Acylation-dependent Export of *Trypanosoma cruzi* Phosphoinositide-specific Phospholipase C to the Outer Surface of Amastigotes

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Vicente de Paulo Martins¹, Michael Okura¹, Danijela Marić³, David M. Engman⁴, Mauricio Vieira⁵, Roberto Docampo⁶, and Silvia N. J. Moreno⁶,³

From the ¹Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, Athens, Georgia 30602, the ²Department of Pathobiology, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802, and the ³Department of Pathology, Northwestern University, Chicago, Illinois 60611

Phosphoinositide phospholipase C (PI-PLC) plays an essential role in cell signaling. A unique *Trypanosoma cruzi* PI-PLC (TcPI-PLC) is lipid-modified in its N terminus and localizes to the plasma membrane of amastigotes. Here, we show that TcPI-PLC is located onto the extracellular phase of the plasma membrane of amastigotes and that its N-terminal 20 amino acids are necessary and sufficient to target the fused GFP to the outer surface of the parasite. Mutagenesis of the predicted acylated residues confirmed that myristoylation of a glycine residue in the 2nd position and acylation of a cysteine in the 4th but not in the 8th or 15th position of the coding sequence are required for correct plasma membrane localization in *T. cruzi* epimastigotes or amastigotes. Interestingly, mutagenesis of the cysteine at the 8th position increased its flagellar localization. When expressed as fusion constructs with GFP, the N-terminal 6 and 10 amino acids fused to GFP are predominantly located in the cystosol and concentrated in a compartment that co-localizes with a Golgi complex marker. The N-terminal 20 amino acids of TcPI-PLC associate with lipid rafts when dually acylated. Taken together, these results indicate that N-terminal acylation modulations serve as a molecular addressing system for sending TcPI-PLC to the outer surface of the cell.

Phosphoinositide-specific phospholipase C (PI-PLC) hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) to generate two second messengers, inositol 1,4,5-trisphosphate and diacylglycerol (1, 2). Inositol 1,4,5-trisphosphate and diacylglycerol initiate signal transduction pathways through intracellular calcium release (1, 2) and protein kinase activation (3), respectively.

*T. cruzi*, the etiologic agent of Chagas disease, possesses a novel and rather unusual PI-PLC that, unlike other PI-PLCs, does not have a pleckstrin homology domain to bind to the plasma membrane, has a highly charged linker region between the catalytic X and Y domains, and is N-myristoylated and palmitoylated in vivo (4, 5). It has been postulated that this lipid modification is important for its plasma membrane localization and for stimulation of differentiation of the infective trypomastigote into the intracellular amastigote forms (5).

More than 100 N-myristoylated proteins have been predicted as encoded in the genome of *T. cruzi*, of which about 30 have N-terminal sequences commonly observed in dually acylated proteins (6). N-Myristoylation generally occurs during protein synthesis. After removal of the initiating methionine by a methionine aminopeptidase, myristate is added through an amide bond to the N-terminal glycine in a reaction catalyzed by N-myristoyltransferase (7). After myristoylation is completed, palmitate or other medium or long chain fatty acids can be attached through a thioester bond to one or more cysteine residues in a reaction catalyzed by a number of palmitoyltransferases, many of which have been identified in the related parasite *Trypanosoma brucei* (8). Attachment of palmitate or the presence of a polybasic domain in the N-terminal region could provide a second signal, in addition to N-myristoylation, necessary for membrane binding (7).

The N-terminal region of TcPI-PLC has a glycine at position 2, which is known to be myristoylated (5), and several cysteines at positions 4, 8, and 15. In this work, we investigated the plasma membrane localization of TcPI-PLC and whether these cysteines are also acylated and contribute to membrane binding or targeting to different membranes of the parasite.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—HRP-labeled goat anti-rabbit IgG, HRP-labeled goat anti-mouse IgG, the protein assay, and SDS were from Bio-Rad. All primers and oligonucleotides were from Integrated DNA Technologies (Coralville, IA). GeneTailor™ site-directed mutagenesis system, pCR2.1-TOPO cloning vector, pcDNA™4/T expression vector, pcDNA™6/TR vector, Opti-MEM®, and restriction enzymes were from Invitrogen. BODIPY® TR C₅-ceramide (Invitrogen) complexed to BSA, Alexa Fluor 546, and Alexa Fluor 488 were from Invitrogen. The plasmid maxi kit and QIAquick gel extraction kit were

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³ Present address: Real Time Genomics, San Francisco, CA 94105.

⁴ Present address: Dept. of Veterinary Biosciences, University of Illinois at Urbana-Champaign, Urbana, IL 61802.

⁵ To whom correspondence should be addressed: University of Georgia, Athens, GA 30602. Tel.: 706-542-4736; Fax: 706-542-9493; E-mail: smoreno@cb.uga.edu.

⁶ The abbreviations used are: PI-PLC, phosphoinositide-specific phospholipase C; TcPI-PLC, *T. cruzi* phosphoinositide-specific phospholipase C; LIT, liver infusion tryptose medium; PIP₂, phosphatidylinositol 4,5-bisphosphate; GPL, glycosylphosphatidylinositol; TRITC, tetramethylrhodamine isothiocyanate.
from Qiagen Inc. (Valencia, CA). Protein-A plus agarose was from Roche Applied Science. The plasmid pXG-GFP+2’ was a gift from Stephen Beverley (Washington University, St. Louis, MO). An antibody against \( T. brucei \) BiP was a gift from Jay Bangs (University of Wisconsin, Madison, WI). Epimastigotes expressing \( \beta \)-galactosidase were a gift from Frederick Buckner (University of Washington, Seattle). [9,10-\(^3\)H]Stearic acid (52 Ci/mmol) was from Moravek Biochemicals (Brea, CA). EN\(^3\)HANCE, [9,10-\(^3\)H]myristate (10 Ci/mmol), and [9,10-\(^3\)H]palmitate (30 Ci/mmol) were from PerkinElmer Life Sciences. All other reagents were analytical grade.

**Culture Methods—** \( T. cruzi \) amastigotes and trypomastigotes, Y strain, were obtained from the culture medium of \( L_6E9 \) myoblasts by a modification of the method of Schmatz and Murray (9) as we have described previously (10). The contamination of trypomastigotes with amastigotes and intermediate forms or of amastigotes with trypomastigotes or intermediate forms was always less than 5%. \( T. cruzi \) epimastigotes (Y strain) were grown at 28°C in liver infusion tryptose medium (LIT) (11) supplemented with either 10% newborn calf serum under normal conditions or 20% newborn calf serum after cell transfections. Epimastigotes were differentiated into intermediate forms or metacyclic trypomastigotes and isolated using a complement selection procedure (12). Differentiated cells were resuspended in 100 \( \mu \)l of PBS and used to infect \( L_6E9 \) myoblasts cultures grown in 25-cm\(^2\) cell culture flasks.

**Generation of \( TcPI-PLC \) Expression Constructs**—The full-length \( TcPI-PLC \) gene (GenBank\textsuperscript{TM} accession number AF093565) was amplified from genomic DNA, and the GFP gene was amplified from the vector pXG-GFP+2’ and inserted into the pCR2.1-TOPO alone or following in-frame the 3’ end of the \( TcPI-PLC \) insert. The GeneTailor\textsuperscript{TM} site-directed mutagenesis system was used to remove an EcoRI site present upstream or differentiation, respectively, and washed twice with cold PBS and once with 100 mM sodium cacodylate buffer, pH 7.3. The samples were fixed with 0.5% glutaraldehyde, 4% paraformaldehyde in cacodylate buffer on ice for 1 h and washed three times with cacodylate buffer, embedded in LR White\textsuperscript{TM} resin, sectioned, and stained. Immunogold electron microscopy experiments were performed as described previously (13). Images were acquired on a Phillips CM-200 transmission electron microscope operating at 120 kV.

**Electron Microscopy—**Infected \( L_6E9 \) cells or in vitro differentiated amastigotes were harvested at different times of infection or differentiation, respectively, and washed twice with cold PBS and once with 100 mM sodium cacodylate buffer, pH 7.3. The samples were fixed with 0.5% glutaraldehyde, 4% paraformaldehyde in cacodylate buffer on ice for 1 h and washed three times with cacodylate buffer, embedded in LR White\textsuperscript{TM} resin, sectioned, and stained. Immunogold electron microscopy experiments were performed as described previously (13). Images were acquired on a Phillips CM-200 transmission electron microscope operating at 120 kV.

**Cell-surface Biotinylation and Immunoprecipitation—**In vitro differentiated amastigotes were washed and resuspended in 1 mM sulfon-N-hydroxysuccinimidobiotin (Pierce) prepared in 1 ml of PBS, pH 8.0. After incubating the cells at room temperature for 30 min, they were washed five times with ice-cold PBS supplemented with 100 mM glycine, pH 8.0.

For immunoprecipitation, all procedures were carried out at 4°C. Labeled cells were lysed in radioimmunoprecipitation analysis buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholic acid, 50 mM Tris-HCl, pH 7.5, 0.02% sodium azide and protease inhibitor mixture Set III from Sigma) for 1 h. The lysates were clarified by centrifugation at 20,000 x g for 20 min,
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and the supernatants were preadsorbed with protein A-agarose (Roche Applied Science) overnight. After centrifugation at 6,000 × g for 30 s, the supernatants were divided into 3 aliquots of 0.4 ml in fresh tubes and immunoprecipitated for 4 h with anti-TcPI-PLC, anti-T. cruzi Ssp4, and anti-Enterococcus coli β-galactosidase. Immunocomplexes were allowed to bind to protein A-agarose overnight, and subsequent procedures were as described previously (14). To confirm that the specific proteins were pulled down, samples were analyzed by Western blot with the same primary antibodies used in the immunoprecipitation and secondary antibodies conjugated to HRP. Protein concentration was determined using the BCA™ protein assay kit from Pierce.

Quenching of Surface-localized GFP Fusion Protein—A modified procedure was adapted from Denny et al. (15). Aliquots of 5 × 10^6 of T. cruzi epimastigotes were pelleted, washed twice with PBS, and resuspended in 2 ml of PBS at either pH 5.0 or 8.0. The suspension was incubated on ice for 30 min, and excitation spectra were measured using a Hitachi F-2000 fluorescence spectrophotometer and measurement software version 1.03 (Hitachi America, Chula Vista, CA). Emission was detected at 508 nm, and excitation was scanned at 400–485 nm at 500 nm/min. After the initial measurements, cells were permeabilized by adding Triton X-100 (0.1% final concentration) and measured using the same conditions.

SDS-Electrophoresis and Western Blot Analyses—Parasite lysates were obtained by sonication (four times for 15 s with 30-s intervals at 4 °C) and processed as described previously (5).

Metabolic Labeling and Immunoprecipitation—For dual myristate and palmitate labeling, ~1 × 10^8 mid log phase epimastigotes were incubated in minimum essential medium with 2% dialyzed fetal calf serum containing [9,10-3H] myristate (25 μCi/ml) or [9,10-3H] palmitate (50 μCi/ml). The cells were labeled for 16 h at 35 °C and processed as described previously (4) using anti-GFP antibodies. Palmitate gels were treated with 1 M hydroxylamine, pH 7.5 or 1 M Tris-HCl, pH 7.5, and rinsed three times for 5 min each with water.

Identification of Fatty Acids—Immunoadsorbed 3H-acylated GFP fusion proteins were deacylated and extracted as described previously (4). The organic phases were pooled and dried in a Savant SpeedVac SVC 100 (Global Medical Instrumentation Inc., Albertville, MN) and analyzed by thin layer chromatography using Adsorbosil reverse phase HPTLC plates (Alltech, Deerfield, IL) (10 × 10 cm) with acetonitrile/acetic acid (1:1, v/v) as the mobile phase. Methyl ester standards were prepared by methylation of [9–10-3H]palmitic acid, [9,10,13H]myristic acid, and [9,10,13H]stearic acid as described above.

Lipid Raft Isolation—An Optiprep gradient centrifugation (sucrose float) procedure was used to isolate lipid rafts from T. cruzi epimastigotes expressing various TcPI-PLC::GFP fusions. 2.4 × 10^6 mid-log phase epimastigotes were washed twice in PBS, then suspended in 1.1 ml of cold lysis buffer (1% Triton X-100, 25 mM Tris-HCl, 150 mM NaCl, EDTA-free protease inhibitor (Roche Applied Science)), and rotated at 4 °C for 30 min. Afterward, a 100-μl aliquot was removed as a control, and the remaining 1 ml was added to 2 ml of a 60% Optiprep solution to form a final density of 40% Optiprep and transferred to the bottom of a 10-ml ultracentrifuge tube. 5 ml of a 35% Optiprep solution was layered on top of the 40% layer. 1.5 ml of a 5% Optiprep solution was layered on top of the 35% layer. All Optiprep solutions were made by dilution of a 60% Optiprep solution with lysis buffer precooled to 4 °C and prepared in a cold room. Tubes were centrifuged continuously at 4 °C in a Beckman L8-80 ultracentrifuge with a Beckman SW41 rotor (Beckman Coulter Inc., Palo Alto, CA) at 35,000 rpm (210,056 × g) for 5 h and then 25,000 rpm (107,170 × g) for 8 h. Gradient fractions were removed from the top down in 1-ml aliquots. The fraction corresponding to the interface between the 5 and 35% Optiprep gradient contained lipid rafts. A 50-μl aliquot of each fraction was mixed with 50 μl of 2× SDS-PAGE loading buffer, boiled for 10 min, and processed for SDS-PAGE and Western blot analysis as above.

RESULTS

N Terminus of TcPI-PLC Is Sufficient for Plasma Membrane Localization in Epimastigotes—TcPI-PLC localizes to the plasma membrane of the three different stages of T. cruzi when expressed in sufficient amounts, and lipid acylation is necessary for this localization (5). With the aim of studying which amino acids from the N terminus are important for this localization, several GFP-containing gene fusions were generated for overexpression in T. cruzi epimastigotes. The full-length protein (TcPI-PLC::GFP) or the first 6 (6-TcPI-PLC::GFP), 10 (10-TcPI-PLC::GFP), or 20 (20-TcPI-PLC::GFP) amino acids of TcPI-PLC followed by GFP were ligated into the T. cruzi expression vectors pTEX and pTREX. In addition, GFP alone was used as control. Epimastigotes were transfected, and drug selection was accomplished with G418. After approximately 5 weeks of selection, expression of fusion proteins in the transfectants was analyzed by direct fluorescence and indirect immunofluorescence analysis. Both the 6-TcPI-PLC::GFP (Fig. 1C) and 10-TcPI-PLC::GFP (Fig. 1D) fusion proteins were predominantly cytosolic with only weak labeling of the plasma membrane in the case of 10-TcPI-PLC::GFP, and strong labeling in the perinuclear region close to where the flagellum emerges (Fig. 1, C and D, arrows). On the other hand, the 20-TcPI-PLC::GFP (Fig. 1E) fusion protein localized to the plasma membrane. Full-length TcPI-PLC::GFP also localized to the plasma membrane (Fig. 1B) as has been reported before (5). The GFP-positive control localized to the cytosol (Fig. 1A), whereas no fluorescence was detected in nontransfected cells (data not shown). These results suggest that the first 20 amino acids of the N terminus are sufficient for targeting of TcPI-PLC to the plasma membrane of epimastigotes. The expression of these constructs was also confirmed by Western blot analyses with polyclonal anti-GFP (see Fig. 4B). When TcPI-PLC was overexpressed, it was possible to detect plasma membrane localization in the epimastigotes (Fig. 1B). However, it was not possible to see this localization when using antibodies with wild type parasites (4). This difference could be explained because of the low level of expression of the enzyme in epimastigotes, probably below the limit of detection for the antibody (4).

When overexpressed, the 6-TcPI-PLC::GFP (Fig. 1C) and 10-TcPI-PLC::GFP (Fig. 1D) fusions showed perinuclear localization. To investigate the subcellular compartment where these fusion proteins accumulate, flagellar pocket and Golgi

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complex markers were used for co-localization studies. TRITC-labeled concanavalin A, which has been used before to label the flagellar pocket and cytostome of \textit{T. cruzi} epimastigotes (16), and BODIPY TR ceramide, which labels the Golgi stacks in mammalian cells (17) and trypanosomes (18), were used (supplemental Fig. S1 and Fig. 2). No co-localization with concanavalin A was detected in the structure labeled by GFP (supplemental Fig. S1). In contrast, very good co-localization was detected when BODIPY TR ceramide was used (Fig. 2), suggesting that traffic of these short fusion proteins to the plasma membrane is diminished, and they remain in the Golgi complex.

\textbf{Lipid Modification of TcPI-PLC Is Necessary for Plasma Membrane Localization—}Acylation of the 20-TcPI-PLC::GFP and the full-length TcPI-PLC::GFP was studied by analyzing fusion proteins as described previously (4). Epimastigotes were metabolically labeled with both [3H]myristate and [3H]palmitate, and the fusion proteins were immunoprecipitated using antibodies against GFP and deacylated as described under “Experimental Procedures.” The resultant fatty acid methyl esters were then analyzed by thin layer chromatography. Using fatty acid methyl esters standards, myristate, palmitate, and stearate were detected (Fig. 3). Furuya \textit{et al.} (4) determined that the ratio of the myristate/palmitate/stearate was 1:3:0.9 when the cells were labeled with [3H]myristate and 1:1.9:0.9 when cells were labeled with [3H]palmitate. Palmitate labeling predominated in both cases (4), suggesting metabolism of the fatty acids before their incorporation into TcPI-PLC. We confirmed the incorporation of these fatty acids to the full-length TcPI-PLC::GFP (Fig. 3, lane 4) and to the 20-TcPI-PLC::GFP (Fig. 3, lane 5) proteins. The detection of lower quantities of stearate probably indicates low metabolization activity or that stearate is a minor component of TcPI-PLC.
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To demonstrate that lipid modification was essential for plasma membrane localization of TcPI-PLC, we generated several GFP fusion protein mutants containing alanine substitutions at either glycine (20-TcPI-PLC-G2A::GFP) (5) or one of several cysteines of the N terminus (20-TcPI-PLC-C4A::GFP, 20-TcPI-PLC-C8A::GFP, and 20-TcPI-PLC-C15A::GFP). This was done to determine whether these residues are acylated in vivo and, if so, to examine the importance of the acylation in plasma membrane localization. Transfected expressing these proteins were metabolically labeled for short periods ranging from 1 to 4 h with either [3H]myristate or [3H]palmitate and immunoprecipitated with antibodies against GFP, separated by SDS-PAGE, followed by Western blot analyses with anti-GFP and fluorography as described under "Experimental Procedures." It was necessary to use GFP antibody for immunoprecipitation, as the polyclonal antibody did not have the specificity for the fusion proteins that was used in the present study. However, when parasites overexpressed the control Tris buffer, the [3H]myristate- or [3H]palmitate-labeled immunoprecipitation was lost by neutral hydroxylamine treatment, whereas 20-TcPI-PLC-C4A::GFP is myristoylated but not palmitoylated. Control cells (transfected with GFP alone or wild type parasites) do not show labeling at the same position of the constructs. B, gels were transferred to nitrocellulose membranes and probed with anti-GFP antibodies to show labeling of GFP and the different fusion proteins. C, after electrophoresis, gels in C were treated with buffer containing hydroxylamine (NH2OH) or Tris-HCl buffer (Tris). Hydroxylamine treatment cleaves the thioester palmitate linkages but not the myristate covalent linkages.

For palmitoylation (Fig. 4A, right panels), which suggests that these two cysteines are not normally palmitoylated. The results of myristate and palmitate labeling suggest that Gly-2 is myristoylated; Cys-4 is palmitoylated, and neither Cys-8 nor Cys-15 is palmitoylated. Myristoylation and palmitoylation of 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP were also detected although palmitoylation was very weak, especially for the 6-TcPI-PLC::GFP fusion protein (Fig. 4A). Western blot analyses using anti-GFP antibodies (Fig. 4B) showed the amount of protein that was used in each lane of Fig. 4A and that some apparent changes in intensity of the bands were due to slightly different amount of protein loaded, although this was not true for 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP fusion proteins that were weakly labeled with palmitate. To test for the presence of thioester linkages formed upon acylation, the [3H]myristate- or [3H]palmitate-labeled immunoprecipitated 20-TcPI-PLC::GFP, 20-TcPI-PLC-C8A::GFP, and 20-TcPI-PLC-C15A::GFP fusion proteins were separated by SDS-PAGE, and the gels were incubated in 1 M hydroxylamine at a neutral pH (7.5). Under these conditions, S-ester but not O-ester linkages are broken (23). As shown in Fig. 4C, the radioactivity incorporated from [3H]palmitate on the different fusion proteins was lost by neutral hydroxylamine treatment, although the radioactivity incorporated from [3H]myristate remained on the protein after the treatment. These results suggest that [3H]myristate is linked to the fusion proteins by an amide bond, whereas [3H]palmitate could be linked to the fusion proteins by a thioester bond. The control Tris buffer wash did not result in any loss of labeling (Fig. 4C).

Acylation of Gly-2 and Cys-4 Is Required for Proper Membrane Targeting in Epimastigotes—Analysis of GFP targeting revealed that when mutating Gly-2 and Cys-4 to Ala (as in 20-TcPI-PLC-G2A::GFP and 20-TcPI-PLC-C4A::GFP; Fig. 5, A and B, respectively) the proteins do not localize to the
These results are in agreement with previous localization studies of amastigotes. The full-length TcPI-PLC was also labeled with PI-PLC::GFP and more intense labeling in the region close to the plasma membrane (Fig. 5D). Arrows in B show labeling of a region close to the flagellum emergence. Epimastigotes expressing 20-TcPI-PLC-C8A::GFP (C) and 20-TcPI-PLC-C15A::GFP (D) show predominant labeling of the plasma membrane. Epimastigotes expressing 20-TcPI-PLC-C8A::GFP also show intense labeling of the flagellum. Bottom panels show differential interference contrast images of A–D, respectively. Bars, 5 μm.

FIGURE 5. Amino acid residues that are lipid-modified are required for plasma membrane localization of TcPI-PLC. Epimastigote transformants expressing 20-TcPI-PLC-G2A::GFP (A) and 20-TcPI-PLC-C4A::GFP (B) show labeling of the cytosol. Arrows in B show labeling of a region close to the flagellum emergence. Epimastigotes expressing 20-TcPI-PLC-C8A::GFP (C) and 20-TcPI-PLC-C15A::GFP (D) show predominant labeling of the plasma membrane. Epimastigotes expressing 20-TcPI-PLC-C8A::GFP also show intense labeling of the flagellum. Bottom panels show differential interference contrast images of A–D, respectively. Bars, 5 μm.

plasma membrane, indicating that acylation of these residues is necessary for plasma membrane localization. The 20-TcPI-PLC-C4A::GFP protein strongly labeled a structure in the perinuclear region of epimastigotes (Fig. 5B, arrows) that co-localized with BODIPY TR ceramide and probably corresponds to the Golgi complex (Fig. 2) but not with the flagellar pocket, which is labeled by concanavalin A (supplemental Fig. S1). The 20-TcPI-PLC-C8A::GFP mutant was targeted to the plasma membrane and the flagellum (Fig. 5C), although the 20-TcPI-PLC-C15A::GFP was targeted to the plasma membrane (Fig. 5D). This pattern of localization appeared very similar to the T. cruzi protein FCaBP (20) and suggests that the absence of cysteine at position 8 makes the construct more similar to the N-terminal region of FCaBP. Moreover, the differential targeting of 20-TcPI-PLC::GFP (Fig. 1E) and 20-TcPI-PLC-C8A::GFP (Fig. 5C) suggests that N-terminal modifications serve as a molecular addressing system for sending modified proteins to different regions of the cell.

TcPI-PLC Localizes to the Plasma Membrane of Amastigotes—We then investigated whether the localization of the fusion proteins was maintained in amastigote stages (Fig. 6). Fusion proteins 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP localized predominantly to the cytosol of amastigotes with weak labeling of the plasma membrane in the case of 10-TcPI-PLC::GFP and more intense labeling in the region close to the flagellar pocket (Fig. 6A, 6-TcPI-PLC and 10-TcPI-PLC, arrows) as occurred in epimastigotes (Fig. 1C and D). The short flagellum of amastigotes was also labeled with 10-TcPI-PLC::GFP (Fig. 6A). GFP alone localized to the cytosol of amastigotes. The full-length TcPI-PLC::GFP and also the 20-TcPI-PLC::GFP fusion proteins were targeted to the plasma membrane (Fig. 6A, TcPI-PLC and 20-TcPI-PLC, respectively). These results are in agreement with previous localization studies using fluorescent antibody staining against TcPI-PLC (4). In summary, these results establish that, when expressed in sufficient amounts, TcPI-PLC localized to the plasma membrane of both epimastigote and amastigote stages of T. cruzi.

Modification of Gly-2 and Cys-4 Is Required for Proper Membrane Targeting in Amastigotes—We also investigated whether the localization of the mutated fusion proteins was maintained in amastigote stages (Fig. 6B). Mutation of the Gly-2 and Cys-4 to Ala (fusion proteins 20-TcPI-PLC-G2A::GFP and 20-TcPI-PLC-C8A::GFP) led to targeting of the fusion proteins to the cytosol of amastigotes and in the case of 20-TcPI-PLC-C8A::GFP to the perinuclear area (Fig. 6B), as in epimastigotes (Fig. 5B). Mutations of the Cys-8 (20-TcPI-PLC-C8A::GFP) and Cys-15 (20-TcPI-PLC-C15A) did not affect plasma membrane targeting. The short flagellum was also labeled with 20-TcPI-PLC-C8A::GFP (Fig. 6B), as occurs with epimastigotes (Fig. 5C). In conclusion, a similar localization pattern was conserved in epimastigotes and amastigotes.

TcPI-PLC-GFP Is Targeted to Lipid Rafts—Dually acylated proteins with both myristate and palmitate and dually acylated proteins involved in cell signaling are known to be targeted to detergent-resistant membrane microdomains known as lipid rafts (24). Lipid rafts have many putative functions, but the principal function of rafts is thought to be platforms for cell signaling proteins to congregate and interact with other components of signaling pathways (24). Rafts contain a higher concentration of sphingolipids and cholesterol making them resistant to detergent solubilization under cold temperature. To determine whether TcPI-PLC associates with rafts, a detergent extraction of epimastigotes expressing different fusion constructs followed by density gradient centrifugation in an Optiprep gradient was used to isolate detergent-insoluble raft fractions. To determine whether rafts contained the fusion proteins, detergent-insoluble fractions were separated using SDS-PAGE and analyzed by Western blotting with anti-GFP antibody. In previous work (25), dually acylated proteins associated to detergent-resistant membrane in trypansomes were shown to float to the top of these Optiprep gradients. Using this technique, we observed that dually acylated 6-TcPI-PLC::GFP, 10-TcPI-PLC::GFP, 20-TcPI-PLC::GFP, 20-TcPI-PLC-C8A::GFP, and 20-TcPI-PLC-C4A::GFP floated to the top of the Optiprep gradient (Fig. 7). As expected, the cytosolic fusion proteins 20-TcPI-PLC-G2A::GFP, 20-TcPI-PLC-C4A::GFP, and GFP did not associate with detergent-resistant membrane (Fig. 7).

TcPI-PLC Localizes to the Outer Surface of Amastigotes—Considering that TcPI-PLC is N-myristoylated and palmitoy-
lated, it was of interest to investigate where in the plasma membrane the enzyme localizes. Fig. 8A shows immunofluorescence assays of amastigotes labeled with an affinity-purified antibody against TcPI-PLC (4). The labeling is clearly on the surface of these parasites (TcPI-PLC, Fig. 8A, top panels), which also express a cytosolic β-galactosidase (26) for further control experiments. Control wild type parasites show a similar pattern of expression (supplemental Fig. S2) (4). Under identical experimental conditions, when using anti-β-galactosidase (β-Gal, Fig. 8A), the reaction shows a cytosolic distribution. When using intact cells without permeabilization (−Triton, Fig. 8A, bottom panels), the anti-TcPI-PLC antibody still showed a clear reaction on the surface of the parasites. Under similar conditions, the anti-β-galactosidase antibody, which would react against the cytoplasmic β-galactosidase, showed no detectable reaction. Similar results were obtained using amastigotes not expressing β-galactosidase, using BiP as internal marker (supplemental Fig. S2). These results suggest that the TcPI-PLC localizes to the outer surface of the cells.

A more detailed picture of the subcellular localization of TcPI-PLC was obtained by immunoelectron microscopy. We investigated the localization of the TcPI-PLC in in vitro differentiated amastigotes (see “Experimental Procedures”) at 3 and 24 h after differentiation (Fig. 8B, top panels) or in intracellular parasites 3 and 12 h after infection (Fig. 8B, bottom panels). The immunogold reaction with anti-TcPI-PLC shows labeling at

FIGURE 6. N terminus of TcPI-PLC is sufficient, and its lipid modification is required for plasma membrane localization in amastigotes. A, T. cruzi amastigotes expressing various fusion proteins were analyzed by fluorescence microscopy. TcPI-PLC::GFP and 20-TcPI-PLC::GFP localize predominantly to the plasma membrane of amastigotes, whereas 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP fusion proteins as well GFP alone localize throughout the cell. 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP also localize in close proximity to the emergence of the flagellum (arrows) or in the case of 10-TcPI-PLC::GFP to the residual flagellum and the plasma membrane. No fluorescence was detected in nontransfected cells (data not shown). B, amastigote transfectants expressing 20-TcPI-PLC-G2A::GFP and 20-TcPI-PLC-C4A::GFP show labeling of the cytosol. Arrows in 20 C4A show labeling of a region close to the flagellar pocket. Amastigotes expressing 20-TcPI-PLC-C8A::GFP and 20-TcPI-PLC-C15A::GFP show predominant labeling of the plasma membrane, whereas 20-TcPI-PLC-C8A::GFP also show labeling of the short flagellum. A and B, bottom panels are the merge of differential interference contrast fluorescence images. DAPI staining is shown in blue. Bars, 5 μm.
the periphery with most of the gold particles deposited on the external face of the plasma membrane. Gold particles also accumulated in the region of the Golgi stacks during the early stages of in vitro differentiation of trypomastigotes into amastigotes (3 h after starting the incubation at pH 5.0; Fig. 8B, top panel) and were also detected in the flagellar pocket area (12 h) and nucleus (3 h) of intracellular amastigotes (Fig. 8B, bottom panel, white arrows).

The outer surface localization of the TcPIPLC was also shown by biotinylation experiments (Fig. 8C). The surface proteins of amastigotes were labeled with biotin succinimidyl ester, a reagent that couples biotin to lysine residues of exposed proteins. The cells were incubated with the reagent and lysed, and TcPI-PLC was immunoprecipitated with the anti-TcPI-PLC antibody. As controls, the lysates were also immunoprecipitated with preimmune serum, with antibodies against β-galactosidase (a cytosolic marker) or antibodies against the amastigote-specific surface antigen Ssp4, a protein that is GPI-anchored to the plasma membrane and therefore exposed to the outer surface of the cells (27). The precipitated proteins were electrophoresed and blotted, and biotinylated proteins were visualized by peroxidase-conjugated streptavidin and ECL. An 85-kDa polypeptide corresponding to TcPI-PLC was detected (Fig. 8C, lane TcPI-PLC, top panels), although no bands were detected with the negative controls immunoprecipitated with β-galactosidase antibody (Fig. 8C, top panels, β-Gal) or preimmune serum (data not shown). Fig. 8C, top panels, lane Ssp4, shows a band of 84 kDa and other bands ranging in size from 80 to 90 kDa corresponding to the Ssp4 protein family (27). Western blot analysis shows the presence of the proteins investigated (Fig. 8C, bottom panels).

We then used transgenic parasites to study the localization to the outer surface of the plasma membrane and the role of the N-myristoylation in this localization. We used a polyclonal antibody against GFP and epimastigotes expressing TcPI-PLC::GFP (transfected with pTREX/TcPI-PLC::GFP) or TcPI-PLC-G2A::GFP (transfected with pTREX/TcPI-PLC-G2A::GFP) or GFP alone (transfected with pTREX/GFP). Fluorescence analysis was done in cells permeabilized or not with Triton X-100. Only when the cells express the full-length TcPI-PLC::GFP was it possible to observe reaction with anti-GFP in the plasma membrane of nonpermeabilized cells (Fig. 9A, GFP, top panel), thus confirming its association with the outer surface of the cells. The parasites expressing the mutant version of TcPIPLC (TcPI-PLC-G2A::GFP) have the enzyme in their cytoplasm because of the missing N-myristoylation site. These parasites (Fig. 9B, central panels) show reaction against the anti-GFP only when permeabilized with Triton (Fig. 9B, compare central panels plus and minus Triton). A similar result was obtained with parasites expressing GFP alone (Fig. 9C, bottom panels). In all cases, the direct fluorescence of GFP is shown on the left panels as controls. Overlays are shown on the right panels of Fig. 9.

The GFP-emitted fluorescence is sensitive to low pH values, and if it is expressed at the outside surface of the cells, it is possible to measure its quenching by exposing intact cells to low pH values (15, 28, 29). Epimastigotes expressing membrane-bound TcPI-PLC::GFP or the cytosolic proteins TcPI-PLC-G2A::GFP and GFP were washed in PBS and resuspended in PBS adjusted to pH 5.0 or 8.0 for 30 min at room temperature, and fluorescence emission was detected at 508 nm (see “Experimental Procedures”). The fluorescence of cells expressing GFP was unchanged at pH 5.0 (Table 1), which is consistent with the protein being located in the cytosol and not exposed to the extracellular environment. Moreover, TcPI-PLC-G2A::GFP was not affected by a reduction in the extracellular pH, which is also consistent with a cytoplasmic localization (Table 1). Permeabilization with 0.1% Triton X-100 led to a loss of fluorescence at pH 5.0 but not at pH 8.0, confirming the sensitivity of the fusion proteins to low pH. However, exposure of nonpermeabilized cells expressing TcPI-PLC::GFP to pH 5.0 resulted in a significant reduction in fluorescence suggesting outer membrane association. These results confirmed the pH-sensitive location of TcPI-PLC::GFP in the external surface of the plasma membrane.

In conclusion, immunofluorescence assay, immunoelectron microscopy, surface biotinylation, and cell surface fluorescence quenching assays all indicate that TcPI-PLC is localized to the outer surface of the parasites, and the experiments using TcPI-PLC fused with GFP (Fig. 9) indicate that lipid modification of the N-terminal region is necessary for targeting TcPI-PLC to the outer surface of the plasma membrane.

DISCUSSION

In this study, we have shown that TcPI-PLC is localized to the extracellular phase of the plasma membrane of amastigotes as indicated by immunofluorescence experiments, immunoelectron microscopy, and biotinylation studies in wild type parasites, as well as by immunofluorescence analysis and assays of the sensitivity to extracellular pH of epimastigotes transfected with GFP fused to wild type or mutated TcPI-PLC. In addition,
FIGURE 8. TcPI-PLC localizes to the outer surface of the plasma membrane. A, indirect immunofluorescence analysis of TcPI-PLC and β-galactosidase in amastigotes obtained by in vitro differentiation at pH 5.0. Fluorescence images of fixed and permeabilized (Triton +) or nonpermeabilized (Triton −) amastigotes stained with antibodies against TcPI-PLC (green) or β-galactosidase (red). B, immunocytochemical localization of TcPI-PLC in amastigotes obtained by in vitro differentiation at pH 5.0 (top panels) or in intracellular amastigotes (bottom panels). Labeling of the Golgi stacks (G) is detected at early time points in in vitro differentiated parasites (3 h, top panel, white arrow), and labeling at the plasma membrane predominates after 24 h of initiation of differentiation (24 h, black arrowheads). In intracellular amastigotes (bottom panels) it is possible to note labeling of the outer surface of the cells (black arrowheads), the nucleus (N, 3 h, white arrows), and the flagellar pocket (FP, 12 h, white arrow, right panel). N, nucleus, K, kinetoplast, F, flagellum. Arrowheads in bottom panels show the parasite plasma membrane in close contact with the parasitophorous vacuole (3 h). C, biotinylation of cell surface proteins in amastigotes obtained by in vitro differentiation at pH 5.0. Amastigotes were incubated with biotin succinimidyl ester. After lysis of the cells, TcPI-PLC was immunoprecipitated by the polyclonal antibody, and the immunoprecipitate was subjected to Western blot analysis (bottom panel). Detection of biotinylation was by streptavidin-peroxidase conjugate and ECL (top panel). A band of 85 kDa was detected (TcPI-PLC). Lane Ssp4 shows a positive control immunoprecipitated with anti-Ssp4, and lane β-Gal shows a negative control immunoprecipitated with anti-β-galactosidase instead of anti-TcPI-PLC. Migration of prestained molecular weight mass standards is shown to the left of the gel. The presence of the immunoprecipitated proteins was demonstrated by Western blot analysis (bottom panel). Bars in A, 10 μm, and in B, 0.5 μm.
we demonstrated that the N-terminal 20 amino acids have the necessary and sufficient information to target GFP to the outer surface of the plasma membrane of T. cruzi. Through the study of trypanosome transfectants expressing various deletion mutants of this N-terminal region fused to GFP, we found that myristoylation of a glycine residue in the 2nd position and acyl modification of a cysteine in the 4th position of the coding sequence are required for correct plasma membrane localiza-

tion. Myristate, palmitate, and stearate were present in the N-terminal region of TcPI-PLC, but only myristate formed an amide bond with glycine 2.

The N terminus of TcPI-PLC differs in amino acid composition from other membrane-targeted acylated proteins in T. cruzi such as FcABP, which localizes to the flagellum (20). The attachment of myristate to Gly-2 and palmitate to Cys-4 is necessary and sufficient to target TcPI-PLC to the plasma membrane. Any change in the amino acids involved in the lipid modifications (G2A and/or C4A) renders the fusion proteins cytosolic, and the C8A substitution additionally targets the fusion protein to the flagellum. Although cysteine 8 is not acylated, this modification probably makes the N terminus of TcPI-PLC more similar to the N terminus of FcABP suggesting that both FcABP and 20-TcPI-PLC-C8A::GFP are being processed through the same targeting mechanism.

The 20-TcPI-PLC-C4A::GFP mutant, which is myristoylated but not palmitoylated, was in part cytosolic and accumulated in a compartment stained with the Golgi-specific probe BODIPY TR ceramide, suggesting that palmitoylation is necessary for trafficking from the Golgi to the plasma membrane. The 6-TcPI-PLC::GFP and 10-TcPI-PLC::GFP fusion proteins, which were shown to be weakly palmitoylated, were in part cytosolic but predominantly localized to the compartment stained with BODIPY TR ceramide. The 10-TcPI-PLC::GFP fusion protein also showed partial plasma membrane localization. Impaired plasma membrane targeting of a short fusion protein immediately proximal to GFP has been noted in Leishmania major (15) and mammalian cells (30). It is tempting to speculate that only the fusion proteins containing short peptides of 6 and 10 amino acids that are palmitoylated can reach the plasma membrane, whereas those that are not palmitoylated remain in the Golgi complex, and that the length of the fusion peptide determines their accessibility to palmitoyltransferases and their potential palmitoylation. Taken together, these results indicate that palmitoylation of carbon 4 and a certain length of amino acids in the N-terminal region of TcPI-PLC are necessary for adequate trafficking to the plasma membrane.

The dually acylated fusion proteins were targeted to lipid rafts in agreement with their content in saturated fatty acid chains, such as myristate, palmitate, and stearate. Lipid rafts are membrane subdomains highly enriched in cholesterol (or ergosterol), glycosphingolipids, and phospholipids containing saturated fatty acid chains (24). Lipid rafts are formed in the Golgi complex (24), in agreement with the suggested traffic of TcPI-PLC fusion protein through this organelle.

Because lipid modification alters signaling-protein function, the enzymes that catalyze these modifications have become attractive targets. N-Myristoyltransferase inhibitors have been shown to have activity as antifungal (31) and trypanocidal (32) agents. Palmitoyltransferase inhibitors are also likely to be attractive targets (8), and identification of the enzymes involved in lipid modification of TcPI-PLC, which is an essential enzyme in T. cruzi (5), could lead to the discovery of potential chemotherapeutic agents.

TcPI-PLC is the only known PI-PLC and the only known enzyme that is dually acylated and targeted to the outer surface of the plasma membrane, and this has implications for both the

![Figure 9. Role of the N-myristoylation in the surface localization of TcPI-PLC. A–C, direct and indirect fluorescence analysis of epimastigotes. Fluorescence images of permeabilized (Triton +) or intact (Triton −) cells expressing TcPI-PLC::GFP (A), TcPI-PLC-G2A::GFP (B), or GFP alone (C). Other experimental conditions are described under “Experimental Procedures.” Bars, 10 μm.](image-url)
Phospholipase C in Amastigotes of T. cruzi

function and regulation of the enzyme. TcPI-PLC possesses an extremely high Ca\(^{2+}\) sensitivity of its PIP\(_2\) hydrolyzing activity (4). TcPI-PLC exhibits 50% maximal activity at 100 nm Ca\(^{2+}\) (4) that is usually the basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) level in mammalian cells (33). The basal [Ca\(^{2+}\)]\(_i\) level in amastigotes is much lower (around 10–20 nm (34)). The localization of TcPI-PLC on the surface of intracellular amastigotes and in contact with the cytoplasm of mammalian cells would be sufficient to stimulate its activity, although it is associated with its plasma membrane. In this regard, TcPI-PLC has high similarity with a new type of PI-PLC that is expressed in mammalian sperm and was named PI-PLC\(_\varepsilon\). As PI-PLC\(_\varepsilon\), TcPI-PLC lacks a pleckstrin homology domain, has an extended X-Y linker sequence with a high proportion of charged residues (35), and has high Ca\(^{2+}\) sensitivity (36). PI-PLC\(_\varepsilon\) triggers Ca\(^{2+}\) oscillations when expressed in mouse eggs and has been proposed to be the “sperm factor” that triggers Ca\(^{2+}\) release from the endoplasmic reticulum when introduced into the ooplasm at fertilization (35, 36). We recently reported that surface expression of TcPI-PLC coincides with PIP\(_2\) depletion in the host cell membrane and with increases in the levels of its product, inositol 1,4,5-trisphosphate. Maximal early expression of TcPI-PLC on the surface of amastigotes and PIP\(_2\) depletion coincides with the host cytoskeletal changes, Ca\(^{2+}\) signaling, and transcriptional responses described previously (37).

A protein that is modified in its N terminus by N-myristoylation and palmitoylation, and that is known as the hydrophilic acylated surface protein B (HASPB), has also been shown to translocate onto the surface of L. major promastigotes, although no enzymatic activity has been described for this protein (15). A “flipfase” model was proposed as a potential mechanism of translocation of this protein to the outer surface of the parasites or the mammalian cells transfected with the HASPB gene. In agreement with this proposal, a mutant CHO cell line apparently deficient in the plasma membrane resident machinery involved in this translocation was incapable of exporting HASPB (38).

Another functional implication of the extracellular localization of TcPI-PLC is the possibility that it could be involved in shedding of GPI-anchored proteins from the surface of the parasite. A number of GPI-anchored proteins from T. cruzi (trans-sialidase from tissue culture-derived trypanomastigotes, mucins from metacyclic trypanomastigotes, and Ssp4 from amastigotes) are shed into the medium (or inside the host cell) (39) by the action of an endogenous PI-PLC (27, 40, 41). It has been demonstrated that the presence of ceramide in the lipid portion of the GPI anchor of these glycoproteins is related to their shedding by endogenous PLCs (42). This shedding results in release of ceramide, which is particularly important because ceramide has been shown to cause differentiation in many cell types (42). In this regard, it has been demonstrated (43) that TcPI-PLC is equally capable of recognizing and cleaving phosphatidylinositol and inositol phosphoceramide, which is the lipid portion of the GPI-anchored of Ssp4, and that the concentration of ceramide increases after 24 h of differentiation of trypanomastigotes into amastigotes (44). Interestingly, a phospholipase C activity is responsible for the release of Ssp4 from the surface of amastigotes (27), and TcPI-PLC could be that enzyme. In addition, it was also shown that exogenous bacterial PI-PLC treatment induced differentiation of trypomastigote forms (45). Further work will be needed to investigate the function of TcPI-PLC in shedding surface molecules from the parasite.

In summary, this is the first example of a PI-PLC that requires acyl modification for proper targeting to the outer leaflet of the plasma membrane. Targeting to the outer face of the membrane supports a potential role in both cell surface remodeling and cell signaling in the host.

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REFERENCES

1. Irvine, R. (1996) Nature 380, 581–583
2. Berridge, M. J. (1987) Annu. Rev. Biochem. 56, 159–193
3. Nishizuka, Y. (1988) Nature 334, 661–665
4. Furuya, T., Kashuba, C., Docampo, R., and Moreno, S. N. (2000) J. Biol. Chem. 275, 6428–6438
5. Okura, M., Fang, J., Salto, M. L., Singer, R. S., Docampo, R., and Moreno, S. N. (2005) J. Biol. Chem. 280, 16235–16243
6. Mills, E., Price, H. P., Johner, A., Emerson, J. E., and Smith, D. F. (2007) Mol. Biochem. Parasitol. 152, 22–34
7. Resh, M. D. (2006) Nat. Chem. Biol. 2, 584–590
8. Emmer, B. T., Souther, C., Torriolo, K. M., Olson, C. L., Epting, C. L., and Engman, D. M. (2009) J. Cell Sci. 122, 867–874
9. Schmutz, D. M., and Murray, P. K. (1982) Parasitology 85, 115–125
10. Moreno, S. N., Silva, J., Vercesi, A. E., and Docampo, R. (1994) J. Exp. Med. 180, 1535–1540
11. Bone, G. J., and Steinert, M. (1956) Nature 178, 308–309
12. Caler, E. V., Vaena de Avalos, S., Haynes, P. A., Andrews, N. W., and Burleigh, B. A. (1998) EMBO J. 17, 4975–4986
13. Vieira, M., Rohlhoff, P., Luo, S., Cunha-e-Silva, N. L., de Souza, W., and Docampo, R. (2005) Biochem. J. 392, 467–474
14. Benchimol, M., De Souza, W., Vanderheyden, N., Zhong, L., Lu, H. G., Moreno, S. N., and Docampo, R. (1998) Biochem. J. 332, 695–702
15. Denny, P. W., Gokool, S., Russell, D. G., Field, M. C., and Smith, D. F. (2000) J. Biol. Chem. 275, 11017–11025
16. Pagano, P. F., de Souza, W., Souto-Padrón, T., and Pinto da Silva, P. (1989) Eur. J. Cell Biol. 50, 263–271
17. Field, H., Sherwin, T., Smith, A. C., Gull, K., and Field, M. C. (2000) Mol. Biochem. Parasitol. 106, 21–35
18. Resh, M. D. (1999) Biochim. Biophys. Acta 1451, 1–16
19. Godsel, L. M., and Engman, D. M. (1999) EMBO J. 18, 2057–2065
20. Hallak, H., Brass, L. F., and Manning, D. R. (1994) J. Biol. Chem. 269, 4571–4576
21. Munday, S. M., Kleuss, C., and Gilman, A. G. (1994) Proc. Natl. Acad. Sci. U.S.A. 91, 2800–2804
22. Bizzozero, O. A. (1995) Methods Enzymol. 250, 361–379
23. Simons, K., and Toomre, D. (2000) Nat. Rev. Mol. Cell Biol. 1, 31–39
24. Tyler, K. M., Fridberg, A., Torriolo, K. M., Olson, C. L., Cieslak, J. A., Hazlett, T. L., and Engman, D. M. (2009) J. Cell Sci. 122, 859–866
25. Buckner, F. S., Verlinde, C. L., La Flamme, A. C., and Van Voorhis, W. C. (1996) Antimicrob. Agents Chemother. 40, 2592–2597
26. Andrews, N. W., Robbins, E. S., Ley, V., Hong, K. S., and Nussenzweig, V. (1988) J. Exp. Med. 167, 300–314
27. Bokman, S. H., and Ward, W. W. (1981) Biochem. Biophys. Res. Commun.
37. Martins, V., Galizzi, M., Salto, M. L., Docampo, R., and Moreno, S. N. (2010) *Infect. Immun.*, in press
38. Stegmayer, C., Kehlenbach, A., Tournaviti, S., Wegehingel, S., Zehe, C., Denny, P., Smith, D. F., Schwappach, B., and Nickel, W. (2005) *J. Cell Sci.* **118**, 517–527
39. Frevert, U., Schenkman, S., and Nussenzweig, V. (1992) *Infect. Immun.* **60**, 2349–2360
40. Pollevick, G. D., Di Noia, J. M., Salto, M. L., Lima, C., Leguizamón, M. S., de Lederkremer, R. M., and Frasch, A. C. (2000) *J. Biol. Chem.* **275**, 27671–27680
41. Agusti, R., Couto, A. S., Campetella, O. E., Frasch, A. C., and de Lederkremer, R. M. (1997) *Glycobiology* **7**, 731–735
42. Hannun, Y. A., and Luberto, C. (2004) *Curr. Biol.* **14**, R163–R165
43. Salto, M. L., Furuya, T., Moreno, S. N., Docampo, R., and de Lederkremer, R. M. (2002) *Mol. Biochem. Parasitol.* **119**, 131–133
44. Bertello, L. E., Andrews, N. W., and de Lederkremer, R. M. (1996) *Mol. Biochem. Parasitol.* **79**, 143–151
45. Mortara, R. A., Minelli, L. M., Vandekerckhove, F., Nussenzweig, V., and Ramalho-Pinto, F. J. (2001) *J. Eukaryot. Microbiol.* **48**, 27–37