A Lipid-modified Phosphoinositide-specific Phospholipase C (TcPI-PLC) Is Involved in Differentiation of Trypomastigotes to Amastigotes of Trypanosoma cruzi*

Received for publication, December 23, 2004
Published, JBC Papers in Press, February 14, 2005, DOI 10.1074/jbc.M414535200

Michael Okura‡, Jiamin Fang‡§, Maria Laura Salto‡, Randall S. Singer‡¶, Roberto Docampo‡§, and Silvia N. J. Moreno‡§§

From the ‡Department of Pathobiology and Center for Zoonoses Research, University of Illinois at Urbana-Champaign, Urbana, Illinois 61802 and the ¶Center for Tropical and Global Emerging Diseases and Department of Cellular Biology, University of Georgia, Athens, Georgia 30602

The phosphotidylinositol-specific phospholipase C (PI-PLC) is an important component of the inositol phosphate/diacylglycerol signaling pathway. A newly discovered Trypanosoma cruzi PI-PLC (TcPI-PLC) is lipid modified in its N terminus, targeted to its plasma membrane, and believed to play a role in differentiation of the parasite because its expression increases during the differentiation of trypomastigote to amastigote stages. To determine whether TcPI-PLC is involved in this differentiation step, antisense inhibition using phosphorothioate-modified oligonucleotides, and overexpression of the gene were performed. Antisense oligonucleotide-treated parasites showed a reduced rate of differentiation in comparison to controls, as well as accumulation of intermediate forms. Overexpression of TcPI-PLC led to a faster differentiation rate. In contrast, overexpression of a mutant TcPI-PLC that lacked the lipid modification at its N terminus did not affect the differentiation rate. Therefore, TcPI-PLC is involved, when expressed in the plasma membrane, in the differentiation of trypomastigotes to amastigotes, an essential step for the intracellular replication of these parasites.

Phosphatidylinositol-specific phospholipase C (PI-PLC) catalyzes the hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP₂) to the second messengers diacylglycerol and inositol 1,4,5-trisphosphate (IP₃) (1). Diacylglycerol is the physiological activator of protein kinase C (2), and IP₃ induces the release of Ca²⁺ from internal stores (3). Together, these second messengers cause an increase in phosphorylation of proteins that result in a cellular response. This pathway, also known as the inositol phosphate/diacylglycerol pathway, is known to regulate a large array of cellular processes, including metabolism, secretion, contraction, neural activity, and proliferation (1).

Previous research has indicated that some of the signaling components of the inositol phosphate/diacylglycerol signal pathway are present in Trypanosoma cruzi, the etiologic agent of Chagas disease, and seem to be associated with cell differentiation. T. cruzi has three main developmental stages, the epimastigote that is found in the insect vector and can be grown in axenic culture, the amastigote or intracellular form, which lives in the cytosol of nucleated cells, and the trypomastigote, which is the terminal differentiation stage in the vector (metacyclic form) or is found in the bloodstream of mammalian hosts (bloodstream form). Ca²⁺ stimulated IP₃ and diacylglycerol formation in digitonin-permeabilized epimastigotes of T. cruzi, thus suggesting the presence of a PI-PLC (4). Protein kinase C activities were characterized in T. cruzi epimastigotes (5, 6) and could be resolved into three subtypes in hydroxylapatite columns (6). The enzyme isoforms require phosphatidylinerine and Ca²⁺ for activity and are stimulated by diacylglycerol (5, 6). The present of significant amounts of inositol phosphates in amastigotes (7) and much lower amounts in trypomastigotes (8) was also reported. Characterization of the recombinant T. cruzi PI-PLC (TcPI-PLC) (9, 10) confirmed the presence of a functional enzyme in T. cruzi.

The PI-PLC described in T. cruzi is, like all PI-PLC described so far in unicellular eukaryotes, similar to the δ-type PI-PLC of mammalian cells and plants. However, the T. cruzi enzyme possesses an N-myristoylation and palmitoylation consensus sequence that had not been described previously in any other PI-PLC, and has been shown to be myristoylated and palmitoylated (10). It has been postulated that this lipid modification could be important for plasma membrane attachment (10).

Evidence for the involvement of T. cruzi PI-PLC in differentiation of these parasites is based on the increase in the IP₃ levels and in the expression of the enzyme during the trypomastigote to amastigote differentiation. Within amastigotes, TcPI-PLC localized to the plasma membrane although the protein was not observed in detectable amounts in trypomastigotes (10). Differentiation of trypomastigotes to amastigotes was also shown to be stimulated by nanomolar concentrations of the protein phosphatase 1 and 2A inhibitor calyculin A (11), and inhibited by the protein phosphatase 2A inhibitor okadaic acid (12) implying the intervention of phosphorylation signaling cascades in this process. In the present study, we report that...
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Antisense inhibition using phosphorothioate-modified oligonucleotides inhibits, although overexpression of TcPI-PLC stimulates, the differentiation of trypanostigote to amastigote forms, and that lipid modification is essential for its plasma membrane localization, and stimulation of differentiation.

**Experimental Procedures**

**Culture Methods—**T. cruzi trypanostigotes and amastigotes (Y strain) were obtained from the culture medium of L<sub>3</sub>k<sub>4</sub> myoblasts by a modification of the method of Schmutz and Murray (13) as we have described before (7, 8). The contamination of trypanostigotes with amastigotes and intermediate forms of or amastigotes with trypanostigotes or intermediate forms was always less than 10% unless otherwise stated. T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose medium (14) supplemented with either 10% newborn calf serum under normal conditions or 20% newborn calf serum after cell transfections. Protein concentration was determined using the Bio-Rad protein assay. Trypanostigotes were induced to transform into amastigotes axenically as described previously (15).

Briefly, trypanostigotes were washed once with Dulbecco's modified Eagle's medium (DMEM) containing 0.4% bovine serum albumin (DMEM/BSA) at pH 7.5. The cells were resuspended at a concentration of 5 to 10<sup>5</sup> parasites/mL in DMEM/BSA at either pH 5 or 7.5, subcultured into a sterile 6-well plate, and buffered with 20 mM Mes (pH 5.0) or 20 mM Hepes (pH 7.5). Parasites were incubated at 35 °C and harvested at different time points. For overnight incubations, parasites previously incubated at acidic pH for 4 h were centrifuged and resuspended in DMEM/BSA at pH 7.5.

**Chemicals—**ECL chemiluminescence detection kits, [α-<sup>32</sup>P]dCTP (3,000 Ci/mmol) and nylon membranes were from Amersham Biosciences. The protein assay was from Bio-Rad. All primers and oligonucleotides were ordered from Integrated DNA Technologies (Coralville, IA). G418 sulfate, Gene Tailor Site-directed Mutagenesis System, pcER2.1-TOPO cloning vector, protein prenatored mammalian weight markers, restriction enzymes, Tag polymerase, and TRIzol reagent were from Invitrogen (Carlsbad, CA). Anti-green fluorescent protein (GFP) mouse monoclonal antibody 3E6 was from Molecular Probes Gent were from Invitrogen (Carlsbad, CA). Anti-green fluorescent protein (GFP) mouse monoclonal antibody 3E6 was from Molecular Probes. The plasmid pTEX was accomplished using the Gene Tailor Site-directed Mutagenesis System in accordance with the manufacturer's instructions. The Gly<sup>-</sup>Ala<sup>-</sup> mutation was generated using forward primer 5'-GTCCTGCTTCTGCTGGAGCTG-3' and reverse primer 5'-GTCCTGCTTCTGCTGGAGCTG-3' and sequenced to confirm the identity and orientation of the insert.

Large-scale purification of plasmid DNA was accomplished using the Qiagen Plasmid Maxi kit for purification of large phase culture volumes of 200–500 ml in Luria-Bertani medium. After plasmid isolation, a restriction fragment containing each targetting construct was removed by digestion with appropriate restriction enzymes and electrophoresis using the Ultrafree-DA extraction system, followed by concentration using ethanol precipitation. Targetting constructs were transfected into wild-type epimastigotes (Y strain) by electroporation (19, 20) and cultured overnight in liver infusion tryptose medium. After 48 h incubation at 28 °C, 400 µg/ml G418 sulfated was added to the culture. Culture medium was changed every 1–3 days. Three-6 weeks after transfection cloning by limited dilution was observed. A cDNA containing the glyceraldehyde-3-phosphate dehydrogenase intergenic region (GAP) (18) into the HindIII site separated by agarose gel electrophoresis, extracted from the gel using the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA), and cloned into the pcER2.1-TOPO cloning vector. Constructs were removed from pcER2.1-TOPO by restriction digest with XbaI and Xhol, gel purified, and ligated into the XbaI and XhoI sites of the T. cruzi epimastigote expression vector pEFV (5).

Site-directed mutagenesis of the full-length TcPI-PLC inserted between the EcoRI and HindIII sites of pTEX and TcPI-PLC/GFP with GFP inserted in the HindIII site following the TcPI-PLC resistance gene (Neo<sup>+</sup>) orientation of all constructs was confirmed by sequencing.

**Transfection of T. cruzi—**Approximately 1 × 10<sup>6</sup> mid-log phase epimastigotes were washed twice with 5 ml of Hepes-buffered saline (21 mm Hepes, 137 mm NaCl, 5 mm KCl, 0.7 mm Na<sub>2</sub>HPO<sub>4</sub>, and 6 mm glucose), resuspended in 500 µl of Hepes-buffered saline, and mixed with 50–100 µg of plasmid DNA. The suspension was placed in a 2-mm Gene Pulser Cuvette (Bio-Rad) and pulsed twice at room temperature with a Gene Pulser II electroporator (Bio-Rad) set at 1.5 KV and 50 µF microfarad capacitance. Cuvettes were cooled on ice for 5 min and the parasites transferred to 5 ml of liver infusion tryptose medium containing 20% newborn calf serum. After 48 h incubation at 28 °C, 400 µg/ml G418 sulfate was added to the culture. Culture medium was changed every 2–3 days. Transfected epimastigotes were differentiated to mammalian forms as described before (17).

**Fluorescence Microscopy—**To observe GFP-tagged proteins, ~1 × 10<sup>7</sup> parasites were fixed with 4% formaldehyde in PBS for 1 h at room temperature, washed three times with PBS, and allowed to adhere to poly-L-lysine-coated glass slides for 10 min. Images of GFP expressing parasites were obtained with a Nikon Eclipse E800 microscope (Nikon).
Inc., Melville, NY) using settings for fluorescent microscopy and equipped with a Spot RT Slider system consisting of a charge-coupled device camera (Diagnostic Instruments, Inc.). Acquired images were evaluated using Meta Morph 5.0 software (Universal Imaging Corp., Downingtown, PA). Slides intended for phase-contrast microscopy were treated in the same manner but images were obtained using an Olympus BX-60 fluorescence microscope. The images were collected with a charge-coupled device camera (model CH250; Photometrix Ltd., Tucson, AZ), an electronic unit (model CE 200A, equipped with a 5-Hz 16-bit A/D converter), and a controller board (model NU 200; both from Photometrix Ltd.). These images were evaluated using Adobe Photoshop 6.0 (Adobe Systems Inc., San Jose, CA).

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Approximately 5 × 10^7 trypomastigotes were harvested from L_E9 myoblast cells. Only cultures containing >90% trypomastigotes were used for treatment. Parasites were considered >90% trypomastigotes each. Two aliquots were used for treatment groups Antisense and Sense and these were resuspended in 400 μl of DMEM (pH 7.5) (DMEM containing 0.4% BSA and buffered with 20 mM Hepes with a final pH adjustment to 7.5) (15) containing 20 μg of either antisense or sense oligonucleotides. The two remaining aliquots were used for Controls, pH 5.0 and pH 7.5, and were resuspended in 400 μl of DMEM (pH 7.5). The aliquots were incubated overnight at 35 °C in closed 1.5-ml microcentrifuge tubes (including intermediate forms). The supernatant was removed and high-speed centrifugation was used to reduce the number of amastigotes, differential centrifugation was used to obtain a final concentration of 20 μg of TcPI-PLC. Oligonucleotides were rehydrated using sterile water at a concentration of 1 μM.

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Phosphorodiamidate Antisense Inhibition of TcPI-PLC—High performance liquid chromatography-purified phosphorodiester oligonucleotides were used containing the following sequences, antisense G^*A*ATC-ATCTACAACTCTCTC^*A*G*C, and sense G*C*T*GAAGACTTT-GTATAGAT*T*T*C (* = phosphorothioate modification). The sense oligonucleotide contains the nucleotide sequence for TcPI-PLC bases 61 to 85 in the coding region of the gene and the antisense is complimentary. Oligonucleotides were rehydrated using sterile water at a concentration of 1 μM.

Cells were taken at time points 1, 2, and 4 h after the initial incubation with the appropriate oligonucleotide. The two remaining aliquots were used for Controls, pH 5.0 and pH 7.5. The Control pH 5 groups were combined with the appropriate oligonucleotide. The sense oligonucleotide was rehydrated using sterile water at a concentration of 20 μg and the appropriate oligonucleotide was added to obtain a final concentration of 20 μg. The samples were collected, individually fixed by combining with an equal volume of PBS containing 4% formaldehyde and processed for microscope counting. An additional sample of 10^7 cells was removed and fixed with 100 μl of PBS within 1 h after fixing and resuspended in 100 μl of PBS or diluted 1:10 with PBS. A 100-μl aliquot was smeared on poly-lysin slides and allowed to air dry. All slides were observed by phase-contrast microscopy under the ×100 oil immersion objective. Images were obtained with an Olympus BX-60 fluorescence microscope containing a contrast riduce with 1-mm index squares (Edmund Industrial Optics) installed in the eyepiece. At least 300 cells were counted on each slide in 3 or more randomly chosen fields of view. The images were collected with the system described above. The percentage of trypomastigotes (including intermediate forms), and amastigotes were calculated for each treatment group at each time point sampled.

Northern Blotting—The total RNA from 1 × 10^7 cells trypomastigotes (transfected or wild-type) was extracted using TRIzol reagent according to the manufacturer’s instructions. Total RNA was electrophoresed in 1.0% agarose gels with 2.2% formaldehyde, 20 mM Mops (pH 7.0), 1 mM EDTA, 8 mM sodium acetate, and transferred to nylon membranes. The DNA probe consisting of the full-length TcPI-PLC was labeled with [α-32P]dCTP using random hexanucleotide prides and Klenow fragment of DNA polymerase I (Prime-A-Gene labeling stringency, and exposed to x-ray film (Midwest Scientific, St. Louis, MO) at ~80 °C for 1–7 days.

Quantitative Real-time PCR—Approximately 1 × 10^7 parasites were washed in PBS and total RNA was purified using the RNeasy Kit (Qiagen) following the procedure for animal cells including the optional DNA digestion step. DNA elimination was accomplished using the iScript cDNA synthesis kit (Bio-Rad) with 100 ng of total RNA used per reaction. Real-time PCR was done using an ABI Prism 7000 sequence detector (Applied Biosystems, Foster City, CA) with software version 1.1 and the Quantitect SYBR Green PCR Kit (Qiagen). The primers for TcPI-PLC amplification were: forward primer 5’-CAGTGAATCTGCT-GGATGTGA-3’ , and reverse primer 5’-ATGCACCTGCTA-CCATCCTG-3’. The primers for amastin ribosomal gene TcP0 were forward primer 5’-CCTCCGGTGTCGCA-CATGAA-3’, and reverse primer 5’-CGTCACTCATCCTCTCCT-3’. The primers for amastin amplification were forward primer 5’-ATCTT-TTG CGC CAC GTG TGT-3’, and reverse primer 5’-AGCACGGGCGGAA AATATCAG-3’. (21). Reaction mixtures contained 12.5 μl of 2 × QuantiTect SYBR Green master mix, 0.6 μl of each primer, 10 ng total RNA, and water to 25 μl of the final reaction volume. PCR cycling conditions were 1 cycle (50 °C for 2 min/95 °C for 10 min) and 40 cycles (95 °C 15 s/60 °C for 1 min). All PCR reactions were performed in duplicate. Separate reactions for amplification of TcPI-PLC, TcP0, and amastin were performed for each sample. Relative expression of TcPI-PLC or amastin was calculated by comparing the Ct values from each amplification with the Ct values from each amplification with the primers for amastin. The Ct values were determined from the following equation: relative TcPI-PLC or amastin expression = 2^ΔΔCt, where ΔΔCt = (ΔCt treatment group (CtTcPI-PLC or amastin) – ΔCt reference gene (CtTcPI-PLC or amastin)) – ΔCt control group (CtTcPI-PLC or amastin).

Metabolic Labeling and Immunoprecipitation—For myristate and palmitate labeling, cells were individually labeled with either [9,10-3H]myristate (25 μCi/ml) or [9,10-3H]palmitate (50 μCi/ml) using a modified procedure developed by Godsel and Engman (22). Briefly, epimastigotes were grown to a density of 5–7 × 10^5/ml and 2 × 10^5 cells were suspended in 5 ml of minimal essential medium with 2% dialyzed fetal calf serum containing 1 mCi of [9,10-3H]myristate (25 μCi/ml), or 1 mCi of [9,10-3H]palmitate (50 μCi/ml). Cultures were incubated at room temperature for 4 h (myristate labeling), or 1 h (palmitate labeling) with gentle shaking and then washed 3 times with 20 ml of sterile PBS to remove excess label. Cells were then lysed in 1 ml of radioimmunoprecipitation analysis (RIPA) buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, and 1 mM MgCl2) containing protease inhibitor mixture for use with mammalian cell and tissue cultures. Lysates were divided into 200-μl aliquots, and stored at -20 °C until use. Aliquots of labeled samples were taken for immunoprecipitation using monoclonal antibody 36E6. After a 2-h incubation at 4 °C with agitation, the protein-antibody complex was selectively adsorbed by incubation with 25 μl of protein A plus agarose overnight under the same conditions. The beads were collected by centrifugation at 14,000 × g for 2 min and washed four times with RIPA buffer and once with 50 mM Tris-HCl (pH 7.5). The collected beads were used for SDS-PAGE analysis and fluorography by mixing beads with 100 μl of 2 × SDS-electrophoresis buffer without...
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β-mercaptoethanol (125 mM Tris, 20% glycerol, 6.0% SDS (w/v), and 0.4% (w/v) bromphenol blue) to avoid deacylation of the protein at high temperature and heated at 96 °C for 2 min. Gels were treated with EN3HANCE according to the manufacturer’s instructions, dried, and exposed to blue-sensitive x-ray film at −80 °C for 7–15 days.

Preparation of T. cruzi Lysates—One × 10⁶ epimastigotes were hypotonically lysed by rapid vortexing in 50 μL of lysis solution (10 mM Hepes, pH 7.9, 0.5 mM dithiotheriol, 1 mM EGTA, 0.5 mM NaHCO₃, and protease inhibitor mixture for use with mammalian and tissue culture cells, at a 1:500 dilution) for 15 min at 0 °C. Further disruption was performed by sonicating 3 times for 10 s with 30-s intervals in ice. The whole cell lysates were used directly to determine phospholipase activity.

PI-PLC and IP₃ Assay—A PI-PLC assay adapted from Furuya et al. (10) was used. PI-PLC activity was measured as the release of water-soluble radioactive (2⁻³²P)inositol-labeled PIP₂. Stock solutions of PIP₂, in organic solvents were dried just before using under a stream of nitrogen and suspended in reaction buffer by vortexing for 10 s. Reaction mixtures contained 20 μL of cold PIP₂, 15,000–20,000 cpm of [³²P]PIP₂, 1 mg of sodium deoxycholate/ml, 50 mM Hepes-HCl (pH 7.0), 2.5 mM EGTA, 10 μM CaCl₂, and 5–10 μM of protein in a total volume of 100 μL. Reactions were initiated by the addition of cell lysates, carried out for 5 or 10 min at 30 °C, and terminated by the addition of 0.5 μL of chloroform/methanol/HCl (100:100:6) followed by 0.15 μL of 5 mM EGTA and 1 N HCl. Samples were subjected to vigorous vortexing for 20 s and centrifuged at 21,000 × g for 2 min to separate the organic and aqueous phases. The aqueous phase (0.25 ml) was removed, dissolved in 5 ml of a liquid scintillation fluid, and counted in a scintillation counter. Protein amount was adjusted in each assay so that a linear time course could be obtained during the reaction.

The quantitation procedure of IP₃ in T. cruzi was identical to that used by Furuya (10). IP₃ measurements were made 1 h after acid-induced (pH 5.0) differentiation of trypanosomes. Approximately 8.3 × 10⁶ cells were collected and washed in pH 7.5 medium. A 10-μL aliquot was removed for protein determination and the remainder used for IP₃ measurement using the n-myo-inositol 1,4,5-trisphosphate (IP₃) [³²P]Biotrak System (Amersham Biosciences) following the perchloric acid (50%) and perchlome (50%) extraction procedures from cells.

Statistical Analysis—The data from the antisense experiments were analyzed in two different ways. First, the proportion of amastigotes was compared between the various treatment groups after 22 h. This was accomplished using the Wilcoxon nonparametric rank test (SPSS version 11.5 software, SPSS, Chicago, IL) for dependent samples (23). A p value <0.05 was considered statistically significant. In addition to comparing the proportion of amastigotes between the groups, the numbers of amastigotes and trypanosomes in the different groups were compared. Mantel-Haenszel odds ratios (OR₉₉) were calculated from contingency tables (EpiInfo, CDC) for each pairwise comparison of treatment groups (24). This analysis provides the odds of a parasite being an amastigote (versus a trypanomastigote) in one group compared with the odds of being an amastigote in the second group. 95% confidence intervals were calculated around each OR₉₉. Confidence intervals that did not contain 1.0 (the null value of the OR₉₉) were considered statistically significant. Wilcoxon nonparametric rank test analyses were also used to compare the ability of sense and antisense phosphorothioate oligonucleotides to reduce the expression of TcPI-PLC. A p value <0.05 was considered statistically significant.

For the overexpression experiments, logistic regression was used (SPSS version 11.5) to model the probability that a trypanomastigote would differentiate into an intermediate form or amastigote (25). Each cell was thus coded as 1 (differentiated form) or 0 (trypanomastigote). These models included variables for treatment group and pH (5.0 versus 7.0), which were categorized as indicator variables. In addition, an interaction term was included in the model. Thus, the relationship was modeled as,

\[
\log\left(\frac{p_i}{1-p_i}\right) = \beta_0 + \beta_1 X_{1i} + \beta_2 X_{2i} + \beta_3 X_{1i} X_{2i} \quad (\text{Eq. 1})
\]

where \( p_i \) is the probability that the \( i \)-th cell was differentiated and \( \beta \) is the regression coefficient indicating the effect of \( X \) on the probability of differentiation. Variables included in the model were treatment group (1 if treatment and 0 if control) and pH (1 if pH 5.0 and 0 if pH 7.0). Significance of each variable and model selection were assessed by the Wald statistic and likelihood ratio test in which a p value <0.05 was considered significant. Fit of the model was assessed using the Hosmer and Lemeshow goodness-of-fit test in which a p value >0.1 was considered a good fit. The probabilities of differentiation represented by various combinations of variables were calculated on the basis of the formula,

\[
\frac{\rho_i + \rho X_i + \rho X_{1i} + \rho X_{2i} + \rho X_{1i} X_{2i}}{1 + \rho_i + \rho X_i + \rho X_{1i} + \rho X_{2i} + \rho X_{1i} X_{2i}} \quad (\text{Eq. 2})
\]

by use of the estimated regression coefficients. Corresponding 95% confidence intervals for the probability of differentiation were obtained.

RESULTS

Attempts to Generate a TcPI-PLC Knock-out—Three initial attempts were made to produce a heterozygous knock-out of TcPI-PLC using a construct that contained Neo⁵. After electroporation, parasites were allowed to recover 1–3 days and selection was started with 400 μg/ml G418 thereafter. During the first two attempts, the parasite concentration decreased over a 3-week period until no living parasites could be detected in the 5-ml culture flasks. During the third attempt, efforts were made to reduce the stress of selection. The concentration of G418 was reduced to 50 μg/ml and the culture volume was reduced during the selection period, as the number of live parasites became less numerous. Volumes started at 5 ml but were progressively reduced to 0.5 ml after 3 weeks. Also, medium was replaced more frequently and was done every day or every 2 days during the entire selection procedure. Epimastigotes survived 3 weeks and began to increase in numbers although the medium concentration was increased from 0.5 to 2 ml. The culture continued to grow but at a very slow rate and consisted entirely of epimastigotes with abnormal morphologies. Two distinct morphologies were observed. The first consisted on two epimastigotes that were fused into one long slender form with one flagellum exposed on each end. The parasites appeared to have stopped dividing. This suggested that division was taking place at a very slow rate. The other form was epimastigotes with rounded amastigote-like shape and a thin wire-like flagellum. Both forms were nonmotile.

At approximately 6 weeks after transfection the concentration of cells was still below the number needed to clone by limited dilution or to analyze them by Southern blotting. At this time the parasite numbers began to decrease until all cells died. No changes in the culture conditions were made. Abnormal morphology, slow growth, and cell death under standard culture conditions suggested that the transfected epimastigotes may have exhibited an impaired phenotype after transfection with a knock-out construct for TcPI-PLC. This suggests that TcPI-PLC is an important or essential gene in T. cruzi epimastigotes. The knock-out experiment was repeated twice additional times using a revised knock construct containing a splice acceptor sequence upstream of the drug resistance marker. Again, all transfectants died during the selection process indicating that failure of selection was not because of lack of expression of the drug resistance marker.

Inhibition of Differentiation by Antisense Phosphorothioate-modified Oligonucleotides—We used antisense phosphorothioate-modified oligonucleotides to inhibit the expression of TcPI-PLC in trypanosomes. Antisense oligonucleotides are incorporated into the cytosol of target cells by an unknown mechanism and in some cases are able to suppress gene expression (26). Phosphorothiotate modification is done to increase the half-life of oligonucleotides in solution (27). Phosphorothioate antisense oligonucleotides were designed spanning the 5‘ initiation codon region (nucleotides 61–85) of the TcPI-PLC gene. Sense oligonucleotides of the same length were prepared as controls. Both oligonucleotides were purified by high performance liquid chromatography as described under “Experimental Procedures.” Trypanosomes were subjected to a 5-h pretreatment with 20 μM antisense or sense oligonucleotides in pH 7.5 medium, then stimulated to differentiate by

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transfer to the same medium at pH 5.0 containing the same concentration of oligonucleotides, for 4 h, followed by transfer to medium at pH 7.5 containing oligonucleotides, for 18 h. The Control pH 5.0 was treated in the same manner without oligonucleotides present, and the Control pH 7.5 remained at pH 7.5 for the entire 22 h. Twenty-two hours after the initiation of differentiation, parasites were collected, fixed, and the percentage of trypomastigotes and amastigotes was determined by microscope counting. Although the percent of trypomastigotes at time 0 before treatment was greater than 90% for all groups (Fig. 1, panels A–D), after 22 h the proportion of trypomastigotes to amastigotes was visibly different with a larger proportion of amastigotes in the Sense group (asterisk) was significantly different than both the Sense and Control pH 5.0 groups (p = 0.043). The Sense and Control pH 5.0 groups were not significantly different (p > 0.1). The proportion of amastigotes in the Control pH 7.5 group was significantly lower than in all of the other groups (p = 0.043). The analysis using ORMH demonstrated a similar relationship. The Sense group had significantly more amastigotes than the Antisense group, with an ORMH of 4.48 (95% confidence interval 3.94–5.44). Thus, a trypomastigote was almost 4.5 times more likely to differentiate into an amastigote in the Sense group than in the Antisense group.

To confirm that antisense treatment caused a reduction in TcPI-PLC expression, real-time quantitative PCR was performed to measure the relative levels of TcPI-PLC mRNA in comparison to the reference gene TcF0 (Fig. 3). Two hours after starting acid-induced differentiation, total RNA was extracted from $1 \times 10^7$ parasites and used for cDNA synthesis followed by real-time PCR. The results of three experiments are shown. The relative expression level of the Antisense group (asterisks) was significantly lower than both Sense and Control pH 5 groups in every experiment (p < 0.043; n = 5).

FIG. 1. Effect of antisense phosphorothioate oligonucleotides on T. cruzi morphology. The percent trypomastigotes at time 0 was greater than 90% for all groups as observed by bright field microscopy (panels A–D). Twenty-two h after the initiation of differentiation the proportion of trypomastigotes to amastigotes was visibly different with a larger proportion of amastigotes in the Sense group (asterisk) and Control pH 5 (G) and an accumulation of intermediate forms (circled) in the Antisense (E) and Control pH 7.5 (H) groups. Bar = 10 µm.

FIG. 2. Antisense phosphorothioate oligonucleotides inhibits T. cruzi differentiation. A total of 5 antisense phosphorothioate oligonucleotide inhibition assays were performed. The combined mean of the percent of amastigotes in each of the treatment groups 22 h after acid-induced differentiation is shown. Error bars represent the S.E. (n = 5). The Antisense group (asterisk) was significantly different than both the Sense and Control pH 5.0 groups (p = 0.043).

FIG. 3. Real-time quantitative PCR confirms a reduction in TcPI-PLC expression upon antisense phosphorothioate oligonucleotide treatment of trypomastigotes. Two hours after starting acid-induced differentiation, total RNA was extracted from $1 \times 10^7$ parasites and used for cDNA synthesis followed by real-time PCR. The results of three experiments are shown. The relative expression level of the Antisense group (asterisks) was significantly lower than both Sense and Control pH 5 groups in every experiment (p < 0.043; n = 5).

FIG. 4. Effect of TcPI-PLC overexpression on T. cruzi morphology and Northern blot analysis of TcPI-PLC. Wild-type trypomastigotes (A–C) or trypomastigotes overexpressing TcPI-PLC (D–F) were examined at time 0 (A and D) or after 1 h at pH 7.5 (B and E) or pH 5.0 (C and F). After 1 h the proportion of trypomastigotes in the TcPI-PLC overexpressing group was greatly reduced to a 12–14% range with accumulation of intermediate forms (F). An increase in intermediate forms is also observed at pH 7.5 (E). The inset in A shows a Northern blot of TcPI-PLC from overexpressing and Wild-type trypomastigotes at time 0 and confirms that TcPI-PLC is being expressed at higher levels in the TcPI-PLC overexpressing group. Bar = 10 µm.
and given the expression level of 1. For all experiments the relative level of TcPI-PLC expression was not statistically different for Sense and Control pH 5.0 groups. In contrast, the relative expression level of the Antisense group was significantly lower than both Sense and Control pH 5 groups in every experiment (Fig. 3, asterisks). In fact, the relative expression in the Antisense group was reduced to levels that were greater than 70% less than in the Control pH 5.0 (Fig. 3, experiment 1) and similar to the Control pH 7.5 (Fig. 3, experiments 2 and 3). Error bars represent the standard deviation of relative gene expression for each experiment. These results confirm that antisense treatment reduces the expression level of TcPI-PLC, whereas sense treatment has little effect.

**Stimulation of Differentiation by Overexpression of TcPI-PLC**—To determine whether overexpression of TcPI-PLC has an effect on the differentiation of trypomastigotes to amastigotes, trypomastigotes obtained by differentiation of epimastigotes (17) transfected with TcPI-PLC inserted in the expression vector pTEX were obtained from myoblast cultures and induced to differentiate to amastigotes using acid treatment in 3 different experiments (Fig. 4). The time of infection of myoblasts, and the harvesting of trypomastigotes were synchronized to collect trypomastigotes as soon as they were released from the host cells. Whenever possible parasite cultures containing greater than 90% trypomastigotes were used and differential centrifugation was utilized to increase the proportion of trypomastigotes. However, even after purification by centrifugation the percentage of trypomastigotes in the TcPI-PLC overexpressing group was less than 90% for three experiments and ranged from 86.5 to 89.2% at time 0 (Fig. 4, panel D). G418 was not added to these cultures because its addition considerably decreased the proportion of trypomastigotes released (data not shown).

Trypomastigotes were exposed to pH 5.0 medium and then collected for counting after 1 h. Control groups of Wild-type and TcPI-PLC overexpressing cells remained in pH 7.5 medium. After 1 h the proportion of trypomastigotes in the TcPI-PLC overexpressing group was greatly reduced from the 80% range to a 12–14% range with the accumulation of intermediate forms (Fig. 4, panel F). In contrast, the Wild-type pH 5.0-treated group changed from the 90% range to a 70–80% range. In the Control pH 7.5 groups, the trypomastigote proportion remained stable in the Wild-type group at the 90% range (Fig. 4, panel B), although the TcPI-PLC overexpressing group changed from the 80% range to a 50–70% range (Fig. 4, panel E). These results suggested that overexpression of TcPI-PLC during acid treatment increased the rate of differentiation of trypomastigotes to amastigotes and that even without acid treatment TcPI-PLC overexpression was inducing trypomastigotes to differentiate at a faster rate. The induction of differentiation without acid treatment reduced the number of trypomastigotes obtained at time 0. The inset in Fig. 4A shows a Northern blot of TcPI-PLC from overexpressing and Wild-type trypomastigotes at time 0 and confirms that TcPI-PLC was being expressed at higher levels in the TcPI-PLC overexpressing group. Statistical analysis of the results of the three experiments, as described under “Experimental Procedures,” is shown in Fig. 5. Fig. 5A, for example, shows the probability of differentiation in the Wild-type and TcPI-PLC overexpressing groups for pH 5 and 7.5. The probability of differentiation is about three times greater in the presence of acid (8 versus 25% for the Wild-type group and 32 versus 88% in the TcPI-PLC overexpressing group). Fig. 5A also shows that the differentiation of the TcPI-PLC overexpressing line is significantly higher than that of the Wild-type line at both pH levels. This demonstrates that the TcPI-PLC overexpressing group has a significantly higher probability of differentiation than the Wild-type group. Even more interesting is that the slopes of the two lines are not parallel. This is the influence of the interaction term. TcPI-PLC overexpression and pH 5.0 are acting synergistically, and the result is an even greater probability by either effect alone (interaction). In other words, the presence of the acid is affecting the probability of differentiation more for the TcPI-PLC overexpressing group than for the Wild-type group.
amastin TcPI-PLC overexpressing showing that expression of proteins from the membrane is likely necessary to induce were treated with pH 5.0 medium for 1 h followed by IP3 detection as acid (pH 5.0)-induced differentiation of bloodstream trypomastigotes to els in the after acid-induced differentiation of trypomastigotes.

level of expression in the indicated under “Experimental Procedures.” The cellular content of IP3 expression of stage-specific proteins, real-time quantitative PCR was performed to measure the relative levels of amastin expression was not statistically different from that of homogenates of wild-type cells grown in medium without G418. In two experiments the activity was 67 ± 2.5% higher in TcPI-PLC overexpressing cells than in wild-type cells. In agreement with these results, overexpression of TcPI-PLC led to increased turnover of the phosphatidylinositol 4,5-bisphosphate substrate. This was demonstrated by the more than 5-fold increase in the concentration of IP3 after 1 h of acid-induced differentiation (Fig. 6B).

TcPI-PLC Needs to Be Overexpressed in the Plasma Membrane to Stimulate Trypomastigote to Amastigote Transformation—TcPI-PLC has been shown to be lipid modified at its N terminus and localized to the plasma membrane of amastigotes (10). We therefore investigated whether this lipid modification was important for plasma membrane localization of TcPI-PLC. Gene fusions coding the full-length protein or the full-length protein with a mutation of the N-terminal glycine to contrast, the relative expression level of amastin expression and given the gene expression for each experiment. These results, together with previous localization studies using fluorescent antibody staining against TcPI-PLC (10). In summary, these results establish that, when expressed in sufficient amounts, TcPI-

Morphological changes that occur during trypomastigote to amastigote transformation, both in vivo and in vitro, are associated with the expression of stage-specific proteins (15). To relate the morphological changes described above with the expression of stage-specific proteins, real-time quantitative PCR was performed to measure the relative levels of amastin (an amastigote-specific protein (21, 28)) mRNA in comparison to the reference gene TcP0 (Fig. 6A). The results of two experiments are shown with the Wild-type group (Fig. 6A, gray bars) used as the standard level of amastin expression and given the expression level of 1. For both experiments the relative level of amastin expression was not statistically different from that of cells transfected with GFP alone or TcPI-PLC G2A-GFP (full-length protein with a mutation of the N-terminal glycine to alanine, the site for N-myristoylation (10), and see below). In contrast, the relative expression level of TcPI-PLC overexpressing cells (white bars) was significantly higher than in the other groups. Error bars represent the standard deviation of relative gene expression for each experiment. These results, together with the real-time quantitative PCR studies reported before (Fig. 3) showing decreased expression of TcPI-PLC (another specific marker of amastigotes, Ref. 21) after antisense treatment, indicate that the morphological characteristics of amastigotes are correlated with the expression of amastigote-specific markers.

To confirm that overexpressed TcPI-PLC was properly folded and active we measured the PLC activity of homogenates from 10^8 mid-log phase epimastigotes overexpressing TcPI-PLC and grown in medium containing 400 μg/ml G418, and compared it to that of homogenates of wild-type cells grown in medium without G418. In two experiments the activity was 67 ± 2.5% higher in TcPI-PLC overexpressing cells than in wild-type cells. In agreement with these results, overexpression of TcPI-PLC led to increased turnover of the phosphatidylinositol 4,5-bisphosphate substrate. This was demonstrated by the more than 5-fold increase in the concentration of IP3 after 1 h of acid-induced differentiation (Fig. 6B).

The full-length TcPI-PLC/GFP fusion protein localized to the plasma membrane (Fig. 7A, panel a) as evidenced by a fluorescent border around the cells. The G2A mutated TcPI-PLC/GFP (Fig. 7A, panel d) and the GFP positive control (data not shown) localized to the cytosol, whereas no fluorescence was detected in cells that were not transfected (data not shown). Although we were previously unable to detect the plasma membrane localization of TcPI-PLC in epimastigotes using antibodies (10), this difference could be attributed to overexpression of the fusion proteins in the present work, and to the low expression of TcPI-PLC in the log phase epimastigotes used in the previous study (10).

We then investigated whether the plasma membrane localization of the full-length fusion protein was maintained in trypomastigote and amastigote stages. Metacyclic trypomastigotes were obtained from stationary phase cultures of epimastigotes by complement lysis selection (17). Tissue culture trypomastigotes were generated by infection of LbE9 myoblasts with metacyclic trypomastigotes to establish the intracellular cycle, and were maintained by weekly passage in the presence of 400 μg/ml G418. Tissue culture-derived trypomastigotes were fixed in 4% formaldehyde solution, smeared on polylysine slides, and observed by fluorescence microscopy. Trypomastigotes transfected with GFP alone (data not shown) or with the G2A mutant displayed cytosolic localization (Fig. 7A, panel e). The full-length TcPI-PLC/GFP localized to the plasma membrane (Fig. 7A, panel b). Trypomastigotes grown in myoblast cultures containing G418 were maintained another 4–5 days until amastigotes were obtained. Parasites were collected for viewing by fluorescence microscopy. Full-length TcPI-PLC/ GFP (Fig. 7A, panel c) was targeted to the plasma membrane as evidenced by a ring around the cell. Cells transfected with GFP alone (data not shown) or the G2A mutant (Fig. 7A, panel f) displayed cytosolic localization. These results are similar to localization in trypomastigotes and they are also in agreement with previous localization studies using fluorescent antibody staining against TcPI-PLC (10).
PLC localized to the plasma membrane of all three developmental stages of T. cruzi.

To confirm the proper acylation of the full-length TcPI-PLC/FP fusion protein, transfectants expressing this protein, the mutated protein (G2A), or GFP alone were metabolically labeled with either [3H]myristate or [3H]palmitate (Fig. 7B) and immunoprecipitates were analyzed by SDS-PAGE and fluorography. Endogenous TcPI-PLC (all lanes) was both myristoylated and palmitoylated, as was the full-length TcPI-PLC/GFP, which is slightly larger because of the GFP tag (arrowheads). G2A was neither myristoylated nor palmitoylated, indicating that glycine is required for these two modifications. It has been described before that the palmitoylation step requires the presence of a nearby myristoyl (22, 29, 30). For the full-length TcPI-PLC/GFP transfectants, gels were treated with buffer containing or lacking hydroxylamine to confirm that the labeled protein was indeed palmitoylated in a cysteine (10). The [3H]palmitate (Fig. 7C, lane 1) or [3H]myristate (Fig. 7C, lane 2) labeled immunoprecipitated TcPI-PLC/GFP from epimastigotes were separated by SDS-PAGE and the gels were incubated in 1 M hydroxylamine at neutral pH (7.5). Under these conditions S-ester but not O-ester linkages are broken (10, 31). As shown in Fig. 7C, lane 1, the radioactivity incorporated from [3H]palmitate on TcPI-PLC/GFP was greatly reduced by neutral hydroxylamine treatment, although the radioactivity incorporated from [3H]myristate (Fig. 7C, lane 2) remained on the protein after treatment. These results strongly suggest that [3H]myristate is linked to TcPI-PLC/GFP by an amide bond (Fig. 5D). Taken together, these results established that only overexpression of TcPI-PLC in the plasma membrane is able to stimulate differentiation of trypomastigotes to amastigotes.

**DISCUSSION**

*T. cruzi* trypomastigotes can invade a wide variety of host cells by a process of parasite-directed endocytosis (32). Although *T. cruzi* internalization and formation of a parasitophorous vacuole is not strictly dependent upon lysosome fusion with the plasma membrane (33), as previously suggested (34, 35), lysosomes rapidly fuse with the parasitophorous vacuole making it acidic (32). Acidification is apparently the trigger for differentiation of trypomastigotes into amastigotes because low pH also accelerates their extracellular transformation (15, 36). Acidification also induces the expression of TcPI-PLC in vitro (10). Because differentiation of trypomastigotes into amastigotes in vitro was accompanied by an increase in IP3 formation, a role for TcPI-PLC in this process was suggested (10). The involvement of the inositol phosphate/diacylglycerol pathway and of phosphorylation cascades in this transformation was also suggested by its stimulation by very low concentrations (1 mM) of the protein phosphatase inhibitor calyculin A (11).

In this report we provide genetic evidence that TcPI-PLC is important for differentiation of trypomastigotes into amastigotes. Two complementary approaches were used to alter TcPI-PLC expression in *T. cruzi*: RNA oligonucleotides designed to target the TcPI-PLC sequence, and overexpression of the TcPI-PLC gene.

Although antisense RNA oligonucleotides have been used extensively to reduce the expression of target genes in mammalian cells (26, 27), their use in trypanosomatids has been limited to the targeting of the mini-exon sequence in *Leishmania amazonensis* as a form of chemotherapy (37), and to reduce the expression of a surface glycoprotein in *T. cruzi* (38). Our results demonstrate that their use to reduce the expression of functional enzymes in *T. cruzi* is feasible and that the technique could therefore have wider application when other techniques, such as gene targeting disruption, are not feasible. The
advantage of the phosphorothioate antisense oligonucleotides is that they can be tested directly on trypanamastigotes and the differentiation step from epimastigotes is not needed as in gene targeting disruption experiments (17). Inhibition of gene expression by RNA oligonucleotides targeted against TcPI-PLC, as verified by real-time quantitative PCR experiments (Fig. 3), resulted in a significant inhibition of trypanamastigote to amastigote differentiation (Figs. 1 and 2).

Overexpression of genes has been used before in T. cruzi to give insight into the function of genes. Overexpression of superoxide dismutase resulted in increased sensitivity to the trypanocidal agents crystal violet and benzidazole (39), although overexpression of cruzipain, the major cysteine proteinase of T. cruzi, was associated with enhanced metacyclogenesis (40). We found that overexpression of TcPI-PLC resulted in an increased rate of differentiation of trypanamastigotes into amastigotes (Figs. 4 and 5).

Our results using GFP fusion proteins (Fig. 6) indicate that, when expressed in sufficient amounts, TcPI-PLC is localized in the plasma membrane of the three different stages of T. cruzi, lipid modification is necessary for this localization and overexpression of TcPI-PLC stimulates differentiation only when the enzyme localizes to the plasma membrane. In this regard, it has been reported that exogenous bacterial PI-PLC treatment induces differentiation of trypanamastigote forms (41), thus suggesting a critical role of phospholipid hydrolysis in the plasma membrane to trigger this process.

In summary, our results demonstrate an important role of TcPI-PLC in the differentiation of trypanamastigotes to amastigotes of T. cruzi, an essential step for the intracellular replication of this parasite, and provide evidence of the utility of antisense RNA oligonucleotides and overexpression techniques in the study of the functional role of genes in T. cruzi.

Acknowledgments—We thank David Engman (Northwestern University, North Chicago) for vector pTEX, Stephen Beverley (Washington University, St. Louis) for plasmid pXG-GFP+2’, Mark Kuhlenschmidt for the use of the Nikon microscope, and Linda Brown for technical assistance.

REFERENCES

1. Irvine, R. (1996) Nature 380, 582–583
2. Nishizuka, Y. (1988) Nature 334, 661–665
3. Berridge, M. J. (1987) Annu. Rev. Biochem. 56, 159–193
4. Docampo, R., and Pignataro, O. P. (1991) Biochem. J. 275, 407–411
5. Gomez, M. L., Erijman, L., Araujo, S., Torres, H. N., and Telles-Iníon, M. T. (1989) Mol. Biochem. Parasitol. 36, 101–108
6. Gomez, M. L., Ochatt, C. M., Kazanietz, M. G., Torres, H. N., and Telles-Iníon, M. T. (1999) Int. J. Parasitol. 29, 981–989
7. Moreno, S. N. J., Vercesi, A. E., Pignataro, O. P., and Docampo, R. (1992) Mol. Biochem. Parasitol. 52, 251–262
8. Docampo, R., Moreno, S. N. J., and Vercesi, A. E. (1993) Mol. Biochem. Parasitol. 59, 305–314
9. Nozaki, T., Toh-e, A., Fujii, M., Yagisawa, H., Nakazawa, M., Takeuchi, T. (1999) Mol. Biochem. Parasitol. 102, 283–295
10. Furuya, T., Kashiwab, C., Docampo, R., and Moreno, S. N. J. (2000) J. Biol. Chem. 275, 6428–6438
11. Grellier, P., Blum, J., Santana, J., Byelen, E., Mourgery, E., Sinou, V., Teixeira, A. R., and Schrevel, J. (1999) Mol. Biochem. Parasitol. 98, 239–252
12. Gonzalez, J., Cornejo, A., Santos, M. R. M., Cordero, E. M., Gutierrez, B., Porcel, F., Martora, R. A., Sagua, H., Da Silveira, J. F., and Araya, J. E. (2000) Biochem. J. 374, 855–860
13. Schmata, D. M., and Murray, P. K. (1982) Parasitology 85, 115–125
14. Bone, G. J., and Steinert, M. (1956) Nature 178, 308–309
15. Tomlinson, S., VanDekerek, F., Prevert, U., and Nussenzweig, V. (1995) Parasitology 110, 547–554
16. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Mol. Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
17. Caler, E. V., de Avalos, S. V., Haynes, P. A., Andrews, N. W., and Burleigh, B. A. (1998) EMBO J. 17, 4975–4986
18. Kendall, G., Wilderspin, A. F., Ashall, P., Miles, M. A., and Kelly, J. M. (1990) EMBO J. 9, 2751–2758
19. Cooper, R., Ribeiro de Jesus, A., and Cross, G. A. M. (1993) J. Cell Biol. 122, 149–156
20. Kelly, J. M., Ward, H. M., Miles, M. A., and Kendall, G. (1992) Nucleic Acids Res. 20, 3963–3969
21. Minning, T. A., Bua, J., Garcia, G. A., McGraw, R. A., and Tarleton, R. L. (2003) Mol. Biochem. Parasitol. 131, 55–64
22. Godel, L. M., and Engman, D. M. (1999) EMBO J. 18, 2057–2065
23. Zar, J. H. (1984) in Biostatistical Analysis, 2nd Ed., pp. 718, Prentice Hall, Englewood Cliffs, NJ
24. Rothman, K. J. (1986) Ann. Int. Med. 105, 445–447
25. Homs, D. W., Taber, S., and Lemeshov, S. (1991) Am. J. Public Health 81, 1630–1635
26. Takeuchi, K., Sato, N., Kasahara, H., Funayama, N., Nagafuchi, A., Yonemana, S., Tsukita, S., and Tsukita, S. (1994) J. Cell Biol. 125, 1371–1384
27. Akhtar, S., Kole, R., and Juliano, R. L. (1994) J. Biol. Chem. 269, 16280–16288
28. Tardieux, I., Webster, P., Ravesloot, J., Boron, W., Lunn, J. A., Heuser, J. E., and Donelson, J. E. (1994) J. Biol. Chem. 269, 20509–20516
29. Hallak, H., Brass, L. F., and Manning, D. R. (1994) J. Biol. Chem. 269, 4571–4576
30. Munsky, S. M., Kleuss, C., and Gilman, A. G. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 2800–2804
31. Bizzozero, O. A. (1995) Methods Enzymol. 250, 361–379
32. Hall, B. F. (1993) Seminars Cell Biol. 4, 323–333
33. Woolsey, A. M., Sunwoo, L., Petersen, C., Brachmann, S. M., Cantley, L. C., and Burleigh, B. A. (2003) J. Cell Sci. 116, 3611–3622
34. Tardieux, I., Webster, P., Ravesloot, J., Boren, W., Lunn, J. A., Heuser, J. E., and Andrews, N. W. (1992) Cell 71, 1117–1130
35. Burleigh, B. A., and Andrews, N. W. (1995) Annu. Rev. Microbiol. 49, 175–200
36. Kanbara, H., Uemura, H., Nakazawa, S., and Kukama, T. (1990) Jpn. J. Parasitol. 39, 226–228
37. Ramazizelles, C., Mishra, R. K., Moreau, S., Pascolo, E., and Toumine, J. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7859–7863
38. Malagà, S., and Yoshida, N. (2001) Infect. Immun. 69, 353–359
39. Temperton, N. J., Wilkinson, S. R., Meyer, D. J., and Kelly, J. M. (1998) Mol. Biochem. Parasitol. 96, 167–176
40. Tomas, A. M., Miles, M. A., and Kelly, J. M. (1997) Eur. J. Biochem. 244, 457–465
41. Mortara, R. A., Minelli, L. M., Vanderkolkthove, F., Nussenzweig, V., and Ramalho-Pinto, F. J. (2001) J. Eukaryot. Microbiol. 48, 27–37