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

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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

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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