The Pathogenic Fungus Paracoccidioides brasiliensis Exports Extracellular Vesicles Containing Highly Immunogenic α-Galactosyl Epitopes

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Exosome-like vesicles containing virulence factors, enzymes, and antigens have recently been characterized in fungal pathogens, such as Cryptococcus neoformans and Histoplasma capsulatum. Here, we describe extracellular vesicles carrying highly immunogenic α-linked galactopyranosyl (α-Gal) epitopes in Paracoccidioides brasiliensis. P. brasiliensis is a dimorphic fungus that causes human paracoccidioidomycosis (PCM). For vesicle preparations, cell-free supernatant fluids from yeast cells cultivated in Ham's defined medium-glucose were concentrated in an Amicon ultrafiltration system and ultracentrifuged at 100,000 × g. P. brasiliensis antigens were present in preparations from phylogenetically distinct isolates Pb18 and Pb3, as observed in immunoblots revealed with sera from PCM patients. In an enzyme-linked immunosorbent assay (ELISA), vesicle components containing α-Gal epitopes reacted strongly with anti-α-Gal antibodies isolated from both Chagas' disease and PCM patients, with Marasmius oreades agglutinin (MOA) (a lectin that recognizes terminal α-Gal), but only faintly with natural anti-α-Gal. Reactivity was inhibited after treatment with α-galactosidase. Vesicle preparations analyzed by electron microscopy showed vesicular structures of 20 to 200 nm that were labeled both on the surface and in the lumen with MOA. In P. brasiliensis cells, components carrying α-Gal epitopes were found distributed on the cell wall, following a punctuated confocal pattern, and inside large intracellular vacuoles. Lipid-free vesicle fractions reacted with anti-α-Gal in ELISA only when not digested with α-galactosidase, while reactivity with glycoproteins was reduced after β-elimination, which is indicative of partial O-linked chain localization. Our findings open new areas to explore in terms of host-parasite relationships in PCM and the role played in vivo by vesicle components and α-galactosyl epitopes.

Paracoccidioides brasiliensis is the fungus responsible for paracoccidioidomycosis (PCM). It is a thermomorphic fungus that grows in the yeast phase at 37°C and as mycelium at temperatures below 26°C. One of the best-acknowledged molecules that parallels this morphological change is an alteration in structural cell wall glucans from molecular features that has recently been achieved using the gp43 and gp70 antigens as targets (11, 15). gp43 is a secreted glycoprotein that elicits both cellular and humoral immune responses; it is the main diagnostic antigen so far characterized in P. brasiliensis (38).

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** Mycobacterium tuberculosis is a dimorphic fungus that causes human tuberculosis (TB). For vesicle preparations, cell-free supernatant fluids from yeast cells cultivated in Ham's defined medium-glucose were concentrated in an Amicon ultrafiltration system and ultracentrifuged at 100,000 × g. M. tuberculosis antigens were present in preparations from phylogenetically distinct isolates Mt18 and Mt3, as observed in immunoblots revealed with sera from TB patients. In an enzyme-linked immunosorbent assay (ELISA), vesicle components containing α-Gal epitopes reacted strongly with anti-α-Gal antibodies isolated from both Chagas' disease and TB patients, with Marasmius oreades agglutinin (MOA) (a lectin that recognizes terminal α-Gal), but only faintly with natural anti-α-Gal. Reactivity was inhibited after treatment with α-galactosidase. Vesicle preparations analyzed by electron microscopy showed vesicular structures of 20 to 200 nm that were labeled both on the surface and in the lumen with MOA. In M. tuberculosis cells, components carrying α-Gal epitopes were found distributed on the cell wall, following a punctuated confocal pattern, and inside large intracellular vacuoles. Lipid-free vesicle fractions reacted with anti-α-Gal in ELISA only when not digested with α-galactosidase, while reactivity with glycoproteins was reduced after β-elimination, which is indicative of partial O-linked chain localization. Our findings open new areas to explore in terms of host-parasite relationships in TB and the role played in vivo by vesicle components and α-galactosyl epitopes.

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debris (33). In C. neoformans, membranous vesicles carry glucuronoxylomannan (GXM), the major capsular polysaccharide, to the extracellular space. The polysaccharide is then released and reincorporated into the cell surface as a parallel mechanism of capsule growth (32, 33, 42). Vesicles containing GXM have also been shown to be produced during macrophage infection, suggesting a role in the pathogenesis of the microorganism (26). Indeed, proteomic and lipidomic analyses of vesicles isolated from culture supernatants from both C. neoformans (32) and H. capsulatum (3) have evidenced the presence of virulence factors and enzymes. Microscopic analysis identified a heterogeneous population of extracellular vesicles, not only in C. neoformans (32, 33), but also in H. capsulatum, Saccharomyces cerevisiae, Candida albicans, Candida paedalispsis, and Sporothrix schenckii (3, 27), pointing to the existence of sophisticated mechanisms of vesicle biogenesis in fungi (14). Troccoli Torrecillas et al. (37) described the role of extracellular vesicles from Trypanosoma cruzi in the pathogenesis of Chagas’ disease. Major components of extracellular vesicles from T. cruzi tryptomastigotes are glycoproteins of the gp85 trans-sialidase superfamily and glycoconjugates bearing α-linked galactosyl epitopes (α-Gal). Cell components bearing α-Gal epitopes are recognized by anti-α-Gal IgG antibodies that can be isolated from patients with chronic Chagas’ disease by affinity chromatography using immobilized Galα1-3Galβ1-4GlcNAc (6). Chagasic (Ch) anti-α-Gal IgG is a lytic antibody, i.e., it can agglutinate and cause parasite lysis by a complement-mediated (6) or -independent (4, 5, 29) mechanism.

In the present work, we characterized extracellular vesicles from P. brasiliensis isolates Pb18 and Pb3. Pb18 represents the major paraplagyrlic group S1 of the Paracoccidioides complex (23, 36) and has been widely used by researchers due to its virulence (12); Pb3 represents a cryptic PS2 species. We have previously shown that the progression of experimental PCM in B10.A mice differed when infections caused by isolates from S1 (more virulent) and PS2 isolates were compared (13). We show here that vesicles from both isolates carry antigenic molecules that are recognized by total sera and anti-α-Gal antibodies from PCM patients. We also demonstrate that P. brasiliensis is enriched with glycoconjugates containing α-galactosyl epitopes, which are largely expressed on the fungal cell wall and are stored in intracellular vacuoles.

MATERIALS AND METHODS

P. brasiliensis isolates and growth conditions. P. brasiliensis isolates Pb3 and Pb18 (described in reference 24) were used in this work. The isolates were maintained in the yeast phase at 36°C in slants to Erlenmeyer flasks containing 200 ml of defined Ham’s F-12 medium (0.5% yeast extract, 0.5% casein peptone, and 1.5% glucose, pH 6.5). Cultures were maintained in the yeast phase at 36°C in slants of modified YPD (mYPD) 
Pb18 (described in reference 24) were used in this work. The isolates were

4,000 culture supernatants following subtle modifications of the protocol described by Rodrigues et al. (33). All the steps were carried out in ice or at 4°C to avoid vesicle rupture and/or fusion. Sequential centrifugations of liquid cultures at 4,000 x g (15 min) and 15,000 x g (30 min) were followed to remove whole cells and smaller debris. The pellets were discarded, and the cell/debris-free supernatants were concentrated 20-fold using an Amicon ultrafiltration system (100-kDa cutoff). Concentrated supernatants containing high-molecular-weight components were centrifuged at 15,000 x g (30 min) to remove aggregates, and the resulting supernatant was then ultracentrifuged at 100,000 x g for 1 h to precipitate vesicles. The pellets were either suspended in phosphate-buffered saline (PBS) (250 to 300 µl) for Western blotting and enzyme-linked immunosorbent assay (ELISA) analyses or processed for cryosections and immunogold assays.

Preparation of whole-cell lysates. P. brasiliensis yeast cell pellets were washed twice in PBS containing proteinase inhibitors (1 nM phenylmethylsulfonyl fluoride, 1 µM pepstatin A, 1 mM o-phenanthroline, and 10 µM E-64 [trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane]), mixed with similar volumes of glass beads (425 to 600 µm; Sigma) and double the volume of PBS, and then vigorously agitated in a vortex (15 times for 30 s with 30-s intervals in ice). The cell pellet was pelleted by centrifugation (5,600 x g), and the supernatant was stored in aliquots at −20°C for further analysis.

Vesicle fractionation and analysis by HPTLC. For sterol analysis by high-performance thin-layer chromatography (HPTLC), extracellular vesicle pellets from P. brasiliensis Pb18 were processed as described by Rodrigues et al. (33). The pellets were suspended in methanol, and two volumes of chloroform were added. The mixture was vortexed and centrifuged to discard precipitates, and the supernatants were dried by vacuum centrifugation and then partitioned according to the method of Folch et al. (16). The lower phase, containing neutral lipids, was loaded into HPTLC silica plates (Si 60F254s; LiChrospher, Germany) and separated using a system containing hexane-ether-acetic acid (80:40:2 [vol/vol/vol]). The sterol spots were identified after the plate was sprayed with a solution of 50 mg ferric chloride (FeCl3) dissolved in a mixture of 90 ml H2O, 5 ml acetic acid, and 5 ml sulfuric acid. The sprayed plates were incubated at 100°C for 5 min. For ELISA, analysis of lipid and lipid-free vesicle fractions, vesicle preparations were extracted with methanol-chloroform, as described previously; both the pellet (lipid free) and the dried supernatant (lipids) were then analyzed.

Isolation of anti-α-galactosyl IgG from patients’ sera. CH and PCM anti-α-Gal were isolated through affinity chromatography on Synsorb 115 resin containing the trisaccharide Galα1-3Galβ1-4GlcNAc (Chemiobiomed, Edmonton, Canada) and subsequently on protein A-Sepharose (Amersham Biociences), as described by Almeida et al. (6). Anti-α-Gal antibodies from healthy individuals (normal human serum [NHS] anti-α-Gal) were isolated similarly. IgG was quantified by optical density readings (1.0 A280 unit = 750 µg/ml IgG).

TEM and immunogold labeling. For transmission electron microscopy (TEM) analysis of P. brasiliensis yeasts, cells from logarithmic-phase cultures grown in liquid mYPD medium were processed in fixative solutions and embedded in hydrophobic Spurr resin exactly as described by Batista et al. (9). For immunogold labeling, both P. brasiliensis Pb18 cells and vesicle pellets obtained after centrifugation at 100,000 x g were processed as described by Rodrigues et al. (33). Briefly, cryosections were obtained in a temperature range from −70°C to −90°C using an Ultracut cryoultramicrotome (Reichert). After being blocked in acetylated bovine serum albumin (BSA) (Aurion)-PBS and 50 mM NH4Cl, the cryosections were incubated overnight at 4°C with 20 µg/ml of gold-labeled Malusus sarmatula agglutinin (MOA) lectin (EY Laboratories; 5 nm) or Ch anti-α-Gal antibodies from healthy individuals (normal human serum [NHS] anti-α-Gal) were isolated similarly. IgG was quantified by optical density readings (1.0 A280 unit = 750 µg/ml IgG).

Confocal microscopy. P. brasiliensis yeast cells from isolate Pb18 growing in F-12/glc aerated liquid cultures in logarithmic phase were collected, washed twice in PBS, adjusted to 3 x 107 viable cells/ml, and fixed for 30 min in cold methanol (80%), 4% formaldehyde, 1% glutaraldehyde, which also made the cells permeable. Cell pellets were incubated with 3% (wt/vol) BSA in PBS (blocking buffer) for 4 h at room temperature, washed three times with PBS, and then incubated with 100 µg/ml of CH, PCM, or NHS anti-α-Gal IgG for 16 h at 4°C. The cells were then incubated for 2 h in the dark with Alexa Fluor 488 goat anti-human IgG (Invitrogen) in blocking buffer at 1:20 dilution. Between incubation steps with antibodies, the fungal cells were washed six times with PBS. Each suspension (10 µl) was prepared on glass slides with Vectashield mounting medium (Vector Laboratories, Inc., Burlingame, CA) and analyzed using a laser scanning confocal microscope (LSM-510 Axiovert; Carl Zeiss, Jena, Germany).

ELISA and immunoblotting. To analyze the reactivity of both biotinylated MOA and CH anti-α-Gal IgG with vesicle preparations and whole-cell extracts from P. brasiliensis Pb3 and Pb18, 96-well microplates (Nunc) were coated with 1 nM vesicle sterol contents (estimated with the Amplex Red Cholesterol Assay
kit) or cell extracts expressed as protein contents (estimated with Bradford’s protein assay [10]). When applied, treatment with α-galactosidase was carried out with substrate immobilized to the microplates. The microplates were incubated overnight at 4°C, and nonspecific interactions were blocked with 5% skim milk in PBS for 4 h at 36°C or with 0.1% BSA-PBS overnight at 4°C. A concentration of 70 μg/ml of biotinylated MOA or 2.5 to 10 μg/ml Ch anti-α-Gal diluted in 0.1% BSA-PBS was used to react with vesicle preparations upon incubation for 4 h at 36°C or overnight at 4°C. This step was followed by a 1-h incubation with peroxidase-conjugated sheep anti-human IgG or peroxidase-conjugated streptavidin (1:1,000 in 0.1% BSA-PBS). The reactions were visualized using a chemiluminescent substrate (Pierce or Millipore ECL substrate), and the results were recorded in a microplate luminometer. ELISA with lipid fractions extracted from vesicles was carried out as described previously (25), with solvent-washed 96-well plates coated with lipids diluted in methanol and evaporated to dryness at room temperature. ELISA data were recorded (in relative luminescence units [RLU]) as mean values from triplicates, with standard deviations. The values of negative controls in the absence of either antibodies or substrate were subtracted from the values obtained for test samples.

For immunoblotting, about 6% of one vesicle preparation batch (500 ml culture) from P. brasiliensis was subjected to electrophoresis in a 10% SDS-polyacrylamide gel (21) and electrotransferred to nitrocellulose membranes. The incubation conditions with antibodies and conjugates were as described above for ELISA and elsewhere (17). For silver staining (8), about half of a vesicle preparation (500 ml culture) was necessary for band visualization.

Deglycosylation with endoglycosidase H and β-elimination. Vesicle and whole-cell extract preparations of P. brasiliensis Pb18 were treated with endoglycosidase H (Endo H; Sigma Aldrich) to remove high-mannose N-linked oligosaccharides in 50 mM sodium citrate buffer, pH 5.0, for 48 h at 36°C. Control samples were incubated in the absence of enzyme. Chemical treatment with 1 M NH4OH for 48 h at 36°C was used to remove O-linked glycan chains (20). The proteinase inhibitors E-64 (15 μM) and pepstatin (1 μM) were added in all reactions.

Statistical analysis. We used Student’s t test to analyze the significance of our results.

RESULTS

Characterization of extracellular vesicles. The presence of extracellular vesicles produced by P. brasiliensis was evidenced by TEM. In rare images, vesicles could be seen at the surface of the cell wall in preparations of yeast cells growing in both mYPD medium (Fig. 1A) and F-12/glc (not shown). In order to isolate extracellular vesicles from culture supernatants, yeast cells were cultivated for 48 h in F-12/glc medium starting from heavy preinocula constituted of cell precipitates from 5-day-old logarithmic cultures. The estimated average sterol yields from two different preparations were 38 ng sterol/ml of cells (wet pellet) for Pb3 and 65 ng sterol/ml of cells for Pb18. Preparations from 500-ml cultures resulted in wet cell pellets of about 6 ml for Pb3 and 8 ml for Pb18. Figure 1B shows the presence of vesicles smaller than 100 nm in a representative 100,000 g pellet. The lipid bilayer membrane can be seen in a representative vesicle (Fig. 1C and D, labeled with MOA-gold particles).

To confirm that the vesicles originated from exosomal bilayer formation and not from membranes released from dead cells, we analyzed a control 100,000 g pellet preparation obtained from culture supernatants of heat-killed P. brasiliensis yeast cells, in parallel with live cells. HPTLC detected sterols migrating next to ergosterol only in preparations from live-cell cultures (Fig. 2), suggesting that membranes did not result from cell debris. Similar results have previously been obtained for C. neoformans (33).

Preliminary evidence of extracellular vesicles in P. brasiliensis cultures has been obtained by our group from a series of studies with the Pb339 isolate (A. L. Matsuo, L. Ganiko, I. C. Almeida, and R. Puccia, unpublished data). In the present work, we used Pb18 as a model but compared some antigenic characteristics with Pb3 vesicle preparations. Silver-stained SDS-PAGE profiles of vesicle preparations from Pb18 and Pb3 were similar, with visible components migrating within molecu-
ular masses between 98 and 50 kDa (Fig. 3). The stacking gel did not stain, suggesting that most components had indeed been resolved in the running gel. Some vesicle components were recognized specifically by sera from a pool of PCM patients in immunoblotting (Fig. 3), as opposed to the complete lack of reactivity with a pool of sera from healthy individuals (NHS). A reactive broad smear was clearly seen in both Pb18 and Pb3 preparations, as well as three other bands with masses estimated as 49 kDa, 64 kDa, and 75 kDa. A high-molecular-mass antigenic band was observed in Pb3 and only faintly in Pb18; a 47-kDa component was revealed only in Pb3, whereas two weak and thin bands slower than 82 kDa were observed only in Pb18 preparations.

Finding α-Gal epitopes in *P. brasiliensis* vesicles. While looking for vesicle markers, we surprisingly detected strong reactivity in ELISA of *P. brasiliensis* preparations from both Pb3 and Pb18 with anti-α-galactosyl IgG isolated from patients with chronic Chagas’ disease (Fig. 4, left). Immunoreactivity with Pb3 and Pb18 preparations decreased about 45% upon treatment with green coffee bean α-galactosidase in two sets of experiments. Figure 4 shows one of these experiments, where the enzyme was used to compete with binding of the anti-α-galactosyl IgG. These results suggested the involvement of terminal α-galactosyl epitopes in the reaction and were corroborated by intense reactivity in ELISA with MOA lectin (Fig. 4, right), which specifically binds to terminal nonreducing Gal1,3Gal moieties (41). The reactivity with MOA lectin decreased about 75% upon treatment of *P. brasiliensis* vesicles from both Pb3 and Pb18 with α-galactosidase (Fig. 4, right) in single experiments, supporting similar results previously obtained with vesicles from Pb339.

TEM images confirmed the ELISA data and revealed immunogold labeling with MOA lectin on the surfaces and in the lumen of Pb18 vesicles (Fig. 5A, B, and C). Negative controls—vesicles treated with α-galactosidase—had decreased reactivity (Fig. 5D), as indicated by particle counts (Fig. 5E), once again suggesting the presence of vesicular components carrying α-galactosyl residues that are involved in lectin binding.

Isolation of PCM α-Gal IgG and confocal and immunogold microscopy. We managed to isolate anti-α-Gal IgG from a pool of PCM patients’ sera using the same methodology employed to purify Ch anti-α-Gal (6). Since we did not have access to large volumes of PCM patients’ sera, only a few experiments were carried out with PCM anti-α-Gal. In Fig. 6, left, note that it reacted in ELISA with Pb18 vesicle preparations in a dose-dependent manner, while at comparable antibody concentrations, NHS anti-α-Gal purified from healthy individuals reacted poorly. For both Pb3 and Pb18 vesicles, the reactivity with PCM anti-α-Gal was significantly inhibited by
α-galactosidase between 34% and 38%, pointing to the involvement of terminal α-galactosyl epitopes in the reaction.

When PCM anti-α-Gal was used to localize the corresponding epitopes in *P. brasiliensis* yeast cells by confocal microscopy, we observed intense labeling that was comparable to that obtained with Ch anti-α-Gal (Fig. 7). In both cases, fluorescence was distributed in a punctuated pattern, which is compatible with vesicular distribution. The same pattern was observed on the cell surface and inside the cell (Fig. 7). NHS anti-α-Gal stained the *P. brasiliensis* cells poorly and diffusely, following a background pattern. Intracellular localization of α-galactosyl epitopes was also suggested by ELISA carried out with cell lysates, for which the patterns of dose-response reactivity with PCM and NHS anti-α-Gal were similar to those observed in Fig. 6 (not shown). When Pb18 yeast cells were incubated with Ch anti-α-Gal and analyzed by TEM, immunogold particles were abundantly observed on the cell wall (Fig. 8A) and inside vacuoles (Fig. 8B), but not in negative controls (Fig. 8C and D). Labeling in these compartments was specific, as indicated by particle counting (Fig. 8E). Labeling in vesicle-like structures inside the vacuoles is suggested in Fig. 8B.

**Nature of α-Gal-containing components.** In *T. cruzi*, α-galactosyl epitopes are part of O-linked glycans found in mucins (5). In *P. brasiliensis*, we investigated the nature of protein moieties carrying α-Gal epitopes by treating vesicle and cell lysate preparations of *P. brasiliensis* Pb18 either with Endo H, to remove N-linked mannose chains, or mildly with NH₄OH (β-elimination), to remove O-linked glycans. The treated samples were compared with controls by ELISA reactivity with both PCM and Ch anti-α-Gal. Figure 9A shows percentages of inhibition up to 35% after β-elimination, suggesting that at least part of the *P. brasiliensis* epitopes recognized by PCM and Ch anti-α-Gal are found in oligosaccharides O linked to proteins. Smaller percentages of inhibition were obtained with Pb18 vesicles, and differences in the nature of α-Gal epitopes between Pb18 and Pb3 have to be better investigated. Our results with Endo H Pb3 vesicles also suggest that some α-Gal epitopes could be expressed in N-linked chains, since inhibitions of about 10% were seen.

To better investigate the localization of α-Gal epitopes, we fractionated Pb3 and Pb18 vesicle preparations into lipid and lipid-free fractions and tested the products by ELISA with anti-α-Gal before and after treatment with α-galactosidase. Figure 9B shows that lipid-free fractions reacted with both PCM and Ch anti-α-Gal, and this reaction was highly inhibited by α-galactosidase. Lipid fractions were also tested, but the results were not reproducible, probably because of the small amounts of substrate used in the reactions. We did, however, observe reactivity with lipid fractions at least once, which was completely abolished after α-galactosidase treatment. Therefore, α-Gal epitopes in *P. brasiliensis* glycolipids are likely to occur, but this has to be better investigated.

**DISCUSSION**

The present work described the presence of extracellular vesicles in the yeast phase of *P. brasiliensis* for the first time by using isolate Pb18 as a model and comparing some antigenic characteristics of vesicle preparations with the phylogenetically distinct isolate Pb3. We showed that *P. brasiliensis* extracellular vesicles carry antigenic components bearing highly immuno-

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**FIG. 5.** TEM images of two distinct vesicle preparations (panel A and panels B, C, and D) incubated with MOA-labeled gold before (A, B, and C) and after (D) treatment with α-galactosidase. (E) Quantification of the gold particles showed decreased labeling after treatment with the enzyme. The error bars indicate standard deviations. Scale bars of 100 nm (A) and 50 nm (B, C, and D) are included.

**FIG. 6.** (A) Reactivity in ELISA of a Pb18 vesicle preparation with different concentrations of either PCM or natural (NHS) anti-α-Gal. (B) Reactivity in ELISA of 2.5 µg/ml of PCM anti-α-Gal with Pb3 and Pb18 vesicle preparations treated (+) or not (−) with α-galactosidase. Similar percentages of inhibition were obtained in replicates. The asterisks indicate statistical significance (*P* < 0.05). The error bars indicate standard deviations.
genic α-linked galactosyl epitopes. In the cells, components bearing α-Gal epitopes were found abundantly distributed on the cell wall, following a confocal punctuated pattern, but they were also stored inside large intracellular vacuoles. The α-galactosyl-containing components from *P. brasiliensis* reacted strongly in ELISA with anti-α-Gal antibodies isolated from both Chagasic and PCM patients and with MOA lectin, but only weakly with natural anti-α-Gal from healthy individuals.

Natural immunoglobulins specifically recognizing nonreducing terminal α-galactosyl epitopes are prevalent in sera from healthy individuals and account for about 1% of the total IgG. These antibodies preferentially bind to terminal Galα1,3Galβ1,4GlcNAc-R trisaccharide (Galili epitope), which is absent in humans, apes, and Old World monkeys (reviewed in reference 22). By eliminating self α-Gal epitope through mutation in the α-1,3-galactosyltransferase gene, protective antibodies would be produced against microorganisms. Indeed, viruses, bacteria, and protozoa can express a variety of α-Gal epitopes; reactivity with the host’s anti-α-Gal might cause elimination of the infectious agent (22). In *T. cruzi*, the causative agent of Chagas’ disease, α-galactosyl epitopes

![Fig. 7](image_url)  
**FIG. 7.** Confocal microscopy of *P. brasiliensis* yeast cells comparatively labeled with natural (NHS), PCM, and Ch anti-α-Gal, as indicated. Both immunofluorescence and merged images with phase-contrast are shown. Scale bars are included.

![Fig. 8](image_url)  
**FIG. 8.** (A and B) Cell wall (A) and vacuolar (B) localization of components bearing α-Gal epitopes in *P. brasiliensis* yeast cells incubated with Ch anti-α-Gal. (C and D) Negative controls lacked the primary Ch anti-α-Gal antibody. (E) Quantification of gold particles was negligible in the Formvar grids and in the cytoplasm. Higher labeling was detected on the cell wall (CW) and vacuole. The error bars indicate standard deviations. Scale bars of 500 nm (A and C), 250 nm (B), and 200 nm (D) are included.
are part of long and complex sugar chains that are O linked mainly to threonine residues of glycosylphosphatidylinositol (GPI)-anchored mucin-like glycoproteins (4, 5). Chagasic anti-α-Gal and about 35% for PCM anti-α-Gal, while almost complete inhibition was achieved when lipid-free vesicle fractions were tested with anti-α-Gal. These differences may be attributed to different degrees of accessibility of the enzyme to the cleavable residues under our ELISA conditions. It is also noteworthy that Ch anti-α-Gal antibodies can recognize a broader variety of linkages than does the MOA lectin. The latter is highly specific for terminal Galα1,3Gal disaccharides of blood type B, with enhanced affinity achieved after addition of β1,4GlcNAc or α1,2-L-Fuc; however, it does not bind to Galα1,2Gal and Galα1,6Gal disaccharides (41).

In T. cruzi, α-Gal epitopes are part of long, mostly branched O-linked glycans of mucins (5). In P. brasiliensis, a broad smear characterized immunoblotting reactions of anti-α-Gal with both vesicle preparations and cell extracts, suggesting antibody binding to highly glycosylated components (not shown). We found evidence for the localization of α-Gal epitopes in glycoproteins, where they are at least partially distributed in O-linked glycan chains and possibly also in N-linked chains in Pb3. Localization in glycolipids seems to occur, but those results should be better investigated. Eukaryotic O-glycans are usually attached to the β-hydroxyl group of serines or threonines. Unlike those of mammals and parasites, fungal O-glycans, such as those from C. albicans, S. cerevisiae, and Pichia pastoris, tend to be short (2 to 5 residues) and generally linear, and the main component is mannose (reviewed in reference 19). Nevertheless, terminal or side α-1,2- and α-1,3-galactopyranosyl (Galp) residues linked to mannose have been found in A. paradoxus and Schizosaccharomyces pombe, where an α-1,2-galactosyltransferase uses UDP-galactose as a sugar donor (19). Examples of fungal galactose residues linked to other galactoses can be found in polysaccharides (1, 18).

In P. brasiliensis, we have found two annotated galactosyltransferase sequences in the Pb3 and Pb18 genomes released by the Broad Institute (http://www.broadinstitute.org/annotation/genome/paracoccidioides_brasilensis). In this fungus, a high-molecular-mass extracellular galactomannan bears terminal β-Galp residues linked to mannose (30). Galactomannans extracted from the mycelium cell wall have α-Galp1,6α-Manp1,2 side chains, which in the yeast phase contain mostly β-Galp instead of the rare α-Galp (2). Terminal β-galactofuranosyl residues are commonly found in galactomannans of pathogenic fungi and are responsible for cross-reactivity in serum diagnosis, as exemplified in P. brasiliensis by the main diagnostic glycoprotein gp43 (7, 31). It is also noteworthy that β-Galp is an essential part of the carbohydrate epitope Galβ1,6-Manpα1,3Manpβ1,2-inositol contained in P. brasiliensis glycosphingolipids (35).

In C. neoformans, vesicles containing GXM are produced during macrophage infection, suggesting a role in the pathogenesis of the microorganism (33). In addition, recent in vitro data showed that vesicles can be incorporated by macrophages and stimulate expression of tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin 10 (IL-10), and nitric oxide, and hence, they increase the phagocyte microbicidal capacity (26). When α-Gal-rich vesicles secreted by T. cruzi infective trypomastigote forms were administered in vivo to BALB/c mice, increased tissue parasitism and a severe inflammatory reaction by stimulation of IL-4 and...
IL-10 synthesis were observed (37). In *P. brasilensis*, we analyzed extracellular vesicles from both Pb18 and Pb3, which evoke experimental PCM in B10.A mice that have distinct outcomes (13). We observed that the immunoblotting anti-gp43 from our group suggested that vesicle preparations from these two isolates are able to stimulate cytokine expression of macrophages in vitro and that these responses can vary according to the isolate (M. C. Vallejo, L. Ganiko, I. C. Almeida, and R. Puccia, unpublished data); however, the participation of *P. brasilensis* α-Gal epitopes in the host’s cellular immune response is so far speculative.

Fungal components carrying α-Gal epitopes recognized by both anti-α-Gal and MOA lectin could be observed, not only in *P. brasilensis* vesicles, but also along the cell wall and in intracellular vacuoles, where labeling seemed to target vesicle-like structures distributed inside and near the vacular membrane. Vacuoles are important fungal components where diverse membrane-trafficking pathways can converge. They contain molecules coming from the secretory pathway on their way to be secreted, as well as molecules that have been endocytosed or derived from autophagy that are meant to be disposed of or degraded (40). The biogenesis of fungal extracellular vesicles has recently been addressed using *S. cerevisiae* mutants with defects in conventional secretion pathways and multivesicular body trafficking (27, 28). Vesicular export has not been affected in any of the mutants analyzed; however, the relative peptide abundances varied in a considerable number of vesicular proteins. In mutants with affected Golgi-dependent secretion, glycosylation, and that of *Apodus deciduus* histoplasma, we analyzed the glycoproteins of *H. capsulatum* and that of *Trypanosoma cruzi* pathogenicity of *P. brasilensis* vesicles. Cell Biol. Mol. Biol. 16:1095–1110.

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