Characterization *of Leishmania (Viannia) braziliensis* membrane microdomains, and their role in macrophage infectivity

Kelly A. G. Yoneyama,* Ameria K. Tanaka,* Thais G. V. Silveira,† Helio K. Takahashi,* and Anita H. Straus*,[1]

Department of Biochemistry,* Universidade Federal de São Paulo/Escola Paulista de Medicina, São Paulo, SP 04023-900, Brazil; and Departamento de Análises Clínicas,† Universidade Estadual de Maringá, Maringá, PR 87020-900, Brazil

Abstract Detergent-resistant membranes (DRMs) from *Leishmania (Viannia) braziliensis* promastigotes, insoluble in 1% Triton X-100 at 4°C, were fractionated by sucrose density gradient ultracentrifugation. They were composed of glycoinositolphospholipids (GIPLs), inositol phosphorylceramide (IPC), phosphatidylinositol (PI), phosphatidylethanolamine (PE), and sterols. In contrast, 1% Triton X-100-soluble fraction was composed of PE, phosphatidylcholine, phosphatidylserine, PI, IPC, sterol, and lyso-PI. High-performance thin-layer chromatography (HPTLC) immunostaining using monoclonal antibody SST-1 showed that 85% of GIPLs are present in DRMs, and immunoelectron microscopic analysis showed that SST-1-reactive components are located in patches along the parasite surface. No difference in GIPL pattern was observed by HPTLC between Triton X-100-soluble versus -insoluble fractions at 4°C. Analysis of fatty acid composition in DRMs by GC-MS showed the presence of GIPLs containing an alkylacylglycerol, presenting mainly saturated acyl and alkyl chains. DRMs also contained sterol, IPC with saturated fatty acids, PI with at least one saturated acyl chain, and PE with predominantly oleic acid. Promastigotes treated with methyl-β-cyclodextrin to disrupt lipid microdomains showed significantly lower macrophage infectivity, suggesting a relationship between lipid microdomains and the infectivity of these parasites.—Yoneyama, K. A. G., A. K. Tanaka, T. G. V. Silveira, H. K. Takahashi, and A. H. Straus. **Characterization *of Leishmania (Viannia) braziliensis* membrane microdomains, and their role in macrophage infectivity. *J. Lipid Res.* 2006.** 47: **2171–2178.**

Supplementary key words glycolipid • inositol phosphorylceramide • lipid raft • promastigote • detergent-resistant membranes • gas chromatography-mass spectrometry • methyl-β-cyclodextrin

In the fluid bilayer of eukaryotic plasma membranes, various lipid species are asymmetrically distributed within the exoplasmic and cytoplasmic layers, and specific subsets of proteins and glycolipids are organized as specialized microdomains (1). In mammalian cells, certain plasma membrane proteins, such as receptors and proteins anchored by glycosylphosphatidylinositol (GPI) or acyl moieties, are associated with sphingolipid/cholesterol-rich microdomains termed "lipid rafts." Such microdomains have also been termed "detergent-resistant membranes" (DRMs) because they can be isolated by density gradient ultracentrifugation on the basis of their low density and insolubility in cold, nonionic detergent (2).

DRMs containing GPI-anchored proteins have also been isolated from trypanosomatids such as *Leishmania* (3, 4). *Leishmania* is a digenetic parasite whose life cycle includes motile promastigotes in the alimentary tract of its insect vector, the phlebotomine sandfly, and intracellular nonmotile amastigotes in mononuclear phagocytes of mammalian hosts. Numerous studies during the past 15 years indicate that the infection process in parasites depends on surface glycoconjugates such as lipophosphoglycans, GPI-anchored proteins, and glycolipids (5–12).

The purpose of this study was to characterize the lipids present in lipid raft microdomains in promastigote forms and to analyze the involvement of these microdomains in parasite-macrophage interaction in a particular *Leishmania* species, *Leishmania (Viannia) braziliensis*. This species belongs to *the L. Viannia* subgenus that causes human cutaneous and mucocutaneous leishmaniasis in the New World (13). Members of this subgenus express lipophosphoglycan levels at only 5–10% of those found in species of the *L. Leishmania* subgenus (14, 15). In contrast,

Manuscript received 18 July 2005.

Published, JLR Papers in Press, July 22, 2006.
DOI 10.1194/jlr.M600285-JLR200

Abbreviations: AGPB, phosphate buffer containing 0.5% bovine serum albumin and 0.1% gelatin; DRM, detergent-resistant membrane; FAME, fatty acid methyl ester; GIPL, glycoinositolphospholipid; GPI, glycosylphosphatidylinositol; HPTLC, high-performance thin-layer chromatography; IPC, inositol phosphorylceramide; MAb, monoclonal antibody; MβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

[1] To whom correspondence should be addressed.
e-mail: straus.bioq@epm.br

glycoinositolphospholipids (GIPLs) are highly expressed in *L. (V.) panamensis* and *L. (V.) braziliensis* promastigotes (16, 17). Therefore, it is important to evaluate the distribution and functional role of these molecules in cell membranes of these parasites.

MATERIALS AND METHODS

Parasites

L. (V.) braziliensis (WMHOM/BR/1987/M11272) promastigotes were cultured at 23°C by several passages of log-phase parasite in Medium 199 supplemented with 10% heat-inactivated fetal calf serum (Cultilab, Campinas, Brazil).

Triton X-100 detergent extraction

L. (V.) braziliensis promastigotes (1×10^8 cells) were harvested by centrifugation (2,000 *g*, 4°C, 10 min), washed three times with 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS), resuspended in 1 ml of ice-cold 1% Triton X-100 (Sigma) in PBS, and maintained for 30 min at 37°C or on ice. Detergent-soluble (supernatant) and -insoluble (pellet) fractions were separated by centrifugation at 14,500 *g* for 10 min at 4°C or 25°C. Lipids present in the detergent-soluble fraction were recovered directly by partition with the same volume of 1-butanol (3); the butanol fraction was dried, resuspended in chloroform-methanol (2:1), and analyzed by high-performance thin-layer chromatography (HPTLC). Lipids present in the detergent-insoluble fraction were extracted with 2 ml of isopropanol-hexane-water (55:20:25; solvent A), dried, resuspended in chloroform-methanol (2:1), and analyzed by HPTLC (17).

Sucrose density gradient centrifugation

L. (V.) braziliensis promastigotes (1×10^8 cells) were washed in ice-cold PBS, resuspended in 50 mM Tris-HCl, 150 mM NaCl, and 5 mM EDTA, pH 7.4 (TNE buffer), and stirred with glass beads (5 mm; Reidel-deHäen, Seeize, Germany) for 5 min on ice to disrupt the parasites. The suspension was centrifuged at 800 *g* for 10 min at 4°C. The supernatant was collected and adjusted to 1% Triton X-100 in TNE buffer and maintained for 30 min on ice. The extract was adjusted to 45% sucrose and then overlaid with a discontinuous sucrose density gradient consisting of 30% sucrose (3 ml) and 5% sucrose (1.75 ml) in an ultracentrifugation tube. Gradients were centrifuged with a Sorvall AH-629 rotor at 100,000 *g* for 30 h at 4°C, and fractions (950 μl) were collected from the top. The fractions were dialyzed against water and dried. Lipids were recovered from the fractions by extraction with isopropanol-hexane-water (55:20:25) and analyzed by HPTLC.

Lipid analysis

GIPLs were analyzed by HPTLC on Silica Gel 60 plates (E. Merck, Darmstadt, Germany) using chloroform-methanol-0.02% $CaCl_2$ (60:40:9; solvent B) as the mobile phase. Glycolipids were visualized by staining with orcinol/H_2SO_4. The standard used was GIPL-1 purified from *L. (L.) major*, as described by Suzuki et al. (18). Phospholipids were analyzed by HPTLC, developed in chloroform-methanol-40% methylamine (63:35:10; solvent C), and visualized by Dittmer-Lester reagent as blue spots (19). HPTLC plates were stained, and phospholipids were quantified by densitometry (Shimadzu CS9301) at 525 nm. The standard mixture contained 1 mg/ml each of six phospholipids: phosphatidylinositol (PI), egg lecithin phosphatidylcholine (PC), phosphatidylethanolamine (PE; Matreya, Pleasant Gap, PA), sphingomyelin, phosphatidylserine (PS; Sigma), and inositol phosphorylceramide (IPC) purified from *Saccharomyces cerevisiae* as described by Smith and Lester (20).

Individual GIPLs and phospholipids were further purified by a combination of HPLC and preparative HPTLC as follows. GIPL bands were purified by HPLC (Varian; model 9010) on an Iatrobeads column (4.6 × 300 mm, 6R-8010; Iatron, Tokyo, Japan) and eluted on a gradient of isopropanol-hexane-water from 55:43:2 to 55:30:15 (175 min, flow rate of 1.0 ml/min) (17). GIPLs were quantified by phenol-sulfuric acid reaction, a colorimetric method for carbohydrate determination (21). Phospholipids were purified by preparative HPTLC in solvent C. Separated phospholipids were visualized under ultraviolet light after spraying with 0.01% primulin in 90% aqueous acetone. Individual phospholipids were isolated from HPTLC plates by scraping and sonication in solvent A (22). Sterols were analyzed by HPTLC developed in chloroform-ethyl ether-acetic acid (97:2.3:0.5; solvent D) and visualized as gray spots using copper acetate reagent (10% $CuSO_4 \cdot 5H_2O$ in 3% H_3PO_4) (23). Ergosterol and cholesterol (Sigma) were used as standards for the quantification of HPTLC by densitometry at 525 nm.

HPTLC immunostaining

Immunostaining of GIPLs by monoclonal antibody (MAb) SST-1, which specifically recognizes glycolipids of *L. (V.) braziliensis* promastigotes, was performed as described previously (17). After HPTLC development, plates were dried, soaked in 0.5% polymethacrylate in hexane, dried, blocked with 1% BSA in PBS for 2 h, incubated with MAb SST-1 overnight at 4°C, and incubated sequentially with rabbit anti-mouse IgG and ^{125}I-labeled protein A (4×10^5 cpm/ml).

Solid-phase RIA

For solid-phase RIA binding assay, glycolipids present in the fractions obtained after sucrose gradient ultracentrifugation were recovered directly by partition with the same volume of 1-butanol (950 μl). The butanol fractions were dried and resuspended in ethanol (400 μl), and 20 μl of each fraction was adsorbed on 96-well plates (Falcon Microtest III, Oxnard, CA) (24). The ethanol was dried at 37°C, and the wells were blocked with 1% BSA in PBS (200 μl/well) for 2 h. Plates were incubated overnight with MAb SST-1 (100 μl) at 4°C. The amount of MAb bound to glycolipid was determined by reaction with 50 μl of rabbit anti-mouse IgG. Plates were washed three times with PBS and incubated with 50 μl of ^{125}I-labeled protein A in 1% BSA in PBS (~10^5 cpm/well) for 1 h. Radioactivity in each well was measured by γ counter.

Analysis of phospholipids and GIPLs by GC-MS

Purified phospholipids or glycolipids (20 μg) were dried in Pyrex tubes with Teflon-lined screw caps. Methanolysis was performed by adding 1.0 ml of 1 M HCl in anhydrous methanol and incubating at 80°C for 16–22 h. The cooled lysate was partitioned three times with an equal volume of hexane. Combined hexane phases containing fatty acid methyl esters (FAMEs) were dried under N_2 at 37°C, resuspended in 100 μl of hexane, and analyzed by GC-MS. For analysis of inositols and sphingoid bases, the anhydrous methanol phase was dried under N_2 at 37°C, washed three times with anhydrous methanol, converted to trimethylsilyl derivative by reaction with Tri-Sil reagent (Pierce, Rockford, IL) for 30 min at 80°C, and analyzed by GC-MS (25). GC-MS analyses were performed on a Varian CP-3800/1200L using a CP-Sil 8 CB column with splitless injection. The temperature program was from 40°C to 300°C at 5°C/ min, followed by a 5 min plateau at 300°C. Derivatives were identified by their

retention times and mass spectra, in comparison with authentic standards and published data.

Indirect immunofluorescence

Parasites (1×10^8) were fixed with 1% formaldehyde in PBS for 10 min. Cells were washed and resuspended in 1 ml of PBS, and 20 μl of the solution was added to coverslips. Air-dried preparations were extracted with 1% Triton X-100 in PBS at 4°C or 37°C. Coverslips were blocked with 5% BSA in PBS and incubated sequentially with primary antibody (1 h) and with 1% BSA containing 0.01 mM 4,6-diamidino-2-phenylindole (Sigma) and anti-mouse IgG conjugated to FITC (Dako Corp., Carpinteria, CA). Coverslips were washed five times with PBS after each incubation and examined by epifluorescence microscopy (Nikon).

Transmission electron microscopy

Parasites (3×10^8) were fixed with 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M Na-cacodylate buffer (CB), pH 7.6, for 20 min at 4°C. Cells were washed (3,400 *g*, 3 min) in 0.1 M phosphate buffer (PB), pH 7.4, and incubated with 0.05% sodium borohydride and 0.1% glycine in PB for 10 min to block free aldehyde groups. Parasites were washed with phosphate buffer containing 0.5% BSA and 0.1% gelatin (AGPB), and blocked with 5% goat serum (Sigma) in AGPB for 1 h. Approximately 1×10^7 parasites were incubated with 0.5 ml of MAb (culture supernatant, 90 min at 25°C), washed with AGPB, and incubated with goat anti-mouse Ig conjugated with colloidal gold (10 nm; E-Y Laboratories, San Mateo, CA) for 90 min. Parasites were washed with AGPB and PB, fixed with 2.5% glutaraldehyde in PB (10 min), washed in PB, and embedded with 4% agarose. Fragments (2–3 mm) were treated with 2% osmium tetroxide in CB (1 h) for contrast, washed with water, and incubated with 0.5% uranyl acetate in 0.3 M sucrose (30 min). Fragments were washed with water, dehydrated in ethanol (70, 90, and 100% successively), and infiltrated sequentially with propylene oxide (two times for 15 min), Araldite in propylene oxide (1:2, 1 h), Araldite in propylene oxide (1:1, overnight under agitation), and Araldite in a desiccator (5 h). After polymerization, the material was sectioned (70–90 nm thickness). Sections were treated with uranyl acetate (8 min) and with lead citrate (4 min) for contrast and examined using a JEOL 1200 EX-II transmission electron microscope at 80 kV.

Preparation of peritoneal macrophages

Peritoneal macrophages were harvested by washing the peritoneal cavity of BALB/c mice with PBS. Macrophages were washed three times with cold PBS by centrifugation at 400 *g*, and the pellet was resuspended in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, and penicillin/streptomycin (100 U/ml and 100 μg/ml, respectively). Approximately 5×10^5 macrophages were placed on sterile glass coverslips on 24-well plates for 1 h at room temperature. Nonadherent cells were removed by several washes with RPMI 1640, and the plates were kept at 37°C in a CO_2 incubator.

Disruption of lipid microdomains

Promastigotes (1×10^8) from the stationary growth phase were washed three times with sterile PBS by centrifugation at 600 *g* for 10 min at 4°C and maintained in Medium 199 without serum for 12 h. The cells were washed three times in PBS and incubated with 20 or 40 mM methyl-β-cyclodextrin (MβCD) (Sigma) in PBS for 1 h at room temperature. After treatment, viability was analyzed by culturing the parasites in complete medium containing 10% fetal calf serum for cholesterol replacement in the parasite membrane. Growth rates of parasites were measured relative to those of control cells incubated without MβCD.

Leishmania infectivity in macrophages after MβCD treatment

Promastigotes preincubated with MβCD (20 or 40 mM) and control promastigotes were incubated with peritoneal macrophages (10 parasites/macrophage, 5×10^6 parasites/well) for 1 h in RPMI 1640 medium without serum at room temperature. Nonadherent parasites were removed by washing the monolayers with medium. Infected macrophages were maintained in RPMI 1640 with 10% fetal calf serum in a CO_2 incubator for 24 h. Macrophages were fixed with 2% formaldehyde in PBS for 10 min and stained with Giemsa's solution. The phagocytic index was determined by multiplying the percentage of macrophages that had phagocytosed at least one parasite by the parasite average per infected macrophage (300 cells were examined), as described by Straus et al. (5).

RESULTS

Immunolocalization of glycolipids recognized by MAb SST-1

MAb SST-1 specifically recognizes glycolipids of *L. (V.) braziliensis* promastigotes and does not cross-react with the lipophosphoglycan fraction or with other parasite glycoconjugates, as shown by Silveira et al. (17). We observed strong reactivity in all parasites by indirect immunofluorescence (**Fig. 1A**). When promastigotes were incubated

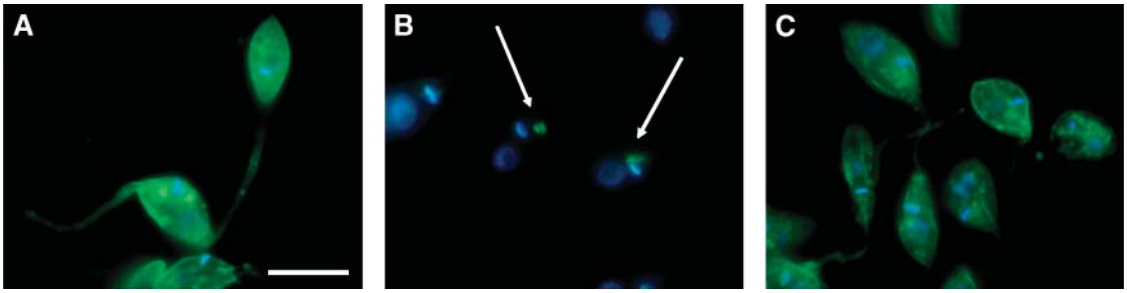

Fig. 1. Indirect immunofluorescence of detergent-extracted promastigotes probed with monoclonal antibody (MAb) SST-1. *L. (V.) braziliensis* promastigotes were analyzed as described in Materials and Methods. A: Fixed parasites. B: Coverslips containing fixed promastigotes, extracted with 1% Triton X-100 at 37°C before SST-1 labeling. C: Same as B, but Triton X-100 extraction was carried out at 4°C. Green areas, SST-1 reactivity; blue areas, nucleus and kinetoplast stained with 4,6-diamidino-2-phenylindole. Bar = 5 μm.

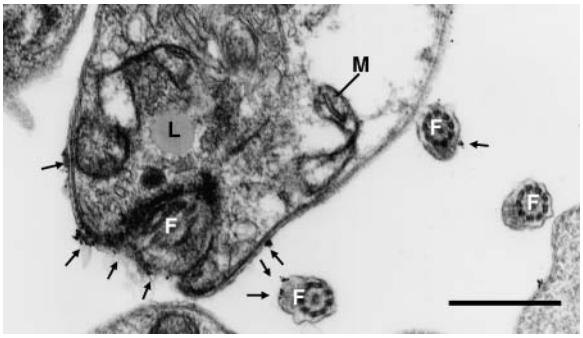

Fig. 2. Transmission electron microscopy of *L. (V.) braziliensis* promastigotes using MAb SST-1. Promastigotes were fixed, incubated sequentially with SST-1 and gold-conjugated anti-mouse IgG, fixed with Araldite, and processed as described in Materials and Methods. F, flagella; L, lipid vesicles; M, mitochondria. Bar = 0.5 μm.

with 1% Triton X-100 at 37°C, surface fluorescence was abolished and only a small fluorescent stain remained near the kinetoplast (Fig. 1B, arrows). In contrast, when parasites were treated with 1% Triton X-100 at 4°C, SST-1 immunofluorescence labeling was unchanged (Fig. 1C), indicating that the glycolipid antigens recognized by SST-1 are detergent-insoluble at 4°C.

We also analyzed MAb SST-1 reactivity with *L. (V.) braziliensis* promastigotes by transmission electron microscopy to localize and determine the organization of glycolipids in the plasma membrane. Gold particles were visualized mainly in patches at the surface of the promastigotes (**Fig. 2**, arrows). No labeling was detected in the cytoplasm or other organelles. In control experiments using an irrelevant MAb, no labeling was observed (data not shown).

GIPL and phospholipid composition of detergent-soluble and -insoluble fractions

L. (V.) braziliensis promastigotes were extracted with 1% Triton X-100 at 4°C and 37°C, and GIPL composition was determined by HPTLC and quantified by densitometry. When promastigotes were extracted in ice-cold 1% Triton X-100, ~85% of total GIPLs were recovered in the detergent-insoluble fraction (**Fig. 3A**, 4°C, lane I). In contrast, at 37°C, almost 99% of GIPLs were soluble (37°C, lane S). Six GIPL bands, termed B1–B6, were present in both fractions as visualized by orcinol staining. Bands B3 and B5 correspond to SST-1-reactive GIPLs (Fig. 3B). Traces of GIPLs in the 37°C insoluble fraction were detected only after SST-1 im-

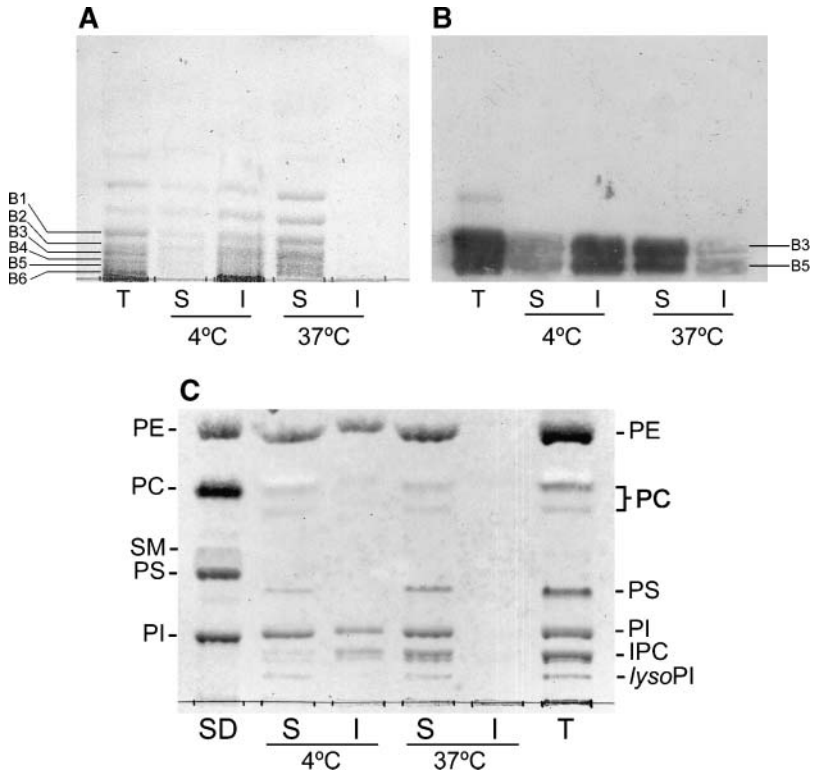

Fig. 3. Temperature-dependent detergent extraction of *L. (V.) braziliensis* lipids. Soluble (S) and insoluble (I) fractions after parasite extraction with 1% Triton X-100 at 4°C or 37°C were analyzed by high-performance thin-layer chromatography (HPTLC). A: HPTLC gel developed in chloroform-methanol-0.02% $CaCl_2$ (60:40:9) and stained with orcinol/H_2SO_4 for glycolipids. B: HPTLC gel developed in the same solvent and immunostained with SST-1. C: HPTLC gel developed in chloroform-methanol-40% methylamine (63:35:10) and phospholipids visualized by Dittmer-Lester reagent. SD, standard phospholipid (1 mg/ml) containing phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (SM), phosphatidylserine (PS), and phosphatidylinositol (PI); T, total promastigote lipid content after extraction with isopropanol-hexane-water and chloroform-methanol.

munostaining. Approximately 64% of phospholipids were recovered in the 4°C Triton X-100-soluble fraction, as determined by HPTLC densitometry (Fig. 3C). Phospholipid compositions of the soluble versus insoluble fractions were conspicuously different. The 4°C soluble fraction contained mainly phospholipids with chromatographic migration of PE, PC, PS, PI, IPC, and a slow-migrating component corresponding to lyso-PI, as determined by GC-MS. Approximately 36% of phospholipids remained in the 4°C insoluble fraction (4°C, lane I), including PE, PI, and IPC, which corresponds to the major sphingolipids of these parasites. When parasites were incubated with 1% Triton X-100 at 37°C, all phospholipids were recovered in the soluble fraction (37°C, lane S). Concentrations of glycolipids and phospholipids recovered in the 1% Triton X-100-soluble fraction at 37°C and the insoluble fraction at 4°C were determined by densitometry of HPTLC plates stained with orcinol (for GIPLs) or Dittmer-Lester reagent (for phospholipids) (**Fig. 4**).

Fractionation by sucrose density gradient centrifugation of promastigote membranes incubated with ice-cold 1% Triton X-100

To confirm that the 4°C insoluble fraction described above represents DRMs, low-density membrane microdomains were obtained by sucrose density gradient centrifugation, and GIPL and phospholipid content was analyzed by HPTLC. GIPLs were present mainly in fractions 3 and 4 (**Fig. 5A**), which correspond to low-density membranes. GIPLs were also present in fractions 5 and 6, which correspond to soluble GIPLs. Cholesterol/ergosterol was present predominantly in fractions 3 and 4 (Fig. 5B), whereas fractions 5 and 6 contained other yet-unidentified sterol components with lower relative mobility. Phospholipid composition, analyzed by HPTLC, varied among the different fractions (Fig. 5C). Fractions 3 and 4 contained mainly phospholipids with chromatographic migration of PE, PI, and IPC, whereas fractions 5 and 6 (corresponding to 4°C Triton X-100-soluble phospholipids) contained PE, PS, PI, IPC, lyso-PI, and traces of PC. Percentages of each phospholipid present in fractions 3 and 4 and fractions 5 and 6, calculated by densitometry of the HPTLC plate, are shown in Fig. 5D. PE and PI were distributed equally in

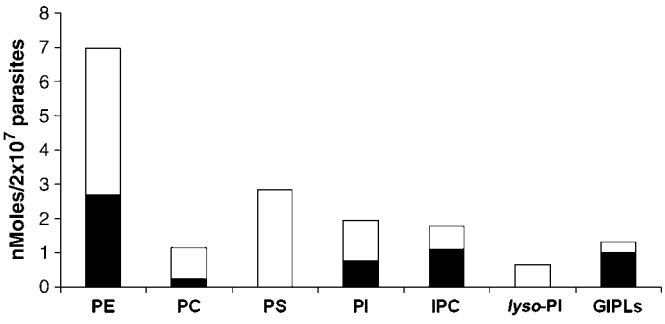

Fig. 4. Glycoinositolphospholipid (GIPL) and phospholipid composition of *L. (V.) braziliensis* promastigotes. Parasites were extracted with 1% Triton X-100 at 37°C and subjected to HPTLC. Concentrations of GIPLs and phospholipids were determined by densitometry using a standard solution of 1 mg/ml commercial phospholipids containing PI, PC, PE, SM, and PS, inositol phosphorylceramide (IPC) purified from *S. cerevisiae*, and GIPL-1 purified from *L. major*. Black columns, concentration of phospholipids and GIPLs that remained insoluble in 1% Triton X-100 at 4°C.

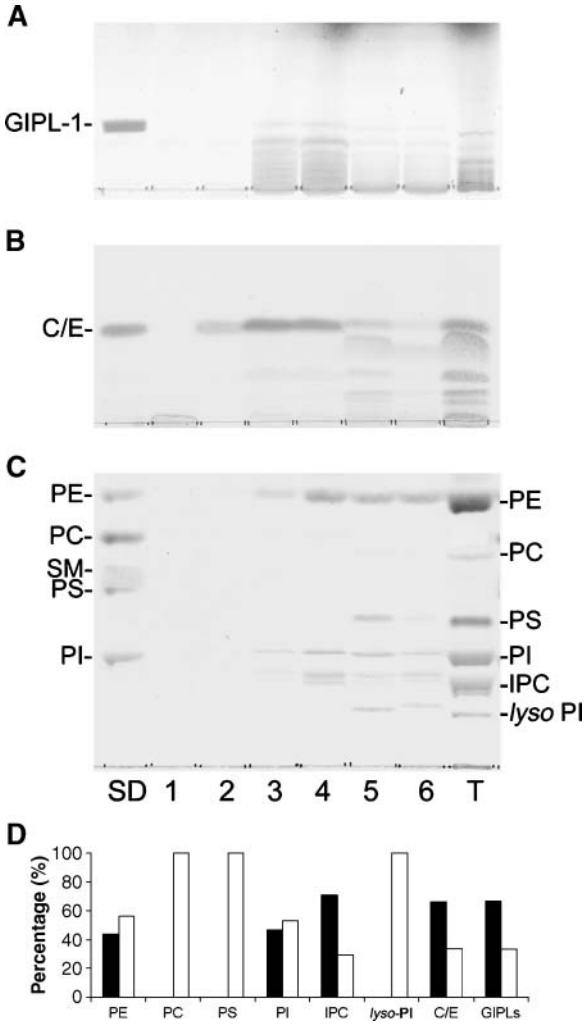

Fig. 5. Distribution pattern of GIPLs, phospholipids, and sterols in various fractions from sucrose density gradient centrifugation of *L. (V.) braziliensis* promastigote lysate. The membrane suspension was placed at the bottom of a centrifuge tube, and a sucrose gradient was established as described in Materials and Methods. After ultracentrifugation (100,000 *g* for 30 h), 950 µl fractions were collected and analyzed for lipid content by HPTLC. Abscissa, fraction number (1 = top, 6 = bottom). A: HPTLC gel developed in solvent B and stained with orcinol/H_2SO_4 for GIPLs. SD, standard GIPL-1 purified from *L. major*. B: Distribution of sterols in gradient centrifugation fractions by HPTLC as developed in solvent D and stained with copper acetate reagent. C/E, cholesterol/ergosterol; SD, standard ergosterol; T, total promastigote lipid content after extraction with isopropanol-hexane-water and chloroform-methanol. C: Phospholipid distribution determined by HPTLC as developed in solvent C and stained with Dittmer-Lester reagent. SD, standard phospholipids. D: Distribution of phospholipid, GIPL, and sterol in fractions 3 and 4 (corresponding to detergent-resistant membrane; black columns) and fractions 5 and 6 (corresponding to detergent-soluble fraction; white columns). Lipid concentration in each fraction was estimated as the recovered percentage of each lipid, based on the densitometry of HPTLC gels shown in A–C.

fractions 3 and 4 and fractions 5 and 6. On the other hand, 78% of total IPC was present in fractions 3 and 4, whereas 79% of total PS and all lyso-PI and PC were concentrated in fractions 5 and 6. For glycolipids, 68% of total GIPLs were present in fractions 3 and 4.

Fatty acid and sphingosine composition of *L. (V.) braziliensis* promastigote GIPLs and phospholipids

Phospholipids were purified by preparative HPTLC in solvent C and subjected to methanolysis as described in Materials and Methods. Percentages of peaks corresponding to fatty acids detected by GC-MS as FAME derivatives are summarized in **Fig. 6A**. The main fatty acid detected was oleic acid, except for IPC, in which 69% of molecules present myristic acid and 23% present palmitic acid. The presence of sphingoid base and inositol in IPC was confirmed by GC-MS analysis of trimethylsilyl derivatives (data not shown).

The various GIPLs purified by HPLC were analyzed for FAMEs and found to preferentially present saturated fatty acid, mainly stearic acid, and also palmitic acid and oleic acid (Fig. 6B). GC-MS analysis after acid methanolysis and trimethylsilylation showed the presence in all six glycolipid bands of peaks corresponding to fully saturated unbranched 1-*O*-monoalkylglycerol with a chain length of C18:0 and *myo*-inositol (data not shown), indicating that these glycolipids correspond to GIPLs presenting alkylacylglycerols.

Role of membrane microdomains in *L. (V.) braziliensis* infectivity to macrophage

A critical event in *Leishmania* pathogenesis is the invasion of macrophages by the parasite. To assess the possible role of *L. (V.) braziliensis* membrane microdomains in the infectivity of the macrophage monolayer by promastigotes, parasites were treated with a sterol binding reagent, MβCD (26). Treatment of promastigotes with 20 or 40 mM MβCD for 1 h caused a significant reduction (40% or 70%, respectively) of parasite sterol levels, as determined by densitometry of HPTLC plates containing parasite sterols stained by copper acetate reagent. MβCD treatment had no effect on the parasite concentration of phospholipids and GIPLs. Membranes of MβCD-treated parasites, and of control parasites, were incubated with ice-cold 1% Triton X-100 and fractionated by sucrose gradient ultracentrifugation, and GIPL distribution in the fractions was analyzed

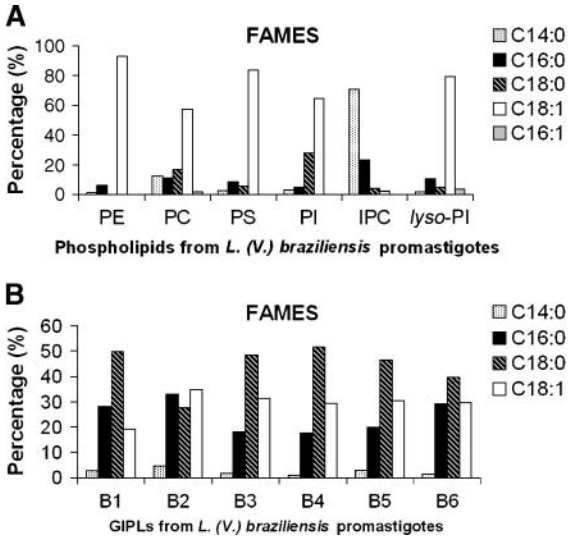

Fig. 6. Fatty acid composition (percentage abundance) of phospholipids (A) and GIPLs (B) of *L. (V.) braziliensis* promastigotes. Fatty acid methyl esters (FAMEs) were analyzed by GC-MS as described in Materials and Methods.

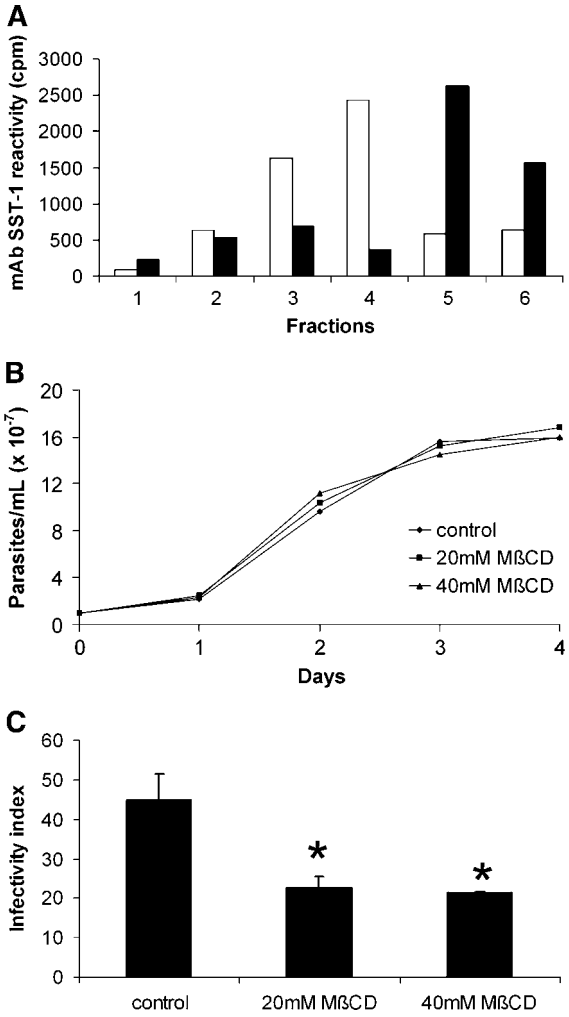

Fig. 7. Effect of the disruption of membrane microdomains on *L. (V.) braziliensis* growth and infectivity. Parasites were preincubated with 20 or 40 mM methyl-β-cyclodextrin (MβCD) in PBS for 1 h, as described in Materials and Methods. A: GIPL distribution in six fractions obtained after ultracentrifugation of parasite lysate incubated with ice-cold 1% Triton X-100. GIPL distribution was determined by RIA probed with MAb SST-1. Black columns, fractions from parasites treated with 40 mM MβCD; white columns, fractions from control parasites (not treated with MβCD). B: MβCD-treated parasites were cultured in complete Medium 199, and concentration was determined daily. C: MβCD-treated parasites were incubated with macrophage monolayers for 1 h. Nonadherent parasites were removed, and infected macrophages were maintained in a CO_2 incubator for 24 h as described in Materials and Methods. Values shown are means ± SD from triplicate experiments. * Values for MβCD-treated parasites were significantly lower ($P < 0.05$ by Student's *t*-test) than values for controls.

by RIA using MAb SST-1. MβCD-treated parasites showed a clear displacement of GIPLs from low-density fractions (fractions 3 and 4) to soluble fractions (fractions 5 and 6) (**Fig. 7A**), indicating that MβCD treatment led to a reduction of sterol content and a disruption of membrane microdomains. MβCD treatment did not affect parasite viability (i.e., treated vs. control parasites had similar growth rates) (Fig. 7B). However, macrophage infectivity by MβCD-treated parasites was significantly lower than infectivity by control parasites (Fig. 7C).

DISCUSSION

This study was designed to elucidate the membrane organization of glycolipids in *L. (V.) braziliensis* promastigotes. We found that these parasites present GIPLs containing alkylacylglycerol, composed mainly of saturated fatty acid chains, primarily stearic acid. We estimate that there are $\sim 4 \times 10^7$ GIPL molecules/parasite and that 85% of the GIPLs are present in DRMs. Approximately 36% of phospholipids are also present in DRMs, indicating that *L. (V.) braziliensis* promastigotes present an extensive liquid order or gel lipid bilayer, which may be essential for the survival/infectivity of parasites in the host.

The presence of DRMs in mammalian cells can be explained by the formation of a detergent-resistant liquid-ordered phase of sterols and sphingolipids containing saturated fatty acid chains (27) surrounded by a fluid-disordered phase presenting higher concentrations of phospholipids with unsaturated fatty acids. We found that *L. (V.) braziliensis* promastigote DRMs contain GIPLs presenting mainly saturated acyl and alkyl chains. The DRMs also contained sterol, IPC with saturated fatty acid, PI with at least one saturated acyl chain, and PE with predominantly oleic acid. In contrast, the 1% Triton X-100 (4°C)-soluble fraction contained phospholipids presenting almost exclusively oleic acid as PE, PC, PS, and lyso-PI.

Our findings indicate that GIPLs, IPC, and sterols are distributed preferentially in *L. (V.) braziliensis* membrane microdomains. This is the first demonstration that membrane microdomains of *Leishmania* are involved in macrophage infectivity. GIPLs and GPI-anchored proteins are presumably organized in membrane microdomains, as demonstrated in other species of *Leishmania* (3, 4), and may be associated with signal transducer molecules and carbohydrate-mediated parasite-host cell adhesion/recognition mechanisms. Carbohydrate-mediated interaction may initiate a series of signals in these parasites to affect cellular interactions and modulate host response, in a manner similar to that proposed for glycosynapses in mammalian cells by Hakomori and Handa (28). Our results show clearly that membrane microdomains of *Leishmania* play a role in parasite infectivity. Molecules present in these microdomains are potential targets for leishmaniasis therapy.

The authors thank Dr. Stephen Anderson for editing of the manuscript and Edna Freymuller of Centro de Microscopia Eletronica (CEME) for assistance with electron microscopy. This study was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES).

REFERENCES

1. Tamm, L. K., V. Kiessling, and M. L. Wagner. 2002. Membrane dynamics. *In* Encyclopedia of Life Sciences. McMillan Publishers, Nature Publishing Group, London. 1–7.
2. Simons, K., and D. Toomre. 2000. Lipid rafts and signal transduction. *Nat. Rev.* **1:** 31–39.
3. Ralton, J. E., K. A. Mullin, and M. J. McConville. 2002. Intracellular trafficking of glycosylphosphatidylinositol (GPI)-anchored proteins and free GPIs in *Leishmania mexicana*. *Biochem. J.* **363:** 365–375.
4. Denny, P. W., and D. F. Smith. 2004. Rafts and sphingolipid biosynthesis in the kinetoplastid parasitic protozoa. *Mol. Microbiol.* **53:** 725–733.
5. Straus, A. H., S. B. Levery, M. G. Jasiulionis, M. E. K. Salyan, S. J. Steele, L. R. Travassos, S. Hakomori, and H. K. Takahashi. 1993. Stage-specific glycosphingolipids from amastigote forms of *Leishmania (L.) amazonensis*. *J. Biol. Chem.* **268:** 13723–13730.
6. Descoteaux, A., and S. J. Turco. 1999. Glycoconjugates in *Leishmania* infectivity. *Biochim. Biophys. Acta.* **1455:** 341–352.
7. Handman, E. 1999. Cell biology of *Leishmania*. *Adv. Parasitol.* **44:** 1–39.
8. Ritting, M. G., and C. Bogdan. 2000. *Leishmania*-host-cell interaction: complexities and alternative views. *Parasitol. Today.* **16:** 292–297.
9. Guha-Niyogi, A., D. R. Sullivan, and S. J. Turco. 2001. Glycoconjugate structures of parasitic protozoa. *Glycobiology.* **11:** 45R–59R.
10. Silveira, T. G. V., E. Suzuki, H. K. Takahashi, and A. H. Straus. 2001. Inhibition of macrophage invasion by monoclonal antibodies specific to *Leishmania (Viannia) braziliensis* promastigotes and characterisation of their antigens. *Int. J. Parasitol.* **31:** 1451–1458.
11. Suzuki, E., H. K. Takahashi, and A. H. Straus. 2002. Role of beta-D-galactofuranose in *Leishmania major* macrophage invasion. *Infect. Immun.* **70:** 6592–6596.
12. Späth, G. F., L. A. Gerraway, S. J. Turco, and S. M. Beverley. 2003. The role(s) of lipophosphoglycan (LPG) in the establishment of *Leishmania major* infections in mammalian hosts. *Proc. Natl. Acad. Sci. USA.* **100:** 9536–9541.
13. Herwaldt, B. L. 1999. Leishmaniasis. *Lancet.* **354:** 1191–1199.
14. Muskus, C., I. Segura, R. Oddone, S. J. Turco, D. A. Leiby, L. Toro, S. Robledo, and N. G. Saravia. 1997. Carbohydrate and LPG expression in *Leishmania Viannia* subgenus. *J. Parasitol.* **83:** 671–678.
15. Soares, R. P. P., T. L. Cardoso, T. Barron, M. S. S. Araujo, P. F. P. Pimenta, and S. J. Turco. 2005. *Leishmania braziliensis*: a novel mechanism in the lipophosphoglycan regulation during metacyclogenesis. *Int. J. Parasitol.* **35:** 245–253.
16. Zawadzki, J., C. Scholz, G. Currie, G. H. Coombs, and M. J. McConville. 1998. The glycoinositolphospholipids from *Leishmania panamensis* contain unusual glycan and lipid moieties. *J. MoL. Biol.* **282:** 287–299.
17. Silveira, T. G. V., K. A. G. Yoneyama, H. K. Takahashi, and A. H. Straus. 2005. Specific glycolipids of *Leishmania (Viannia) braziliensis* promastigotes recognized by mAb SST-1. *Parasitology.* **131:** 737–745.
18. Suzuki, E., M. S. Toledo, H. K. Takahashi, and A. H. Straus. 1997. A monoclonal antibody directed to terminal residue of β-galactofuranose of a glycolipid antigen isolated from *Paracoccidioides brasiliensis*: cross-reactivity with *Leishmania major* and *Trypanosoma cruzi*. *Glycobiology.* **7:** 463–468.
19. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin-layer chromatography. *J. Lipid Res.* **5:** 126–127.
20. Smith, S. W., and R. L. Lester. 1974. Inositol phosphorylceramide, a novel substance and chief member of a major group of yeast sphingolipids containing a single inositol phosphate. *J. Biol. Chem.* **249:** 3395–3405.
21. Dubois, M. K., K. A. Gilles, J. K. Hamilton, P. Bebers, and F. Smith. 1956. Colorimetric method for determination of sugar and related substances. *Anal. Chem.* **28:** 350–356.

22. Toledo, M. S., E. Suzuki, A. H. Straus, and H. K. Takahashi. 1995. Glycolipids from *Paracoccidioides brasiliensis*. Isolation of a galactofuranose-containing glycolipid reactive with sera of patients with paracoccidioidomycosis. *J. Med. Vet. Mycol.* **33:** 247–251.
23. Fewster, M. E., B. J. Burns, and J. F. Mead. 1969. Quantitative densitometric thin-layer chromatography of lipids using copper acetate reagent. *J. Chromatogr.* **43:** 120–126.
24. Toledo, M. S., E. Suzuki, S. B. Levery, A. H. Straus, and H. K. Takahashi. 2001. Characterization of monoclonal antibody MEST-2 specific to glucosylceramide of fungi and plants. *Glycobiology*. **11:** 105–112.
25. Levery, S. B., M. S. Toledo, A. H. Straus, and H. K. Takahashi. 1998. Structure elucidation of sphingolipids from the mycophatogen *Paracoccidioides brasiliensis*: an immunodominant β-galactofuranose residue is carried by a novel glycosylinositol phosphorylceramide antigen. *Biochemistry.* **37:** 8764–8775.
26. Klein, U., G. Gimpl, and F. Fahrenholz. 1995. Alteration of the myometrial plasma membrane cholesterol content with beta-dextrin modulates the binding affinity of the oxytocyn receptor. *Biochemistry.* **34:** 13784–13793.
27. Zajchowski, L. D., and S. M. Robbins. 2002. Lipid rafts and little caves. Compartmentalized signalling in membrane microdomains. *Eur. J. Biochem.* **269:** 737–752.
28. Hakomori, S., and K. Handa. 2002. Glycosphingolipid-dependent cross-talk between glycosynapses interfacing tumor cells with their host cell: essential basis to define tumor malignancy. *FEBS Lett.* **531:** 88–92.