosphatase-conjugated goat anti-mouse IgG and *p*-nitrophenyl phosphate solutions. Reactions were quantified by the transfer of the soluble, colored products to the wells of 96-well plates and absorbance reading at 405 nm as described above.

## 2.8. Monosaccharide analysis

Monosaccharide components of the *P. brasiliensis* glycan fraction were determined by gas chromatography/mass spectrometry (GC/MS) analysis of the per-*O*-trimethylsilyl (TMS) derivatized monosaccharides. The dry sample (0.3 mg) was submitted to methanolysis in methanol/1 M HCl at 80 °C (18–22 h) for preparation of methyl glycosides. The sample was then treated with Tri-Sil (Pierce) at 80 °C (0.5 h) for per-*O*-trimethylsilylation. GC/MS analysis of the per-*O*-TMS derivatives was performed on an HP 5890 gas chromatograph interfaced to a 5970 MSD mass spectrometer, using a Supelco DB-1 fused silica capillary column (30 m × 0.25 mm ID). Carbohydrate standards included arabinose, rhamnose, fucose, xylose, glucuronic acid, galacturonic acid, mannose, galactose, glucose, mannitol, dulcitol and sorbitol.

## 2.9. Effective diameter determination of glycan fractions

Effective diameter and size distribution of molecules in GXM preparations were measured by Quasi elastic light scattering in a 90Plus/BI-MAS Multi Angle Particle Sizing analyzer (Brookhaven Instruments Corp., Holtsville, NY), according with the method described by Frases and colleagues (Frases et al., 2009). Multimodal size distribution analysis of polysaccharides was calculated from the values of intensity weighted sizes obtained from the non-negatively constrained least squared (NNLS) algorithm for further processing with GraphPad Prism software (version 5.0).

## 2.10. Glycan detection in extracellular vesicles

Vesicles were isolated from *P. brasiliensis* culture supernatants by sequential centrifugation as recently described (Vallejo et al., 2011, 2012a, 2012b). Vesicle pellets were lyophilized and suspended in 100 µl of absolute methanol, resulting in the immediate formation of a precipitate. The suspension was then supplemented with 900 µl of chloroform and the organic fraction was recovered by centrifugation. The residual material that was not soluble in the chloroform–methanol mixture was then dried under a nitrogen stream, suspended in PBS, and assayed for the presence of GXM-related glycans by ELISA with mAb 18B7. The organic phase, containing lipids, was recovered for analysis by high-performance thin-layer chromatography (HPTLC) aiming at the detection of sterols, which were previously characterized as extracellular vesicle lipid markers in fungi (Oliveira et al., 2010b; Rodrigues et al., 2007; Vallejo et al., 2012a). Sterol analysis by HPTLC was performed as described (Oliveira et al., 2010a, 2010b, 2009; Rodrigues et al., 2007).

## 2.11. Coating acapsular *C. neoformans* cells with fungal glycans

Acapsular *C. neoformans* cells (strain Cap67, 10<sup>6</sup> cells) were suspended in solutions of purified GXM or of the *P. brasiliensis* glycan fraction (100 µl, 10 µg/ml) and incubated for 18 h at 25 °C, followed by extensive washing with PBS. Control systems consisted of Cap67 cells incubated in minimal medium.

## 2.12. Scanning electron microscopy (SEM)

Acapsular cells of *C. neoformans* or the glycan-coated mutant were washed in PBS and fixed in 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde for 1 h and prepared for SEM as recently described (Ramos et al., 2012). Briefly, the cells were 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 dehydrated in alcohol (30%, 50%, 70% and 100% for 5 min and 95% and 100% for 10 min), and submitted to critical point drying and metallization. The cells were observed in a scanning electron microscope (JEOL JSM-5310).

## 2.13. Phagocytosis

The murine macrophage cell line RAW 264.7 (American Type Culture Collection, Rockville, MD) was grown to confluence in 25 cm<sup>2</sup> culture flasks containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), at 37 °C in a 5% CO<sub>2</sub> atmosphere. For interaction with glycan coated- or uncoated yeast cells, the macrophages were cultivated in 24-well plates, in the same conditions described above. The cell wall of fungal cells was stained with 0.5 mg/ml fluorescein isothiocyanate (FITC, Sigma) in PBS (25 °C) for 10 min. FITC reacts with amino groups on surface proteins, generating fluorescent yeast cells. This approach has been consistently used for the quantitative analysis of phagocytosis of yeast cells by flow cytometry (Barbosa et al., 2006; Nogueira et al., 2010). FITC-labeling does not impact yeast viability. Yeast suspensions were prepared in DMEM, to generate a ratio of 10 yeasts per macrophage-like cell. Interactions between fungal and host cells occurred at 37 °C in a 5% CO<sub>2</sub> atmosphere for 18 h. After removal of non-adherent fungi by washing, yeast-host cell complexes were treated for 10 min at 25 °C with trypan blue (200 µg/ml) and washed. Trypan blue is a quenching agent of FITC-derived fluorescence and is useful to discriminate between surface-associated and intracellular yeast cells, because it is not capable of penetrating the intracellular compartment of viable cells. Thus this dye quenches the fluorescence of non-internalized cells. Cells were gently removed from the plastic surface with a cell scraper. The cells were analyzed by flow cytometry on a FACSCalibur (BD Biosciences, San Jose, CA) flow cytometer. Data were processed with Cyflogic software ([www.cyflogic.com](http://www.cyflogic.com)). The index of infection was determined as the percentage of fluorescent host cells. Control preparations were developed as described above using uninfected cells and infecting with non-stained yeast. Each experiment was repeated twice.

## 3. Results

### 3.1. The *P. brasiliensis* genome contains homologous genes to those involved in GXM synthesis

We first sought to evaluate whether *P. brasiliensis* and *C. neoformans* would share protein sequences related to the metabolic steps involved in GXM synthesis, capsule formation and polysaccharide export. We first selected proteins coded by genes that directly affected capsular structures in *C. neoformans* (Chang et al., 1997, 1996, 1995; Chang and Kwon-Chung, 1998, 1999; Cottrell et al., 2007; Janbon et al., 2001; Klutts and Doering, 2008; Kmetzsch et al., 2011; Moyrand et al., 2002; Panepinto et al., 2009; Sommer et al., 2003; Yoneda and Doering, 2009) and classified them as involved in (i) capsule formation and GXM synthesis and (ii) GXM export (Table 1), based on the literature on the generation of capsule-deficient mutants. Nine of the 12 candidates we selected had

homologous sequences in *P. brasiliensis*. Searches for proteins coded by two genes required for capsule formation in *C. neoformans* (*CAP64* and *CAP60*) and by one gene required for GXM O-acetylation (*CAS1*) produced negative results. *P. brasiliensis* had homologs for all proteins required for polysaccharide export in *C. neoformans* and also for building the non-acetylated GXM backbone. We therefore hypothesized that *P. brasiliensis* may have the metabolic apparatus required for the synthesis of polysaccharides with antigenic similarity to the *C. neoformans* GXM, but not necessarily for the formation of a large, physical capsule.

### 3.2. *P. brasiliensis* produces glycans that share serologic and structural properties with cryptococcal GXM

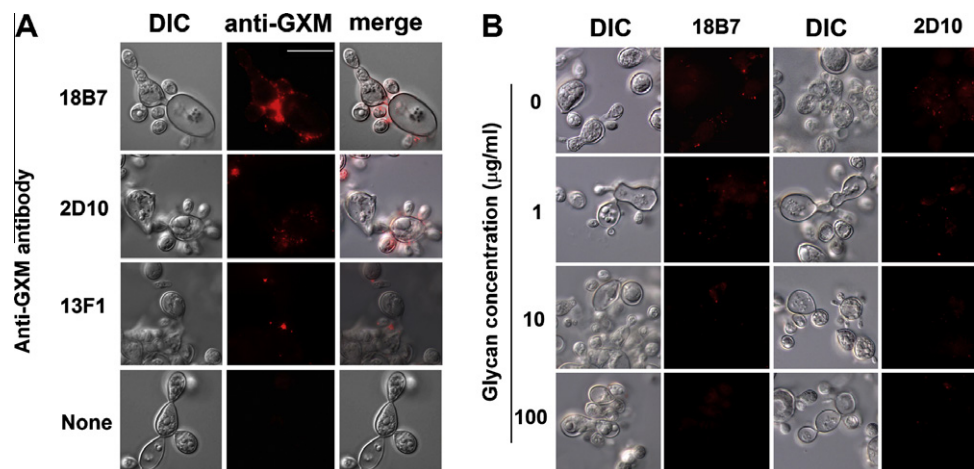
The hypothesis that *P. brasiliensis* can synthesize molecules with serologic similarity with cryptococcal GXM was first tested by immunofluorescence using mAbs 18B7, 2D10 and 13F1 (Fig. 1A). All antibodies reacted with the surface of yeast cells in a cell wall-associated punctate pattern. In addition, the GXM-binding mAbs recognized extracellular structures that accumulated in the budding neck and in intercellular spaces formed by dividing cells. Fluorescent reactions were not observed when primary antibodies were replaced with isotype-matched irrelevant antibodies. To test the specificity of the reactions between *P. brasiliensis* molecules and the GXM-binding antibodies, we selected mAbs 18B7 and 2D10 for immunofluorescence reactivity in the presence of varying concentrations of the *Paracoccidioides* glycan obtained by DMSO extraction (Maxson et al., 2007a, 2007b). Fluorescent reactions were efficiently inhibited when glycan fractions were used at 10 and 100  $\mu\text{g/ml}$  (Fig. 1B). We estimated the efficacy of the glycan-mediated inhibition of antibody binding by counting the number of surface-associated fluorescence spots in at least 100 yeast cells. According to this calculation, the *P. brasiliensis* glycan fraction inhibited mAb binding by approximately 70% and 90% at 10 and 100  $\mu\text{g/ml}$ , respectively. This inhibitory profile was similar for both mAbs. Altogether, these results suggested the existence of surface structures in *P. brasiliensis* that share serologic properties with cryptococcal GXM.

To exclude the possibility that the results observed in Fig. 1 originated from unspecific reactivity of GXM-binding mAbs with

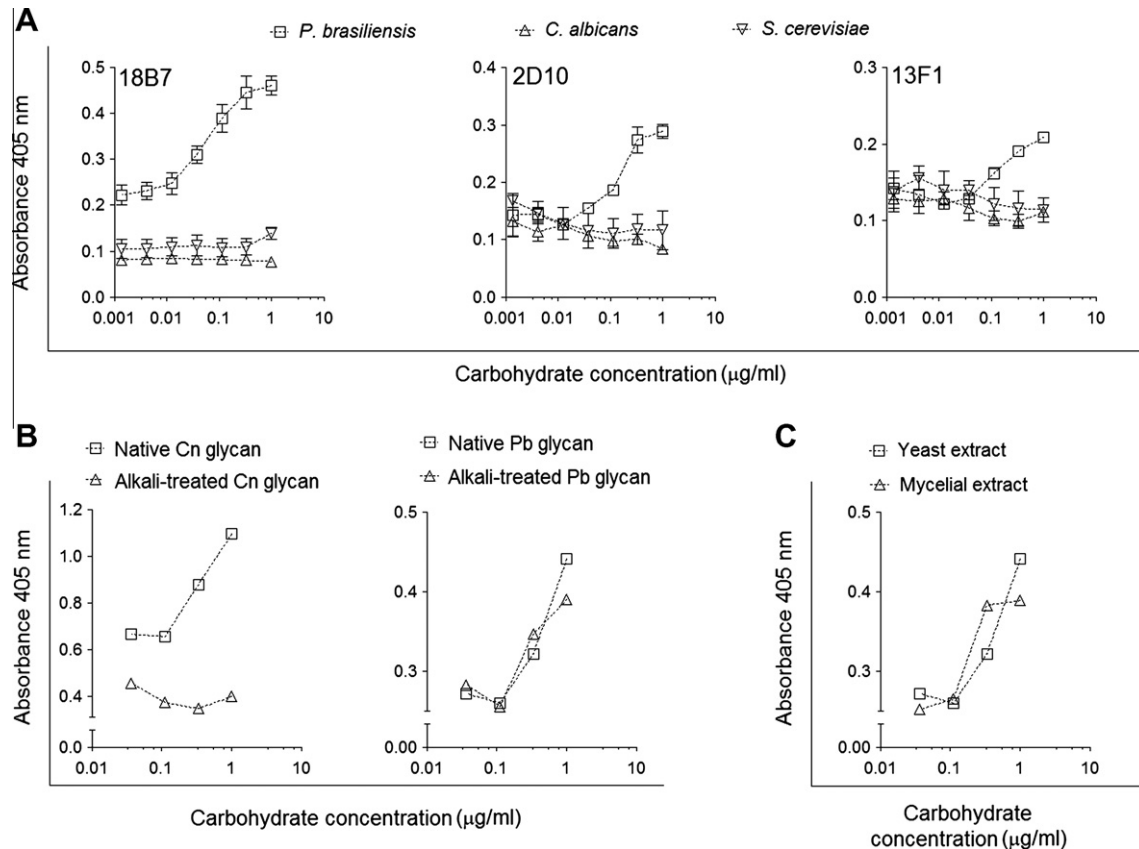
surface *P. brasiliensis* structures, we extended the serologic approach for detection of GXM-related molecules to another model of antigen–antibody reactivity. Glycan fractions extracted from *P. brasiliensis* yeast cells were used in ELISA with different mAbs raised to cryptococcal GXM. All mAbs showed dose-dependent serologic reactivity with the *P. brasiliensis* extract with variable affinities (Fig. 2A), with the IgG1 18B7 having the best binding. The GXM-binding antibodies showed negligible reactivity with DMSO extracts obtained from either *C. albicans* or *S. cerevisiae* yeast cells.

O-acetylation is a serologic marker of cryptococcal GXM (Kozel et al., 2003), which led us to evaluate whether the presence of this group would affect antibody recognition of the fungal glycans. As expected, GXM de-O-acetylation caused a marked reduction in its reactivity with mAb 18B7 (Fig. 2B). The *P. brasiliensis* glycan fraction, however, was not affected by alkali treatment, which is in agreement with the lack of mannosyl O-acetyltransferases in this fungus (Table 1). We finally tested whether the saprophytic form of *P. brasiliensis* would produce similar molecules. Immunofluorescence experiments were inconclusive due to high backgrounds (data not shown), but serologic tests with DMSO extracts from mycelial cells revealed that the filamentous forms of *P. brasiliensis* also produce molecules that share epitopes with cryptococcal GXM (Fig. 2C).

Crude culture supernatants of *P. brasiliensis* were also tested for reactivity with GXM-binding mAbs, showing negative results (data not shown). This observation could imply that the glycan is not released extracellularly. However, we could not rule out the possibility that the *P. brasiliensis* molecules sharing serologic reactivity with GXM were present in culture supernatants at concentrations below the detection limit of our ELISA protocol. Considering that GXM is exported in extracellular vesicles in the *C. neoformans* model (Oliveira et al., 2009; Rodrigues et al., 2007), we then evaluated whether concentrated vesicle fractions isolated from *P. brasiliensis* supernatants would show reactivity with mAb 18B7, the most efficient antibody in serologic tests with the *Paracoccidioides* glycan. The presence of vesicles in ultracentrifugation fractions was confirmed by the positive detection of sterols in lipid extracts (Fig. 3A). ELISA with mAb 18B7 revealed a dose-dependent recognition of the extracellular vesicle fraction (Fig. 3B), suggesting that



**Fig. 1.** A surface glycan of *P. brasiliensis* (Pb18) yeast cells shares serologic properties with cryptococcal GXM. (A) Reactivity of *P. brasiliensis* surface components with different monoclonal antibodies (mAbs 18B7, 2D10 and 13F1) generated to *C. neoformans* GXM is shown. Fluorescent reactions were not observed when primary antibodies were replaced with isotype-matched irrelevant antibodies, as illustrated for IgG1 in the 'None' panel. Similar results were obtained with irrelevant IgMs and TRITC-labeled anti-IgM antibodies (not shown). *P. brasiliensis* cells yeast cells are shown under differential interference contrast (DIC) and fluorescence (anti-GXM) modes. Image merging ('merge' panel) demonstrates that the molecules recognized by the anti-GXM antibodies are externally associated with the cell wall. (B) Immunofluorescence analysis of *P. brasiliensis* yeast cells incubated with mAbs 18B7 and 2D10 in the presence of varying concentrations of the GXM-related glycan fraction. Increasing concentrations of the glycan efficiently inhibited recognition of yeast cells by the GXM antibodies. *P. brasiliensis* cells yeast cells are shown under DIC and fluorescence (mAbs 18B7 and 2D10) modes. Scale bar, 20  $\mu\text{m}$ .



**Fig. 2.** Serologic reactivity of *P. brasiliensis* glycans with antibodies originally raised to cryptococcal GXM. (A) Dose-dependent serologic reactivity of a DMSO extract from *P. brasiliensis* with different monoclonal antibodies raised to cryptococcal GXM (18B7, IgG; 2D10 and 13F1, IgMs) as measured by ELISA. Negative controls included similar serologic tests with DMSO extracts obtained from *S. cerevisiae* or *C. albicans*. (B) Reactivity of fungal glycans with mAb 18B7 after de-O-acetylation by alkali treatment. De-O-acetylation affected the recognition of cryptococcal GXM (Cn glycan) by mAb 18B7. The *P. brasiliensis* fraction (Pb glycan) was not affected by treatment with alkali. (C) DMSO extracts from yeast cells and mycelia show similar profiles of recognition by mAb 18B7. Results in B and C are representative of three independent dot blot analyses.

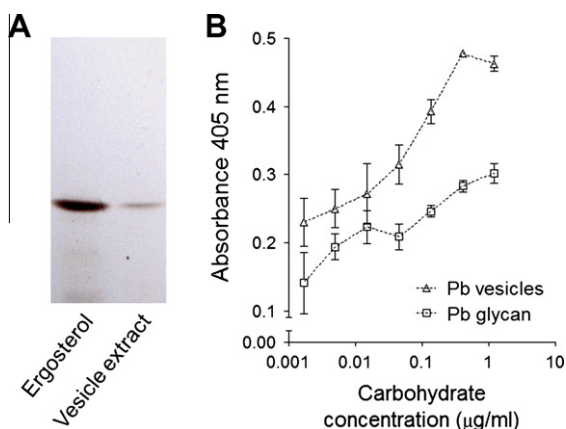
the GXM-related *P. brasiliensis* glycan is also exported extracellularly in secretory vesicles.

The serologic reactivity of molecules in the *P. brasiliensis* extract with antibodies to *C. neoformans* GXM was suggestive of structural similarities in glycans produced by both fungal species. To address this possibility, the carbohydrate composition of the *P. brasiliensis* DMSO extract was analyzed by biochemical methods. After acidic

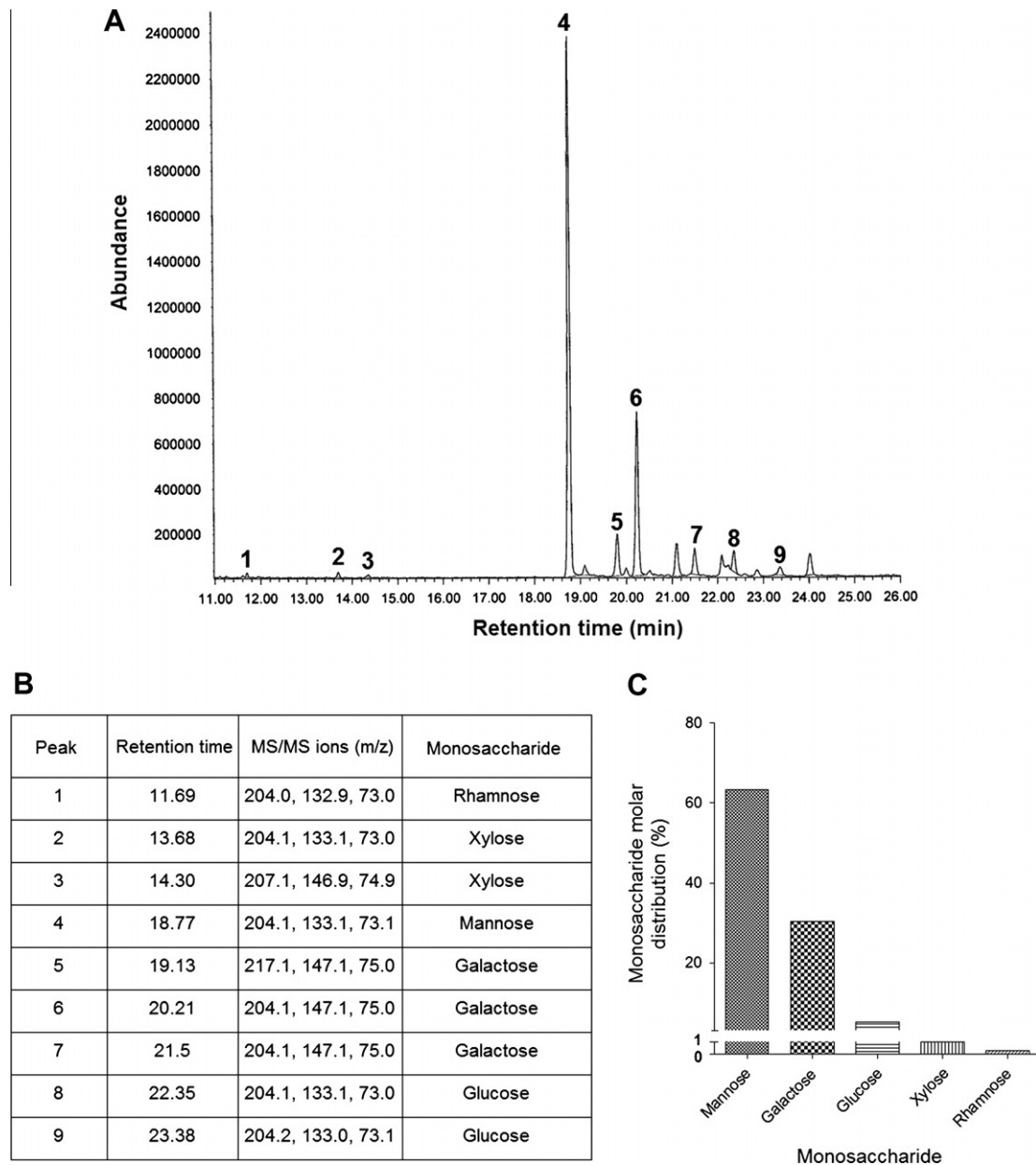
methanolysis of the sample, monosaccharide constituents were analyzed by GC. Classic criteria were used to interpret the results of the GC analysis allowing for the potential detection of four different peaks for each sugar derivative, corresponding to the  $\alpha$ - and  $\beta$ -forms of furanose and pyranose rings. In combination with MS analysis, each peak could be precisely identified, based on the profile of fragmentation observed. Fragmentation of TMS-derivatives of hexoses usually generates diagnostic peaks at  $m/z$  217 and 214 (Kamerling et al., 1975; Vliegthart and Kamerling, 1975). Pyranose rings give rise to a ratio  $(m/z\ 204)/(m/z\ 217) > 1$ , whereas furanose rings show a ratio  $< 1$ . Based on the principles detailed above, peaks with retention times and fragmentation profiles corresponding to mannopyranose (63.3%; relative intensity) and anomers of galactose (30.3%; relative intensity), glucose (5.2%; relative intensity), xylose (1%; relative intensity) and rhamnose (0.3%; relative intensity) were identified (Fig. 4). Therefore, the glycan nature of the *P. brasiliensis* DMSO extract was confirmed, suggesting the presence of polysaccharides constituted primarily of mannosyl and galactosyl units and smaller amounts of glucose, xylose and rhamnose.

### 3.3. Molecular dimensions of *C. neoformans* and *P. brasiliensis* glycans

The fact that *P. brasiliensis* lacks a true capsule, but has the ability to synthesize molecules that are similar to cryptococcal GXM, led us to evaluate whether the dimensions of glycans from this fungus and *C. neoformans* GXM were comparable. Glycans from both species were analyzed by dynamic light scattering.



**Fig. 3.** Analysis of *P. brasiliensis* extracellular vesicles. (A) Detection of sterols by HPTLC in extracellular vesicle fractions isolated from culture supernatants. (B) Dose-dependent recognition of *P. brasiliensis* vesicle (Pb vesicles) and cellular glycan (Pb glycan) fractions by mAb 18B7.



**Fig. 4.** Monosaccharide composition of the polysaccharide fraction obtained from *P. brasiliensis* Pb18 by DMSO extraction. Methanolysis and derivatization of the components extracted with DMSO followed by separation by gas chromatography (A) revealed the presence of different peaks (1–9) corresponding to carbohydrate units. Mass spectrometry analysis of each of these peaks (B) resulted in the identification of abundant mannose and galactose, as well as of trace amounts of glucose, xylose and rhamnose. The relative molar distribution of each monosaccharide in the DMSO extract is shown in C.

*P. brasiliensis* produced glycan fractions with a size distribution ranging from 1500 to 2500 nm, while the *C. neoformans* molecules ranged from 6000 to 9000 nm (Fig. 5). This finding is consistent with the surface morphological features of each pathogen in the sense that larger fibrils are used to form the cryptococcal capsule (Frasers et al., 2009).

#### 3.4. Binding of *P. brasiliensis* glycans to the cell surface of an acapsular mutant of *C. neoformans*

The Cap67 mutant of *C. neoformans* is believed to lack a capsule due to the inability to export GXM but retains the ability to incorporate exogenously provided capsular components onto its cell wall (García-Rivera et al., 2004; Reese and Doering, 2003). Based on this property, the ability of the *P. brasiliensis* glycans to interact with surface components of *C. neoformans* was tested in an immu-

nofluorescence model. Incubation of acapsular *C. neoformans* cells in the presence of the *P. brasiliensis* glycan fraction extracted with DMSO resulted in binding to the cryptococcal surface (Fig. 6). Glycan incorporation by acapsular cryptococci differed when purified GXM or the *P. brasiliensis* extract were used. The use of mAbs 18B7 and 2D10 as detection probes revealed that the acapsular mutant bound to GXM to form an annular pattern of surface coating. In contrast, mAb 13F1 produced a punctate pattern of antibody, which is in agreement with a previous report (Cleare and Casadevall, 1998). However, punctate binding occurred with all of the mAbs when incubated with Cap67 cells coated with the *P. brasiliensis* glycan fraction. These results indicate that, although cryptococcal cell wall components interact with polysaccharide fractions obtained from both *C. neoformans* and *P. brasiliensis*, this process varies depending on what glycan is used, possibly suggesting structural differences.



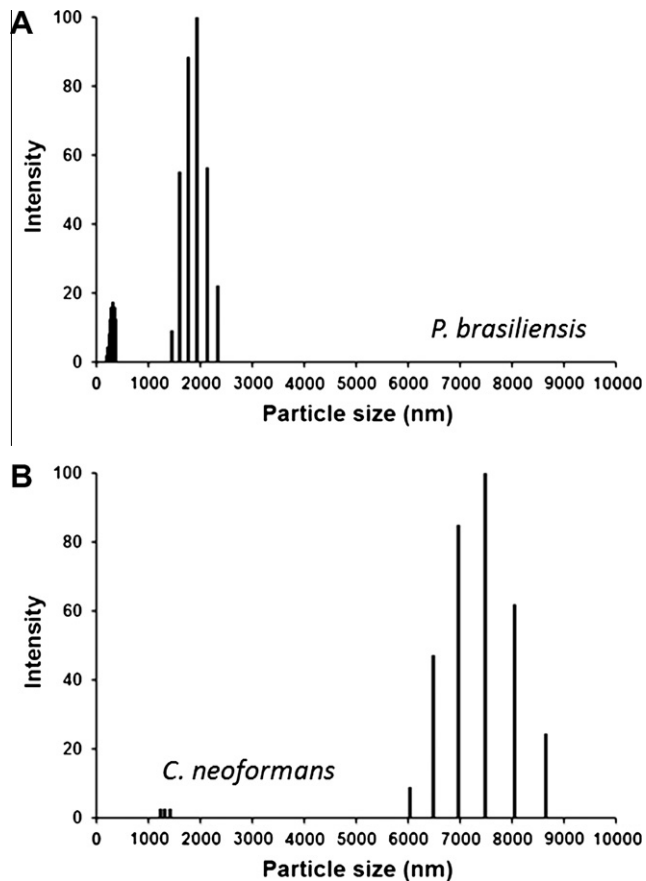


Fig. 5. Size distribution of glycan fractions obtained after DMSO extraction of *P. brasiliensis* Pb18 (A) and encapsulated *C. neoformans* H99 (B) cells.

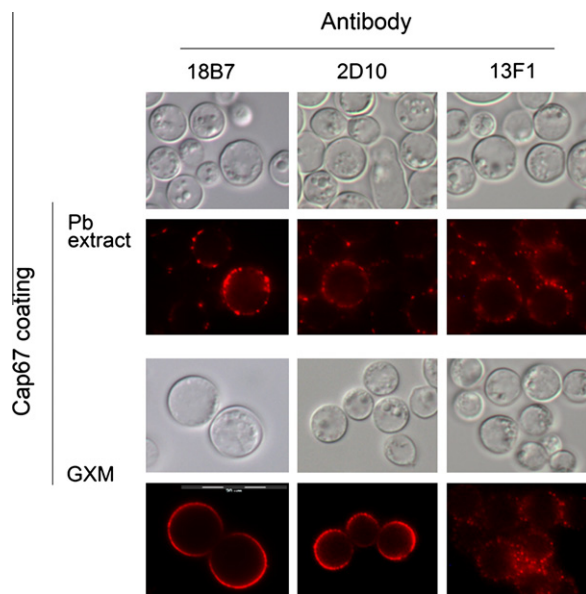


Fig. 6. Incorporation of *P. brasiliensis* Pb18 (Pb) molecules by an acapsular mutant (Cap67) of *C. neoformans*. Cap67 cells were treated with the Pb extract or with GXM purified from *C. neoformans* H99 cultures followed by incubation with mAbs raised to GXM. Positive reactions were observed for all antibodies, but the pattern of reactivity depended on which polysaccharide fraction was used to coat Cap67 cells. Scale bar, 20  $\mu$ m.

In the vast majority of the coating experiments, the *P. brasiliensis* material was found to be tightly associated to the cell wall of

Cap67 cells (Fig. 6). However, different patterns of interaction of the *P. brasiliensis* glycans with acapsular cryptococci were occasionally observed. In these rare cases, DIC images suggested the formation of extracellular aggregates that were not closely attached to the cell wall of the acapsular *C. neoformans* mutant (Fig. 7). Analysis of these cells by immunofluorescence with mAb 18B7 revealed the existence of surface structures that resemble a capsular network, which was confirmed by SEM. Although most of the cells did not contain these surface structures, we found that approximately 5% of the cells formed surface networks that resembled fungal capsules. We therefore concluded that the *P. brasiliensis* glycan is recognized by components of the cryptococcal cell wall with possible formation of glycan networks that may be related to fungal capsules.

### 3.5. Binding to *P. brasiliensis* glycans reduces the efficacy of association of the acapsular mutant of *C. neoformans* with host phagocytes

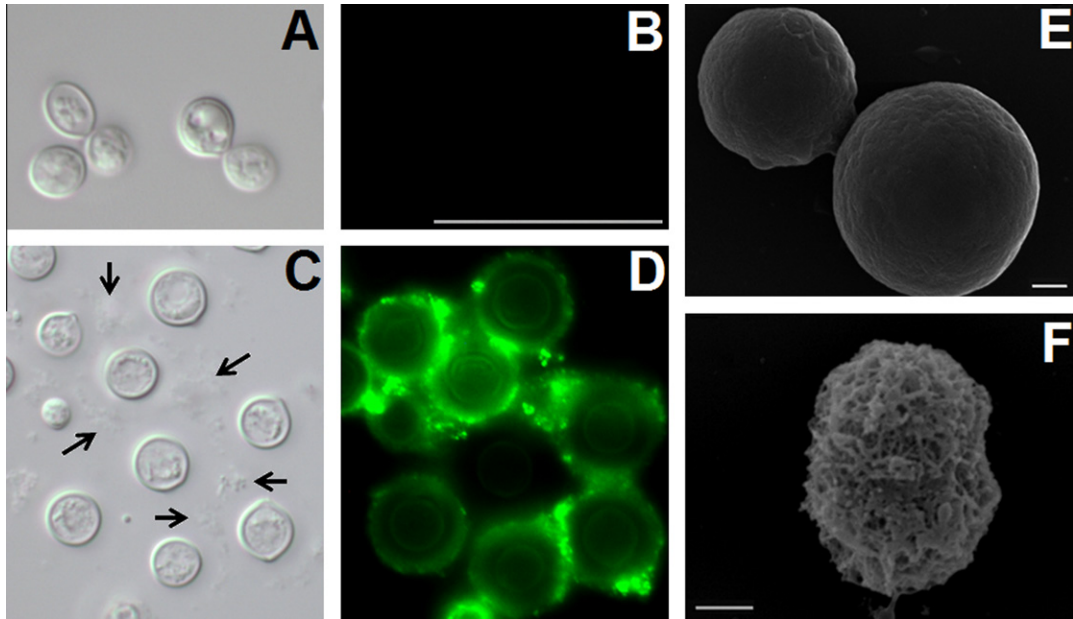
Based on the results presented in Figs. 6 and 7, we asked whether coating Cap67 cells with the *P. brasiliensis* glycan fraction would affect the interaction of the mutant with macrophages, since protection against phagocytosis is one of the primary functions of cryptococcal GXM. We then compared the ability of uncoated Cap67 cells to interact with phagocytic cells relative to that observed for acapsular cells coated with the *P. brasiliensis* glycan fraction. As a control, we used Cap67 cells coated with *C. neoformans* H99 GXM. As expected, GXM-coated Cap67 cells were about 40% less efficient in their interactions with phagocytic cells relative to the uncoated acapsular cells (Fig. 8A). Coating of the mutant with the *P. brasiliensis* glycan resulted in the ability of the *C. neoformans* mutant to associate with RAW macrophages, in comparison with uncoated yeast cells (Fig. 8B). Therefore, in quantitative terms, the *P. brasiliensis* glycan fraction was approximately 50% more effective than GXM in inhibiting the interaction of *C. neoformans* with RAW phagocytes (Fig. 8C).

To compare indices of uncoated and glycan-coated Cap67 adhesion to and internalization by phagocytes, infected macrophages were treated with trypan blue after infection with *C. neoformans*. Trypan blue extinguishes extracellular fluorescence, which implies that the relative fluorescence reduction of the macrophages after exposure to this dye will correspond to the percentage of yeast cells that adhered to but were not internalized by the macrophages. Fluorescence intensity was reduced by about 20% after exposure of macrophages that were infected with uncoated Cap67 cells to trypan blue (Fig. 8D), which is in agreement with the observation that acapsular variants of *C. neoformans* are efficiently phagocytized (Kozel and Gotschlich, 1982). When macrophages that were infected with GXM-coated Cap67 cells were treated with trypan blue, a decrease in fluorescence intensity in the range of 30% was observed (Fig. 8E). In similar systems where the acapsular mutant was coated with the *P. brasiliensis* glycan, fluorescence reduction corresponded to 13% (Fig. 8F). Therefore, in all cases most of the fungal cells were internalized after incubation in the presence of host phagocytes, although the variation in fluorescence reduction fluctuated in the three different systems.

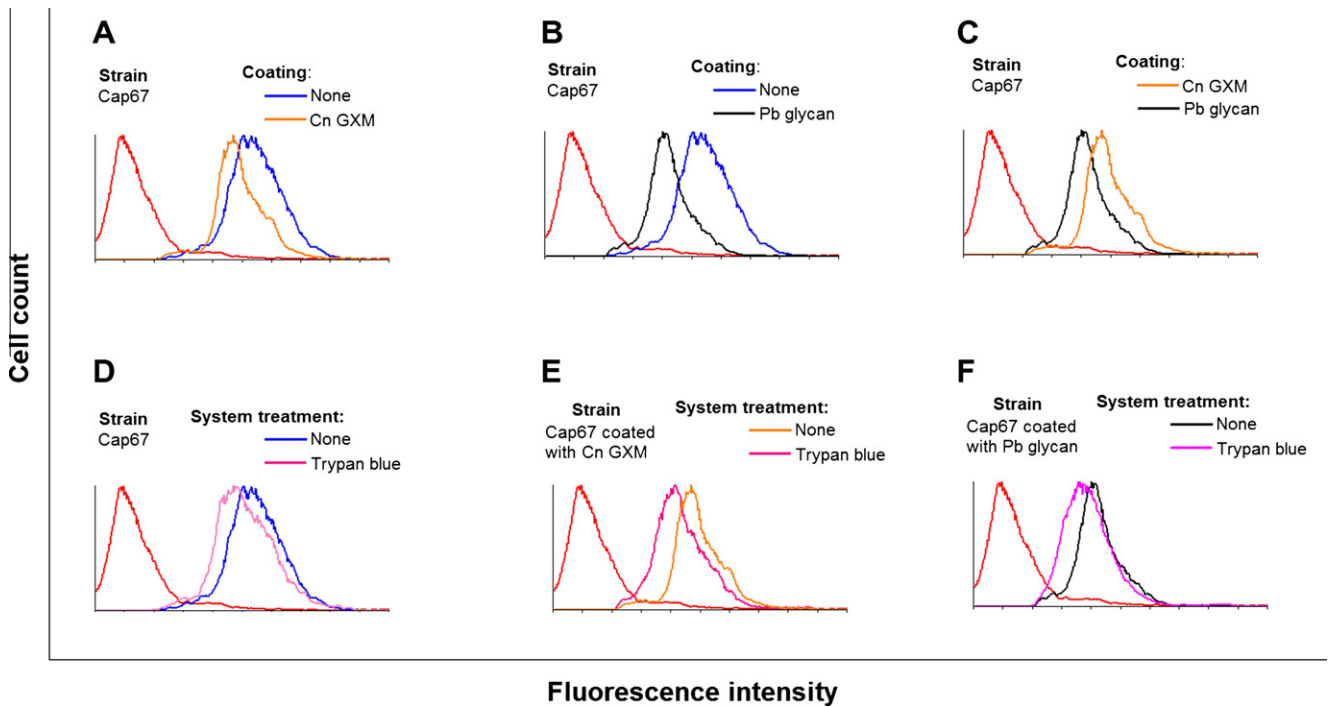
## 4. Discussion

Polysaccharides are key regulators of fungal pathogenesis. In different fungal pathogens, neutralization of polysaccharides with antibodies, as well as the induction of immune responses to polysaccharides, results in modification of the course of infection in favor of the host (Casadevall et al., 1998; Casadevall and Pirofski, 2006; Pirofski, 2001). In this context, it seems clear that





**Fig. 7.** Coating of Cap67 *C. neoformans* cells with the *P. brasiliensis* Pb18 extract results in the formation of capsule-like structures. (A and B) Analysis of the *C. neoformans* acapsular mutant after incubation in the absence of polysaccharide fractions (control) followed by exposure to mAb 18B7 by fluorescence microscopy. No serologic reactivity was observed. Incubation of Cap67 cells with the DMSO extract from *P. brasiliensis* results in the formation of extracellular aggregates (C, arrows) that resembled capsular structures when observed by fluorescence microscopy (D). Differential interference contrast (A and C) and fluorescence (B and D) modes are shown. Results shown in C–D were confirmed by scanning electron microscopy of control (E) or glycan-coated (F) Cap 67 cells. Scale bars correspond to 20  $\mu$ m in panels for A–D, 1  $\mu$ m in E and 2  $\mu$ m in F.



**Fig. 8.** Coating of *C. neoformans* Cap67 (Cn) acapsular cells with *P. brasiliensis* Pb18 (Pb) molecules renders yeast cells less efficient in their ability to associate with macrophage-like cells. To measure the association of the Cap67 mutant with host cells, fungi were stained with FITC and incubated with the phagocytes, which were then analyzed by flow cytometry. (A) Comparison between the indices of association of macrophages with control Cap67 cells (no coating) or with the GXM-coated mutant. (B) Coating of Cap67 cells with the *P. brasiliensis* glycan fraction caused a decrease in the index of association of the acapsular mutant with macrophages. (C) Analysis of the efficacy of coating Cap67 cells with *C. neoformans* GXM or with the *P. brasiliensis* glycan fraction to inhibit the association of yeast cells with phagocytes. To differentiate adhered and internalized yeast cells, infected macrophages were treated with trypan blue, an extinguisher of extracellular FITC-derived fluorescence (D–F). The shift of histograms to regions of lower fluorescence after exposure to trypan blue denotes the presence of extracellularly-associated yeast cells. This figure is representative of three different experiments producing similar results.

understanding the role of polysaccharides in fungal infections is a key strategy for the design of new therapeutics.

$\alpha$ 1,3-Glucans are found in the cell walls of several pathogenic fungi, including *C. neoformans* (Reese and Doering, 2003; Reese

et al., 2007), *H. capsulatum* (Klimpel and Goldman, 1988), *Blastomyces dermatitidis* (Hogan and Klein, 1994) and *P. brasiliensis* (San-Blas and Vernet, 1977). In *H. capsulatum*, gene depletion studies have demonstrated that the virulence of certain strain types requires  $\alpha$ 1,3-glucan (Rappleye et al., 2004). Both RNA interference (RNAi)-mediated reduction in the synthase for  $\alpha$ 1,3-glucan (*AGS1*) and traditional allelic replacement substantially reduced the ability of the fungus to proliferate and kill macrophages in culture and decreased lung fungal burden in a murine infection model. In *P. brasiliensis*, a similar relationship between virulence and  $\alpha$ 1,3-glucan has been described, as concluded from the observation that spontaneous loss of the polysaccharide correlated with decreased virulence (San-Blas and San-Blas, 1977). In *C. neoformans*, the lack of  $\alpha$ 1,3-glucan synthesis correlates with an acapsular phenotype, which was accompanied by disorganization of the cell wall, altered polysaccharide composition, and an avirulent phenotype (Reese and Doering, 2003; Reese et al., 2007). The lack of visible capsules in the strain lacking  $\alpha$ 1,3-glucan was not a consequence of altered GXM synthesis, since cells with altered  $\alpha$ 1,3-glucan synthesis continued to shed capsule material into the environment (Reese and Doering, 2003). This observation led to the conclusion that  $\alpha$ 1,3-glucan was responsible for anchoring surface GXM. In fact, capsule material from *C. neoformans* efficiently bound only to  $\alpha$ 1,3-glucan-producing *H. capsulatum* strains (Reese and Doering, 2003). These cells showed a blurred and very intense staining with a monoclonal antibody to GXM, resembling capsular structures (Reese and Doering, 2003). Apparently, no direct staining of *H. capsulatum* with antibodies to GXM was performed by the authors, implying that the possibility that *H. capsulatum* also produces GXM-like polymers could not be ruled out at present.

*P. brasiliensis* is a non-encapsulated fungal pathogen. An early report, however, indicates the occurrence of cell wall-associated fibrillar material in this species (Carbonell, 1967; Carbonell et al., 1970). Nevertheless, it is inaccurate to state that *P. brasiliensis* synthesizes a true capsule. Our results, however, suggest that a *P. brasiliensis* glycan fraction shares structural, functional and serologic properties with *C. neoformans* GXM. The *P. brasiliensis* glycan sharing epitopes with cryptococcal GXM was surface-associated, but its detection in DMSO extracts raised additional possibilities. Considering that (i) concentrated DMSO is very efficient in damaging the plasma membrane by solubilizing lipids and (ii) *C. neoformans* GXM is synthesized intracellularly and then exported to the extracellular space (Rodrigues et al., 2007, 2011b), we cannot rule out the possibility that the GXM-related components of the *P. brasiliensis* glycan are also found in intracellular compartments.

Glycans sharing serologic properties with *C. neoformans* GXM were detected in both mycelia and yeast cells. Given that  $\alpha$ 1,3-glucan is found exclusively at the cell wall of yeast forms of *P. brasiliensis* (San-Blas and Vernet, 1977), this observation suggests that other molecules interact with the GXM-related structures in *Paracoccidioides* yeast cells, as was previously described for chitin in *C. neoformans* and *T. asahii* (Fonseca et al., 2009a, 2009b; Ramos et al., 2012; Rodrigues et al., 2008a). The molecular dimensions of the *P. brasiliensis* glycan were smaller than those reported for *C. neoformans* H99 GXM and similar to those from hypocapsular *C. neoformans* (Kmetzsch et al., 2011), which is consistent with the acapsular morphology of *P. brasiliensis*. Nevertheless, acapsular *C. neoformans* cells were able to use the *P. brasiliensis* glycans to construct surface structures that resembled capsular networks, indicating that both species used similar polysaccharides to protect themselves against the immune defense and environmental predators. In fact, several mechanisms of pathogenicity and immunological escape are shared by *C. neoformans* and *P. brasiliensis*, including pigmentation (Gomez et al., 2001; Wang et al., 1995), production of extracellular matrix-degrading proteases (Puccia et al., 1998;

Rodrigues et al., 2003), sialylation of surface glycoproteins (Rodrigues et al., 2002; Soares et al., 1998), synthesis of immunogenic glycosylceramides (Bertini et al., 2007; Rodrigues et al., 2000), ability to attach to host cells in the lung (Barbosa et al., 2006; Gonzalez et al., 2008) and vesicular secretion of virulence factors (Eisenman et al., 2009; Rodrigues et al., 2008b, 2007; Vallejo et al., 2011, 2012b). Our results are indeed suggestive that extracellular vesicles are involved in export of the currently studied *P. brasiliensis* glycan. In *C. neoformans* and *P. brasiliensis*, extracellular vesicles have been demonstrated to be immunologically active (Oliveira et al., 2010a; Rodrigues et al., 2008b; Vallejo et al., 2011), which is likely influenced by the presence of GXM and other glycans in these secretory compartments.

Our analysis of the *P. brasiliensis* homologs corresponding to proteins involved in *C. neoformans* GXM synthesis, capsule formation and polysaccharide export was consistent with our experimental findings and with the literature in the field. For instance, two of the three proteins involved in capsule formation in *C. neoformans* (Chang and Kwon-Chung, 1998; Chang et al., 1996) were not found in *P. brasiliensis*, which is consistent with the lack of a capsule in this pathogen and with the reduced dimensions of its glycans that have antigenic similarities to GXM. In fact, polysaccharide enlargement was demonstrated to be essential for capsule growth in *C. neoformans* (Frasen et al., 2009). On the other hand, *P. brasiliensis* had homologs for the genes required for *C. neoformans* GXM synthesis and export, except *O*-acetylation. These observations are in agreement with serologic, biochemical and microscopic data suggesting that *P. brasiliensis* produces a surface glycan likely representing a substituted mannan. The synthesis of mannans does not necessarily translate to capsule formation, since it is well known that, in *C. neoformans*, capsular assembly depends also on the interaction of GXM with other components, including glucuronoxylomannogalactan (GXMGal) (De Jesus et al., 2009). This observation may be also related to the fact that some of the acapsular *C. neoformans* cells used the *P. brasiliensis* glycan fraction to form surface networks that resembled fungal capsules. The acapsular mutant used in this study is known to be an efficient producer of GXMGal (Vaishnav et al., 1998), which could interact with the *P. brasiliensis* glycan to form capsule-like structures. Based on our current data, it is impossible to affirm that these structures form true capsules, but the possibility that *P. brasiliensis* formed extracellular aggregates that would then be incorporated onto the cryptococcal cell surface with consequent formation of capsule-like structures cannot be ruled out, considering the ability of GXM to self-aggregate (Nimrichter et al., 2007). Another important issue particularly related to this model is the suggestion that, despite the similarities detected in our studies, *P. brasiliensis* and *C. neoformans* glycans are likely to differ in certain structural aspects, as concluded from the different patterns of serologic reactivity after incorporation into the surface of acapsular cells, as well as lack of glucuronic acid as a monosaccharide constituent and absence of *O*-acetylation.

The complete structure of the *P. brasiliensis* glycan still remains to be elucidated. The abundance of mannose and galactose suggests a galactomannan, which has been previously described in this fungus (San-Blas and San-Blas, 1982) and characterized in detail in *Aspergillus* species (Latge, 2009). Interestingly, preliminary data from our laboratory shows that *A. fumigatus* conidia were efficiently labeled by mAb 18B7 (P.C. Albuquerque, E. Barreto-Bergter and M.L. Rodrigues, unpublished). *H. capsulatum* glycans are also recognized by GXM-binding antibodies (A. Guimarães, unpublished) and this fungus also produces antigens that cross-react with galactomannan (Zancope-Oliveira et al., 1994). These observations are all consistent with the notion that the *P. brasiliensis* GXM-related glycan identified in this study is a galactomannan. However, the detection of glucose, xylose and rhamnose may sug-

gest a more complex polysaccharide or, more likely, the presence of different polysaccharides in the same fraction. Improved methods for purification of these *P. brasiliensis* molecules and complete structural elucidation are underway in our laboratory. Serologic epitopes in the *P. brasiliensis* glycan also remain to be determined. Using a set of mAbs that were not related to those used in this study, Kozel and colleagues demonstrated that GXM de-*O*-acetylation affected the reactivities of five of seven anti-GXM antibodies (Kozel et al., 2003). In addition, loss of xylosylation in *C. neoformans* produced a substantive alteration in the binding behavior of one mAb. These results demonstrated that polysaccharide *O*-acetylation and xylosylation are determinant for the serologic reactivity of cryptococcal GXM and support the hypothesis that additional epitopes exist in the *P. brasiliensis* glycan, considering the lack of genes required for mannose *O*-acetylation, resistance to alkali treatment and the trace amounts of xylose found in the glycan fraction obtained from this fungus. Regardless, it seems plausible to suggest, based on our current data, that the synthesis of GXM-related polymers is not restricted to pathogenic *Cryptococcus* species. In fact, different reports demonstrate that fungal species that include *C. liquefaciens* (Araujo Gde et al., 2012) and *Trichosporon asahii* (Fonseca et al., 2009a) produce GXM-like polysaccharides that share structural, antiphagocytic and serologic properties with the polysaccharides produced by *C. neoformans* and *C. gattii*.

Protection against phagocytosis is a principal function of cryptococcal GXM (Kozel and Gotschlich, 1982). In our study, coating acapsular *C. neoformans* cells with exogenous *P. brasiliensis* glycans suggested that the latter molecules could also impact *P. brasiliensis* yeast cell interactions with host effector cells. The *C. neoformans* polysaccharide has diverse additional functions, most of them related to modulation of the immune response (Zaragoza et al., 2009). GXM is severely deleterious to a number of host's immunological mechanisms but, depending on structural particularities and molecular dimensions, the polysaccharide may exert paradoxical functions to stimulate the host response (Fonseca et al., 2010; Rodrigues et al., 2011a). The clear relevance of GXM for disease progress and the uniqueness in its structure and functions make this polysaccharide an attractive target for antifungal therapy. The observation that, as previously described for *Trichosporon* species (Fonseca et al., 2009a), molecules with properties that resembled those described for cryptococcal GXM are found in non-*Cryptococcus* fungal species suggest that antibodies to GXM and/or similar tools may be therapeutic in different models of fungal infections. Since the ability of the *P. brasiliensis* glycan fraction to stimulate the immune response is still unknown, its use in vaccine preparations could be also possible.

Our results add *P. brasiliensis* to the group of fungal pathogens producing GXM-related molecules. Considering the major impact that this polysaccharide exerts in infections caused by *C. neoformans*, *C. gattii* and, possibly, *Trichosporon* species, the possibility that the currently identified glycan impacts the pathogenesis of PCM is intriguing from an evolutionary standpoint as well as representing potential targets for diagnostics and therapeutics. Significantly, the use of mimetic peptides and passively administered protective antibodies to prevent and treat animal and human PCM may represent new promising alternatives to control this fungal infection.

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