Identification of Novel Serine/Threonine Protein Phosphatases in Trypanosoma cruzi: a Potential Role in Control of Cytokinesis and Morphology

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We cloned two novel Trypanosoma cruzi proteins by using degenerate oligonucleotide primers prepared against conserved domains in mammalian serine/threonine protein phosphatases 1, 2A, and 2B. The isolated genes encoded proteins of 323 and 330 amino acids, respectively, that were more homologous to the catalytic subunit of human protein phosphatase 1 than to those of human protein phosphatase 2A or 2B. The proteins encoded by these genes have been tentatively designated TcPP1α and TcPP1β. Northern blot analysis revealed the presence of a major 2.3-kb mRNA transcript hybridizing to each gene in both the epimastigote and metacyclic trypomastigote developmental stages. Southern blot analysis suggests that each protein phosphatase 1 gene is present as a single copy in the T. cruzi genome. The complete coding region for TcPP1β was expressed in Escherichia coli by using a vector, pTACTAC, with the trp-lac hybrid promoter. The recombinant protein from the TcPP1β construct displayed phosphatase activity toward phosphorylase a, and this activity was preferentially inhibited by calyculin A (50% inhibitory concentration [IC₅₀], ~2 nM) over okadaic acid (IC₅₀ ~ 100 nM). Calyculin A, but not okadaic acid, had profound effects on the in vitro replication and morphology of T. cruzi epimastigotes. Low concentrations of calyculin A (1 to 10 nM) caused growth arrest. Electron microscopic studies of the calyculin A-treated epimastigotes revealed that the organisms underwent duplication of organelles, including the flagellum, kinetoplast, and nucleus, but were incapable of completing cell division. At concentrations higher than 10 nM, or upon prolonged incubation at lower concentrations, the epimastigotes lost their characteristic elongated spindle shape and had a more rounded morphology. Okadaic acid at concentrations up to 1 μM did not result in growth arrest or morphological alterations to T. cruzi epimastigotes. Calyculin A, but not okadaic acid, was also a potent inhibitor of the dephosphorylation of 32P-labeled phosphorylase a by T. cruzi epimastigotes and metacyclic trypomastigote extracts. These inhibitor studies suggest that in T. cruzi, type 1 protein phosphatases are important for the completion of cell division and for the maintenance of cell shape.

Trypanosoma cruzi, a hemoflagellate and the causative agent of Chagas’ disease, has a complex life cycle involving four major morphogenetic stages (24). The epimastigote and metacyclic trypomastigote are insect-specific stages, whereas the trypomastigote and amastigote are mammalian host-specific extracellular and intracellular stages, respectively. Each developmental stage can be distinguished morphologically, and there are also stage-specific differences in surface and intracellular components (2, 7). The molecular mechanisms involved in the various stage-specific transformations, however, remain ill defined. In higher eukaryotes, the reversible phosphorylation of proteins on serine, threonine, and tyrosine residues plays a key role in the integration of the signals involved in cellular proliferation and differentiation (5, 7). It is possible that similar regulatory pathways also exist in T. cruzi and are involved in the various developmental transformations. However, information concerning such pathways is limited. Cyclic AMP, an important second messenger in higher eukaryotes, has been reported to be involved both in epimastigote-to-metacyclic trypomastigote transformation within the insect vector (13) and in the control of proliferation and differentiation of amastigotes (9). Several serine/threonine protein kinases, including protein kinase A (20, 26), protein kinase C (12), and a calcium- and calmodulin-dependent protein kinase (21), have been detected in T. cruzi epimastigotes. However, information on serine/threonine protein phosphatases in this organism is limited.

Four major classes of protein phosphatases have been identified in eukaryotic cells: protein phosphatase 1 (PP1), PP2A, PP2B, and PP2C (3, 6). This classification is based on the use of specific activators and inhibitors, substrate specificity, and divalent cation requirements of these enzymes. Subsequent amino acid and cDNA sequencing studies have revealed that PP1, PP2A, and PP2B are members of the same gene superfamily, termed the PPP family. PP2C is structurally and mechanistically unrelated to the PPP family and has been classified as a member of the PPM family of Mg²⁺-dependent protein phosphatases.

In this paper, we provide evidence for a critical role for PP1-like phosphatases in the T. cruzi life cycle. Two protein phosphatase genes from T. cruzi have been isolated by homology cloning. The encoded proteins, designated TcPP1α and TcPP1β, were found to be more homologous to human PP1 than to human PP2A or PP2B. The availability of highly specific inhibitors of PP1 and PP2A has provided the opportunity...
to investigate the role of these enzymes in cellular processes (11, 15, 16, 28). Okadaic acid is a potent inhibitor of PP2A (50% inhibitory concentration [IC50] = 2 nM), whereas higher concentrations are necessary for inhibition of PP1 (IC50 = 60 to 200 nM) and PP2B (IC50 = 10 nM). PP2C is unaffected by okadaic acid. In contrast, calyculin A inhibits both PP1 and PP2A, but not PP2B or PP2C, with high potency (IC50 = 0.5 to 1 nM). Calyculin A, but not okadaic acid, had marked effects on *T. cruzi* epimastigote growth and morphology. In the presence of low concentrations of calyculin A (1 to 10 nM), epimastigotes underwent growth arrest. Microscopic analysis indicated that the calyculin A-treated epimastigotes had undergone flagellar duplication and both kinetoplast and nuclear divisions but were incapable of successfully completing cytokinesis. Calyculin-treated cells also lost their characteristic elongate, spindle-shaped trypomastigone morphology and adopted a more rounded morphology. Our studies suggest that in *T. cruzi*, PP1-like phosphatases are important for the completion of cell division and the maintenance of cell shape.

**MATERIALS AND METHODS**

Epimastigote and metacyclic trypomastigote culture conditions. *T. cruzi* epimastigotes were grown at 26°C in liver infusion tryptose broth supplemented with 10% fetal calf serum (GIBCO BRL) (2, 3). *T. cruzi* metacycitic trypomastigotes (HO 3/15), kindly provided by James Dvorak (National Institutes of Health), were produced by allowing epimastigote cultures to reach stationary phase as indicated by a change in the rate of DNA synthesis (22). Prior to induction, the culture was diluted 1:10 into fresh medium. When DNA synthesis was arrested (as indicated by by on September 12, 2008) the culture was transferred to a 37°C incubator and this was repeated daily until the highest 37°C synchronization was achieved.

Epimastigotes and metacyclic trypomastigotes were checked for viability by trypan blue exclusion. Metacyclic forms were identified by their characteristic needle-shaped body and a large central kinetoplast. The rate of DNA synthesis as monitored by thymidine incorporation was similar in epimastigotes and metacyclic trypomastigotes.

Amplification of sequences encoding PP1 and PP2A homology domains. Three oligonucleotide primers corresponding to conserved domains in protein phosphatases were synthesized: primer 1, CCGGATCA ATG GAG CAG ATG GAT TTA G; primer 2, 5'-GGCA TCT TGA CTG CTT TGC C-3', and primer 3, 5'-CTG TCT CGC AGG CGG TCT TGA-3'. The PCR mixture (100  μL of each primer, and 2.5 U of Taq polymerase, PCR conditions were 1 min each at 94 and 50°C followed by 1.5 min at 72°C for 35 cycles. The last cycle was 10 Min at 72°C. Bands of the appropriate size, i.e., 220 bp for primers 1 and 2 and 648 bp for primers 1 and 3, were isolated, subcloned into pBluescript II KS(+) (Stratagene), and sequenced by the dideoxynucleotide termination procedure of Sanger et al. (24), using a combination of pBluescript and synthetic oligonucleotide primers.

Genomic library construction and screening. *T. cruzi* genomic DNA was partially digested with SnaI and electrophoresed on a 0.7% agarose gel. The region of the gel between 2 and 9 kb was excised and electroeluted, and the resultant DNA was ligated and packaged into the ZAP Express vector (Stratagene) in accordance with the manufacturer's instructions. The library was screened with a 32P-labeled probe prepared by PCR using primers 1 and 3. Positive clones were plaque purified and sequenced as described above.

**Northern and Southern blot analyses.** Total RNA (20  μg), isolated from *T. cruzi* epimastigotes and metacyclic trypomastigotes, was electrophoresed on 1% agarose-formamide gels and calibrated by hybridization to nylon membranes prepared by standard procedures. The membranes were probed with the 648-bp insert labeled with 32P by PCR amplification with primers 1 and 3. Hybridization was performed at 42°C overnight; then the membranes were washed once with 1 x SSC (0.15 M sodium chloride, 0.015 M sodium citrate) at 50°C, twice with 0.5 x SSC at the same temperature and then three times with 0.2 x SSC-0.5% SDS for 20 min each at 65°C.

For Southern analysis, DNA (40  μg) was digested with various restriction enzymes (50  Ej) at 37°C overnight. The restriction fragments were separated by electrophoresis on 0.7% agarose gels and transferred to nylon membranes. Probe labeling, hybridization, and washing conditions were as for Northern blot analysis.

**Protein phosphatase assay.** PP1 and PP2A activities in *T. cruzi* epimastigotes and metacyclic trypomastigotes extracts were determined by using a [32P]labeled phosphorylase assay system (GIBCO BRL) in accordance with the manufacturer's instructions. One unit of phosphatase activity releases 1 nmol of [32P]phosphate from [32P]labeled phosphorylase a per minute. The concentrations of calyculin A and okadaic acid in the assay mixture ranged from 0 to 1  M. Cell extracts were prepared as previously described (22). In the case of the axenically propagated *Escherichia coli* lysates, samples were serially diluted twofold in 0.5 mM imidazole, pH 7.0, containing 2 mM MnCl2, 1 mM EDTA, 2 mM dithiothreitol, and 1% Triton X-100 prior to assay.

**Bacterial expression of *T. cruzi* protein phosphatases.** PCR primers incorporating the initiating ATG into the *NdeI* recognition sequence or placing a BamHI site immediately downstream of the stop codon of both phosphatase genes were synthesized; the primer sequences for TcPP1 were 5'-CCGCCCATATGCACTCCAAACGAGTGAGAATCGGTTACC and 3'-CCGGATCCATTTAATACCTTTATTTATTCGACCACCGCCTGTTG, while those for TcPP2 were 5'-CCGCGCATATGGCGCCTGTTGCCGGAAACATTG and lacking the T. cruzi 3' untranslated region, was cloned directly into *NdeI*- and BamHI-chipBstS1208x and from, there as a NdeI-HindIII insert into pTACAT. A second PCR product of TcPP1, based on the revised 3' sequence (GCAGGATCCTACACGTGTTCCCAGGAACATG and lacking the T. cruzi 3' untranslated region, was cloned directly into *NdeI* and BamHI-chipBstS1208x and from, there as a NdeI-HindIII insert into pTACAT. The PCR products were digested with *BamH*I and *EcoR*I and cloned into pTACAT vector containing 32P-labeled phosphorylase a as a marker. The insert was sequenced to confirm these events.

**Fluorescence microscopy.** For light microscopic examination, organisms were re-suspended in the medium by collision at 800  rpm for 2 min, spun, resuspended in PBS, and placed on slides. After air drying, the slides were fixed in absolute methanol for 5 min and stained first with May-Grünwald stain (Harelco) for 5 min and then with Giemsa stain for 13 min. Slides were rinsed five times in PBS, and then incubated in 10% methanol (vol/vol) in PBS followed by Triton X-100 to a final concentration of 1%. The bacterial lysate was centrifuged at 20,000 γ g for 20 min, and the supernatant stored at −70°C until use.

**Transmission electron microscopy (TEM).** Trypanosome suspensions were fixed in 2.5% (vol/vol) glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2, for 30 min at room temperature (20°C). The fixed trypanosomes were centrifuged (1,000 x g) for 30 s, and the supernatant was removed. The pellet was resuspended in warm (45°C) 0.1 M sodium cacodylate, pH 7.2, containing 2% (wt/vol) agar and allowed to cool to room temperature. All subsequent procedures utilized cold (4°C) solutions through 95% ethanol, 100% ethanol and propylene oxide were used at room temperature. The solidified agar, containing the suspended trypanosomes, was cut into small (1-2 mm) cubes and fixed for 24 h in PBS-2.5% glutaraldehyde. The agar cubes were rinsed several times in PBS and then postfixed in 2% (wt/vol) osmium tetroxide for 4 h. The cubes were subsequently rinsed in PBS, dehydrated through a graded ethanol series, treated with propylene oxide (transitional fluid), and embedded in Araldite 502. Thin sections (silver) were placed on copper grids, stained with uranyl acetate and lead citrate, and examined with a Philips CM-10 transmission electron microscope operated at 80 kV.

**SEM.** Trypanosome suspensions were spread on acid-washed 18-mm-diameter circular cover glasses and allowed to partially air dry. The cover glasses were subsequently rinsed with 0.2% (vol/vol) glucose in 100% ethanol (pH 7.2) for 12 h at 4°C, rinsed several times in PBS, postfixed in PBS-1% (vol/vol) osmium tetroxide for 2 h, rinsed several times in PBS, and dehydrated...
in a graded ethanol series. The cover glasses were then placed in acetone and
critical-point dried in a Tousimis Samdri-790 critical-point drier (Tousimis Re-
search Corp., Rockville, Md.), using liquid carbon dioxide for the transition.
Cover glasses were mounted on scanning electron microscopy (SEM) aluminum
stubs with silver paint and sputter coated in a Denton Du-502 vacuum evaporator
(Denton Vacuum, Inc., Cherry Hill, N.J.) equipped with a gold target. The
critical-point-dried and sputter-coated trypanosomes were then examined with a
Super IIIA or an SS-40 scanning electron microscope (ISI International Scien-
tific Instruments, Santa Clara, Calif.) operated at 15 kV.

RESULTS

Identification and characterization of genes encoding
serine/threonine protein phosphatases in T. cruzi. We have
used PCR to isolate T. cruzi genomic fragments corresponding
to conserved domains in the catalytic subunits of PP1, PP2A,
and PP2B. The lack of introns in the T. cruzi genome made
PCR an ideal tool for this type of analysis. Initially, two de-
generate primers were designed against conserved sequences
common to all three isotypes (4). The sequences chosen were
CGDIHGQ (sense primer) and LRGNHE (antisense primer).
Amplification of T. cruzi genomic DNA with these primers
gave rise to a 220-bp fragment, the expected size based on the
sequences of the mammalian enzymes. After subcloning of the
PCR fragment into pBluescript, examination of over 30 indi-
vidual clones revealed the presence of two distinct nucleotide
sequences. Both sequences contained an open reading frame
which, in addition to the conserved primers, possessed residues
diagnostic for PP1, PP2A, and PP2B. For example, the se-
quence GDXVDRG is found in all three isotypes throughout
different phyla. Overall comparison of the deduced amino acid
sequences indicated that both amplified sequences were more
homologous to mammalian PP1 than to PP2A or PP2B. To
confirm that both sequences encoded a PP1-type enzyme, an
additional degenerate primer corresponding to a C-terminal
domain (NYCGEFD) which is highly conserved in all charac-
terized PP1 enzymes was designed (4). This sequence is not
conserved in either PP2A or PP2B. Amplification with primers
1 and 3 gave rise to a 648-bp fragment. When this fragment was
amplified with primers 1 and 2, a 220-bp fragment was ob-
tained (data not shown). After subcloning of the 648-bp frag-
ment into pBluescript, analysis of the purified clones again
revealed the presence of two unique nucleotide sequences.
Each sequence contained an open reading frame, and each
open reading frame contained one of the previously character-
ized N-terminal sequences obtained with the previous primer
pair.

The remaining 5' and 3' sequences of both phosphatase
genes were obtained by screening a T. cruzi genomic library in
lZap Express with the 32P-labeled 648-bp PCR-generated
fragment of each gene. The complete nucleotide sequence and
the deduced amino acid sequence of the two genes are shown
in Fig. 1. Computer searches of the databases revealed signif-
icant homology to the mammalian PP1 isotype. The two T.
cruzi genes encode proteins of 323 and 330 amino acids that
have been tentatively designated TcPP1a and TcPP1b, respec-
tively. TcPP1a exhibits 61% identity to the conserved catalytic
core of mammalian PP1. If conservative amino acid substitu-
tions are included, this number increases to 77%. In contrast,
TcPP1b is 54% identical and 72% similar to the mammalian

DNA and Protein Sequence of TcPP1a

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FIG. 1. Nucleotide and deduced amino acid sequences of TcPP1a and TcPP1b.
The levels of identity of both TcPP1α and TcPP1β to the corresponding regions of either mammalian PP2A or PP2B are considerably lower. TcPP1α is 43 and 34% identical to mammalian PP2A and PP2B, respectively. The corresponding levels of identity for TcPP1β are 42 and 38%.

Expression of TcPP1α and TcPP1β in E. coli. Since the glutathione S-transferase and β-galactosidase fusion proteins of the putative PP1 and PP2A from Trypanosoma brucei failed to show phosphatase activity (10), we decided to include only the native coding sequences of the T. cruzi phosphatases in these bacterial expression studies. Taking advantage of the fact that the initiator methionine codon ATG forms the 3’ half of the NdeI recognition site, we have inserted the coding sequence of both genes in frame into the NdeI site of pTACTAC, a lac-inducible expression vector. This vector was used successfully to express the rabbit muscle PP1 catalytic subunit as a soluble and active protein in E. coli (29). After induction and cell fractionation, we observed that TcPP1β expressed in E. coli DH5α possessed phosphatase activity against 32P-labeled phosphorylase a. In contrast, E. coli transformed with the vector alone possessed no such phosphatase activity (data not shown). SDS-polyacrylamide gel electrophoresis PAGE analysis revealed the presence of an ~37-kDa polypeptide in lysates from E. coli transformed with TcPP1β-pTACTAC that was absent in bacteria transformed with the empty vector (Fig. 2A). The predicted molecular weight of TcPP1β is 37,557. Lysates were assayed for protein phosphatase activity, using 32P-labeled phosphorylase as a substrate. Protein phosphatase activity was detected in E. coli expressing TcPP1β (0.5 to 1 U/ml of supernatant) but not in E. coli transformed with vector alone. The recombinant TcPP1β phosphatase activity was inhibited in a concentration-dependent manner by calyculin A, with an estimated IC50 of ~2 nM (Fig. 2B). The IC50 for inhibition of phosphatase activity by okadaic acid was ~100 nM. Attempts to achieve high-level expression of phosphatase activity directed against 32P-labeled glycogen phosphorylase by using TcPP1α in pTACTAC were unsuccessful.

Northern and Southern blot analyses of TcPP1α and TcPP1β. Northern blot analysis using RNA isolated from both epimastigotes and metacyclic trypomastigotes of T. cruzi was performed to determine whether the mRNAs encoding TcPP1α and TcPP1β were differentially expressed in the two vector-specific developmental stages. In these experiments, hybridization was performed with each 32P-labeled 648-bp PCR-generated fragment under high-stringency conditions. Both probes hybridized with a major 2.3-kb mRNA transcript present in

FIG. 2. Bacterial expression of TcPP1β and inhibition by calyculin A and okadaic acid. (A) SDS-PAGE and Coomassie blue staining of extracts from E. coli transformed with pTACTAC alone (lane 1) or with TcPP1β-pTACTAC (lane 2). (B) Recombinant TcPP1β assayed using 32P-labeled phosphorylase as substrate in the absence or presence of various concentrations of either calyculin A or okadaic acid. The extract was diluted 1:128 prior to use. No phosphatase activity was detected in lysates of E. coli transformed with empty vector alone.

FIG. 3. Northern analysis of TcPP1α and TcPP1β mRNA expression in T. cruzi epimastigotes and metacyclic trypomastigotes. RNA from epimastigotes or metacyclic trypomastigotes was separated on a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with the appropriate probe as described in Materials and Methods. Lanes 1 and 3, epimastigote RNA probed with TcPP1α; lanes 2 and 4, metacyclic trypomastigote RNA probed with TcPP1β; lanes 5 and 6, ethidium bromide-stained gels of epimastigote and metacyclic trypomastigote RNA, respectively, showing the characteristic triplet pattern of T. cruzi rRNA and demonstrating equal loading and integrity of the samples.
Effect of calyculin A and okadaic acid on protein phosphatase activity in *T. cruzi* epimastigotes and metacyclic trypanastigotes. To assess directly the effect of each inhibitor on phosphatase activity, the abilities of extracts isolated from both vector developmental stages to dephosphorylate 32P-labeled phosphorylase *a* in the presence of each inhibitor were measured. In higher eukaryotes, both PP1 and PP2A, but not PP2B or PP2C, can catalyze this reaction. Calyculin A, but not okadaic acid, was a potent inhibitor of the phosphorylase phosphatase activity present in both developmental stages (Fig. 8). The IC50 for calyculin was estimated to be 10 nM. In contrast, the IC50 for okadaic acid was greater than 500 nM. These data strongly suggested that PP1-type enzymes are the major phosphatases catalyzing the dephosphorylation of phosphorylase *a* in *T. cruzi* epimastigotes and metacyclic trypanastigote extracts.

**DISCUSSION**

Phosphorylation and dephosphorylation of serine/threonine residues constitute major regulatory pathways in higher eukaryotes, controlling a wide range of intracellular processes (5, 17). It has become increasingly apparent that protein phosphatases, in addition to kinases, play a dynamic role in regulating complete cytokinesis (Fig. 6A). Multiple nuclei, flagella, and kinetoplasts were observed in treated cells (Fig. 6A and B). At this concentration, loss of cell shape, i.e., spheromastigote-like or rounded forms, became evident early (1 day), but by 7 days this also was evident at concentrations as low as 1 nM (Fig. 6C). The parasites were also examined by SEM after 3 days of exposure to calyculin A (10 nM). Figure 7A is a scanning electron micrograph of untreated normal dividing epimastigotes, each with a single flagellum. Figure 7B is an SEM view of an extreme example of a calyculin A-treated epimastigote that has initiated but failed to complete cytokinesis two or three times. Figure 7C is an SEM view of a spheromastigote-like form of a calyculin A-treated epimastigote that possesses two, or possibly three, flagella. When examined by transmission electron microscopy (Fig. 7D and E) after 3 days of exposure to calyculin (5 nM), *T. cruzi* was again seen to contain multiple copies of normal-appearing organelles, compared with untreated *T. cruzi*.

**FIG. 5.** Effect of calyculin A on growth of *T. cruzi* epimastigotes. Epimastigotes were cultured at a density of 2.5 × 10^6/ml (total volume, 10 ml) in the presence or absence of calyculin A or okadaic acid as described in Materials and Methods. Cell number was determined at various times after the additions.
these cellular events (3, 6). Mammalian serine/threonine protein phosphatases are classified into four major types, 1, 2A, 2B, and 2C, depending on substrate and inhibitor specificities and on metal ion dependencies (3, 6). It has been established that PP1, PP2A, and PP2B are members of the PPP gene family which share a conserved catalytic core of approximately 280 amino acids (4). PP2C bears no sequence homology to the other three phosphatase types and has been classified as a PPM phosphatase. We have performed PCR with degenerate oligonucleotide primers against conserved domains in PP1-, PP2A-, and PP2B-type enzymes to catalog and characterize the genes encoding related phosphatases in T. cruzi. 

The initial sequences used for primer design were C 62GDIHGQ (sense) and L 121RGNHE (antisense). The numbering is based on mammalian PP1. This primer pair was designed to amplify all members of the PP1/PP2A/PP2B family of protein phosphatases. We have performed PCR with degenerate oligonucleotide primers against conserved domains in PP1-, PP2A-, and PP2B-type enzymes to catalog and characterize the genes encoding related phosphatases in T. cruzi. The initial sequences used for primer design were C 62GDIHGQ (sense) and L 121RGNHE (antisense). The numbering is based on mammalian PP1. This primer pair was designed to amplify all members of the PP1/PP2A/PP2B family of protein phosphatases. An additional set of primers, designed to amplify specifically the PP1-type enzyme, used the sense primer mentioned above plus an additional antisense primer based on the sequence N277YCGEFD. This sequence is highly conserved in all characterized PP1s from mammals to higher plants (4). Analysis of the amplified products with either primer set revealed the presence of two unique nucleotide sequences. The sequences obtained using the PP1/PP2A/PP2B “universal” primer pair were also contained in one of the two sequences obtained by using the PP1-specific primer pair. The complete nucleotide sequences for both phosphatase genes were obtained by screening a T. cruzi genomic library. One gene, containing an open reading frame that has a higher level of homology to that of human PP1 than to that of human PP2A or PP2B, encodes a protein designated TcPP1α. The other gene product also was more related to human PP1 than to human PP2A or PP2B. In a comparison of 21 PP1-like enzymes from separate phyla, it was found that the level of sequence identity ranged from 54 to 100%. The product of this T. cruzi phosphatase gene falls within the lower range of sequence identity for PP1-like enzymes and was designated TcPP1β. Barton et al. (4) identified 42 invariant residues in 44 eukaryotic PP1-PP2-PP2B by multiple sequence alignment. In TcPP1α, one of these invariant residues is not conserved (E139 to a D), whereas in TcPP1β there are three changes: S100 to G, F235 to A, and P270 to S. However, that fact that all of these residues are either not conserved or absent in the λ bacteriophage phosphatase ORF221 suggests that they are not essential for phosphatase activity. Importantly, the residues in mammalian PP1 (N277YCGEFD) responsible for the interaction with a variety of toxins, including calcullin A, are totally conserved in both T. cruzi sequences (30).

Both putative phosphatase genes were expressed as recombinant proteins in E. coli by using pTACTAC, a vector with a trp-lac hybrid promoter that was used successfully to express the human PP1 isoforms (29). Recombinant TcPP1β was shown to catalyze the dephosphorylation of 32P-labeled phosphorylase a and exhibited inhibitor sensitivities similar to those of its mammalian counterpart; i.e., it was preferentially inhibited by calcullin A over okadaic acid. Efforts to obtain high-level phosphatase activity against 32P-labeled phosphorylase a by using the TcPP1α-pTACTAC construct were unsuccessful. It is unclear whether the lack of phosphatase activity is due to poor protein expression or inappropriate folding or whether TcPP1α has a restricted substrate specificity. In contrast to TcPP1β, upon SDS-PAGE, there was no distinct band of the appropriate size in E. coli containing this expression vector.

No other phosphoserine/threonine phosphatase genes were...
FIG. 7. Electron microscopy of Calyculin A-treated *T. cruzi* epimastigotes. (A) SEM of a normal untreated epimastigote. (B) SEM view of an extreme example of arrested cytokinesis in a calyculin-treated epimastigote that has initiated but failed to complete cytokinesis two or three times. (C) SEM view of a treated spheromastigote-like organism with three flagella. (D) TEM photograph of a treated epimastigote, showing three kinetoplasts; the large kinetoplast appears to be preparing to undergo replication. (E) TEM photograph of a treated epimastigote; two kinetoplasts and a single nucleus are evident.
amplified from *T. cruzi* genomic DNA with the universal PP1/PP2A/PP2B primer pair under the conditions employed. Since trypanosomids diverged early in the eukaryotic lineage, it is possible that the sequences chosen for the amplification primers, although extremely highly conserved across different phyla, have been modified to such an extent in other *Trypanosoma* species in the way that amplification will not occur. An alternative explanation is that PP2A/PP2B-type phosphatases do not exist in lower-eukaryotic protozoa. However, PP2A-type phosphatases have been identified in the African trypanosome *T. brucei* (10) and in *Plasmodium falciparum* (18).

We used calyculin A and okadaic acid to explore the role of the PP1- and PP2A-type phosphatases in *T. cruzi*. The differences in inhibitor specificity have allowed investigation of the role of PP1 and PP2A in intact cells (11, 15, 16, 28). Calyculin A, but not okadaic acid, had profound effects on the growth and morphology of *T. cruzi* epimastigotes. In the presence of 1 to 10 nM calyculin A, there was cessation of cell replication accompanied by the formation of morphologically abnormal organisms. Microscopic studies revealed that duplication of major organelles, including the flagellum, kinetoplast, and nucleus, occurred in the presence of calyculin A; cytokinesis, however, was arrested. The loss of the typical trypanosomal morphology suggested that major alterations to the subpellicular microtubular network had occurred. In mammalian fibroblast and epithelial cell lines, both calyculin A and okadaic acid caused the selective breakdown of stable, but not dynamic, microtubules, suggesting that PP1 and PP2A are involved in the regulation of microtubule stability (15). It was recently reported that calyculin A promoted the extracellular transformation of *T. cruzi* trypomastigotes to amastigote-like forms (14). Calyculin A caused trypomastigotes to lose their characteristic spindle shape and adopt a spherical shape typical of amastigotes. In addition to these morphological alterations, calyculin A also induced the expression of amastigote-specific epitopes and caused a repositioning of the kinetoplast.

In the African trypanosome *T. brucei*, okadaic acid was used to uncouple nuclear and organelle (i.e., kinetoplast) segregation (8). In these organisms, nuclear DNA duplicated and segregated while kinetoplast DNA duplicated but did not segregate to form new organelles. Moreover, flagellar duplication was incomplete and the organisms retained their elongated morphology. In contrast, in calyculin A-treated *T. cruzi*, both kinetoplast and nuclear DNA duplicated and segregated, forming new organelles, and there was also complete flagellar duplication. Although the African and American trypanosomes are classified in the same genus, vast genetic distances (12% divergence) separate *T. brucei* and *T. cruzi* and may account for the differences observed (27).

The fact that cessation of epimastigote replication and the occurrence of morphological changes were observed with calyculin A but not with okadaic acid suggests that inhibition of a PP1-type enzyme(s) was responsible for both phenotypes. We have also shown that the major phosphatase activity in *T. cruzi* extracts capable of dephosphorylating $^{32}$P-labeled phosphorylase a is inhibited by low concentrations of calyculin A but not by okadaic acid. These in vivo and in vitro inhibitor studies suggest that calyculin A-sensitive PP1-type enzymes are the major cellular phosphatases in *T. cruzi*. It is likely that the two phosphatase genes characterized in this study are responsible for the calyculin A phenotypes observed in this study and by others (14). Moreover, the recombinant protein encoded by the *TcPP1β* gene exhibited calyculin A-sensitive phosphatase activity. Since both PP1-like phosphatases appear to be encoded by single-copy genes, we are in a position to create strains of *T. cruzi* lacking either or both phosphatases by homologous recombination. In addition, transfection studies will allow us to overexpress each phosphatase specifically. Such experiments will allow us to explore the physiological role of each phosphatase in the growth and development of the parasite.

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### REFERENCES


