Vesicular Polysaccharide Export in *Cryptococcus neoformans* Is a Eukaryotic Solution to the Problem of Fungal Trans-Cell Wall Transport

Marcio L. Rodrigues,1,¢ Leonardo Nimrichter,1,‡ Débora L. Oliveira,1,‡ Susana Frases,2 Kildare Miranda,3,‡ Oscar Zaragoza,2‡ Mauricio Alvarez,2 Antonio Nakouzi,2 Marta Feldmesser,2,4 and Arturo Casadevall2,4*

Laboratório de Estudos Integrados em Bioquímica Microbiana, Instituto de Microbiologia Professor Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941590, Brazil1; Department of Microbiology and Immunology2 and Division of Infectious Diseases4 of the Department of Medicine, Albert Einstein College of Medicine, 1300 Morris Park Ave., Bronx, New York 10461; and Laboratório de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro 21941590, Brazil3

Received 6 October 2006/Accepted 6 November 2006

The mechanisms by which macromolecules are transported through the cell wall of fungi are not known. A central question in the biology of *Cryptococcus neoformans*, the causative agent of cryptococcosis, is the mechanism by which capsular polysaccharide synthesized inside the cell is exported to the extracellular environment for capsule assembly and release. We demonstrate that *C. neoformans* produces extracellular vesicles during in vitro growth and animal infection. Vesicular compartments, which are transferred to the extracellular space by cell wall passage, contain glucuronoxylomannan (GXM), a component of the cryptococcal capsule, and key lipids, such as glucosylceramide and sterols. A correlation between GXM-containing vesicles and capsule expression was observed. The results imply a novel mechanism for the release of the major virulence factor of *C. neoformans* whereby polysaccharide packaged in lipid vesicles crosses the cell wall and the capsule network to reach the extracellular environment.

*Cryptococcus neoformans* is a yeast-like pathogenic fungus that is the etiologic agent of human cryptococcosis. Infection is usually asymptomatic and restricted to the lung in immunocompetent individuals, but fungal cells can disseminate to other organs and cause cryptococcal meningitis, a common syndrome in immunosuppressed patients (29, 39). Significant progress in our understanding of how *C. neoformans* causes disease has been made in the last decade, but many aspects of cryptococcal pathogenesis remain poorly understood.

*C. neoformans* represents a unique model in cell biology studies because it is the only eukaryotic pathogen with a polysaccharide capsule, a structure that is essential for virulence (4, 19). The major capsular polysaccharide glucuronoxylomannan (GXM) has an average mass ranging from 1.7 × 10^6 to 7 × 10^6 daltons (22) and is released extracellularly during infection, inducing a number of deleterious effects to the host (39). GXM also represents a potential vaccine component and is the target of therapeutic antibodies that are currently in clinical trial (3, 8, 20, 27).

The fungal cell wall is a compact, albeit dynamic, structure that plays important roles in several biological processes that determine cell shape, morphogenesis, reproduction, cell-cell and cell-matrix interactions, and osmotic and physical protection (25). Although the relevance of the cell wall to fungal physiology and pathogenesis is clear, the mechanisms by which large molecules (molecular weight, >1 million) cross this rigid structure to reach the extracellular environment are largely unknown. In *C. neoformans*, it has been suggested that capsule components containing epitopes recognized by a monoclonal antibody (MAb) to GXM are synthesized intracellularly and exported through the cell wall, possibly inside membrane vesicles (11). These results were validated in a recent study using a secretion mutant of *C. neoformans* in which post-Golgi secretory vesicles containing GXM were accumulated in the plasma membrane region (40). Based on these observations, one could imagine that surface components of *C. neoformans*, including the capsule, are synthesized in the cytoplasm and exported to the exterior of the cell in secreory vesicles that traverse the cell wall. Indeed, *C. neoformans* does produce glucosylceramide (GlcCer)-containing vesicles that are transferred to the cell wall (1, 25, 30). Since *C. neoformans* efficiently releases extracellular capsular material, we hypothesized the existence of extracellular secreted vesicles containing GlcCer and capsule components.

In the present work, we describe for the first time that a fungal cell can produce extracellular vesicles that are secreted across the cell wall. Supernatants of *C. neoformans* cultures contained vesicles with bilayered membranes. Lipid analysis revealed that key fungal lipids, such as GlcCer, ergosterol, and a novel sterol, are present in these membranes. By different approaches, we demonstrated that GXM is packaged inside the vesicles, which cross the cell wall and the capsule network to reach the extracellular environment. A correlation between capsule growth and detec-
tion of vesicle-associated GXM was observed, suggesting that C. neoformans can release the polysaccharide from the vesicles and incorporate it into the cell surface. Accordingly, acapsular cells used vesicle-associated GXM to become encapsulated. GXM-containing vesicles were produced during macrophage infection, suggesting a role in pathogenesis. These findings illustrate a new phenomenon in fungi with potential relevance for such diverse areas as capsule assembly and pathogenesis and reveal new insights into how secreted molecules reach the extracellular environment.

MATERIALS AND METHODS

Culture conditions. The cryptococcal isolates used in this study comprised strains ATCC 24067 (serotype D; American Type Culture Collection), H99 (serotype A; clinical isolate), HEC3393 (serotype A; clinical isolate), and Cap67 (acapsular mutant). In the different analyses performed, similar data were obtained with encapsulated strains. Except for the presence of GXM, results for assays using acapsular cells followed the same profile obtained with HEC3393, H99, and ATCC 24067 cells. For vesicle purification, C. neoformans cells were inoculated into 1,000-ml Erlenmeyer flasks containing 400 ml of a minimal medium designed of dextrose (15 mM), MgSO4 (10 mM), KH2PO4 (29.4 mM), glycine (13 mM), and thiamine-HCl (3 μM). Fungal cells were cultivated for 5 days at 30°C with shaking. The viability of C. neoformans cells after this period of cultivation was analyzed by propidium iodide staining.

Isolation of vesicles. Fungal cells were separated from culture supernatants by centrifugation at 4,000 × g for 15 min at 4°C. The supernatants were collected and again centrifuged at 15,000 × g at 4°C to remove smaller debris. The pellets were discarded, and the resulting supernatant was concentrated approximately 1/100 by lyophilization and again centrifuged at 15,000 × g for 1 h at 4°C. The supernatants were then discarded, and the pellets were washed with five sequential resuspension and centrifugation steps, each consisting of 100,000 × g for 1 h at 4°C with 0.1 M Tris-buffered saline (TBS). The pellets were then resuspended in fixative solution for electron microscopy analysis. Alternatively, 100,000 × g pellets were fractionated by affinity chromatography and sucrose density centrifugation or extracted with organic solvents as detailed below.

TEM. Transmission electron microscopy (TEM) was used to visualize vesicles isolated from supernatants and those that were cell associated, in vitro and in vivo. The pellets obtained after washing and centrifugation at 100,000 × g were fixed in 2% glutaraldehyde in 0.1 M cacodylate at room temperature for 2 h and then incubated overnight in 4% formaldehyde, 1% glutaraldehyde, and 0.1% phosphate buffer (pH 7.2) (PBS). The supernatant was incubated for 90 min with 0.1 ml of 4% aqueous osmium, serially dehydrated in ethanol, and embedded in Spurr’s epoxy resin. Thin sections were obtained on a Reichert Ultratrac and stained with 0.5% uranyl acetate and 0.5% lead citrate. Samples were observed in a JEOL 1200EX transmission electron microscope operating at 80 kV. For immunogold labeling with antibodies to GXM, the vesicles were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4% paraformaldehyde, 0.2% glutaraldehyde, and 1% picric acid, infiltrated in 25% polyvinylpyrrolidone and 2.1 M sucrose, and rapidly frozen by immersion in liquid nitrogen. Cryosections were obtained in a temperature range of ~70 to ~90°C using an Ultracut cryo-ultramicrotome (Reichert). After being blocked in PBS-bovine serum albumin and 50 mM NH4Cl, the cryosections were incubated overnight in the presence of a mouse monoclonal antibody to GXM (MAb 18B7, 1 μg/ml). The antibody is a mouse immunoglobulin G1 (IgG1) with high affinity for GXM of different cryptococcal serotypes and has been extensively characterized previously (3). After incubation with 15-nm (particle size) immunogold-labeled anti-mouse IgG, specimens were visualized by transmission electron microscopy in a JEOL 1200EX transmission electron microscope operating at 80 kV.

For detection of cryptococcal vesicles in vivo, murine infection and lung tissue preparation for transmission electron microscopy were carried out as described previously (10, 12, 26). Briefly, anesthetized C57BL/6 mice (National Cancer Institute, Bethesda, MD) were infected intratracheally via a midline neck incision with 107 and 108 colony-forming units of C. neoformans strain ATCC 24067 and sacrificed 2 h, 48 h, or 7 days after infection. The analysis of vesicle production in vitro was performed using strain Cap67 as previously described (30). Human antibodies to GlcCer were used for lipid detection at the cell wall following previously described experimental conditions (30).

Removal of nonvesicular GXM from 100,000 × g pellets. To remove extravesicular GXM and putative aggregates from vesicle preparations, 100,000 × g pellets were purified by passage through a column packed with an antibody-bound resin. MAb 18B7 was coupled to cyanogen bromide-activated Sepharose according to the manufacturer’s protocols (Sigma, Richmond, CA). Briefly, 1 g of the resin was suspended in 1 mM HCl, washed, and resuspended in carbonate buffer, pH 8.0. MAb 18B7 was dissolved in 5 ml of the same buffer, at 1 mg/ml, and incubated overnight with the resin. At this pH, MAb 18B7 retained its biological properties, such as antigen binding and integrity of chains (not shown). Coupling efficiency was ~90%, as confirmed by spectrophotometric determinations at 280 nm. After being washed with carbonate buffer and blocked with 0.2 M glycine, the resin was sequentially washed with 0.1 M acetic acid (pH 4.0) and Tris (pH 8.0) buffers for final resuspension in PBS. Each vesicle suspension (500 μl) was mixed with the antibody-containing resin (100 μl) and incubated for 1 h (37°C) with shaking. The unbound fraction was recovered by centrifugation. This process was repeated three times, and the GXM contents in different fractions were analyzed by capture enzyme-linked immunosorbent assay (ELISA) as described below.

Sucrose gradient. Sucrose density centrifugation was performed as described by Gutwein et al. (15). The vesicle suspension (0.5 ml) obtained after ultracentrifugation and purification with the antibody-coupled resin was mixed with an equivalent volume of 85% (w/v) sucrose (in TBS), generating a final concentration of 42.5% sucrose. This suspension was transferred to an ultracentrifuge tube, and a step gradient was prepared by overlaying the original suspension with 35% sucrose, followed by a final layer of 5% sucrose in TBS. The gradient was centrifuged for 18 h at 200,000 × g and fractions of 0.25 ml were collected from the top to the bottom of the gradient. Fractions were then extensively dialyzed against TBS, dried under vacuum centrifugation, and suspended in 100 μl of absolute methanol, resulting in the immediate formation of a precipitate. The solution was then filtered through a 0.45-μm filter membrane and 1 μl of chloroform for 1 h at 25°C. The organic fraction was recovered by centrifugation and analyzed by different methods for lipid identification. The residual material that was not soluble in the chloroform-methanol mixture was then dried under nitrogen gas, resuspended in TBS, and assayed for the presence of GXM, as described later in this section.

Lipid analysis. The pellets obtained from centrifugation of cell supernatants at 100,000 × g were first suspended in methanol, and then two volumes of chloroform were added. The mixture was vigorously vortexed and centrifuged to discard precipitates, dried by vacuum centrifugation, and partitioned according to Folch et al. (13). The lower phase, containing neutral lipids, was recovered for analysis by high-performance thin-layer chromatography (HPTLC). For sterol analysis, the lipid extract was loaded into HPTLC silica plates (Si 60F254s; LiChrospher, Germany) and separated using a solvent system containing hexane-ether-acetic acid (80:40:4, vol/vol/vol) solvent. The plate was sprayed with a solution of 50 mg ferric chloride (FeCl3) in 90 ml water, 5 ml acetic ether-acetic acid (80:40:4, vol/vol/vol) solvent. The capillary precipitates, dried by vacuum centrifugation, and partitioned according to Folch et al. (13). The lower phase, containing neutral lipids, was recovered for analysis by high-performance thin-layer chromatography (HPTLC). For sterol analysis, the lipid extract was loaded into HPTLC silica plates (Si 60F254s; LiChrospher, Germany) and separated using a solvent system containing hexane-ether-acetic acid (80:40:4, vol/vol/vol) solvent. The lipids were then extracted with 15-nm (particle size) immunogold-labeled anti-mouse IgG, specimens were visualized by transmission electron microscopy in a JEOL 1200EX transmission electron microscope operating at 80 kV. For immunogold labeling with antibodies to GXM, the vesicles were fixed in 0.1 M sodium cacodylate buffer (pH 7.2) containing 4% paraformaldehyde, 0.2% glutaraldehyde, and 1% picric acid, infiltrated in 25% polyvinylpyrrolidone and 2.1 M sucrose, and rapidly frozen by immersion in liquid nitrogen. Cryosections were obtained in a temperature range of ~70 to ~90°C using an Ultracut cryo-ultramicrotome (Reichert). After being blocked in PBS-bovine serum albumin and 50 mM NH4Cl, the cryosections were incubated overnight in the presence of a mouse monoclonal antibody to GXM (MAb 18B7, 1 μg/ml). The antibody is a mouse immunoglobulin G1 (IgG1) with high affinity for GXM of different cryptococcal serotypes and has been extensively characterized previously (3). After incubation with 15-nm (particle size) immunogold-labeled anti-mouse IgG, specimens were observed in a JEOL 1200EX transmission electron microscope operating at 80 kV.

For detection of cryptococcal vesicles in vivo, murine infection and lung tissue preparation for transmission electron microscopy were carried out as described previously (10, 12, 26). Briefly, anesthetized C57BL/6 mice (National Cancer Institute, Bethesda, MD) were infected intratracheally via a midline neck incision with 107 and 108 colony-forming units of C. neoformans strain ATCC 24067 and sacrificed 2 h, 48 h, or 7 days after infection. The analysis of vesicle production in vitro was performed using strain Cap67 as previously described (30). Human antibodies to GlcCer were used for lipid detection at the cell wall following previously described experimental conditions (30).

Radioactive labeling of vesicles. C. neoformans (strain ATCC 24067) was grown in the presence of L-[3H]serine (20 Ci/mmol) (American Radiolabeled Chemicals, Inc., St. Louis, MO) and [9,10-3H]palmitic acid (50 Ci/mmol) (DuPont NEN, Boston, MA). These ceramide precursors were added to the

GXM-CONTAINING VESICLES IN Cryptococcus neoformans

Vol. 6, 2007
culture medium described in “Culture conditions” to generate final amounts of radioactivity corresponding to 2.0 μCi/ml (14C]serine) and 1.0 μCi/ml (14C]palmitic acid). The culture was incubated for 3 days at 30°C, and then fungal cells were removed by centrifugation. Vesicles were obtained as described above and suspended in methanol (100 μl). Chloroform (200 μl) was added, and the mixture was centrifuged to discard precipitates. The lipid extract was loaded into HPTLC plates and separated using chloroform-methanol-water (65:25:4, vol/vol/vol) as the solvent system. Molecules with migration rates corresponding to that of GlcCer, identified by comparison with an iodine-stained standard of glucocerebroside (Avanti Polar Lipids, Alabaster, AL), were scraped off the plate, suspended in scintillation liquid, and counted for radioactivity. Negative controls included 100,000 × g pellets from sterile medium containing the radioactive precursors and vesicle-depleted culture supernatants.

Fungal killing and lipid detection. To evaluate whether cryptococcal vesicles were physiologically released or were simply an artifact of dead cells, we evaluated the presence of lipid markers in 100,000 × g pellets of living and dead cells. Cryptococci were treated with 10 mM sodium azide in PBS for 60 min at 25°C or, alternatively, suspended in PBS and heated at 50°C for the same period. Control cells (viable) were suspended in PBS and incubated at 25°C for 60 min. Cell viability was evaluated by inoculating control or treated yeasts onto Sabouraud dextrose agar and counting CFU. After incubation under the conditions described above, yeasts were removed as described previously in this section and the supernatants were centrifuged at 100,000 g. The resulting pellets were extracted with mixtures of chloroform-methanol at 2:1 (for GlcCer analysis) or 9:1 (for sterol analysis). Extracts were analyzed by HPTLC following the conditions described in “Lipid analysis.”

Serological assays for GXM detection. The profiles of GXM distribution in the fractions obtained after sucrose density centrifugation were analyzed using a capture ELISA (2). Briefly, each well of a 96-well polystyrene plate was coated with 100 μl of the respective fraction. After removal of unbound antibodies, a solution of MAb 12A1, an IgM MAb with specificity for GXM, was added to the plate, and this step was followed by blocking with 1% bovine serum albumin. The ELISA was used to analyze vesicles after treatment with methanol and chloroform as described in “Lipid analysis.” Purified GXM was used as a positive control. The samples were incubated in the plate overnight at 4°C. The plates were then washed five times with a solution of TBS supplemented with 0.1% Tween 20, followed by incubation with MAB 18B7 for 1 h. The plate was again washed and incubated with an alkaline phosphatase-conjugated goat anti-mouse IgG1 for 1 h. Reactions were developed after the addition of p-nitrophenyl phosphate disodium hexahydrate, followed by reading at 405 nm with a Multiscan MS (Labsystem, Helsinki, Finland). The antibodies in this assay were all used at a concentration of 1 μg/ml.

Extracellular formation of GXM-containing vesicles. To explore the possibility that cryptococcal vesicles were formed as a result of random aggregation of lipid vesicles in the extracellular environment, minimal medium was supplemented with GXM (15 μg/ml) and inoculated with Cap67 cells, which are known to produce vesicles (see Fig. 1, C and S). After growth for 72 h at 30°C, supernatants were collected and evaluated for GXM-containing vesicles. Alternatively, acapsular cells were grown in the absence of GXM and the polysaccharide was added to culture supernatants at the same concentration after removal of yeast cells, followed by incubation for 1 h at 30°C. GXM-containing supernatants were centrifuged at 100,000 × g, and the pellets were purified in Sepharose-4B. GXM in supernatants and 100,000 × g pellets was then quantified by capture ELISA. The negative control consisted of GXM determinations in regular Cap67 cultures.

Capsule induction. Capsule expression in C. neoformans was induced as described previously (24). Briefly, C. neoformans was inoculated in Sabouraud broth diluted 10 times in 50 mM MOPS (morpholinepropanesulfonic acid), pH 7.3. After incubation for 6 or 24 h at 37°C with shaking, the cells were recovered by centrifugation at 2,000 × g for 2% paraformaldehyde in TBS. The fixative agent was removed by washing the cells with PBS, and after India ink staining, capsule expression was analyzed microscopically. Capsule sizes, defined as the distances between the cell wall and the outer border of the capsule, were determined using ImageJ software, elaborated and provided by the National Institutes of Health (NIH) (http://rsb.info.nih.gov/ij). All experiments were performed in triplicate sets and the results analyzed using Student’s t test. Supernatant-associated vesicles produced as described above were then purified by affinity chromatography and different centrifugation steps as described previously in this section. The GXM concentrations in 100,000 × g fractions under the different conditions of capsule induction were normalized to the number of cells in the culture after each condition of stimulation, as measured in a Neubauer chamber.

RESULTS

Production of extracellular vesicles by C. neoformans. Several lines of evidence suggested that secretion of macromolecules by fungi could rely on vesicular transport (11, 14, 30–32, 35, 36, 40). Consequently, we designed experiments to search for vesicles in fungal cells and culture supernatants. In acapsular cells, putative vesicular bodies were observed in association with the cell wall (Fig. 1A to C), providing additional evidence for the existence of an extracellular vesicular transport mechanism. To address whether vesicles could be observed during infection by encapsulated cryptococci, the presence of vesicles in vivo was evaluated by electron microscopy of C57BL/6 mice infected intratracheally with C. neoformans. TEM analysis demonstrated extracellular vesicular structures near the edge of the capsule within 2 h after infection (Fig. 1D) as well as hypoluculent vesicular structures in the cryptococcal cell wall (Fig. 1E). Similar results were observed when mice were killed 48 h or 7 days after infection (data not shown).

Production of extracellular vesicles by C. neoformans was induced in acapsular cells. Acapsular C. neoformans cells (strain Cap67, 105 cells) were suspended in 100 μl of a purified vesicular suspension, with a GXM concentration corresponding to 10 μg/ml. The suspension was incubated for 12 h at 25°C and extensively washed with PBS, followed by fixation with 4% paraformaldehyde. The cells were further blocked for 1 h in PBS-bovine serum albumin and incubated with MAB 18B7 (1 μg/ml) for 1 h at room temperature, followed by a fluorescein isothiocyanate-labeled goat anti-mouse IgG1 (anti-specific) antibody (Sigma). Yeast cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). In control systems, MAB 18B7 was replaced by irrelevant antibodies. In addition, vesicle preparations were simply removed from the experimental system (negative control) or replaced by soluble, nonvesicular GXM (positive control), purified as described elsewhere (6).

GXM binding by acapsular cells. Acapsular C. neoformans cells (strain Cap67, 106 cells) were suspended in 100 μl of a purified vesicular suspension, with a GXM concentration corresponding to 10 μg/ml. The suspension was incubated for 12 h at 25°C and extensively washed with PBS, followed by fixation with 4% paraformaldehyde. The cells were further blocked for 1 h in PBS-bovine serum albumin and incubated with MAB 18B7 (1 μg/ml) for 1 h at room temperature, followed by a fluorescein isothiocyanate-labeled goat anti-mouse IgG1 (anti-specific) antibody (Sigma). Yeast cells were finally observed with an Axioplan 2 (Zeiss, Germany) fluorescence microscope. Images were acquired using a Color View SX digital camera and processed with the software system analySIS (Soft Image System). In control systems, MAB 18B7 was replaced by irrelevant antibodies. In addition, vesicle preparations were simply removed from the experimental system (negative control) or replaced by soluble, nonvesicular GXM (positive control), purified as described elsewhere (6).

GXM production by GXM-containing vesicles during the in vitro infection of macrophages with C. neoformans. The ability of cryptococci to produce GXM-containing vesicles during the infection of host cells was evaluated using the J774.16 cell line, which has been extensively used to study C. neoformans/macrophage interactions. Macrophage-like cells were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum, 10% NCTC109 cells, and 1% nonessential amino acids, at 37°C in a 5% CO2 atmosphere. Cultures were also supplemented with 100 U/ml gamma interferon and 1 μg/ml lipopolysaccharide. The cells were grown to confluence in 75-cm2 flasks and then infected with C. neoformans (10 yeast cells per host cell) in the presence of 10 μg/ml of MAB 18B7, which binds to the capsular polysaccharide and is opsonic (3). After 1 h of incubation at 37°C, free C. neoformans cells were removed by washing them. Based on the observation that J774.16 cells lyse after hosting intracellular replication of cryptococci (38), washed infected cells were incubated for 18 h at 37°C. Host cell lysis and cryptococcal intracellular replication were observed microscopically. Fluids from infected cultures were then analyzed for vesicles as described above.
In different vesicle preparations, bands with migration rates corresponding to the GlcCer standard were detected in lipid extracts from both acapsular and encapsulated C. neoformans cells (Fig. 2A, inset). Vesicular extracts from both encapsulated and acapsular isolates also present molecules with $R_f$ values corresponding to that of ergosterol, the principal fungal sterol. In order to confirm that sterols and GlcCer are in fact the molecules detected by HPTLC analysis, vesicle lipids from encapsulated cells were examined by ESI (MS and MS/MS) in positive mode. Although several molecular ions were detected (Fig. 2), the analysis was focused on sterols and GlcCer, the major molecules detected in HPTLC analysis.

The principal cerebroside produced by C. neoformans is N-2'-hydroxyoctadecanoyl-1-$\beta$-D-glucopyranosyl-9-methyl-4,8-sphingadienine, as demonstrated by our group (30). The full spectra (scanned from 300 to 800 m/z) of neutral lipids obtained after Folchi’s partition in fact suggested the presence of GlcCer with Li$^+$ adducts. Monolithiated peaks at m/z 734 ([M + Li$^+$]$^+$) and 762 ([M + Li$^+$]$^+$) were observed and revealed a profile similar to what has been described by our group and others for fungal GlcCer (1, 24, 30). These molecular ions correspond to N-2'-hydroxyhexadecanoyl- and N-2'-hydroxyoctadecanoyl-1-$\beta$-D-glucopyranosyl-9-methyl-4,8-sphingadienine, as confirmed after ESI-MS/MS analysis (Fig. 2B and C). Loss of water ([M - H$_2$O + Li$^+$]$^+$) generated peaks at m/z 716 and 744. Abundant lithiated aglycon ([M - hexose + Li$^+$]$^+$) peaks with m/z at 572 and 600 corresponded, respectively, to the loss of 162 units from the molecular ions at m/z 734 and 762. The variation of 28 units among these peaks indicates the presence of two species of hydroxylated fatty acids, containing 16 or 18 carbons. The presence of an abundant ion peak at m/z 480 in both spectra, corresponding to the loss of OH C$_{16}$ and OH C$_{18}$ fatty acids ([M - FA + Li$^+$]$^+$), confirmed this structural diversity.

Ergosterol and obtusifoliol are the major sterols produced by C. neoformans (18). The major peaks at m/z 397 and 435 (Fig. 2) supported, respectively, the occurrence of a [M + H$^+$]$^+$ ion corresponding to ergosterol and a [M + Li$^+$]$^+$ peak compatible with a molecule differing from obtusifoliol by 2

FIG. 1. TEM of vesicles in acapsular (A to C) and encapsulated (D to H) C. neoformans cells. The occurrence of vesicles in association with the cell wall of acapsular cryptococci (A and B) or in the extracellular environment (C) is evident after in vitro growth. Vesicle-like structures were also observed in the lung following murine pulmonary infection (D and E). Putative vesicles near the edge of the capsule (D) or in the cryptococcal cell wall (E) were observed 2 h after infection. Bars, 100 nm (A to C) and 500 nm (D and E). Arrows point to vesicles, and asterisks are on the cryptococcal cell wall. (F to H) The pellets obtained by ultracentrifugation were isolated by differential centrifugation, purified from GXM by affinity chromatography, and analyzed by TEM. Extracellular vesicles with bilayered membranes and different profiles of electron density were observed. Bars, 100 nm (F and G) and 50 nm (H).
For structural elucidation, these ions were submitted to an ESI-MS/MS scan (Fig. 2D and E). The molecular ion at m/z 397 ([M + H]+) gave rise to fragments at m/z 285 ([C20H29O]+), 302 ([C22H38]+), 190 ([C14H22]+), 173 ([C13H17]+), and 154 ([C11H22]+), compatible with the sterol profile of fragmentation proposed by Scallen et al. (33) and with a commercial standard of ergosterol (data not shown).

The fragmentation of the molecular ion at m/z 435 ([M + Li]+) yielded daughter ions at m/z 417 ([M – H2O + Li]+), 407 ([C28H48OLi]+), 267 ([C28H32Li]+), and 186 ([C13H24Li]+). Minor ions at m/z 391, 351, 337, 311, 295, 284, and 199 were also observed. All of these peaks were compatible with a monolithiated molecular ion corresponding to an obtusifoliol-like molecule lacking the double bond between carbons 8 and 17.
This structure was therefore identified as 4,14-dimethyl-ergosta-24(24\(^{-}\))-en-3\(^{-}\)ol.

Vesicle production requires viable \textit{C. neoformans} cells. After growth of \textit{C. neoformans} cells for 72 h, the viability of the cryptococcal population was very close to 100\%, as determined by propidium iodide staining (data not shown). However, even with high indices of viability, the possibility that the vesicles originated from a minor fraction of dead cells could not be discarded. In this context, we first evaluated the ability of \textit{C. neoformans} cells to use radioactive precursors of ceramide to produce \[^{3}\text{H}]\text{GlcCer}\)-containing vesicles, based on the evidence that \textit{GlcCer} is present in vesicle extracts. The detection of \[^{3}\text{H}]\text{GlcCer}\) in vesicle fractions from strain ATCC 24067 (Fig. 3A) suggested that \textit{C. neoformans} cells metabolically incorporated the radioactive precursors and secreted their corresponding products in vesicular preparations.

The possibility that the vesicles could be the products of dead cells was also considered, given that lipids are present in the membranes of all organelles. Thus, we compared lipid profiles obtained from regular vesicle fractions with those obtained from dead cells. To avoid false-negative results, several methods of fungal killing were evaluated, including metabolic inhibition (sodium azide treatment) and protein denaturation (mild heat exposure). \textit{C. neoformans} cells were grown under the conditions used for vesicle production, and then the supernatants obtained after fungal killing revealed the detection of \textit{GlcCer} in fractions from living but not heat- or azide-treated cells. Compounds with migration rates corresponding to an ergosterol standard were detected in 100,000 \(\times\) \(g\) fractions of the supernatants obtained after fungal killing revealed the detection of \textit{GlcCer} in fractions from living but not heat- or azide-treated cells. Compounds with migration rates corresponding to an ergosterol standard were detected in 100,000 \(\times\) \(g\) fractions of the supernatants obtained after fungal killing revealed the detection of \textit{GlcCer} in fractions from living but not heat- or azide-treated cells.

The possibility that the vesicles could be the products of dead cells was also considered, given that lipids are present in the membranes of all organelles. Thus, we compared lipid profiles obtained from regular vesicle fractions with those obtained from dead cells. To avoid false-negative results, several methods of fungal killing were evaluated, including metabolic inhibition (sodium azide treatment) and protein denaturation (mild heat exposure). \textit{C. neoformans} cells were grown under the conditions used for vesicle production, and then the supernatant was collected and used for vesicle purification. Cell viability decreased by 83 or 99\%, respectively, after treatment with azide or heat (Fig. 3C). The treated yeast cells were removed as described in Materials and Methods and the supernatants centrifuged at 100,000 \(\times\) \(g\). \textit{GlcCer} was detected in 100,000 \(\times\) \(g\) fractions from supernatants of living and azide-treated cells but not in preparations from heat-killed cryptococci.

We speculate that the detection of sterols in vesicles from azide-treated cells could be the result of stress-associated secretory activity during metabolic inhibition. Also, the sterol contents of...
100,000 × g fractions from living and azide-treated cells were analyzed by densitometry, showing that the detection of sterol in the former was 2.5-fold higher (data not shown). In summary, these results suggest that the vesicles described here are produced and excreted by living C. neoformans cells rather than released from dead cells. The results obtained using the different strains currently studied were very similar.

**GXM is contained inside cryptococcal vesicles.** The presence of GXM in vesicular bodies was evaluated by immunogold labeling of purified vesicles with MAb 18B7, followed by TEM analysis (Fig. 4A). Antibody binding to purified vesicles was concentrated largely in the vesicular matrix. The presence of GXM was confirmed by the fact that the antibody reacted strongly with the vesicles' contents (obtained by solvent-mediated lysis) in ELISAs (Fig. 4B). As an additional control, the supernatant obtained before vesicle lysis was also assayed, and the content of GXM in this preparation was around 10-fold lower than the content of GXM in the vesicle pellet. The most straightforward interpretation of these results is that GXM is contained primarily in the intravesicular compartment but that some material is found in solution, probably as a result of vesicle damage and/or disruption in handling. To confirm the premise that GXM was intravesicular, we removed the extracellular GXM by sequential passage of the vesicle preparation over a column of Sepharose-bound MAb 18B7 (Fig. 4C). In this experiment, the vesicle fraction and a vesicle-depleted supernatant were consecutively passed through the antibody-containing resin. While the GXM content in the supernatant seemed to decrease after each step of incubation with the resin, it stabilized when the vesicle preparation was used. Hence, we conclude that the vesicles contain GXM.

To exclude the possibility that GXM-containing vesicles were formed extracellularly by random incorporation of the polysaccharide into liposomes instead of secretion, the mini-
mal medium was supplemented with GXM and inoculated with Cap67 cells. After 72 h of growth at 30°C, Cap67 supernatants were collected for vesicle purification. Alternatively, acapsular cells were grown in the absence of GXM, and the polysaccharide was added to culture supernatants after the removal of yeast cells. Vesicle purification in these systems followed by polysaccharide determination demonstrated the absence of significant amounts of GXM in the 100,000×g fractions (Fig. 4D), suggesting that the association of GXM and vesicles was not a simple artifact of polysaccharide incorporation into liposomes or polysaccharide-facilitated formation of lipid vesicles. Interestingly, no significant loss of GXM in supernatants was observed when the polysaccharide was added to the culture after fungal growth. When the medium was supplemented with GXM before inoculation of acapsular cells, however, only one-third of the total amount of the polysaccharide initially added was detected in supernatants. This result could be linked to the well-known ability of Cap67 cells to incorporate GXM into their cell walls. In fact, immunofluorescence analysis with MAb 18B7 revealed that after growth of Cap67 cells in the presence of GXM, their surface was stained by the antibody to polysaccharide (data not shown).

**GXM content in vesicles correlates with capsule synthesis.** If GXM were packaged inside vesicles for extracellular transport, we hypothesized that the conditions that promoted capsule growth would also be associated with an increase in supernatant vesicles. To evaluate the possible correlation between capsule synthesis and vesicle secretion, cryptococci were stimulated to produce capsule, and supernatants were evaluated for vesicle content. Vesicles were obtained by ultracentrifugation at 100,000×g, and pellets were resuspended in TBS for GXM analysis by capture ELISA. GXM concentrations in 100,000×g fractions under the different conditions of capsule induction were normalized to the number of cells in the culture under each experimental condition. In parallel, yeast cells were collected by centrifugation, washed in PBS, and fixed in 2% paraformaldehyde for microscopic observation after India ink staining. After 24 h of incubation of cryptococci in diluted Sabouraud broth, capsule expression was approximately twofold higher than in cells incubated for 6 h (Fig. 5A). Similarly, the content of GXM in the vesicle fraction after 24 h was twofold higher than that observed after incubation of cryptococci for 6 h (Fig. 5B).

**Incorporation of vesicular GXM into the surface of acapsular cryptococci.** The correlation between capsule growth and GXM content in the vesicle fraction suggested that *C. neoformans* must have mechanisms to extract the polysaccharide from vesicles. To evaluate this hypothesis, acapsular *C. neofo-
mans cells were incubated in the presence of purified vesicles and then evaluated by immunofluorescence with MAb 18B7. A strong fluorescent reaction of the antibody with acapsular cells was observed after incubation with the vesicle suspension (Fig. 6), suggesting that C. neoformans can release GXM from vesicular bodies and uses it for capsule growth. Control yeasts, which had not been incubated with MAb 18B7 or vesicle preparations, presented very weak levels of fluorescence. Yeast cells incubated with nonvesicular purified GXM also became encapsulated (data not shown), as extensively described in previous studies.

Detection of GXM-containing vesicles from regular cultures or infected macrophages after sucrose gradient separation. Given the size diversity of the vesicles demonstrated in Fig. 1, a more detailed analysis of vesicle production by C. neoformans during its regular growth was performed using ultracentrifugation, followed by flotation on a step sucrose gradient (15). After ultracentrifugation for 18 h, 12 fractions were obtained. HPLC analysis revealed the presence of peaks with retention times similar to that of a standard GlcCer in all fractions (Fig. 7A). The area of each peak varied depending on the sucrose concentration in each fraction. Analysis of the presence of GXM in sucrose gradient fractions was performed using a capture ELISA. The results shown in Fig. 7B indicate that the

**FIG. 6.** Acapsular cells of C. neoformans bind GXM from extracellular vesicles. Cap67 cells were incubated in the presence of purified vesicles and then analyzed by immunofluorescence with MAb 18B7. Control cells, which were not incubated with MAb 18B7, are shown in the upper panels (a and b). Yeast cells that were incubated in the presence of the vesicular preparation reacted strongly with the antibody to GXM, as shown in the lower panels (c and d). The left panels (a and c) represent cryptococci analyzed under differential interference contrast, while the right panels (b and d) show images in the fluorescence mode. Bar, 2 μm.

**FIG. 7.** Analysis of vesicle fractions obtained after ultracentrifugation and sucrose gradient separation. Fractions from supernatants of regular cultures were extracted with chloroform-methanol mixtures and analyzed by HPLC. In each fraction, a single peak with a retention time (Rt) corresponding to a GlcCer standard was detected. Analysis of the same fractions (solid line) or preparations obtained from infected macrophages (dashed line) by capture ELISA revealed different profiles of GXM distribution, although the polysaccharide was always expressively detected in the region of the gradient presenting the highest density. Lipid and polysaccharide analyses were performed at least three times, always presenting similar profiles. O.D. 405 nm, optical density at 405 nm.
maximum indices of polysaccharide detection were measured in the most concentrated sucrose fractions, suggesting that packaging of GXM into lipid membranes results in highly dense vesicles. The influence of experimental conditions on GXM distribution in gradient fractions was analyzed by changing sucrose concentrations. Using different conditions, the highest content of GXM was always found in the most concentrated sucrose fractions (data not shown).

Based on the property that macrophages (J774.16 cells) infected with C. neoformans become lysed after prolonged periods of incubation (38), supernatants of yeast-infected macrophage cells were collected for vesicle purification. After separation of these fluids by ultracentrifugation associated with sucrose gradient, the presence of GXM was analyzed in each fraction. By comparison of the profile observed in infection-derived vesicles with that observed in regular cultures (Fig. 7B), two additional regions containing high levels of polysaccharide were observed in preparations obtained from infected macrophages. These results support the idea that C. neoformans produces GXM-containing vesicles inside host cells or, alternatively, induces the production of host-derived membrane domains filled with polysaccharide, as previously suggested (38).

**DISCUSSION**

The secretion of macromolecules (molecular weight, >1 million) by fungal cells is a puzzling topic. To reach the extracellular environment, secreted molecules must cross the cell wall molecular network, a porous but very rigid complex. In C. neoformans, it was recently demonstrated that GXM is trafficked within cytoplasmic secretory vesicles (40), supporting a model that capsular materials are synthesized in the Golgi and targeted to the plasma membrane for exocytosis. However, it remains unknown how the vesicles would reach the extracellular environment to be used for capsule expression. In this regard, we present evidence that the major virulence factor of C. neoformans is secreted by a novel mechanism involving the release of membrane vesicles through the cell wall. Vesicle secretion is apparently not exclusively related to GXM traffic, since lipid-containing extracellular vesicles were observed in acapsular cells.

The possibility that the cell wall of C. neoformans is permeable to the passage of intact vesicular structures was supported by microscopic analysis of acapsular cells cultivated in vitro and encapsulated cells from infected mice. Putative vesicular bodies in association with the cell wall and in the extracellular milieu were observed in both acapsular and encapsulated cells, suggesting that C. neoformans can use vesicular transport to secrete different compounds. Microscopic analysis of pellets obtained after differential centrifugation of culture supernatants revealed intact vesicles ranging in size from 60 to 300 nm. Despite this heterogeneity, the vesicles had the common appearance of being rounded and defined by a lipid bilayered membrane. Differences in electron density were observed, suggesting heterogeneity in vesicular contents. The sizes of the vesicles were consistent with those shown in Fig. 1 and with the size predicted from the early electron microscopic studies that showed intracellular GXM in what appeared to be vesicular structures (11, 14, 40). In those studies, the vesicular structures were 100 to 300 nm in diameter, which is within the size range of the vesicles described here.

Glycosphingolipids, sterols, and glycosylphosphatidylinositol-anchored proteins form detergent-insoluble lipid rafts on the plasma membrane (23). They are required for the processing of surface proteins in yeasts, making part of the vesicles that link the reticuloendothelial system to the Golgi to the plasma membrane (34). In C. neoformans, it has been suggested that GlcCer-containing vesicles migrate from the cell membrane to the cell wall (1, 25, 30). In the present study, lipid analysis by mass spectrometry revealed that a glycosphingolipid (GlcCer) and sterols are components of extracellular vesicles, supporting the idea that they are enriched in lipid rafts. The physiologic production of GlcCer as a vesicle component was strengthened by the observation that [3H]glucosylceramide was metabolically incorporated in vesicular fractions. Lipids were not detected in supernatants from dead cells, strongly suggesting that C. neoformans physiologically secretes vesicles.

The visualization of GlcCer-containing vesicle-like structures in both cell wall and extracellular spaces of C. neoformans cells supports the view that these structures are active products of fungal cells. The relevance of these findings to the pathogenesis and control of cryptococcosis is supported by different studies, which demonstrated that GlcCer induces the production of antimicrobial antibodies during human infection (30) and is the target of defensins from insect and plant cells (37). More recently, it was demonstrated that GlcCer expression regulates cryptococcal virulence and is essential for fungal growth in neutral/alkaline pH in vitro and in vivo (28).

Sterol derivatives, including ergosterol and an obtusifoliol-like molecule, were also characterized as lipid components of vesicle membranes. Ergosterol and obtusifoliol have previously been described as major sterol components of C. neoformans (18), but to our knowledge, this is the first demonstration of 4,14-dimethylergosta-24(28)-en-3β-ol in cryptococcal membranes. The presence of other classes of hydrophobic compounds is evidently expected, and indeed, additional molecular ions were observed in ESI-MS analysis. The lack of detection of these molecules by HPTLC could be due to either small amounts of each individual lipid component or nonappropriated conditions of separation and staining. The fact that other lipids are clearly relevant to the physiology and pathogenesis of C. neoformans (16, 17, 21) justifies the characterization of other components of vesicle membranes.

GXM, the principal capsular polysaccharide of C. neoformans, was detected in vesicle preparations by serological approaches. The possibility that polysaccharide aggregates were contaminating 100,000 × g fractions was discarded, since vesicle preparations were purified in a MAb 18B7-bound resin. A definitive association between GXM and vesicles was obtained by immunogold labeling of the vesicles with an anti-GXM antibody followed by TEM analysis, which revealed that the polysaccharide is indeed surrounded by extracellular membrane domains. This information was supported by the facts that vesicle formation was not an artifact of polysaccharide-mediated effects on lipids (Fig. 4D) and that the polysaccharide and GlcCer, a lipid marker of cryptococcal vesicles, were concomitantly detected in fractions from a sucrose gradient. In this analysis, the polysaccharide content is directly related to...
vesicular density, which is in agreement with the high viscosity described for GXM (22).

McFadden and coworkers recently provided experimental evidence suggesting that capsule growth involves the production of GXM fibers that are released from, rather than attached to, the cell (22). In this model, capsule construction would depend on the self-association of GXM fibers, in which newly synthesized molecules would be secreted into the extracellular environment and further incorporated throughout the capsule by becoming entangled in existing capsular material. A new model of capsule growth was recently proposed whereby the capsule grows by apical extension (41). Vesicular transport to the capsular edge, followed by polysaccharide unloading and polysaccharide self-assembly into a capsule, would provide a potential mechanism for apical growth. Such a mechanism may have an inherent error rate such that some vesicles could be released to the extracellular space without being unloaded and could accumulate in the culture supernatant. Alternatively, released to the extracellular space without being unloaded and have an inherent error rate such that some vesicles could be potential mechanism for apical growth. Such a mechanism may depend on the self-association of GXM fibers, in which attached to, the cell (22). In this model, capsule construction is indeed able to lyse vesicles and use its internal content, possibly through the activity of exocellular lipases (5).

Our current results and previous reports (11, 14, 40) indicate that vesicles are synthesized intracellularly and transferred to the cell surface, from which they are secreted to the extracellular environment. It remains unknown how vesicles with an average diameter of 160 nm cross the fungal cell wall, a highly complex and dynamic structure (25). One possible explanation comes from the observation that the cell wall of Saccharomyces cerevisiae presents regular pores of around 200 nm, which can be increased to 400 nm under stress conditions (7). Therefore, despite being a rigid and complex structure, the fungal cell wall could allow the passive passage of particles in the range of 60 to 300 nm, dimensions that could accommodate the vesicles described here. Another mechanism for extracellular transport could involve motor proteins. In Aspergillus fumigatus, a 180-kDa polypeptide recognized by antimyosin antibodies was found to be concentrated in cell wall and plasma membrane fractions of conidia (9). Immunoelectron microscopy with an antimyosin antiserum revealed that the antigen is distributed in the cell body and transported extracellularly for capsule assembly by a mechanism that involves the production of vesicles. Hence, our results and other reports (11, 14, 31, 32, 40) suggest that the eukaryotic solution to the problem of capsular assembly takes advantage of a sophisticated trans-cell wall vesicular transport secretory mechanism that is not available in prokaryotes. The discovery that polysaccharide is packaged in vesicles containing immunologically active lipids has the potential to revolutionize our views of capsule assembly, extracellular polysaccharide shedding, and the mechanisms by which GXM mediates immunosuppression.

ACKNOWLEDGMENTS

M.L.R. was the recipient of an International Fellowship for Latin America and is supported by grants from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq-Brazil), Fundação Universitária José Bonifácio (FUJB-Brazil), and Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ-Brazil). K.M. is supported by Programa de Nanociência e Nanotecnologia, MCT-CNPq. L.N. is supported by CNPq-Brazil. A.C. is supported by NIH grants AI033142, AI033774, AI052733, and HL059842.

We thank Eliandro Lima, Yvonne Kress, and the Albert Einstein College of Medicine Analytical Imaging Facility staff for help with electron microscopy. We also thank Igor C. Almeida and Sirlei Dafre for support with mass spectrometry analysis. We are indebted to Jorge José Bastos Ferreira for helpful discussions.

D.L.O. is a M.S. student at Instituto de Bioguímica Medica, UFRJ.

REFERENCES


Downloaded from http://ec.asm.org/ on September 26, 2017 by guest