Biochimica et Biophysica Acta 1818 (2012) 458–466

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Membrane microdomain components of *Histoplasma capsulatum* yeast forms, and their role in alveolar macrophage infectivity

Loriane Tagliari [a,1,2], Marcos S. Toledo [a,1,2], Tanil G. Lacerda [a,2], Erika Suzuki [a,b,2], Anita H. Straus [a,2], Helio K. Takahashi [a,*]

[a] *Division of Glycoconjugate Immunochemistry, Department of Biochemistry, Universidade Federal de São Paulo/Escola Paulista de Medicina, Ed. J.L. Prado, Rua Botucatu, 862, 04023-900, São Paulo, SP, Brazil*

[b] *Department of Microbiology, Immunology and Parasitology, Universidade Federal de São Paulo/Escola Paulista de Medicina, Rua Botucatu, 862, 04023-900, São Paulo, SP, Brazil*

ARTICLE INFO

Article history:
Received 17 May 2011
Received in revised form 2 December 2011
Accepted 5 December 2011
Available online 14 December 2011

Keywords:
Histoplasma capsulatum
Membrane microdomain
Ergosterol
Macrophage infectivity
Glycosphingolipids
Methyl-beta-cyclodextrin

ABSTRACT

Analysis of membrane lipids of *Histoplasma capsulatum* showed that ~40% of fungal ergosterol is present in membrane microdomain fractions resistant to treatment with non-ionic detergent at 4 °C. Specific proteins were also enriched in these fractions, particularly Pma1p a yeast microdomain protein marker (a plasma membrane proton ATPase), a 30 kDa laminin-binding protein, and a 50 kDa protein recognized by anti-α5-integrin antibody. To better understand the role of ergosterol-dependent microdomains in fungal biology and pathogenicity, *H. capsulatum* yeast forms were treated with a sterol chelator, methyl-beta-cyclodextrin (mβCD). Removal of ergosterol by mβCD incubation led to disorganization of ergosterol-enriched microdomains containing Pma1p and the 30 kDa protein, resulting in displacement of these proteins from detergent-insoluble to -soluble fractions in sucrose density gradient ultracentrifugation. mβCD treatment did not displace/remove the 50 kDa α5-integrin-like protein nor had effect on the organization of glycosphingolipids present in the detergent-resistant fractions. Ergosterol-enriched membrane microdomains were also shown to be important for infectivity of alveolar macrophages; after treatment of yeasts with mβCD, macrophage infectivity was reduced by 45%. These findings suggest the existence of two populations of detergent-resistant membrane microdomains in *H. capsulatum* yeast forms: (i) ergosterol-independent microdomains rich in integrin-like proteins and glycosphingolipids, possibly involved in signal transduction; (ii) ergosterol-enriched microdomains containing Pma1p and the 30 kDa laminin-binding protein; ergosterol and/or the 30 kDa protein may be involved in macrophage infectivity.

1. Introduction

Fungal infections have received considerable research attention in the past two decades due to the increasing numbers of immunocompromised patients, and drug-resistant fungal strains. Mycoses range from low morbidity to severe systemic involvement, depending on the etiologic agent and host immune response [1].

Membrane components (proteins, lipids, glycoconjugates) of fungal cells differ from those of mammalian cells. Thus, to better understand fungal infectious processes, and aiming to find new targets for antifungal therapy, we studied *Histoplasma capsulatum* membrane microdomains and their role in host–pathogen interactions. In mammalians it is well known that several membrane proteins such as Lyn, Caveolin 1 or flotillin are preferentially distributed in microdomains and may be involved in a number of biological processes as cell signaling, pathogens binding and infectivity [2,3]. In this context, we also studied the Pma1p (a plasma membrane proton ATPase), a well known yeast microdomain marker, as well as other proteins present in *H. capsulatum* yeast membrane microdomains. Classical histoplasmosis is caused by inhaling fragments of aerosolized hyphae and/or conidia of this species, which are subsequently phagocytized by host alveolar macrophages, followed by mycelium to yeast transformation. In contrast to the usual role of macrophages to eliminate harmful microorganisms, alveolar macrophages present a favorable environment for replication and dissemination of *H. capsulatum*

Abbreviations: BHI, brain heart infusion; BSA, bovine serum albumin; Cho, cholesterol; DRM, detergent-resistant membrane microdomain; ECM, extracellular matrix; Erg, ergosterol; FAMEs, fatty acid methyl esters; Fr., fraction; GC–MS, gas chromatography coupled to mass spectrometry; GIPC, glycoinositol phosphorylceramide; GSLs, glycosphingolipids; HPTLC, high performance thin layer chromatography; HRP, horseradish peroxidase; mβCD, methyl-beta-cyclodextrin; MHC, monohexosylceramide; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Pho, phospholipid; PVDF, polyvinylidene fluoride; TBST, Tris, NaCl, Tween-20 buffer; TNE, Tris–HCl, NaCl, EDTA buffer

* Corresponding author at: Division of Glycoconjugate Immunochemistry, Department of Biochemistry, Ed. J.L. Prado, Rua Botucatu, 862, 04023-900, São Paulo, SP, Brazil. Tel./fax: +55 11 5579 2509.
E-mail address: takahashi.bioq@epm.br (H.K. Takahashi).
[1] Both authors contributed equally to this manuscript.
[2] Tel./fax: +55 11 5579 2509.

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doi:10.1016/j.bbamem.2011.12.008

yeasts, which are able to prevent phagosome–lysosome fusion, and control lysosomal pH [4,5].

1.1. Lipid components of H. capsulatum and other fungi

Sphingolipids are ubiquitous plasma membrane components of eukaryotic organisms, including fungi. These molecules are essential for cell survival [6], and are involved in the processes of cell adhesion, proliferation, differentiation, and apoptosis [7–9]. Studies from our laboratory and others have shown that fungal membranes contain glycosphingolipids (GSLs) of two types: (a) neutral monohexosylceramide (MHC), and (b) glycoinositol phosphorylceramide (GIPC), an acidic GSL [10–12]. In *H. capsulatum*, MHC is represented by glucosylceramide [13,14], while GIPC can be represented by various GSLs, termed compound V (Man*p*α1-3Man*p*α1-2/6Ins), VI (Gal*f*α1-6(Man*p*α1-3)Man*p*α1-2/6Ins), and VIII (Gal*p*β1-4(Man*p*α1-3)Man*p*α1-2/6Ins) [15,16]. GIPCs are not synthesized in mammalian cells, but their expression is essential for fungal viability. Therefore, GIPCs are a class of glycolipids that may be considered molecules with diagnostic value for different mycosis and/or targets for antifungal therapy [17–19].

Ergosterol, another characteristic lipid component in fungal plasma membranes, similar to vertebrate cholesterol, modulates several biological processes and also is organized in membrane microdomains. The absence of ergosterol in mammals and inhibition of fungal proliferation by ergosterol biosynthesis inhibitors point out the ergosterol as a target to antifungal therapy [20].

1.2. Yeast–macrophage interaction

McMahon et al. [21] reported that a 50 kDa protein present in the *H. capsulatum* cell wall is able to bind to laminin, a component of the extracellular matrix (ECM) of host lung. Adhesion of this protein to laminin is presumably a key step in fungal pathogenesis. Adhesion of *H. capsulatum* yeast to ECM promotes its phagocytosis through surface receptors of alveolar macrophages, and this event inhibits production of pro-inflammatory cytokines, thereby facilitating *H. capsulatum* infection [22,23].

We examined the membrane microdomains of *H. capsulatum*, and observed enhanced levels of specific lipid and protein components in fractions corresponding to "detergent-resistant membrane" (DRM). These microdomains in *H. capsulatum* appear to be involved in yeast–macrophage interaction.

2. Materials and methods

2.1. Fungal growth condition

H. capsulatum, strain 496, was provided by Dra. Olga F. Gompetz, Universidade Federal de São Paulo, São Paulo, Brazil. Yeast forms were grown 5–7 days in BHI (brain heart infusion) medium [13], at 37 °C, with rotary shaking (100 rpm).

2.2. Isolation of detergent-resistant membrane microdomains (DRMs) by sucrose density gradient ultracentrifugation

Yeast forms of *H. capsulatum* (2.5 g) were washed in ice-cold 0.01 M phosphate-buffered saline (PBS), pH 7.2. In order to disrupt cell walls, yeast were suspended in TNE buffer (50 mM Tris–HCl, 150 mM NaCl, 5 mM EDTA, pH 7.4) containing 10 μM of leupeptin and 2 mM of phenylmethanesulfonyl fluoride and stirred with glass beads (212–300 μm, Sigma-Aldrich, MO, USA) for 7 min at 4 °C. The suspension was centrifuged at 800×*g* for 5 min at 4 °C. The supernatant was collected, volume adjusted to 1% Brij 98 (Sigma) in TNE buffer, and kept for 30 min on ice. The lysate extract was adjusted to 45% sucrose, and then overlaid with a discontinuous sucrose density gradient consisting of 30% sucrose (5.5 mL) and 5% sucrose (4 mL) in an ultracentrifuge tube. Discontinuous sucrose gradient was achieved after centrifugation in a Sorvall AH-629 swing bucket rotor at 135,000×*g* for 20 h at 4 °C, and 12 fractions of 1 mL each were collected and numbered from top to bottom [24–26]. Alternatively, mammalian cells (NS-1) were incubated for 30 min with 1% Brij 98 at 4 °C or 37 °C, and the lysate was subjected to ultracentrifugation on sucrose gradient in a smaller scale, to obtain 7 fractions of 1 mL each. Aliquots of 300 μL were subjected to protein analysis by SDS-PAGE and Western blotting, and aliquots of 700 μL were subjected to partition with 1-butanol saturated water for lipid analysis by high performance thin layer chromatography (HPTLC).

2.3. Treatment of H. capsulatum yeast forms with methyl-beta-cyclodextrin (mβCD)

Yeast forms of *H. capsulatum* were washed in PBS, kept at 37 °C for 12 h in RPMI medium without serum, and washed twice in PBS by centrifugation. Yeasts were incubated with mβCD (40 mM) (Sigma) in PBS for 30 min at 37 °C, supernatant was removed, and yeasts were washed in PBS again to remove ergosterol extracted by mβCD. Yeasts were then stirred with glass beads as above to disrupt cell walls, and the suspension was incubated at a final concentration of 1% Brij 98 for 30 min at 4 °C. DRMs were isolated by ultracentrifugation on sucrose gradient, as described above, and control cells were suspended in PBS without mβCD [26].

2.4. SDS-PAGE and Western blotting

Twenty-five μL of each fraction obtained by sucrose gradient ultracentrifugation was analyzed by 8% SDS-PAGE. A 2.5 μL volume of fractions 11 and 12 was used for better band visualization; this adjustment was necessary to avoid saturation in SDS-PAGE. Protein profiles were visualized by Coomassie Blue or Silver staining; alternatively, proteins were transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were then blocked with 10% non-fat dry milk in TBST (200 mM Tris buffer, pH 8.0, containing 150 mM NaCl and 0.1% Tween 20) for 1 h, and incubated with antibody anti-Pma1p (Santa Cruz, CA, USA) or anti-α5-integrin (Cell Signaling, MA, USA) diluted in 1% bovine serum albumin (BSA) in TBST (TBST-BSA). Membranes were incubated overnight at 4 °C, then incubated 1 h at room temperature with horseradish peroxidase (HRP)-conjugated anti-goat or anti-mouse antibody (Santa Cruz). After each step, membranes were washed 3× with TBST. Finally, samples were incubated with chemiluminescence reagent (Pierce, IL, USA), and reactive proteins were visualized by the photodocumentation system G-BOX HR-16, analyzed and quantified by GeneTools software (Syngene) [27].

2.5. Binding of laminin to proteins obtained after density sucrose gradient ultracentrifugation

Proteins obtained after ultracentrifugation were transferred to PVDF membranes as described above. Membranes were blocked overnight with 10% non-fat dry milk in TBST, incubated for 1 h in TBST-BSA containing 30 μg/mL laminin (Sigma), and washed 4× in TBST. The PVDF membranes were then incubated for 2 h with anti-laminin antibody (diluted 1:1000) (Sigma) in TBST-BSA, washed in TBST, and incubated with HRP-conjugated anti-rabbit immunoglobulin (Santa Cruz) (diluted 1:1000) [28]. Reactive proteins were visualized and analyzed as described above.

2.6. Immunoprecipitation

Microdomain fractions obtained after sucrose gradient ultracentrifugation (Fr. 4 and 5, about 400 μg) were pre-cleared with Protein A-Sepharose 4B (3 h at 4 °C, under agitation), and the supernatants were incubated with laminin (30 μg, under agitation, overnight at

4 °C). Concomitantly, 50 μL of Protein A-Sepharose 4B was incubated with 6 μg of anti-laminin antibody (Sigma) in the presence of 500 μL of TNE buffer containing 1% Brij 98 (under agitation, overnight at 4 °C). After overnight incubation the Sepharose Protein A containing anti-lamin linked non-covalently was washed three times with TNE buffer and mixed with microdomain fraction pre-incubated with laminin (6 h, under agitation at 4 °C). After incubation the samples were centrifuged, the pellet was washed two times with TNE, suspended in 100 μL of sample buffer, boiled for 3 min, centrifuged, and their supernatant was applied on 12% SDS-PAGE. The gel was stained with SilverSNAP Stain Kit II (Pierce) according to the manufacturer's instructions. Proper controls were carried out omitting laminin incubation with microdomain fraction, or omitting anti-laminin incubation with Sepharose Protein A.

2.7. Lipid analysis

GSLs were analyzed by HPTLC on Silica Gel 60 Plates (Merck, Darmstadt, Germany) using chloroform/ methanol/$CaCl_2$ 0.02% (60:40:9; v/v/v) (Solvent A) as mobile phase. Glycolipids were visualized by staining with orcinol/H_2SO_4. Phospholipids were analyzed by HPTLC, developed in chloroform/ methanol/methylamine 40% (60:35:10, v/v/v) (Solvent B), and visualized as blue spots using Dittmer–Lester reagent [29]. Ergosterol was analyzed by HPTLC using chloroform/ethyl ether/acetic acid (97:2.5:0.5, v/v/v) (Solvent C) as mobile phase, and visualized as gray spots using copper sulfate reagent (10% $CuSO_4.5H_2O$ in 3% H_3PO_4). GSLs, phospholipids, and ergosterol were quantified by densitometric analysis (Scion Image Beta 4.02 and Image J 1.25) of HPTLC plates, in comparison to standard sample (1 mg/mL), phospholipids and ergosterol from Matreya (PA, USA), and GSLs purified from fungi [30–32].

2.8. Gas chromatography coupled with mass spectrometric analysis (GC–MS)

50 μL aliquots from pooled fractions (5–6 and 10–12) were extracted by chloroform/methanol/water partition [33], and dried in Pyrex tubes with Teflon-lined screw caps. Methanolysis was performed by adding 1.0 mL of 1 M HCl in anhydrous methanol (MeOH/HCl) and heating at 80 °C for 16 h. The cooled methanolysate was partitioned 3× with an equal volume of hexane. Combined hexane phases containing fatty acid methyl esters (FAMEs) were dried under N_2 stream at 37 °C, resuspended in 100 μL hexane, and analyzed by GC–MS [34]. Analysis was performed on a Varian CP-3800/1200L using a CP-Sil 8 CB column, with splitless injection. Samples were analyzed with temperature programming from 40 to 300 °C at 5 °C/min, then a 5 min plateau at 300 °C. Derivatives were identified based on their retention times and mass spectra, in comparison to authentic standards and published data [26].

2.9. Preparation of methyl-β-cyclodextrin: sterol complexes

Complex formation between sterol (cholesterol or ergosterol) (Sigma) and mβCD was performed essentially as described by Christian et al. and Zidovetzki and Levitan [35,36], with minor modifications. A 5 mM water-soluble solution of mβCD–sterol complex was prepared as follows. 118 μL of sterol (50 mg/mL) in choroform/methanol 1:1 (v:v) was added to a glass tube, dried under nitrogen stream, and dissolved in 3 mL distilled water. 171 mg mβCD was added, and the mβCD/sterol solution was vortexed and sonicated for 3 min. The saturated solution was placed in a rotating incubator at 37 °C overnight, and soluble sterol was filtered using 0.45 μm cellulose membrane (Millipore, MA, USA). The filtrate contained soluble mβCD–sterol complex (40 mMol: 5 mMol).

2.10. Preparation of alveolar macrophages

Alveolar macrophages were harvested by bronchoalveolar lavage of BALB/c mice with 1.0 mL PBS at 37 °C. Lavage fluid was carefully removed following gentle massage of the chest. Macrophages were washed 3× with cold PBS by centrifugation at 400×*g*, and the pellet was suspended in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, and penicillin (100 U/mL)/streptomycin (100 μg/mL). ~5×10^5 macrophages were placed on sterile glass coverslips on 24-well plates for 1 h at room temperature. Non-adherent cells were removed by washing 3× with RPMI 1640, and the plates kept at 37 °C in a CO_2 incubator. Research ethical approval (CEP 0023/06) was granted by the Ethical Research Committee Board, Universidade Federal de São Paulo.

2.11. Infectivity of yeast forms of H. capsulatum after mβCD treatment

H. capsulatum yeast forms were pre-incubated with or without mβCD (40 mM), and mβCD-treated yeasts were incubated or not with 5 mM exogenous soluble ergosterol or cholesterol at 37 °C for 1 h, in order to replenish membrane sterol. All yeast preparations were washed with PBS twice and cultured with alveolar macrophages in 24-well plates (5 yeasts/macrophage; 2.5×10^6 yeasts/well) for 1 h in RPMI 1640 without serum at room temperature. Non-adherent yeasts were removed by washing the monolayer with medium. Macrophages were fixed with 4% formaldehyde for 10 min, and stained with HEMA-3 solution. Phagocytic index was determined by multiplying the percentage of macrophages that had phagocytized at least one yeast by the average number of yeasts per infected macrophage (300 macrophages were examined) [26]. Alternatively, non-adherent yeasts were removed by washing the monolayer with medium, the infected cells were harvested and lysed by ten strokes through a 24-gauge needle. The lysates were centrifuged and yeasts were suspended in 500 μL of PBS. Samples were diluted 65 times in BHI medium, 50 μL of each solution was plated onto 6-well BHI-agar plates, followed by incubation for 3 days at 37 °C, and colony counting. All experiments were repeated three times in triplicates.

3. Results

3.1. Lipid distribution in detergent-resistant membrane microdomains (DRMs) of H. capsulatum isolated at 4 °C

Membranes of *H. capsulatum* yeast forms treated with 1% Brij 98 at 4 °C were fractionated on a sucrose gradient by ultracentrifugation, and 1.0-mL fractions were collected from top to bottom. (Glyco) lipid profiles of twelve fractions were analyzed by HPTLC. GSLs were found mainly in Fr. 5 and 6 (low-density fractions — raft fractions), and Fr. 9–12 (high-density fractions and detergent) (Fig. 1A). Significant amounts of ergosterol and phospholipids were detected in Fr. 4–7 (low-density membranes) (Fig. 1B, C). Quantification of (glyco) lipid components by densitometry of HPTLC plates showed that Fr. 4–7 contain ~25% of total GSLs, 42% of total ergosterol, and 36% of total phospholipids. These findings indicate that low-density DRMs of *H. capsulatum* are found in Fr. 5 and 6, similar to those observed in mammalian cells [25,37]. Other non-ionic detergents, Tween 20 and Triton X-100, were less efficient than Brij 98 for isolation and recovery of DRMs (data not shown).

In order to analyze the composition of fatty acid of GSLs and phospholipids present in detergent-insoluble and -soluble membranes, Fr. 5 and 6 and Fr. 10 to 12 were pooled. Lipids (50 μL of pooled fractions) were derivatized as fatty acid methyl esters (FAMEs), and analyzed by GC–MS. As shown in Fig. 2, about 80% of fatty acids present in Fr. 5–6, consisted of saturated C16 and C18, and 20% was unsaturated C18:1 and C18:2. In contrast, in Fr. 10–12, only 42% of fatty acids

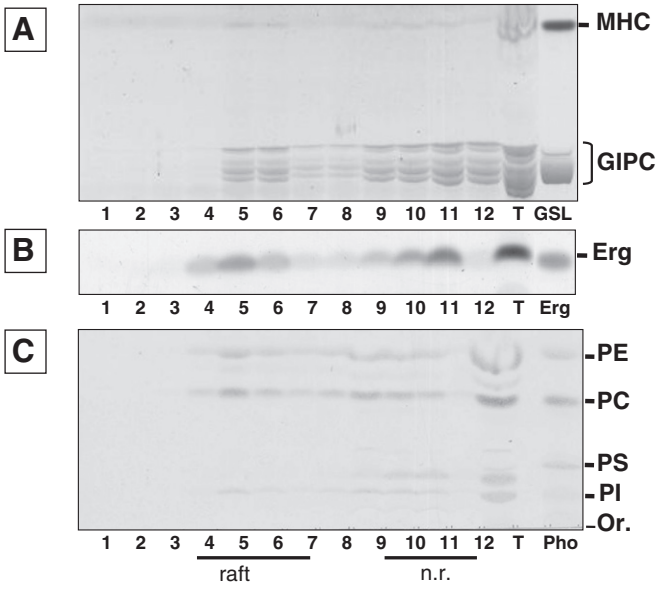

Fig. 1. Lipid profile of fractions obtained by sucrose density gradient ultracentrifugation of *H. capsulatum*.*H. capsulatum* yeast forms were lysed with 1% Brij 98 at 4 °C, and subjected to sucrose density gradient ultracentrifugation to isolate membrane microdomains. Twelve fractions were collected, from top to bottom of the tube. Lipid components were analyzed by HPTLC developed in the following solvents. Panel A: solvent A; GSLs stained with orcinol/H_2SO_4. Panel B: solvent B; ergosterol stained with Cu_2SO_4. Panel C: solvent C; phospholipids stained with Dittmer–Lester reagent. T: total fraction; GSL: GSL standard mixture containing monohexosylceramide (MHC) and glycoinositol phosphorylceramide (GIPC). Erg: ergosterol standard. Pho: phospholipid standard mixture containing phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylserine (PS), and phosphatidylinositol (PI). n.r. = nonraft fractions. Or., origin. Results shown are representative of three or more experiments.

were saturated C16 and C18, while 58% were unsaturated C18:1 and C18:2.

3.2. DRMs isolated at low vs. high temperature

To compare the stability of membrane microdomains of *H. capsulatum* yeast forms, vs. those of mammalian cells, yeast forms were disrupted with glass beads at 4 °C, and incubated with 1% Brij 98 in TNE buffer for 30 min at 4 °C and 37 °C. Stability of membrane microdomains at 37 °C was determined after sucrose gradient ultracentrifugation and HPTLC analysis. Distributions of ergosterol, GSLs, and phospholipids in the fractions are shown in Fig. 3A, B, C. DRMs were observed in Fr. 4 to 7 at both 4 °C and 37 °C. Ergosterol, GSL, and

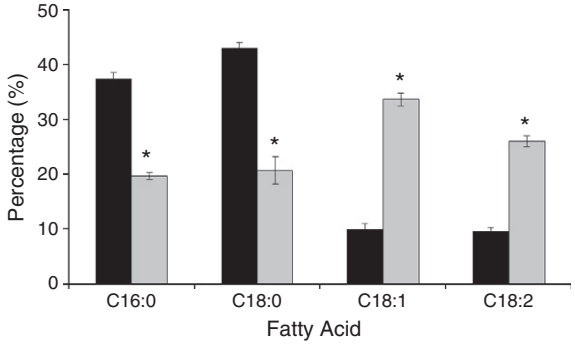

Fig. 2. GC/MS quantification of fatty acids from *H. capsulatum* Fr. 5–6 and Fr. 10–12.50 μL aliquots from pooled Fr. 5–6 (black bars) and Fr. 10–12 (gray bars) as shown in Fig. 1 were analyzed by GC/MS. Fatty acid chains were identified on the basis of retention time and fragmentation pattern.C16:0: hexadecanoic acid (palmitic acid). C18:0: octadecanoic acid (estearic acid). C18:1: cis-9-octadecenoic acid (oleic acid). C18:2: 9,12-octadecadienoic acid (linoleic acid). Results shown are representative of three or more experiments. *Significantly different from Fr.5–6 at $p<0.0001$.

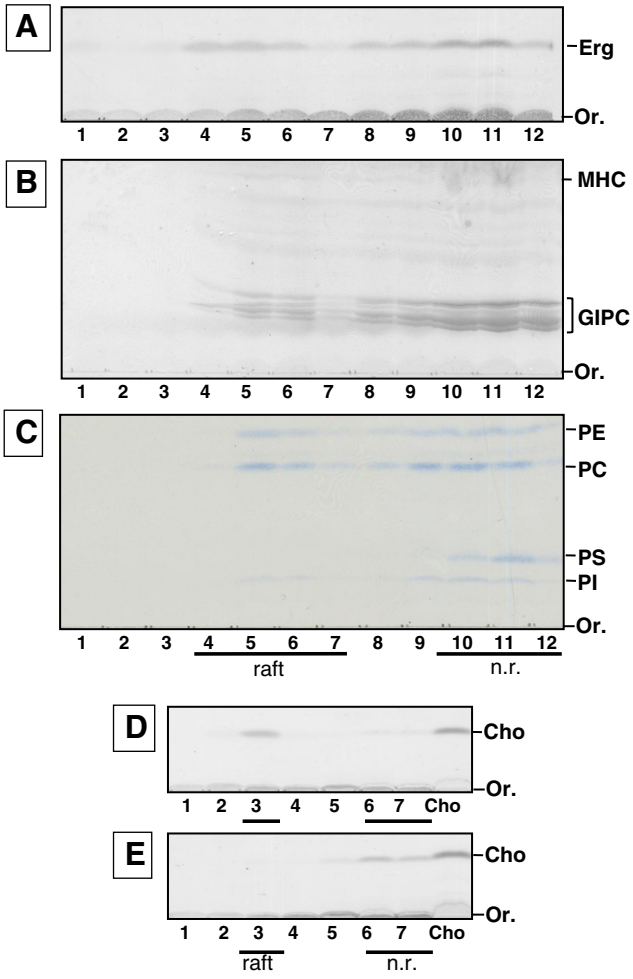

Fig. 3. Lipid profile of ultracentrifugation fractions obtained at 37 °C.Panels A, B, C: *H. capsulatum* yeast forms were lysed with glass beads and incubated with Brij 98 for 30 min at 37 °C. The lysate was subjected to sucrose density gradient ultracentrifugation, and 12 fractions were obtained. Panels D, E: mammalian cells (NS-1) were incubated with Brij 98 for 30 min at 4 °C (D) or 37 °C (E). The lysate was subjected to ultracentrifugation, and 7 fractions were obtained. Lipid components were analyzed by HPTLC, developed in the following solvents. Panels A, D, E: solvent B; ergosterol and cholesterol stained with Cu_2SO_4. Panel B: solvent A; GSLs stained with orcinol/H_2SO_4. Panel C: solvent C; phospholipids stained with Dittmer–Lester reagent. n.r. = nonraft fractions. Abbreviations are the same as in Fig. 1. Results shown are representative of three or more experiments.

phospholipid profiles were similar for DRM fractions isolated at 4 °C (Fig. 1) or 37 °C (Fig. 3). These results suggest that membrane microdomains of *H. capsulatum* are resistant to 1% Brij 98 treatment at 37 °C. In contrast, for mammalian cells, DRMs are isolated only at 4 °C (Fig. 3D); at 37 °C, microdomains are completely solubilized, and cholesterol (Fig. 3E) and GSLs (not shown) are completely displaced from raft- to non raft-fractions.

3.3. Protein distribution in DRMs

Protein distribution in the sucrose gradient fractions was analyzed by SDS-PAGE and visualized by Coomassie Blue, Silver staining or Western blotting. The relative protein distribution was calculated by densitometric analysis. As shown in Fig. 4A ('Control'), by SDS-PAGE, two protein populations were enriched in microdomain Fr. 5 and 6 at 30 kDa ("a") and 50–55 kDa ("b"). Anti-Pma1p antibody (Fig. 4B, left panel), and anti-α5-integrin antibody (Fig. 4D, left) stained preferentially components of ~100 kDa and ~50–55 kDA, respectively. By densitometry it was found that about 90% of the ~100 kDa component and 52% of the ~50–55 kDa component were

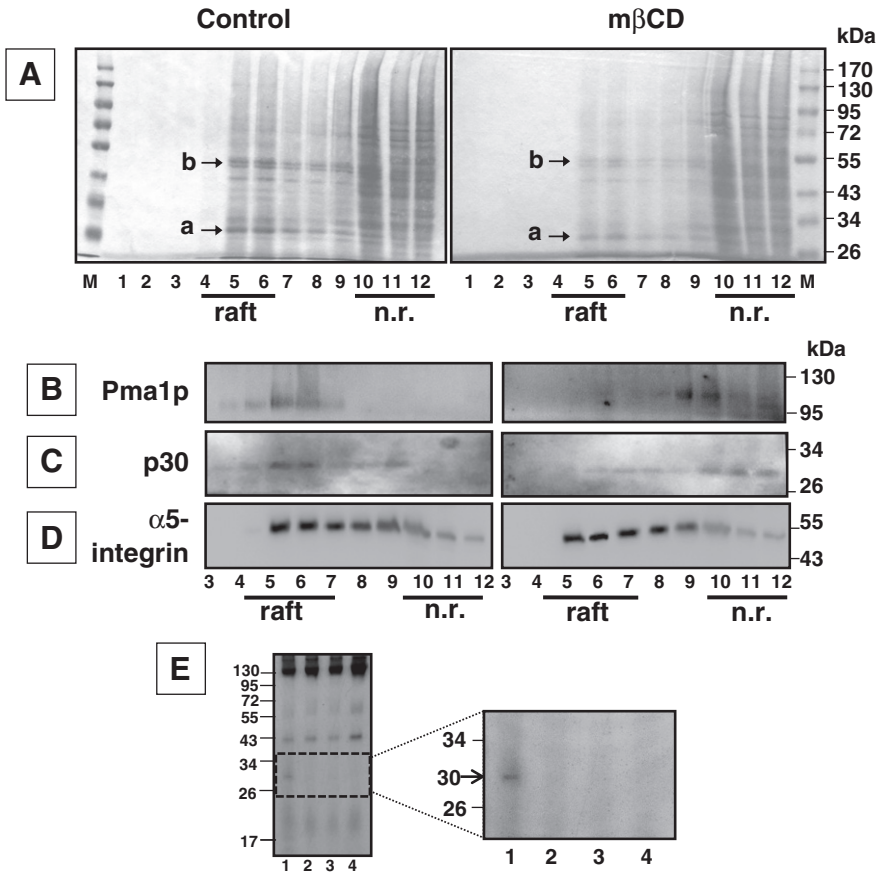

Fig. 4. Microdomain proteins of *H. capsulatum* yeast forms with or without mβCD treatment. *H. capsulatum* yeast forms were treated or not with mβCD. 25 μL of Fr. 1–10 and 2.5 μL of Fr. 11–12 were subjected to 8% SDS-PAGE, transferred to PVDF membrane, and analyzed as follow: Panel A: Coomassie Blue staining. Panel B: Western blotting using anti-Pma1p antibody. Panel C: Western blotting assayed by incubating PVDF membrane with laminin (30 μg/ml) followed by anti-laminin antibody. Panel D: Western blotting using anti-α5-integrin antibody. Panel E: SDS-PAGE Silver staining of laminin immunoprecipitation of microdomain fractions (Fr. 5 and 6) isolated from yeast forms of *H. capsulatum* control (Lane 1) or mβCD treated (Lane 2). Laminin-immunoprecipitation controls were carried out omitting microdomain fractions (Lane 3) or omitting laminin incubation with microdomain fractions (Fr. 5 and 6) isolated from yeast forms of *H. capsulatum* (Lane 4).

present in microdomain Fr. 4–7. Laminin-binding assay and densitometric analysis showed that 77% of the 30 kDa laminin-binding protein was found in Fr. 4–7 (Fig. 4C, left). To confirm the laminin binding specificity to native 30 kDA protein, an immunoprecipitation assay was carried out using laminin, anti-laminin antibody and Sepharose Protein A. Under these conditions only the 30 kDa protein was immunoprecipitated, as shown in Fig. 4E, lane 1.

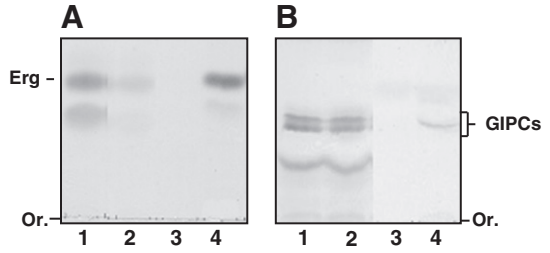

Fig. 5. Extraction of ergosterol from *H. capsulatum* yeast forms with 40 mM mβCD.*H. capsulatum* yeast forms were incubated with 40 mM mβCD for 1 h at 37 °C, centrifuged, and supernatants were collected. To assess the efficiency of lipid extraction by mβCD, supernatant was extracted with 1-butanol, and the pellet was washed with PBS and extracted with isopropanol/hexane/water (55:20:25, v/v/v). Panel A: HPTLC developed in solvent B; ergosterol stained with Cu_2SO_4. Panel B: HPTLC developed in solvent A; GSLs stained with orcinol/ H_2SO_4. Lane 1: lipid fraction extracted from yeast cells not treated with mβCD. Lane 2: lipid fraction from cells treated with 40 mM mβCD. Lane 3: lipid fraction from culture supernatant of cells not treated with mβCD. Lane 4: lipid fraction from culture supernatant of cells treated with 40 mM mβCD. Results shown are representative of three or more experiments.

3.4. Effect of mβCD on DRMs

To investigate the importance of ergosterol in DRMs of *H. capsulatum*, yeast forms were incubated with mβCD at 40 mM, and subsequent viability was tested by: (i) staining with live/dead yeast (Invitrogen, CA, USA); (ii) determination of colony-forming units (CFU) [38]. Untreated vs. treated mβCD-yeast were found to be equally viable by the two methods.

Lipids were extracted from mβCD-treated cells and from culture supernatants and both these extracts were analyzed by HPTLC.

Table 1
Distribution of lipids and proteins of *H. capsulatum* yeast forms with or without mβCD treatment, after sucrose density gradient ultracentrifugation. Fractions were pooled according to HPTLC and SDS-PAGE results.

	Control		mβCD	
Fractions	4–7 (μg)	10–12 (μg)	4–7 (μg)	10–12 (μg)
Erg[a]	49.1 ± 5.9	38.4 ± 6.9	7.3 ± 4.0	15.0 ± 5.7
GIPC[a]	13.9 ± 3.5	24.9 ± 5.1	14.6 ± 3.1	32.1 ± 6.6
MHC[a]	12.2 ± 2.7	15.9 ± 5.1	12.4 ± 2.4	19.8 ± 6.9
Pho[a]	17.8 ± 3.5	15.3 ± 3.9	10.2 ± 2.9	23.1 ± 6.6
Protein[a]	878 ± 29.2	14,421 ± 45	464 ± 25	13,161 ± 51

Erg = ergosterol; GIPC = glycoinositol phosphorylceramide; MHC = monohe xosylceramide; Pho = phospholipid. Lipids were quantified by densitometric parameters using appropriate standard; protein was quantified by Micro BCA Protein Assay Kit (Pierce). Fr. 1–3 and Fr. 8–9 are omitted because of low concentration, or absence.

[a] Components are expressed as μg per pooled fraction.

mβCD treatment removed 85% of membrane ergosterol (Fig. 5A), remaining 91% of membrane GIPCs (Fig. 5B).

DRMs were isolated from cells incubated with 40 mM mβCD by sucrose density gradient ultracentrifugation, as described in Section 2.3, and resulting fractions were analyzed by HPTLC. GSL profiles of detergent-insoluble (Fr. 4–7) vs. -soluble fractions (Fr. 10–12) did not differ significantly between mβCD-treated vs. non-treated cells (Table 1). These findings indicate that mβCD selectively extracts ergosterol from yeast membrane, and ergosterol removal does not affect GSL composition in DRMs.

On the other hand, SDS-PAGE analysis showed that removal of ergosterol did result in a partial change of protein profile. mβCD treatment of yeast cells caused a 45% reduction of protein in Fr. 5 and 6 (Fig. 4A, right), with displacement of Pma1p and 30 kDa laminin-binding protein to soluble fractions (Fr 9–12), as shown by Western blotting (Fig. 4B and C, right). Densitometric analysis revealed that after mβCD treatment only 10% of Pma1p and 16% of 30 kDa laminin-binding protein remained in microdomain Fr. 4–7, comparing to 90% of Pma1p and 77% of the 30 kDa laminin-binding protein found in the Fr. 4–7 of non-treated yeasts. As expected the proportion of Pma1p and 30 kDa protein in soluble Fr. 10–12 increased from 2.7% and 7% to 63% and 69%, respectively. In agreement with this data, no 30 kDa protein was immunoprecipitated when laminin and anti-laminin-Sepharose-Protein A were incubated with mβCD-yeast microdomain fractions (Fig. 4E, lane 2). Reactivity of anti-α5-integrin antibody with the 50 kDa protein of yeast forms treated or not with mβCD was detected mainly in microdomain Fr. 4–7 (50% and 52%, respectively), as shown in

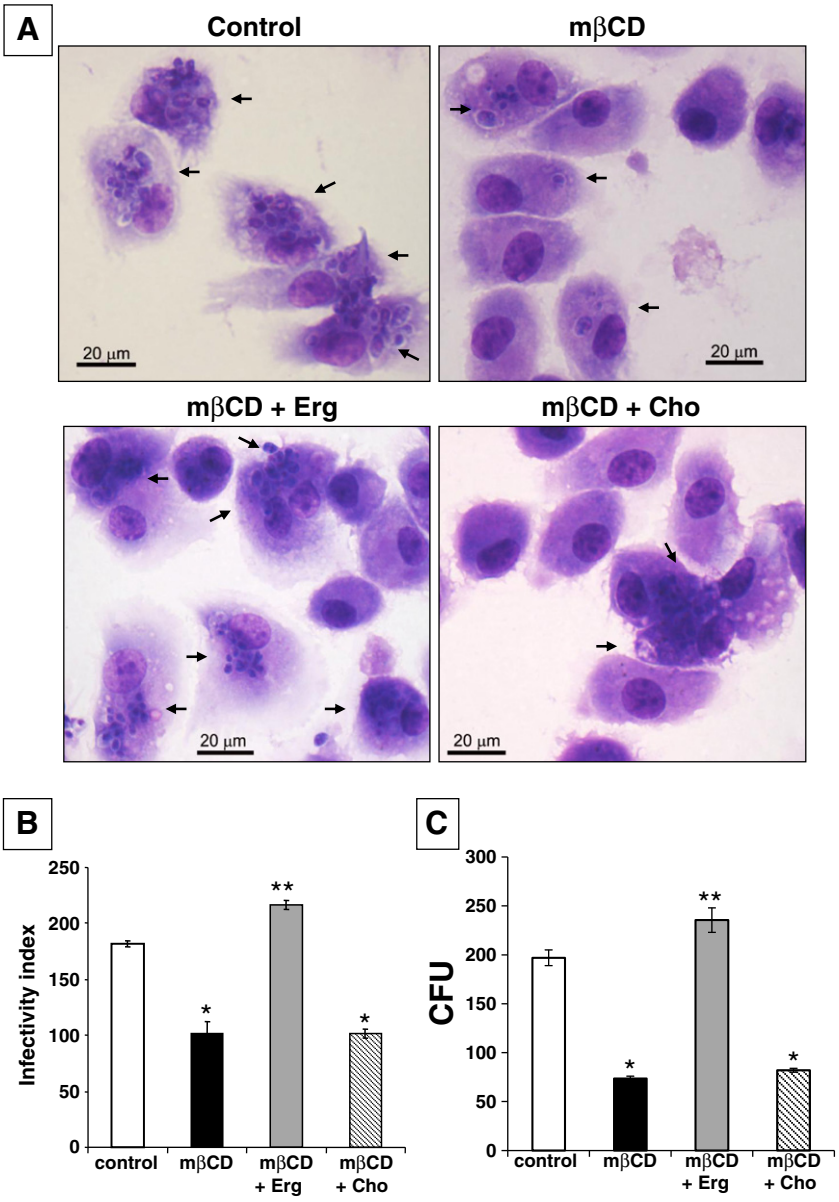

Fig. 6. *H. capsulatum* infectivity assay using alveolar macrophages. Panel A: Alveolar macrophages of BALB/c mice were collected by bronchoalveolar lavage, placed on coverslips, and incubated with *H. capsulatum* yeast forms pre-treated with or without 40 mM mβCD, followed by 1 h incubation with exogenous ergosterol or cholesterol (5 mM mβCD: sterol), in 5:1 proportion of yeasts/macrophage. Coverslips were then washed, fixed with 4% formaldehyde in PBS, stained with HEMA-3, and analyzed by optical microscopy. Infected macrophages are indicated by arrows. Panel B: Phagocytic index was determined by multiplying the percentage of macrophages that had phagocytosed at least one yeast by the average number of yeasts per infected macrophage; 300 cells were examined. Experiments were repeated 3×, in triplicate. Results shown are representative of these experiments. *Significantly different from control at $p<0.0001$; **significantly different from control at $p<0.005$. Panel C: Yeast forms of *H. capsulatum* recovered from infected macrophages were plated on a petri dish containing BHI-agar medium, and incubated for 3 days at 37 °C. Colony forming units (CFUs) were counted. *Significantly different from control at $p<0.0001$; **significantly different from control at $p<0.005$.

Fig. 4D, right and left, suggesting that this protein is present in ergosterol-independent microdomain.

3.5. Importance of microdomain integrity in the infectivity of alveolar macrophages

To determine the role of ergosterol-dependent membrane microdomains in infection of alveolar macrophages, *H. capsulatum* yeast forms were treated with 40 mM mβCD, washed with PBS, and then incubated with macrophages isolated from lungs of BALB/c mice. Treatment of the yeast forms with mβCD reduced macrophage infection by ~45% (Fig. 6A and B, control and mβCD).

Next, the ability of exogenous soluble sterols (cholesterol, ergosterol) incorporated into membrane microdomains to restore macrophage infectivity was evaluated. To ascertain whether exogenous sterols were incorporated into plasma membrane and restored membrane microdomains, mβCD-treated yeasts were incubated with exogenous sterols, lysed, and microdomains were isolated by ultracentrifugation. HPTLC analysis confirmed that exogenous ergosterol (Fig. 7B) and cholesterol (Fig. 7C) were incorporated into DRM-containing Fr. 4–7. In the same set of experiment it was analyzed whether sterol reincorporation was able to reinsert the 30 kDa laminin-binding protein in membrane microdomain and its relative distribution in microdomain and non-microdomain fractions was determined by densitometric analysis. As shown in Fig. 7D, in control experiments 88% of the 30 kDa laminin-binding protein is found in the microdomain Fr. 4–7, upon treatment with mβCD only 13% of the 30 kDa protein remains in the Fr. 4–7, and about 52% of the 30 kDa protein is displaced to non-raft Fr. 10–12 (Fig. 7E). As observed in Fig. 7F, ergosterol-reinserted yeasts show that 76% of the 30 kDa laminin-binding protein is reinserted in microdomain Fr. 4–7. On the other hand, for mβCD-treated yeasts incubated with exogenous cholesterol, no reinsertion of the 30 kDa laminin-binding was observed in microdomain Fr. 4–7, which present about 10% of the 30 kDa laminin binding protein, whereas about 61% of the 30 kDa laminin-binding protein remained in Fr. 10–12 (Fig. 7G).

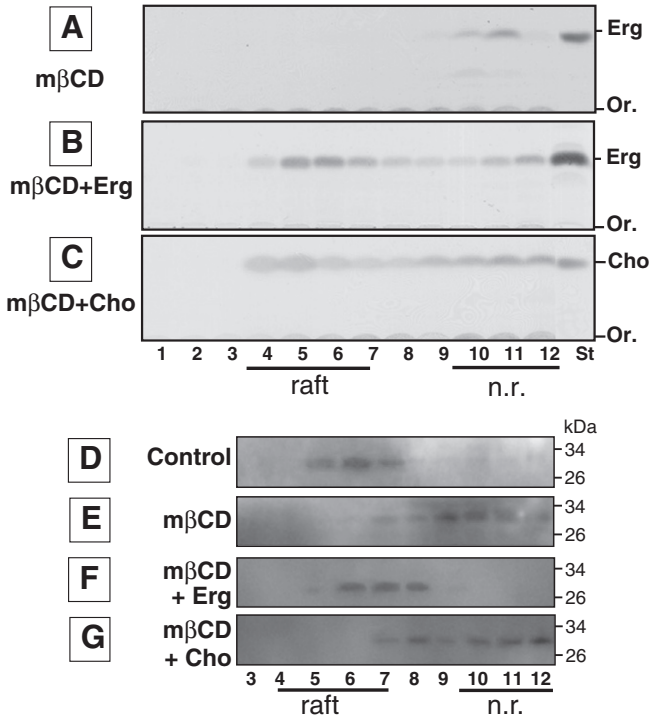

Fig. 7. Sterol and 30 kDa protein profiles of *H. capsulatum* treated with mβCD, and effect of exogenously-added ergosterol and cholesterol.*H. capsulatum* yeast forms were treated with mβCD, then incubated with or without 5 mM exogenous water-soluble ergosterol or cholesterol, washed extensively with PBS, and lysed with glass beads. The lysate was incubated with Brij 98 for 30 min, subjected to sucrose density gradient ultracentrifugation, and 12 fractions were collected from top to bottom of the tube, and analyzed as follows: sterols were analyzed by HPTLC developed in solvent B and stained with Cu_2SO_4. Panel A: Ergosterol profile of mβCD treated yeasts. Panel B: Ergosterol profile of mβCD treated yeasts with exogenous ergosterol addition. Panel C: Cholesterol profile of mβCD treated yeasts with exogenous cholesterol addition. Results shown are representative of three or more experiments. Also, proteins were subjected to 8% SDS-PAGE, transferred to PVDF membrane, and Western blot assayed by incubating PVDF membrane with laminin (30 μg/ml) followed by anti-laminin antibody. Panel D: laminin-binding protein profile of control yeasts. Panel E, laminin-binding protein profile of mβCD treated yeasts. Panel F: laminin-binding protein profile of mβCD treated yeasts with exogenous ergosterol addition. Panel G: laminin-binding protein profile of mβCD treated yeasts with exogenous cholesterol addition. n.r. = nonraft fractions.

Finally, yeast forms treated with mβCD were incubated or not with exogenous ergosterol or cholesterol, and macrophage infectivity was analyzed. Infectivity of mβCD-treated cells was restored by incubation of mβCD-treated cells with ergosterol (Fig. 6A, B, 'mβCD + Erg'), but not with cholesterol ('mβCD + Cho'), indicating that only ergosterol has the structural requirement to restore fungal DRM's organization needed for *H. capsulatum* infectivity. The macrophage infection rate by *H. capsulatum* was also determined by colony formation units (Fig. 6C) for each experimental condition, 'mβCD' and 'mβCD–Cho' treatment reduced colony formation to approximately 37% and 42% of that observed in controls, while 'mβCD–Erg' restores *H. capsulatum* infectivity, corroborating the infectivity index results.

4. Discussion

Isolation of membrane microdomains of fungal species such as *Saccharomyces cerevisiae*[24], *Candida albicans*[39,40], and *Cryptococcus neoformans*[41] has been reported during the past decade. However, components and functions of membrane microdomains of pathogenic fungi remain poorly understood [11,42,43]. In the present study, we characterized membrane microdomains isolated from yeast forms of *H. capsulatum*, and their possible role in macrophage infectivity.

4.1. Components of membrane microdomains of H. capsulatum

Analysis of lipid content showed that ergosterol is the main lipid in the microdomain fraction (42%), followed by phospholipids (36%) and GSLs (25%).

Western blotting showed that Pma1p, the major plasma membrane proton ATPase, is present mainly in DRMs [44], particularly in ergosterol-dependent microdomains. Binding experiments using PVDF membrane containing sucrose gradient fractions, also showed binding of laminin to a ~30 kDa protein localized in DRMs, Fr. 5–6, which was immunoprecipitated by addition of laminin and anti-laminin-Sepharose A. It is feasible that this 30 kDa laminin-binding protein may belong to a family of fungal adhesins, since a similar laminin-binding protein was also described in *Paracoccidioides brasiliensis*[28]. Further studies along this line may reveal relevant new data regarding a possible fungal adhesion protein family related to fungal infectivity. The identification of a possible *H. capsulatum* adhesin located in DRM has important implications for yeast interactions with host cells and other substrates, since adhesion is the first step in the infection process.

4.2. Isolation of membrane microdomains of H. capsulatum at 37 °C

H. capsulatum microdomains were isolated either at 4 °C or 37 °C and presented similar composition of ergosterol and glycosphingolipids. However, microdomains isolated at 37 °C presented reduction of phospholipids, only 23% of phospholipids remain in DRM fractions, vs 36% in microdomains isolated at 4 °C.

In mammalian cells isolation of membrane microdomains is possible using non-ionic detergent at 4 °C [37,45–47], but it is not feasible at 37 °C [48]. One possible explanation for the maintenance of fungal cell microdomains at 37 °C is the finding of Czub and Baginski [49]

that ergosterol-containing membranes are packed more orderly than cholesterol-containing membranes, and thus they are more resistant to detergent. Our results are also consistent with those of Xu et al. [50], who showed that ergosterol is able to form areas that are more stable at high temperatures (e.g., 37 °C) than cholesterol. This could be related to structural differences between ergosterol and cholesterol, i.e., ergosterol has an additional double bond between carbons 7 and 8 of ring B, between carbons 22 and 23, and a methyl group at C-24 aliphatic chain, which may promote stability through a more orderly-packed lipid raft.

4.3. Ergosterol-dependent and -independent membrane microdomains

mβCD treatment resulted in extraction of 85% of ergosterol present in *H. capsulatum* membranes, but did not significantly alter profiles of GSLs or phospholipids. Along this line, Hakomori [47] and Westerlund and Slotte [51] reported the ability of GSLs to form membrane microdomains independently of sterols, possibly due to strong compaction of hydrophobic regions of GSL molecules, and stabilization by hydrogen bonds at the ceramide portion.

mβCD treatment did alter protein profile in DRMs, by shifting proteins, such as the 30 kDa laminin-binding protein and Pma1p, to soluble fractions.

Results from mβCD experiments suggest that both ergosterol-dependent and ergosterol-independent microdomains are present in *H. capsulatum* plasma membranes, since it was detected presence of microdomains enriched in GSLs and α5 integrin-like protein even after ergosterol removal by mβCD. Integrin-like molecules have been also described in *C. albicans* surface [52,53], such as the 37 kDa alcohol dehydrogenase, a protein recognized by antibody directed to α5β1 integrin described by Klotz et al. [54].

An attractive hypothesis for an integrated functionality of fungal membrane, would be different populations of DRMs such as ergosterol-dependent and ergosterol-independent present in yeast forms of *H. capsulatum,* acting in a concerted way allowing key molecules to exert their biological roles in a more efficient way.

4.4. Importance of membrane microdomains in infectivity of alveolar macrophages

H. capsulatum infectivity to BALB/c alveolar macrophages was reduced significantly (45%) after mβCD treatment, suggesting that the infectivity requires integrity of ergosterol-dependent fungal membrane microdomains. Similarly, Cossart et al. [55] demonstrated the importance of host membrane microdomain integrity by showing that infection of cholesterol-depleted Vero cells by the bacterium *Listeria monocytogenes* was reduced by 50%, and that infectivity was restored by addition of cholesterol. In this context we described earlier that treatment of *Leishmania (Viannia) braziliensis* with mβCD decreased macrophage infectivity by 52% [26].

In the present study, we showed that exogenous ergosterol can be re-inserted into membranes of mβCD-treated yeast cells, and restore their ability to infect alveolar macrophages. Addition of exogenous cholesterol did not restore infectivity. Thus, ergosterol, but not cholesterol, is essential for the maintenance of key structural and functional aspects of membrane microdomains necessary for the *H. capsulatum*–host interaction. A possible explanation may be due to the fact that only in microdomain fractions containing ergosterol was observed the presence of a 30 kDa laminin-binding protein, similar to that described in *P. brasiliensis,* as an adhesin able to bind to laminin [28], possibly responsible for *H. capsulatum*–macrophage interaction.

5. Conclusions

Detergent-resistant membrane microdomains (DRMs), obtained after fractionation of *H. capsulatum* yeast forms were isolated by sucrose density gradient ultracentrifugation at 4 °C. These DRMs are enriched in ergosterol, GSLs, and specific proteins, and remain stable at 37 °C.

Protein analysis following treatment of DRMs with mβCD (a sterol chelator, which extracts ergosterol from membranes) showed displacement of Pma1p, and the laminin-binding 30 kDa protein from DRM fractions (Fr. 4–7) to detergent-soluble fractions (Fr. 10–12). Conversely, a 50 kDa integrin-like protein was not displaced from DRMs to soluble fractions after mβCD treatment, suggesting the existence of microdomains whose integrity does not depend on ergosterol. Removal of ergosterol of *H. capsulatum* yeast forms with mβCD reduced the fungus ability to infect alveolar macrophage by 45%. These findings suggest the existence in *H. capsulatum* yeast forms of ergosterol-dependent DRMs where Pma1p and the 30 kDa laminin-binding protein are inserted, and ergosterol-independent DRMs, where 50 kDa integrin-like protein is inserted. It is noteworthy the observation that reinsertion of ergosterol, but not cholesterol, was able to fully restore the *H. capsulatum* ability to infect alveolar macrophages.

Acknowledgements

This work was supported by FAPESP, CNPq, and CAPES. The authors thank Dr Stephen Anderson for editing of the manuscript.

References

[1] E. Brummer, E. Castaneda, A. Restrepo, Paracoccidioidomycosis: an update, Clin. Microbiol. Rev. 6 (1993) 89–117.
[2] S. Kannan, J. Knittel, S. Mullegama, G.F. Gao, M. Wu, Src tyrosine kinase Lyn plays a role in *P. aeruginosa* infection of respiratory epithelium cells, Eur. J. Immunol. 36 (2006) 1739–1752.
[3] C. Hoffmann, A. Berking, F. Agerer, A. Buntru, F. Neske, G.S. Chhatwal, K. Ohlsen, C.R. Hauck, Caveolin limits membrane microdomain mobility and integrin-mediated uptake of fibronectin-binding pathogens, J. Cell Sci. 15 (2010) 4280–4289.
[4] L.G. Eissenberg, W.E. Goldman, *Histoplasma capsulatum* fails to trigger release of superoxide from macrophages, Infect. Immun. 55 (1987) 29–34.
[5] L.A. Gildea, G.M. Ciraolo, R.E. Morris, S.L. Newman, Human dendritic cell activity against *Histoplasma capsulatum* is mediated via phagolysosomal fusion, Infect. Immun. 73 (2005) 6803–6811.
[6] S.B. Levery, M. Momany, R. Lindsey, M.S. Toledo, J.A. Shayman, M. Fuller, K. Brooks, R.L. Doong, A.H. Straus, H.K. Takahashi, Disruption of the glucosylceramide biosynthetic pathway in *Aspergillus nidulans* and *Aspergillus fumigatus* by inhibitors of UDP-Glc:ceramide glucosyltransferase strongly affects spore germination, cell cycle, and hyphal growth, FEBS Lett. 525 (2002) 59–64.
[7] G. Kawai, Y. Ikeda, K. Tubaki, Fruiting of *Schizophyllum commune* induced by certain ceramides and cerebrosides from *Penicillium funiculosum*, Agric. Biol. Chem. 49 (1985) 2137–2146.
[8] K. Hanada, Sphingolipids in infectious diseases, Jpn. J. Infect. Dis. 58 (2005) 131–148.
[9] C.Y. Ywazaki, P.K. Maza, E. Suzuki, H.K. Takahashi, A.H. Straus, Role of host glycosphingolipids on *Paracoccidioides brasiliensis* adhesion, Mycopathologia 171 (2011) 325–332.
[10] M. Leipelt, D. Warnecke, U. Zähringer, C. Ott, F. Müller, B. Hube, E. Heinz, Glucosylceramide synthases, a gene family responsible for the biosynthesis of glucosphingolipids in animals, plants, and fungi, J. Biol. Chem. 276 (2001) 33621–33629.
[11] E. Suzuki, A.K. Tanaka, M.S. Toledo, S.B. Levery, A.H. Straus, H.K. Takahashi, Trypanosomatid and fungal glycolipids and sphingolipids as infectivity factors and potential targets for development of new therapeutic strategies, Biochim. Biophys. Acta 1780 (2008) 362–369.
[12] H.K. Takahashi, M.S. Toledo, E. Suzuki, L. Tagliari, A.H. Straus, Current relevance of fungal and trypanosomatid glycolipids and sphingolipids: studies defining structures conspicuously absent in mammals, An. Acad. Bras. Cienc. 81 (2009) 477–488.
[13] M.S. Toledo, S.B. Levery, E. Suzuki, A.H. Straus, H.K. Takahashi, Characterization of cerebrosides from the thermally dimorphic mycopathogen *Histoplasma capsulatum*: expression of 2-hydroxy fatty *N*-acyl (*E*)-Δ^3-unsaturation correlates with yeast–mycelium phase transition, Glycobiology 11 (2001) 113–124.
[14] M.S. Toledo, E. Suzuki, S.B. Levery, A.H. Straus, H.K. Takahashi, Characterization of monoclonal antibody MEST-2 specific to glucosylceramide of fungi and plants, Glycobiology 11 (2001) 105–112.
[15] K. Barr, R.L. Lester, Occurrence of novel antigenic phosphoinositol-containing sphingolipids in the pathogenic yeast *Histoplasma capsulatum*, Biochemistry 23 (1984) 5581–5588.

[16] K. Barr, R.A. Laine, R.L. Lester, Carbohydrate structures of three novel phosphoinositol-containing sphingolipids from the yeast *Histoplasma capsulatum*, Biochemistry 23 (1984) 5589–5596.
[17] R.C. Dickson, R.L. Lester, Yeast sphingolipids, Biochim. Biophys. Acta 1426 (1999) 347–357.
[18] M.S. Toledo, S.B. Levery, A.H. Straus, H.K. Takahashi, Sphingolipids of the mycopathogen *Sporothrix schenckii*: identification of a glycosylinositol phosphorylceramide with novel core GlcNH(2)alpha1 → 2Ins motif, FEBS Lett. 493 (2001) 50–56.
[19] D. Warnecke, E. Heinz, Recently discovered functions of glucosylceramides in plants and fungi, Cell. Mol. Life Sci. 60 (2003) 919–941.
[20] E.J.A. François, A.M. Aerts, B.P.A. Cammue, K. Thevissen, Currently used antimycotics: spectrum, mode of action and resistance occurrence, Curr. Drug Targets 6 (2005) 895–907.
[21] J.P. McMahon, J. Wheat, M.E. Sobel, R. Pasula, J.F. Downing, W.J. Martin II, Murine laminin binds to *Histoplasma capsulatum.* A possible mechanism of dissemination, J. Clin. Invest. 96 (1995) 1010–1017.
[22] C.A. Rappleye, L.G. Eissenberg, W.E. Goldman, *Histoplasma capsulatum* alpha-(1,3)-glucan blocks innate immune recognition by the beta-glucan receptor, Proc. Natl. Acad. Sci. USA 104 (2007) 1366–1370.
[23] P.R. Taylor, S.V. Tsoni, J.A. Willment, K.M. Dennehy, M. Rosas, H. Findon, K. Haynes, C. Steele, M. Botto, S. Gordon, G.D. Brown, Dectin-1 is required for beta-glucan recognition and control of fungal infection, Nat. Immunol. 8 (2007) 31–38.
[24] M. Bagnat, S. Keranen, A. Shevchenko, A. Shevchenko, K. Simons, Lipid rafts function in biosynthetic delivery of proteins to the cell surface in yeast, Proc. Natl. Acad. Sci. USA 97 (2000) 3254–3259.
[25] M.S. Toledo, E. Suzuki, K. Handa, S. Hakomori, Cell growth regulation through GM3-enriched microdomain (glycosynapse) in human lung embryonal fibroblast WI38 and its oncogenic transformant VA13, J. Biol. Chem. 279 (2004) 34655–34664.
[26] K.A. Yoneyama, A.K. Tanaka, T.G. Silveira, H.K. Takahashi, A.H. Straus, Characterization of *Leishmania* (*Viannia*) *braziliensis* membrane microdomains, and their role in macrophage infectivity, J. Lipid Res. 47 (2006) 2171–2178.
[27] P.K. Maza, A.H. Straus, M.S. Toledo, H.K. Takahashi, E. Suzuki, Interaction of epithelial cell membrane rafts with *Paracoccidioides brasiliensis* leads to fungal adhesion and Src-family kinase activation, Microbes Infect. 10 (2008) 540–547.
[28] P.F. Andreotti, J.L. Monteiro da Silva, A.M. Bailão, C.M. Soares, G. Benard, C.P. Soares, M.J. Mendes-Giannini, Isolation and partial characterization of a 30 kDa adhesin from *Paracoccidioides brasiliensis*, Microbes Infect. 7 (2005) 875–881.
[29] J.C. Dittmer, R.L. Lester, A simple, specific spray for the detection of phospholipids on thin-layer chromatograms, J. Lipid Res. 5 (1964) 126–127.
[30] H.K. Takahashi, R. Metoki, S. Hakomori, *Immunoglobulin G3* monoclonal antibody directed to Tn antigen (tumor-associated alpha-N-acetylgalactosaminyl epitope) that does not cross-react with blood group A antigen, Cancer Res. 48 (1988) 4361–4367.
[31] M.S. Toledo, E. Suzuki, A.H. Straus, H.K. Takahashi, Glycolipids from *Paracoccidioides brasiliensis.* Isolation of a galactofuranose-containing glycolipid reactive with sera of patients with paracoccidioidomycosis, J. Med. Vet. Mycol. 33 (1995) 247–251.
[32] M.L. Zuolo, M.S. Toledo, H.E. Nogueira, A.H. Straus, H.K. Takahashi, Identification of GM3 as a marker of therapy-resistant periradicular lesions, J. Endod. 27 (2001) 107–109.
[33] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues, J. Biol. Chem. 226 (1957) 497–509.
[34] S.B. Levery, M.S. Toledo, A.H. Straus, H.K. Takahashi, Structure elucidation of sphingolipids from the mycopathogen *Paracoccidioides brasiliensis*: an immunodominant β-galactofuranose residue is carried by a novel glycosylinositol phosphorylceramide antigen, Biochemistry 37 (1998) 8764–8775.
[35] A.E. Christian, M.P. Haynes, M.C. Phillips, G.H. Rothblat, Use of cyclodextrins for manipulating cellular cholesterol content, J. Lipid Res. 38 (1997) 2264–2272.
[36] R. Zidovetzki, I. Levitan, Use of cyclodextrins to manipulate plasma membrane cholesterol content: evidence, misconceptions and control strategies, Biochim. Biophys. Acta 1768 (2007) 1311–1324.
[37] J. Pike, Lipid rafts: bringing order to chaos, J. Lipid Res. 44 (2003) 655–667.
[38] M.S. Toledo, L. Tagliari, E. Suzuki, C.M. Silva, A.H. Straus, H.K. Takahashi, Effect of anti-glycosphingolipid monoclonal antibodies in pathogenic fungal growth and differentiation. Characterization of monoclonal antibody MEST-3 directed to Manpα1 → 3Manpα1 → 2IPC, BMC Microbiol. 10 (2010) 47.
[39] K. Malínská, J. Malínský, M. Opekarová, W. Tanner, Visualization of protein compartmentation within the plasma membrane of living yeast cells, Mol. Biol. Cell. 14 (2003) 4427–4436.
[40] S.W. Martin, J.B. Konopka, Lipid raft polarization contributes to hyphal growth in *Candida albicans*, Eukaryot. Cell 3 (2004) 675–684.
[41] A.R. Siafakas, L.C. Wright, T.C. Sorrell, J.T. Djordjevic, Lipid rafts in *Cryptococcus neoformans* concentrate the virulence determinants phospholipase B1 and Cu/Zn superoxide dismutase, Eukaryot. Cell 5 (2006) 488–498.
[42] J.T. Djordjevic, M. Del Poeta, T.C. Sorrell, K.M. Turner, L.C. Wright, Secretion of cryptococcal phospholipase B1 (PLB1) is regulated by a glycosylphosphatidylinositol (GPI) anchor, Biochem. J. 389 (2005) 803–812.
[43] F.J. Alvarez, L.M. Douglas, J.B. Konopka, Sterol-rich plasma membrane domains in fungi, Eukaryot. Cell 6 (2007) 755–763.
[44] M. Bagnat, A. Chang, K. Simons, Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast, Mol. Biol. Cell 12 (2001) 4129–4138.
[45] K. Simons, E. Ikonen, Functional rafts in cell membranes, Nature 387 (1997) 569.
[46] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science 327 (2010) 46–50.
[47] S. Hakomori, Glycosynapses: microdomains controlling carbohydrate-dependent cell adhesion and signaling, An. Acad. Bras. Cienc. 76 (2004) 553–572.
[48] D.A. Brown, Lipid rafts, detergent-resistant membranes, and raft targeting signals, Physiology 21 (2006) 430–439.
[49] J. Czub, M. Baginski, Comparative molecular dynamics study of lipid membranes containing cholesterol and ergosterol, Biophys. J. 90 (2006) 2368–2382.
[50] X. Xu, R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, E. London, Effect of the structure of natural sterols and sphingolipids on the formation of ordered sphingolipid/sterol domains (rafts). Comparison of cholesterol to plant, fungal, and disease-associated sterols and comparison of sphingomyelin, cerebrosides, and ceramide, J. Biol. Chem. 276 (2001) 33540–33546.
[51] B. Westerlund, J.P. Slotte, How the molecular features of glycosphingolipids affect domain formation in fluid membranes, Biochim. Biophys. Acta 1788 (2009) 194–201.
[52] G. Santoni, A. Gismondi, J.H. Liu, A. Punturieri, A. Santoni, L. Frati, M. Piccoli, J.Y. Djeu, *Candida albicans* expresses a fibronectin receptor antigenically related to alpha 5 beta 1 integrin, Microbiology 140 (1994) 2971–2979.
[53] K.S. Gustafson, G.M. Vercellotti, C.M. Bendel, M.K. Hostetter, Molecular mimicry in *Candida albicans.* Role of an integrin analogue in adhesion of the yeast to human endothelium, J. Clin. Invest. 87 (1991) 1896–1902.
[54] S.A. Klotz, M.L. Pendrak, R.C. Hein, Antibodies to alpha5beta1 and alpha(v)beta3 integrins react with *Candida albicans* alcohol dehydrogenase, Microbiology 147 (2001) 3159–3164.
[55] P. Cossart, P.J. Sansonetti, Bacterial invasion: the paradigms of enteroinvasive pathogens, Science 304 (2004) 242–248.