The phosphoinositide (PI)-specific phospholipase C gene (TcPI-PLC) of the protozoan parasite *Trypanosoma cruzi* was cloned, sequenced, expressed in *Escherichia coli*, and the product protein (TcPI-PLC) was shown to have enzymatic characteristics similar to those of mammalian δ-type PI-PLCs. The TcPI-PLC gene is expressed at high levels in the epimastigote and amastigote stages of the parasite, and its expression is induced during the differentiation of trypomastigotes into amastigotes, where TcPI-PLC associates with the plasma membrane and increases its catalytic activity. In contrast to other PI-PLCs described so far, the deduced amino acid sequence of TcPI-PLC revealed some unique features such as an N-myristoylation consensus sequence at its amino-terminal end, lack of an apparent pleckstrin homology domain and a highly charged linker region between the catalytic X and Y domains. TcPI-PLC is lipid modified in vivo, as demonstrated by metabolic labeling with [3H]myristate and [3H]palmitate and fatty acid analysis of the immunoprecipitated protein, and may constitute the first example of a new group of PI-PLCs.

Many pathogenic parasites have developed the ability to live in two distinct hosts, one vertebrate and the other invertebrate. Such parasites include *Trypanosoma cruzi*, the etiologic agent of Chagas’ disease or American trypanosomiasis. During its life cycle, *T. cruzi* has to adapt to environments of different temperature, osmolarity, ionic composition, and pH, and some of these adaptation processes are paralleled by morphological and temperature, osmolarity, ionic composition, and pH, and some of these adaptation processes are paralleled by morphological and functional changes. Very little is known about the signaling mechanisms involved in these processes.

Phosphoinositide-specific phospholipases C (PI-PLCs) catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) to d-myo-inositol-1,4,5-trisphosphate (IP3) and sn-1,2-diacylglycerol (DAG) (1, 2). Both products of this reaction function as second messengers in eukaryotic signal transduction cascades. The soluble IP3 triggers release of calcium from intracellular stores (1). The membrane-resident DAG controls cellular protein phosphorylation states by activating various protein kinase C isoforms (2). Three classes of mammalian PI-PLCs with 10 different isoforms have been characterized (β1-β4, γ1-γ2, and δ1-δ4) (3). The activity of β- and γ-isozymes (145–150 kDa) is regulated by G protein-coupled and tyrosine kinase-linked receptors, respectively. These isoforms are related to the much smaller δ-isozymes (~85 kDa) (3). It seems very likely that PI-PLC-δ evolved first, because every PI-PLC cloned so far from a non-mammalian species (for example, *Dictostelium*, yeast, higher plants, and *Chlamydomonas*) is clearly a δ-isoform (3). It is currently not known how δ-isozymes are regulated in vivo (4). It is possible that they are regulated only by calcium ions (3) although the idea of PI-PLC-δ being regulated by GTP-binding proteins is one which has increasing support (3, 5). Results from several laboratories (6–8) have suggested that, at least in yeasts, PI-PLC-δ is required for a number of nutritional and stress-related responses. It has also been postulated that PI-PLC-δ could have a role in differentiation of *Dictostelium discoideum* (9). Transcription of this PI-PLC-δ appears to be enhanced during cell aggregation, it decreases during slug formation, and increases in the culminating fruit body (9). *D. discoideum* PI-PLC-δ is G protein-coupled (10).

The understanding of factors controlling phospholipid metabolism in parasitic protozoa is very poor, although these lipids could be involved in several important events in these eukaryotic cells. The presence and operation of the inositol phosphatase/diacylglycerol signaling pathway was demonstrated in epimastigotes of *T. cruzi* (11). IP3 and DAG formation was stimulated by Ca2+ in digitonin-permeabilized cells, thus suggesting the presence of a PI-PLC (11). The presence of different inositol phosphates in amastigotes (12) and trypomastigotes (13) was reported later. A shift in the levels of phosphoinositide metabolites after incubation of epimastigotes with carbamoylcholine (14) and the stimulation of IP3 and DAG production and epimastigote proliferation by fetal calf serum (15) were also reported. A PI-PLC activity was also detected in epimastigote lysates using PI as substrate (15). A synthetic peptide corresponding to a chicken α2-globin fragment was able to increase inositol monophosphate (IP1) and IP3 levels in epimastigotes (16) and stimulate transformation of epimastigotes into trypomastigotes (metacyclogenesis) (17). A protein kinase C was also characterized in *T. cruzi* epimastigotes (18). This enzyme requires phosphatidylserine and Ca2+ for activity and is stimulated by DAG (18).
In this work we report the cloning, sequencing, and expression of a gene (TcPI-PLC) encoding a PI-PLC from T. cruzi. The gene is expressed at high levels in the epimastigote and amastigote stages, and its expression is induced during the differentiation of trypanosomatids into amastigotes, where its product (TcPI-PLC) is associated with the plasma membrane and increases its activity. In contrast to other PI-PLCs, T. cruzi PI-PLC is lipid modified in vivo, and has a highly charged linker region between the catalytic X and Y domains.

**EXPERIMENTAL PROCEEDURES**

**Culture Methods**—T. cruzi amastigotes and trypanosomatids (Y strain) were obtained from the culture medium of L_E$_6$ myoblasts by a modification of the method of Schmatz and Murray (19) as we have described before (12, 20). The contamination of trypanosomatids with amastigotes and intermediate forms or of amastigotes with trypanosomatids or intermediate forms was always less than 5% unless otherwise stated. T. cruzi epimastigotes (Y strain) were grown at 28 °C in liver infusion tryptose medium (21) supplemented with 10% newborn calf serum. Protein concentration was determined using the Bio-Rad protein assay. Trypanosomatids were induced to transform into amastigotes by a modification of the method described before (12, 20). Brieﬂy, trypanosomatids or amastigotes axenically as described previously (22). For Southern blotting DNA was electrophoresed in 1.0% agarose gels with 2.2 M formaldehyde. RNA was obtained using a PolyATract mRNA isolation system. The polyadenylation of RNA was performed by using reverse transcriptase, total RNA, and oligo(dT) as a primer. The cDNA was amplified by PCR using primers corresponding to the T. cruzi splice leader sequence (5′-ATAGAACAC- TTTCTGTC-3′) and the product was puriﬁed and cloned into pCR2.1-TOPO. DNA probes were prepared from linearized double-stranded plasmids and the protein assay was performed by using the NEN Life Science Products Inc., Boston, MA. PCR 2.1-TOTO cloning kit was from Promega, Madison, WI. The Zeta Probe GT nylon membranes and the protein assay were from Zeta Probe GT nylon membranes and the protein assay were from Superco, Bellefonte, PA. All other reagents were analytical grade.

**Nucleic Acid Analysis**—All basic recombinant techniques followed standard procedures described previously (23) unless otherwise noted. For Southern blotting DNA was electrophoresed in 1.0% agarose with TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0) buffer and transferred to Zeta Probe GT nylon membranes. DNA was isolated by standard procedures (23). Total RNA was isolated with Trizol reagent following the manufacturer’s recommendations. The polyadenylated RNA was obtained using a PolyATract mRNA isolation system. RNA was electrophoresed in 1.0% agarose gels with 2.2 M formaldehyde, 20 mM Mops (pH 7.0), 8 mM sodium acetate, 1 mM EDTA and transferred to Zeta Probe GT nylon membranes. DNA probes were prepared using random hexanucleotide primers and Klenow fragment of DNA polymerase I (Prime-a-Gene Labeling System) and [α-32P]dCTP. RNA probes were prepared from linearized double-stranded plasmids as described above except the 3′-end of the transcripts was ﬁlled in with BstEII I site followed by T7 RNA polymerase (RiboProbe in vitro Transcription System) and digoxigenin (DIG System). The primers were prepared from Genosys Biotechnologies Inc., Woodlands, TX. The Escherichia coli expression vector pET28c, the Quick 900 cartridge, and the His-bind buffer kit were from Novagen, Madison, WI. The Protease Inhibitor Mixture Set III was from Calbiochem, La Jolla, CA. Protein A/G PLUS-agarose was from Santa Cruz Biotechnology, Santa Cruz, CA. Zeta Probe GT nylon membranes and the protein assay were from Promega, Madison, WI. The Protease Inhibitor Mixture Set III was from Calbiochem, La Jolla, CA. Protein A/G PLUS-agarose was from Santa Cruz Biotechnology, Santa Cruz, CA. BFG-methanol was from Superco, Bellefonte, PA. All other reagents were analytical grade.

**PI-PLC Assay**—PI-PLC activity was measured as the release of water-soluble radioactivity from [3H]inositol-labeled PI or PIP$_2$ by a procedure described before (6) with minor modifications. Brieﬂy, stock solutions containing either PI or PIP$_2$ in organic solvent were dried just prior to use under a stream of nitrogen and suspended in reaction buffer by sonication for 10 s in a Branson digital soniﬁer. Reaction mixtures contained cold PI or PIP$_2$ at various concentrations, 15,000 to 20,000 cpm of [3H]PI or [3H]PIP$_2$, 1 mg of soybean phosphatidylethanolamine/ml, 1 mM NaCl, 20 mM Tris-HCl (pH 7.9). The transparent PI-PLC was transferred into dialysis tubes (M$_r$ cut off 3,500) and concentrated by incubating the tube in polyethylene glycol (M$_r$ 15,000–20,000) powder at 4 °C until the volume was one-tenth of the original. The proteins were analyzed by SDS-polyacrylamide gel electrophoresis and silver staining (Bio-Rad).

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the addition of 0.5 ml of chloroform-methanol-HCl (100:100:0.6) followed by 0.15 ml of 5 mM EGTA in 1 N HCl. Samples were subjected to vigorous vortex mixing for 30 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. Enzyme activity was assayed twice and adjusted in each assay so that a linear time course could be obtained during the 10-min reaction.

Production of Polyclonal Antibody against TcPI-PLC—A polyclonal antibody against TcPI-PLC was generated using the E. coli-expressed recombinant protein. For this purpose, the inclusion bodies in the pellet were mixed with an equal amount of non-reducing T. cruzi different stages of the medium was used as described by Laemmli (29). Aliquots of the supernatant was adjusted to pH 7.5 (DMEM containing 0.4% fatty acid-free BSA and 20 mM Hepes). The cells were labeled for 16 h at 35 °C and harvested for immunoprecipitation. Epimastigotes were metabolically labeled by incubating the cells in MEM with the same radiochemicals as above with 20 μM myristate (3H) or palmitate (3H). After overnight incubation, the protein-antibody complex was selectively adsorbed by incubation with Protein A/G Plus-agarose for 1 h. 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 mixed with an equal volume of 2 × SDS electrophoresis buffer (125 mM Tris-HCl, 62.5 mM sodium dodecyl sulfate, 10% glycerol, 1.5% sodium dodecyl sulfate, 5% β-mercaptoethanol, and 0.05% bromphenol blue) and boiled for 3 min prior to application to SDS-polyacrylamide gels. The antibodies were eluted in 0.2 M glycine (pH 2.8), 1 mM EDTA. The suspension was centrifuged at 90 °C for 2 min. The resulting supernatant was adjusted to a volume of 10 μl of 1% SDS buffer containing 0.1 volume of 1 M Tris-HCl (pH 9.5), supplemented with sodium azide to a final concentration of 0.05% and stored at 4 °C.

SDS Electrophoresis and Western Blotting—The electrophoretic system was essentially as described by Laemmli (30). Briefly, cultures from different stages of T. cruzi (at the protein concentration indicated under “Results”) were mixed with an equal amount of non-reducing T. cruzi different stages of treatment. The cellular content of IP3 was determined with a competitive binding protein assay. This assay was performed on acid-treated (pH 5.0) and control (pH 7.5) trypomastigotes at different incubation times. Aliquots of the parasite suspensions were removed, centrifuged (2,000 × g for 10 min), and washed once in DMEM (pH 7.5). The cells were resuspended at a concentration of 8.3 × 10^5 cells/ml in the same buffer. An aliquot was removed for protein determination followed immediately by addition of ice-cold 20% perchloric acid to a final concentration of 4% HClO4. The lysate was neutralized with KOH, and the precipitate was centrifuged. The supernatants were removed, centrifuged (2,000 × g for 10 min), and washed once in DMEM (pH 7.5). The cells were resuspended at a concentration of 8.3 × 10^5 cells/ml in the same buffer. An aliquot was removed for protein determination followed immediately by addition of ice-cold 20% perchloric acid to a final concentration of 4% HClO4. The lysate was neutralized with KOH, and the supernatant was assayed for IP3 by a competitive binding protein assay as described in the manufacturer’s instructions.

Identification of Fatty Acids—Deacylation of the immunoprecipitated TcPI-PLC was carried out as described previously (32). A complex of [3H]myristate-labeled TcPI-PLC (from 1 × 10^6 cells), was carried out with polyclonal antibody against TcPI-PLC, which was in turn bound to protein A/G-conjugated agarose beads as described above. This complex was resuspended in 100 μl of 2 × SDS-PAGE buffer and heated at 90 °C for 2 min. The resulting supernatant was adjusted to a volume of 750 μl by adding water. A similar complex of [3H]palmitoleic acid-labeled TcPI-PLC, anti-TcPLC, and agarose beads obtained as described above was resuspended in 250 μl of 1 M NH4OH (pH 7.0), and incubated for 20 min at room temperature, followed by centrifugation at 14,000 × g for 1 min and withdrawal of the supernatants. Two more hydroxylamine treatments were performed and the eluates were pooled. Fifty-four μl of concentrated HCl was added to both [3H]-acylated protein preparations to acidify the solutions. Fatty acids released from both [3H]-acylated preparations were recovered into hexane by three extractions, each with 500 μl of the solvent. The hexane phases for each sample were pooled, back-extracted twice with 700 μl of 10 mM HCl, and dried under nitrogen gas. Methyl ester derivatives of the fatty acids were generated by resuspending the dried fatty acids in 200 μl of BF3-methanol and heating for 2 min at 100 °C. Two-hundred μl of 5 M H2SO4 was added to the methyl ester solutions, and water was added to 400 μl (FAMES) extracted three times, each time with 200 μl of toluene. Toluene extracts were combined, dried under nitrogen gas, and resuspended in 15 μl of the solvent. The samples were analyzed by reverse phase-high performance thin layer chromatography using Adsorbosil RP HPTLC plates (10 × 10 cm) (Alttech, Deerfield, IL) with chloroform/methanol/water (15:45:3, v/v/v) as the mobile phase. The developed
Cloning and Sequencing of a PI-PLC Gene of T. cruzi (TcPI-PLC)—To clone the PI-PLC gene of T. cruzi, a region containing a conserved PI-PLC sequence was amplified from T. cruzi genomic DNA. In order to design degenerate oligonucleotide primers, amino acid sequences of three unicellular eukaryotic species (Saccharomyces cerevisiae, Schizosaccharomyces pombe, and Dictyostelium discoideum) were retrieved from GenBank and regions with the highest similarity were located in the catalytic domains (X and Y domains) of the proteins (Fig. 1A). Six degenerate oligonucleotide primers (three primers each for the forward and reverse direction) were selected according to these domains and the PCR was carried out with T. cruzi genomic DNA as a template. Among those used, four combinations with four primers (two for the forward and two for the reverse direction) gave distinctive bands of the expected sizes using PCR conditions of relatively low stringency (annealing temperature of 40°C). One of the PCR products with the primer pair 5B and 5R (the corresponding regions are shown in Fig. 1A) and with a size of 1.0 kb was purified, ligated into the cloning vector pGEM-T Easy, and sequenced. Its deduced amino acid sequence showed marked similarity to those of PI-PLCs of other species (more than 40% identity and 60% similarity) and showed motifs characteristic of the X and Y domains of PI-PLCs. This PCR clone was named PLC-PCR (Fig. 1B).

Southern blotting was performed with PLC-PCR as a probe to confirm the presence of this gene in the T. cruzi genome (Fig. 1C). All restriction enzymes except EcoRI gave single, strong bands, which were distinct from one another, indicating the presence of the PI-PLC gene in the T. cruzi genome. After obtaining the entire ORF of this PI-PLC gene (TcPI-PLC) by screening a T. cruzi subgenomic library, Southern blotting was also performed with the 5’-proximal (from nucleotide 1 to 915) or 3’-proximal (from nucleotide 1716 to 2272) region of TcPI-PLC to confirm that the bands in the Southern blotting represented only one gene. Observation of more than one band with EcoRI was not reproducible (data not shown) and could be due to the star activity of the enzyme at the high concentration used.

In order to clone the entire TcPI-PLC, a subgenomic library of T. cruzi was constructed. Since BamHI digestion gave a strong band at 4.4 kb in the Southern blotting (Fig. 1C), T. cruzi genomic DNA was digested with BamHI and DNA in the 4.4 kb size range was purified from the gel, and ligated into a λZAP Express phage vector. The resulting T. cruzi subgenomic library was screened by plaque hybridization with PLC-PCR as a probe. After screening 5.0 × 10⁴ plaques, 18 positive phage clones were selected. After in vivo excision to obtain the inserts together with the phagemid vector, the DNA was digested with BamHI and EcoRI and three distinct digestion patterns (four, five, and nine clones with each pattern) were observed (Fig. 1B). Nucleotide sequencing results revealed that all clones contained sequences overlapping with the sequence of PLC-PCR (Fig. 1B). Two groups (Fig. 1B, groups I and II) shared identical 5’-regions of the ORF, one of which contained an additional EcoRV site in its intergenic sequence upstream of the ORF, and the third group contained the rest of the 3’-regions of the ORF (Fig. 1B, group III), together forming a contiguous ORF of 2175 base pairs encoding a protein (TcPI-PLC) of 725 amino acid with a calculated molecular mass of 82 kDa (Fig. 2A). The portions containing the initiation and stop codon were sequenced twice in both directions to confirm the beginning and end of the ORF. The variation between groups I and II in the 5’-end flanking region could be due to allelic variation at the same locus because no evidence of multiple copies of the gene or tandem repeats were detected in Southern blots with completely or partially digested genomic DNA. The number of clones obtained also agrees with the molar ratio that would result after BamHI digestion of a diploid DNA with an allelic variation at the same locus (Fig. 1B). This type of allelic variation at the same locus was reported for the polyubiquitin gene of Trypanosoma brucei (33).

The nucleotide sequence and deduced amino acid sequence obtained according to universal codon usage were analyzed to identify homologies with other genes. This analysis revealed several domains characteristic of PI-PLCs (Figs. 2A and 3).
charged, respectively) amino acid residues in the linker region between the X and Y catalytic domains (Fig. 2A). Third, a PH domain at the amino-terminal region, which is present in most PI-PLCs, is not found in the TcPI-PLC amino acid sequence. These unique features may indicate some important differences in regulation of T. cruzi PI-PLC from other PI-PLCs.

Expression, Purification, and Catalytic Activity of Recombinant TcPI-PLC—TcPI-PLC was expressed in E. coli to study its enzymatic activity. The two pieces of the TcPI-PLC ORF sequences (5’ and 3’) from the genomic clones were combined in the cloning vector pBluescript KSI –) to form a contiguous and complete TcPI-PLC ORF. In order to express TcPI-PLC with a hexahistidine tag, an NheI site was introduced at the 5’-terminal end of the ORF by using the PCR. The resulting TcPI-PLC ORF was inserted into the prokaryotic expression vector pET28c in a way that allowed the hexahistidine tag and the TcPI-PLC ORF to be translated in-frame (pET-TcPI-PLC). An E. coli strain, BL21(DE3), was transformed by this plasmid and used for the induction of TcPI-PLC expression.

The TcPI-PLC expression was induced by adding isopropyl-1-β-D-thiogalactopyranoside to the culture of E. coli transformed by pET-TcPI-PLC. After 3 h of induction, the cells were disrupted and separated into pellet and supernatant fractions, and proteins in these fractions were separated using SDS-polyacrylamide gels. In both pellet and supernatant fractions, the expressed TcPI-PLC appeared as a protein with an approximate size of 80 kDa, which is very close to the size predicted by its amino acid sequence (82 kDa) (Fig. 4, lanes 3 and 4).

Taking advantage of the specific binding between the hexahistidine tag and nickel ions, the recombinant TcPI-PLC was purified by a one-step affinity chromatography procedure. The supernatant fraction of the TcPI-PLC-expressing E. coli obtained above was applied to the nickel affinity column and the TcPI-PLC was eluted from the column by a buffer containing a high concentration of imidazole (1.0 M). As shown in the SDS-PAGE (Fig. 4, lane 5), near homogenous TcPI-PLC was obtained by this method. A PI-PLC assay was carried out with PIP_2 as a substrate to measure the specific enzymatic activity in each fraction. PI-PLC activity was found in the fraction of the transformed E. coli obtained after nickel affinity chromatography. This purified recombinant TcPI-PLC was subsequently used for further characterization of the enzyme.

The rate of enzymatic reaction at different PIP_2 substrate concentrations was determined. The enzyme reaction was car-
As shown in Fig. 5, maximum activity was achieved at around 10 μM free calcium concentration of 10 μM and pH 7.15. As shown in Fig. 5A, maximum activity was achieved at around 50 μM PIP2. A Lineweaver-Burk plot (Fig. 5A, inset) gave an apparent K_m value of 2.3 × 10^{-5} M and a V_max value of 0.93 μM/min/mg protein.

The effect of free calcium concentration was examined using both PIP2 and PI as substrate. The enzyme activity was dependent on free calcium in both cases. When PIP2 was used as a substrate, the enzyme activity increased with increasing free calcium concentrations above 10 μM and the activity decreased when free calcium concentration exceeded 100 μM (Fig. 5B). When PI was used as a substrate, the enzyme needed more than 100 μM free calcium to be activated (Fig. 5B). These results showed that TcPI-PLC preferred PIP2 to PI as a substrate especially at low calcium concentrations.

The effect of pH on the enzyme activity was also examined (Fig. 5C). The enzymatic activity was measured only at pH higher than 6.5 because precipitation of the substrate occurred at lower pH values. The enzyme activity decreased very rapidly at pH lower than 6.5 or higher than 7.25 and the optimum pH was between 6.65 and 6.80.

**Transcriptional Analysis of TcPI-PLC**—To examine whether TcPI-PLC was actually transcribed in T. cruzi, Northern blotting was performed using RNA from different forms of the parasite. The 3.0-kb TcPI-PLC mRNA was detected in both total RNA (Fig. 6A) and poly(A) RNA (Fig. 6B) from T. cruzi (amastigote, trypomastigote, and epimastigote forms) indicating that TcPI-PLC mRNA was transcribed in vivo in all stages of the parasite. A higher level of TcPI-PLC mRNA was observed in amastigotes and epimastigotes than in trypomastigotes while the amount of mRNA of a ribosomal protein gene (TcP0) (26) was at comparable levels in all forms of the parasite. It should be noted that an additional 5.3-kb transcript of TcPI-PLC specific sequence was observed in trypomastigote total RNA (Fig. 6A) but not in poly(A) RNA (Fig. 6B), indicating the presence of a premature RNA transcript in trypomastigote forms.

Since amastigotes develop from trypomastigotes and the level of TcPI-PLC mRNA was higher in amastigotes, it was expected that TcPI-PLC expression would be induced at some point during transformation from trypomastigotes to amastigotes. To determine when the induction of TcPI-PLC expression occurred, TcPI-PLC mRNA was examined in T. cruzi trypomastigotes which were undergoing transformation into amastigotes. Transformation was induced by incubation in acidic medium (22) and total RNA was extracted from the cells at different times. Northern blotting (Fig. 6C) showed that TcPI-PLC mRNA expression was induced as early as 1 h after changing the pH to 5.0, while the mRNA expression was very low even after 4 h when the cells were kept at pH 7.5. It should be noted that complete transformation of trypomastigotes into amastigotes took more than 4 h although morphological changes started appearing at a relatively early time (around 2–3 h) as described previously (22). Consistent with the previous Northern blotting (Fig. 6A), the 5.3-kb transcript was also observed. Interestingly, the amount of the 5.3-kb transcript was markedly reduced after 4 h in pH 5.0 medium in contrast to the increase in TcPI-PLC mRNA (Fig. 6C). Therefore, the expression of TcPI-PLC was induced by acidic pH in trypomastigotes at an early stage in the transformation process.

**Localization of TcPI-PLC in T. cruzi**—Total homogenates prepared from different stages of T. cruzi were subjected to Western blotting analysis with the affinity purified antibodies. These antibodies detected a single band of approximately 80 kDa, close to the predicted molecular mass of TcPI-PLC, in epimastigotes (Figs. 7A, left lane), and amastigote (Fig. 7B, left lane) homogenates. No detectable band was observed using preimmune serum (Fig. 7, A and B, right lanes). The amount and localization of TcPI-PLC in different forms of T. cruzi was also determined by indirect immunofluorescence assays using the anti-TcPI-PLC antisera. Different forms of T. cruzi were fixed and permeabilized prior to antibody binding. As Fig. 8 shows, TcPI-PLC was detected in amastigotes (panel A) and epimastigotes (panel E) but not in trypomastigotes (panel C), in agreement with the mRNA expression level results (Fig. 6, A and B). No detectable signal was observed when the preimmune serum was used (data not shown). In amastigotes, fluorescence was detected clearly in association with the plasma membrane (and somewhat weaker and punctate intracellular staining in its close proximity), while in epimastigotes a weaker and more diffuse fluorescence was detected with some intracellular spots of brighter fluorescence (Fig. 8, panels A and E). Interestingly, there was a significant variation in the amount and distribution of TcPI-PLC in amastigotes, even from the same preparation (Fig. 8, panel A). This qualitative and quantitative change was not noticeable in epimastigotes (Fig. 8, panel E). The expression of TcPI-PLC was also observed in intracellular amastigotes in infected myoblasts (Fig. 8, panel G). Here, as occurred with isolated amastigotes, TcPI-PLC expression was heterogeneous in terms of amount and localization.

**Changes in Protein Level and Activity of TcPI-PLC during Trypomastigote to Amastigote Differentiation**—Since Northern blotting indicated early induction of TcPI-PLC in T. cruzi trypomastigotes during transformation into amastigotes, it was of interest to study the TcPI-PLC expression at the protein level. The transformation of T. cruzi trypomastigotes was induced by acidic medium (pH 5.0) and immunofluorescence assays was performed to detect TcPI-PLC. TcPI-PLC expression was induced after 1 h incubation in the acidic medium (pH 5.0) (data not shown) and the signal became obvious after 2 h (Fig. 9, panel C). After overnight incubation, most cells expressed TcPI-PLC at high levels (Fig. 9, panel G). Plasma membrane localization of TcPI-PLC was found in some cells after 4 h (Fig. 9, panel E) and in most cells after overnight incubation (Fig. 9, panel G). Trypomastigotes in neutral pH (pH 7.5) medium did not show any detectable amount of TcPI-PLC at any time point (from time 0 to overnight incubation) (data not shown). These results show that TcPI-PLC expression occurs progressively during the development of amastigotes and that the protein...
tends to traffic to the plasma membrane during this process.

To test whether a change in the activity of TcPI-PLC accompanied trypomastigote to amastigote differentiation, the cellular content of IP₃ was quantified with a competitive binding assay. A significant increase in the level of IP₃ was observed after 2–4 h exposure to pH 5.0 while no changes were detected when trypomastigotes were maintained in the same buffer at pH 7.5 (Fig. 10).

Lipid Modification of TcPI-PLC—The presence of a myristoylation motif within the first 6 amino acids of the TcPI-PLC sequence is unique in the family of PI-PLCs. Protein myristoylation is important for the subcellular localization of a variety of proteins (34). A comparison of the amino-terminal amino acid sequence of TcPI-PLC with the N-myristoylation consensus sequence (35) and the amino-terminal amino acid sequence of an N-myristoylated protein of T. cruzi (flagellar calcium-binding protein, FCaBP, Ref. 36) is shown in Fig. 2B. The essential residues in the consensus sequence are glycine at the amino-terminal end and serine (or threonine) at position 5. Although it has not been considered as essential, lysine at position 6 is also conserved in many myristoylated proteins (25 out of 72 proteins, Ref. 36). Uncharged residues are allowed at position 2 but proline is not allowed at position 6. The amino-terminal sequence of TcPI-PLC matches this consensus sequence unlike any other member of the PI-PLC family. The amino-terminal sequence of TcPI-PLC is also very similar to that of the FCaBP, including glycine at position 1, cysteine at position 3, serine at position 5, and lysine at position 6 (Fig. 2B).

It was therefore of interest to demonstrate that TcPI-PLC was actually modified by myristic acid. For this purpose, parasites were labeled with [3H]myristate, lysed, and subjected to immunoprecipitation with TcPI-PLC polyclonal antibodies (Fig. 7, C-G). Endogenous TcPI-PLC was apparently myristoylated in both epimastigotes (Fig. 7C, left lane) and amastigotes obtained by acid treatment of trypomastigotes (Fig. 7D, left lane) while no labeled protein was immunoprecipitated by the control preimmune serum (Fig. 7, C and D, right lanes).
results suggest that TcPI-PLC is myristoylated in vivo as predicted by the deduced amino acid sequence. No co-immunoprecipitation of other myristoylated proteins with TcPI-PLC was observed in these experiments.

A recent report has shown that the T. cruzi FCaBP is both myristoylated and palmitoylated and that these lipid modifications are essential for flagellar localization of the protein (36). The amino-terminal amino acid sequence of TcPI-PLC is very similar to that of the FCaBP, including the cysteine residue at position 3 that is palmitoylated in the case of FCaBP (Fig. 2B). In order to test whether TcPI-PLC was also palmitoylated, parasites were labeled with [3H]palmitate, lysed, and subjected to immunoprecipitation with TcPI-PLC polyclonal antibodies. As shown in Fig. 7, E and F (left lanes), TcPI-PLC was labeled after incubation of cells with [3H]palmitate while no labeled proteins were immunoprecipitated with the control preimmune serum (Fig. 7, E and F, right lanes). Again, this apparent palmitoylation was found in both epimastigotes (Fig. 7E), and amastigotes obtained by transformation of trypomastigotes by acid treatment (Fig. 7F).

Although myristoylation through amide linkage with an amino-terminal glycine residue is most common, a recent report has shown that the glycosylphosphatidylinositol-specific phospholipase C (GPI-PLC) of T. brucei is myristoylated on cysteine residue(s) of the protein by unusual thioester bonds (S-myristoylation) (32). Thioester linkages formed upon acylation are extremely labile. To test for their presence, the [3H]myristoyl- or [3H]palmitoyl-labeled immunoprecipitated TcPI-PLC from epimastigotes were separated by SDS-polyacrylamide gel electrophoresis 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 (37). As shown in Fig. 7G, lane 2, the radioactivity incorporated from [3H]palmitate on TcPI-PLC was greatly reduced by neutral hydroxylamine treatment while the radioactivity incorporated from [3H]myristate remained on the protein after the treatment (Fig. 7G, lane 1). These results strongly suggest that [3H]myristate is linked to TcPI-PLC by an amide bond while [3H]palmitate could be linked to TcPI-PLC by a thioester bond. The control Tris buffer wash did not result in any loss of labeling.
Fig. 9. Indirect immunofluorescence analysis of TcPI-PLC in T. cruzi trypomastigotes during transformation at pH 5.0. Fluorescence (A, C, E, and G) or bright field (B, D, F, and H) images at 0, 2, 4, or 15 h after suspension of the cells at pH 5.0. Bars, 10 μm.

Fig. 10. IP₃ increases during trypomastigote to amastigote transformation. T. cruzi trypomastigotes were incubated at pH 5.0 or 7.5 for 0, 2, and 4 h and IP₃ was quantitated as indicated under “Experimental Procedures.” The average ± S.D. from two experiments is shown. The cellular content of IP₃ did not vary significantly among parasites incubated at pH 7.5, but increased significantly after 2 and 4 h at pH 5.0 at p < 0.01 as determined by the Student’s t test.

The potential for metabolic interconversion of radioactive fatty acids by the cells makes it necessary to chemically characterize the protein-associated radioactivity. Cells were metabolically labeled with either [³H]myristate or [³H]palmitate, and TcPI-PLC was immunoprecipitated and deacylated as described under “Experimental Procedures.” The resultant FAMES were then analyzed by thin layer chromatography. Using FAMES standards, myristate, palmitate, and an unidentified FAME were detected. The ratio of the myristate:palmitate:unknown was 1:3:0.9 when cells were labeled with [³H]myristate and 1:1.9:0.9 when cells were labeled with [³H]palmitate. Although palmitate labeling predominated in both cases a higher amount of radioactive myristate methyl ester was recovered when cells were labeled with myristate than when cells were labeled with palmitate (Fig. 11, A and B). The formation of myristate from palmitate also explains the residual activity detected after hydroxylamine treatment of [³H]palmitate-labeled TcPI-PLC (Fig. 7G, lane 2). Together these data suggest that T. cruzi metabolizes fatty acids before their incorporation into TcPI-PLC. No labeling of the protein was detected when immunoprecipitated and deacylated [³H]myristate-labeled TcPI-PLC was run in a gel, suggesting that none of the [³H] label was reincorporated into amino acids (data not shown).

DISCUSSION

Our laboratory has described previously the presence and operation of the inositol phosphate/diacylglycerol signaling pathway in different stages of T. cruzi (11–13). In this work, we have demonstrated that a gene, TcPI-PLC, encoding a functional PI-PLC, is present in the T. cruzi genome. We have also shown that the T. cruzi enzyme is developmentally regulated, and has several peculiarities that distinguish it from other known PI-PLCs. In contrast to other PI-PLCs described so far, the deduced amino acid sequence of TcPI-PLC revealed some unique features such as an N-myristoylation consensus sequence at its amino-terminal end, lack of an apparent PH domain, and a highly charged linker region between the catalytic X and Y domains. TcPI-PLC is lipid modified in vivo as demonstrated by metabolic labeling with [³H]myristate and [³H]palmitate and fatty acid analysis of the immunoprecipitated protein, and may constitute the first example of a new group of PI-PLCs.

The protein encoded by TcPI-PLC is most closely related to the mammalian δ isoform and to PI-PLCs found in yeasts, D. discoideum, and plants in terms of sequence identity and arrangement of conserved domains. In addition, among other lower eukaryotic and mammalian PI-PLCs, TcPI-PLC is one of the smallest with 725 amino acids. TcPI-PLC conserves all the amino acid residues which have been found, in mammalian PI-PLC-δ1 (38), to be in contact with IP₃ and Ca²⁺ in the catalytic domains (His²⁶¹, Asn²⁶², Glu²９¹, Asp²⁹³, His³⁰⁶, Glu⁴４０, Lys⁴⁶⁰, Lys⁴⁹２, Ser⁴⁹³, Arg⁴⁹⁵, and Tyr⁵２２) (Fig. 2A). TcPI-PLC also conserves part of the proposed calcium-binding sites in the C2 domain (Ile⁶¹⁶, Val⁶¹⁷, Asp⁶¹⁸, Asp⁶⁷⁶, and Asp⁷₇₈) (Fig. 2A) (38). This conservation of amino acid residues that are important for substrate and calcium binding explains the similar enzymatic activity and calcium requirement of TcPI-PLC as compared with mammalian δ-type PI-PLCs (Fig. 5). In addition, similar to the δ-PI-PLC isozymes from other organisms, TcPI-PLC lacks a carboxyl-terminal extension following the Y domain and contains an EF-hand motif upstream of the X domain. However, no apparent pleckstrin homology (PH) domain at the amino-terminal region, as is found in all δ-PI-PLCs (39–41) reported to date, is present in TcPI-PLC. The presence of unusual clusters of negatively charged and mixed charged amino acids between the X and Y catalytic domains is not common in PI-PLCs of lower eukaryotes or in...
mammalian δ- or γ-PI-PLCs. However, mammalian β-PI-PLCs also contain highly charged clusters of amino acids between the X and Y domain. This is essential for activation by βγ subunits of G proteins. *D. discoideum* δ-type PI-PLC is also postulated to be activated in this manner (42).

Post-transcription regulation has been reported as the major mechanism of regulation of gene expression in trypanosomatids, including regulation by mRNA stability and translation efficiency through the long 3′-end untranslated region of the mRNA (43, 44). Based on the size shown by Northern blotting and the length of the 5′-end untranslated region sequence in the cDNA clone, *TcPI-PLC* possesses a relatively long 3′-untranslated region in its mRNA (~700 bp). The 5.3-kb transcript detected in trypanomastigote RNA (Fig. 6) could also be involved in gene regulation. It was only detected in trypanomastigotes, and its sequence is specific to *TcPI-PLC* since probes specific to other sequences (e.g. rRNA or TcPO) did not bind to this RNA (data not shown). The 5.3 kb does not contain a poly(A) tail since it was not detected in poly(A)-purified RNA (Fig. 6). Moreover, the amount of the 5.3-kb transcript decreased with time when trypanomastigotes transformed into amastigotes at pH 5.0, while it did not change when the cells were maintained at pH 7.5 (Fig. 6). It is tempting to speculate that this 5.3-kb transcript could be an immature *TcPI-PLC* mRNA species that has not undergone splicing to form the mature *TcPI-PLC* mRNA (44). The cell may regulate the amount of mature *TcPI-PLC* mRNA by controlling the rate of splicing of polyestradiolynlated to trans-splicing as reported in other trypanosomatids (44). The cell may regulate the amount of mature *TcPI-PLC* mRNA by controlling the rate of splicing of polyestradiolynlated pre-mRNA. Such mechanism has been reported for some genes of *T. brucei* and Leishmania species (45, 46) although no definitive case has been reported for *T. cruzi*.

The N-myristoylation site found at the amino-terminal end of *TcPI-PLC* has not been found before in any PI-PLC. Metabolic labeling with [3H]myristate and fatty acid analysis of the immunoprecipitated protein demonstrated that *TcPI-PLC* was indeed myristoylated (Figs. 7 and 11). Myristoylation is found in many membrane-associated proteins of eukaryotic cells and viruses and, in many cases, is necessary for the membrane attachment of these proteins (34). Binding of other δ-PI-PLCs to the plasma membrane has been postulated to occur through the PH domain of the protein to PI(3,4,5)P3 (40, 41). Since no PH domain motif was found in *TcPI-PLC* and the protein localized to the plasma membrane (Fig. 8), the myristoyl group may play an important role in this association. In some cases, association of the myristoylated proteins with membranes is regulated by calcium binding to the protein. This mechanism, called calcium-myrstoylation switch, has been found in EF-hand calcium-binding proteins including FCaBP (36), and could be involved in the case of *TcPI-PLC* since this protein also possesses an EF-hand domain near its amino terminus. Interestingly, *TcPI-PLC* (Fig. 7) like FCaBP (36) is also palmitoylated as demonstrated by metabolic labeling with [3H]palmitate and fatty acid analysis of the immunoprecipitated protein (Fig. 11). Although palmitate is the most commonly found S-linked fatty acid, other fatty acids can be incorporated into cellular proteins by a thioester linkage, including stearate, oleate, and arachidonate (47), in agreement with our results (Fig. 11). Palmitoylation can affect a protein’s affinity for membranes, subcellular localization, and interactions with other proteins (47). These results suggest that *TcPI-PLC* may share common mechanisms of membrane attachment or protein-protein interaction with FCaBP.

Our results also demonstrate for the first time that the expression of the *TcPI-PLC* gene is induced and the *TcPI-PLC* activity is increased during transformation of the extracellular, non-replicating form (trypanomastigote), of *T. cruzi* into the intracellular, replicating form (amastigote), of the parasite. This is in agreement with the higher incorporation of inositol into IP3 detected in amastigotes (12). We have found before (48) that under similar conditions to those used in this work to stimulate differentiation (addition of 20 mM MES to trypanomastigotes incubated in DMEM-BSA buffer to obtain a final extracellular pH (pH) of 5.0), the trypanomastigotes’ intracellular pH (pH) decreases almost immediately from a value of 7.35 ± 0.07 to a value of 7.05 ± 0.01 and remains steady at that value for at least 1 h. At this time most of the trypanomastigotes appear to be in the process of differentiation as shown by the accumulation of the amastigote-specific inner membrane complex (60) and the concomitant decrease in the extracellular pH.

In addition, we show that the pH of amastigotes transformed from trypanomastigotes axenically at pH 5.0 for 12 h (7.37 ± 0.04) was similar to that of control trypanomastigotes maintained at pH 7.5 (48). Even though the observed decrease in pH is small, ~0.3 pH units, changes of this magnitude have been associated with a number of metabolic and developmental transitions, including cell cycle changes, in a wide variety of eukaryotic microorganisms (49). It is important to note that the pH attained after extracellular acidification of trypanomastigotes is close to the optimum pH for the *TcPI-PLC* activity (Fig. 5). In Dictyostelium, a similar decrease in pH, which occurs following exposure to acidic pH, has been shown to induce differentiation and up-regulate a plasma membrane H+-ATPase that is more active at acidic pH (50, 51). Activation of the plasma membrane H+-ATPase of *S. cerevisiae* has been linked to protein kinase C-induced phosphorylation in response to increased phosphoinositide turnover stimulated by glucose (52). Interestingly, mild acid stress (incubation at pH 5.5 for 2 h) was found to induce *T. brucei* bloodstream differentiation into procyclic forms and this process appears to involve the GPI-PLC, since null mutants (*GPI-PLC−/−*) appeared to be largely refractory to acid stress-induced differentiation (53). GPI-PLC is different from PI-PLCs and its role in *T. brucei* is still unclear (53). However, like PI-PLC, one of the products of its catalytic activity is also DAG, which is the physiological stimulus of various protein kinase C isozymes (2). A role for phosphorylation cascades in the differentiation process of trypanomastigotes into amastigotes has been postulated recently on the basis of the effects of calyculin A on this process (54). This potent inhibitor of protein phosphatases 1 and 2A induced transformation of *T. cruzi* trypanomastigotes into amastigotes at pH 7.5 (54). Since DAG generated upon activation of PI-PLC leads to activation of protein kinase C with the resulting phosphorylation of a number of proteins, this effect of calyculin A could be due to decreased dephosphorylation of protein kinase C substrates. The same authors found that some low molecular weight proteins were phosphorylated only when trypanomastigotes were incubated at pH 5.0 but not at pH 7.5 (54) and these proteins may represent protein kinase C substrates. Our results therefore suggest that activation of the inositol phosphate/diacylglycerol signaling cascade by acidic conditions could have an important role in the *T. cruzi* differentiation process.

While this paper was in preparation, a report describing the cloning of a *T. cruzi* PI-PLC gene (AB022677), its functional complementation of a yeast PI-PLC mutant, and the detection of a calcium-activated PI(3,4,5)P3 hydrolyzing activity in the lysate of the *T. cruzi* PLC-expressing yeast transformants, appeared (55). Our findings, while generally in agreement and complementary to those of Nozaki et al. (55) differ in three respects. First, our sequence of *TcPI-PLC* differs from the previously published sequence by 4 nucleotide changes; however, these differences do not result in amino acid substitutions. Second, these workers reported that the open reading frame contained

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2178 bp, while the correct number is 2175. Third, Nozaki et al. (55) could not find appreciable PIP$_2$ hydrolyzing activity in the expressed enzyme at Ca$^{2+}$ concentrations below 10$^{-7}$ M. Finally, it has come to our attention that another group (56) has independently and contemporaneously isolated an expressed sequence tag (TENS1347) matching part of the TcPI-PLC (from nucleotides 1172 to 1487).

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