

A novel protein phosphatase 2A (PP2A) is involved in the transformation of human protozoan parasite *Trypanosoma cruzi*

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Here we provide evidence for a critical role of PP2As (protein phosphatase 2As) in the transformation of *Trypanosoma cruzi*. In axenic medium at pH 5.0, trypomastigotes rapidly transform into amastigotes, a process blocked by okadaic acid, a potent PP2A inhibitor, at concentrations as low as 0.1 μ M. 1-Norokadaone, an inactive okadaic acid analogue, did not affect the transformation. Electron microscopy studies indicated that okadaic acid-treated trypomastigotes had not undergone ultrastructural modifications, reinforcing the idea that PP2A inhibits transformation. Using a microcystin–Sepharose affinity column we purified the native *T. cruzi* PP2A. The enzyme displayed activity against ³²P-labelled phosphorylase *a* that was inhibited in a dose-dependent manner by okadaic acid. The protein was also submitted to MS and, from the peptides obtained, degenerate primers were used to clone a novel *T. cruzi* PP2A enzyme by PCR. The isolated gene encodes a protein of 303 amino acids, termed TcPP2A, which

displayed a high degree of homology (86%) with the catalytic subunit of *Trypanosoma brucei* PP2A. Northern-blot analysis revealed the presence of a major 2.1-kb mRNA hybridizing in all *T. cruzi* developmental stages. Southern-blot analysis suggested that the *TcPP2A* gene is present in low copy number in the *T. cruzi* genome. These results are consistent with the mapping of *PP2A* genes in two chromosomal bands by pulsed-field gel electrophoresis and chromoblot hybridization. Our studies suggest that in *T. cruzi* PP2A is important for the complete transformation of trypomastigotes into amastigotes during the life cycle of this protozoan parasite.

Key words: enzyme purification, expression, gene cloning, genomic organization, phosphatase-specific inhibitor, trypanosome transformation.

INTRODUCTION

Trypanosoma cruzi is the causative agent of Chagas' disease that affects 16–18 million people in Latin America. The infection is initiated by metacyclic trypomastigotes present in the faeces of triatomine bugs. Trypomastigotes invade vertebrate host cells and immediately come into contact with the lysosomal compartment [1]. The acidic milieu of lysosomes induces the transformation of trypomastigotes into amastigotes [2], which replicate free in the cytoplasm and a few days later transform back into trypomastigotes that are released into the bloodstream after rupturing the host cell. Thus on two occasions during its intracellular development, *T. cruzi* undergoes dramatic changes in cellular shape and volume, restructures its flagellum and kinetoplast, and synthesizes new sets of surface molecules. These striking modifications are precisely timed, take place in an orderly fashion, and must involve specific proteolytic and signalling pathways.

Little is known about the signals and molecular mechanisms involved in trypanosome differentiation and proliferation. It was shown previously that *T. cruzi* transformation is proteasome-dependent [3]. Like other eukaryotes, protein phosphorylation and signal transduction pathways are associated with the control of this complex process in trypanosomes [4]. In higher eukaryotes, the reversible phosphorylation of proteins (on serine, threonine and tyrosine residues) plays a key role in the integration of signals involved in cellular proliferation and differentiation [5,6]. Similar regulatory pathways also exist in *T. cruzi* and could be in-

involved in parasite differentiation. cAMP, an important second messenger in higher eukaryotes, has been reported to be involved in the transformation of the epimastigote to metacyclic trypomastigote in the insect vector [7], and in the control of proliferation and differentiation of amastigotes [8]. Several serine/threonine protein kinase activities, including protein kinase A [9], protein kinase C [10] and a calcium- and calmodulin-dependent protein kinase [11], have been detected in *T. cruzi* epimastigotes. However, information about serine/threonine PPs (protein phosphatases) in this organism is limited to the report of two type 1 PPs [12].

In eukaryotic cells, four major classes of PP have been identified: PP1, PP2A, PP2B and PP2C [6,13,14]. This classification is based on the use of specific activators and inhibitors, the substrate specificity and the bivalent 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 family, termed the PPP family, which share a conserved catalytic core of approx. 280 amino acids [13]. On the other hand, PP2C is structurally and mechanistically unrelated to the PPP family and has been classified as a member of the PPM family of Mg²⁺-dependent PPs.

In mammalian cells, PP2A is a major protein serine/threonine phosphatase that plays an important role in processes regulated by reversible protein phosphorylation [5,15,16]. PP2A exists as holoenzymes: the basic structure contains an invariant core dimer that is composed of a highly conserved 36-kDa catalytic

Abbreviations used: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulphoxide; mAb, monoclonal antibody; PFGE, pulsed-field gel electrophoresis; PP, protein phosphatase; PP2Ac, catalytic subunit of PP2A; Tos-Lys-CH₂Cl, tosyl-l-lysylchloromethane; Q-TOF, quadrupole time-of-flight.

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The nucleotide sequences data reported are available in the GenBank[®] Nucleotide Sequence Database under the accession numbers AF510320 and AY158228.

subunit (PP2Ac) tightly bound to a 65-kDa regulatory subunit (PR65/A). PR65/A acts as a scaffold protein for the binding of PP2Ac and a large number of B-type regulatory subunits in the heterotrimeric holoenzyme [15,16]. The three major families of B-type regulatory subunits, PR55/B [17], PR61/B' [18] and PR72/B'' [19] share no significant similarity in primary structure. This diversity is believed to determine the enzymic activity and substrate specificity of PP2Ac, as well as intracellular localization and tissue specificity of distinct holoenzyme forms [5,16].

The experiments described below were designed to show the presence and involvement of PP2A in the developmental pathways of the human protozoan parasite *T. cruzi*. One practical advantage of this organism as an experimental model is that trypomastigote forms can be induced to change rapidly into the amastigote form in axenic medium. The resulting amastigote-like parasites cannot be distinguished from intracellular amastigotes by light or electron microscopy, or by stage-specific surface markers. Thus in this model, the effect of phosphatase inhibitors on transformation can be studied independently of their effect on the host cells.

EXPERIMENTAL

Material and reagents

Kinase and phosphatase inhibitors were purchased from Calbiochem (La Jolla, CA, U.S.A.) except as otherwise noted. Staurosporin and genistein were from Sigma (St. Louis, MO, U.S.A.).

Inhibition of transformation of trypomastigotes into amastigotes

T. cruzi (G strain) epimastigotes and metacyclic trypomastigotes were grown at 28 °C in liver-infusion tryptose broth as described previously [3]. Metacyclic trypomastigotes were separated from residual epimastigotes by anion-exchange chromatography [20]. Vero cells were infected with trypomastigotes as described previously [3]. Then, 5 days later, supernatants contained more than 95 % trypomastigotes that were collected by centrifugation. Trypomastigote transformation into amastigotes was induced as reported previously [2]. To assay the effect of kinase and phosphatase inhibitors on *T. cruzi* transformation, 2-fold dilutions of each inhibitor were distributed in 96-microwell plates. Dilutions were made with DMEM (Dulbecco's modified Eagle's medium) buffered with 20 mM Mes (pH 5.0) containing 0.4 % BSA. All inhibitors were dissolved in DMSO at 200 μ M and added to wells to final concentrations of 0.1–10 μ M. Trypomastigotes were centrifuged (3000 *g* for 10 min) and resuspended at 2×10^7 /ml in DMEM (pH 5.0); 50 μ l of this suspension were added to each well, mixed and incubated for 4 h at 37 °C in an atmosphere containing 5 % CO₂. The percentage of transformed parasites was estimated by microscopically scoring 250 cells in each well in a blinded fashion. All experiments were carried out in triplicate.

Microcystin affinity purification of *T. cruzi* PP2A

PP2A purification was performed according to Collins and Sim [21], with slight modifications. Briefly, epimastigotes from 7-day cultures (late logarithmic phase) were collected by centrifugation at 2000 *g* for 20 min and washed three times with Tris-buffered saline. The pellet was then resuspended in 5 vol. of buffer A (50 mM triethanolamine, 0.3 mM EGTA, 5 % glycerol, 0.5 M NaCl, 1 mM MnCl₂ and 0.1 mM dithiothreitol, pH 7.5) containing a mixture of protease inhibitors [25 μ g/ml E-64, 25 μ g/ml leupeptin, 1 mM benzamidine, 1 mM PMSF, 0.5 mM

Tos-Lys-CH₂Cl (tosyl-lysylchloromethane, or 'TLCK'), 10 μ g/ml soya bean trypsin inhibitor, 0.1 μ g/ml aprotinin and 2 μ g/ml pepstatin A] and lysed by 10 cycles of freezing in liquid N₂ and thawing at 30 °C. The homogenate was clarified by centrifugation for 15 min at 8000 *g*, and the supernatant was centrifuged at 100 000 *g* for 1 h. The supernatant was filtered in acrodisc 0.45 μ m (Gelman, Ann Arbor, MI, U.S.A.), poured into a column containing 0.5 ml of microcystin–Sepharose (Upstate Biotechnology, Lake Placid, NY, U.S.A.) and incubated under gentle agitation for 4 h at 4 °C. The column was washed extensively with buffer A containing 1 M NaCl and 0.5 mM MnCl₂, followed by incubation at 4 °C for 2 h with 3 M KSCN in buffer A and 0.5 mM MgCl₂. The bound phosphatase was eluted in the same buffer and dialysed immediately against buffer A containing 150 mM NaCl and 0.5 mM MgCl₂.

Immunoprecipitation studies

For immunoprecipitation studies a native PP2A was purified as described above, where parasites were harvested from 35 l of a 6-day-old *T. cruzi* epimastigote culture. The purified protein was immunoprecipitated as described previously [3] using a commercial mAb (monoclonal antibody) raised against the catalytic domain of human PP2A (Transduction Laboratories, San Diego, CA, U.S.A.). The immunocomplexes were collected by incubation with 100 μ l of a 50 % suspension of Protein G–Sepharose (Amersham Biosciences, Piscataway, NJ, U.S.A.). The immunoprecipitates were washed and PP2A activity measured in the presence or absence of PP inhibitors. In order to demonstrate that native protein was recognized by the mAb, it was separated by SDS/PAGE, transferred to PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.), blocked with PBS containing 5 % skimmed milk and incubated with mAb against human PP2A or an unrelated mAb (25A10, raised against *T. cruzi* proteasome), for 1 h at room temperature. After several washes in PBS containing 0.05 % Tween 20, the membrane was incubated with anti-mouse IgG conjugated with peroxidase. The final reaction was revealed by chemiluminescence using an ECL Western-blotting detection reagent (Amersham Biosciences, Piscataway, NJ, U.S.A.). For co-immunoprecipitation with cytoskeletal proteins, trypomastigotes were dissolved in ice-cold lysis buffer (25 mM Tris, 150 mM NaCl, 0.5 % Nonidet P40, 5 mM EGTA, 25 μ g/ml E-64, 25 μ g/ml leupeptin, 1 mM benzamidine, 1 mM PMSF, 0.5 mM Tos-Lys-CH₂Cl, 10 μ g/ml soya bean trypsin inhibitor, 0.1 μ g/ml aprotinin and 2 μ g/ml pepstatin A) and incubated for 30 min at 4 °C. After centrifugation the supernatant was pre-cleared by incubation with an excess of Protein G–Sepharose. Immunoprecipitation was performed as described above, using commercial mAb raised against α -tubulin, actin and myosin (Sigma). The immunoprecipitates were washed and PP2A activity measured in the presence or absence of PP inhibitors.

Protein determination and SDS/PAGE

Protein concentration was determined using the Bio-Rad protein assay (Bio-Rad). Samples were separated by SDS/PAGE according to [22] in 3 % stacking and 12 % separating gels, and silver stained as described in [23]. Bands were cut and submitted to MS.

Identification of native PP2A by MS analysis

The analysis was performed in the Protein Analysis Facility, Skirball Institute of Biomolecular Medicine (New York University

School of Medicine, NY, U.S.A.), as reported previously [24]. The resulting peptide mixture was analysed by nano-electrospray tandem MS in a Q-TOF (quadrupole time-of-flight) mass spectrometer (Micromass, Beverly, MA, U.S.A.). The amino acid sequences obtained in this way were searched against the NCBI non-redundant protein database using the BLAST search program [25].

Phospho-immunoblot analysis of cytoskeletal proteins in the flagellar fraction of *T. cruzi*

In a standard experiment, 1×10^7 trypomastigotes were incubated at 37 °C in DMEM in the presence or absence of okadaic acid or 1-norokadaone, and aliquots were taken at time 0 (pH 7.2) and 0.5, 1, 2, 3 and 4 h later in transformation medium (pH 5.0). The parasites were centrifuged and the cytoskeletal fraction was extracted from the pellets as described previously [26]. Briefly, pellets were incubated at 4 °C in buffer containing phosphatase and protease inhibitors (60 mM Pipes, 25 mM Hepes, pH 7.4, 10 mM EGTA, 2 mM MgCl₂, 0.5 % Triton X-100, 25 µg/ml aprotinin, 25 µg/ml E-64, 25 µg/ml leupeptin, 1 mM benzamide, 1 mM PMSF, 0.5 mM Tos-Lys-CH₂Cl, 10 µg/ml aprotinin, 2 µg/ml pepstatin A, 1 mM iodoacetamide, 2.5 mM NaVO₄, 50 mM NaF and 20 mM sodium pyrophosphate). Cytoskeleton proteins were washed twice in the same buffer and extracted with the buffer containing 1 M NaCl. The resulting material was dissolved with 6 M urea for 1 h on ice and subjected to SDS/PAGE as described above. The proteins were transferred to PVDF membrane (Bio-Rad, Hercules, CA, U.S.A.), blocked with PBS containing 5 % skimmed milk and incubated with a mouse mAb anti-phosphoserine (Sigma) diluted in PBS/5 % skimmed milk, for 1 h at room temperature. After several washes in PBS containing 0.05 % Tween 20 the membrane was incubated with anti-mouse IgG conjugated to peroxidase and developed as described above.

Transmission electron microscopy

Parasite suspensions were fixed in 3 % (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate, pH 7.2, for 48 h at room temperature (20 °C). The fixed trypanosomes were centrifuged (1000 g) for 30 s, and the supernatant was removed. The pellet was resuspended in previously warm (45 °C) 0.1 M sodium cacodylate, pH 7.2, containing 2 % (w/v) agar and allowed to cool to room temperature. All subsequent procedures employed cold (4 °C) solutions through 95 % ethanol; 100 % ethanol and propylene oxide were used at room temperature. The solidified agar was cut into small cubes (1–2 mm³) and fixed for 24 h in PBS/2.5 % glutaraldehyde. The agar cubes were rinsed several times in PBS and then post-fixed in PBS/1 % (w/v) 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 resin Embed 812. Thin sections were obtained with a Sorvall MT-II microtome and placed on copper grids. Samples were stained with 4 % uranyl acetate in methanol and lead citrate, and examined in a Philips Tecnai 12 Bio Twin transmission electron microscope operated at 80 kV.

Scanning electron microscopy

Parasite suspensions were spread on acid-washed 18-mm-diameter circular coverslips and partially air-dried. The coverslips were placed in 2.5 % (v/v) glutaraldehyde buffered with 0.1 M

sodium cacodylate (pH 7.2) for 12 h at 4 °C, rinsed several times in PBS, post-fixed in PBS/1 % (v/v) osmium tetroxide for 2 h, rinsed several times in PBS, and dehydrated in a graded ethanol series. The coverslips were then placed in acetone and critical-point dried from liquid CO₂ in a Tousimis Samdri-790 critical-point drier (Tousimis Research Corp., Rockville, MD, U.S.A.). Coverslips were mounted on scanning electron microscopy aluminium stubs with silver paint and sputter coated in a Denton Du-502 vacuum evaporator (Denton Vacuum, Cherry Hill, NJ, U.S.A.) equipped with a gold target. The critical-point-dried and sputter-coated trypanosomes were then examined on a Super IIIA or an SS-40 scanning electron microscope (ISI International Scientific Instruments, Santa Clara, CA, U.S.A.) operated at 15 kV.

Isolation of nucleic acids: Northern- and Southern-blot hybridizations

Epimastigotes (4×10^9 cells) were lysed in 10 mM Tris/HCl, pH 8.0, containing 100 mM EDTA, 0.5 % SDS, 20 µg/ml RNase and 100 µg/ml proteinase K for 3 h at 50 °C. After phenol/chloroform extraction, the DNA was precipitated with 0.2 vol. of 10 M ammonium acetate and 2 vol. of ethanol. Total RNA from epimastigotes, tissue-culture trypomastigotes, metacyclic trypomastigotes and *in vitro*-transformed amastigotes were isolated with Trizol (Gibco-BRL, Gaithersburg, MD, U.S.A.) according to the manufacturer's instructions.

Total RNA (12 µg), isolated from different developmental stages of *T. cruzi*, was electrophoresed on 1 % agarose/formaldehyde gels and blotted on to nylon Hybond N membranes (Amersham Biosciences) using a vacuum blotter (Bio-Rad). Hybridization was performed overnight at 42 °C in a solution containing 50 % formamide and 5 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate); then the membranes were washed once with 1 × SSC/0.5 % SDS for 20 min at room temperature and then three times with 0.2 × SSC/0.5 % SDS for 20 min each time at 65 °C. Epimastigote genomic DNA was digested with restriction endonucleases, separated by electrophoresis in a 0.8 % agarose gel, transferred to nylon membrane and hybridized as described for Northern blots.

Chromosome-sized DNA molecules were prepared in agarose blocks and stored at 4 °C in 0.5 M EDTA, pH 8.0 [27]. Aliquots of 10^7 parasites were electrophoresed in 1.2 % agarose gel in 0.5 × TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.3) at 80 V for 132 h at 13 °C, with pulse times varying from 90 to 800 s, using a Gene Navigator apparatus (Amersham Biosciences) with a hexagonal electrode [27]. Chromosomal DNA bands were transferred to nylon membranes and hybridized as described previously [27].

Cloning of the PP2A gene

Based on amino acid sequences obtained by MS analysis, two degenerate oligonucleotide primers corresponding to conserved domains in PP2A were synthesized (Biosynthesis, Lewisville, TX, U.S.A.). The primers were YGSVNVW (Forward) (5'-TAYGGNAGTGTNAAAYGTNTGG-3') and LFEAAPD (Reverse) (5'-GCRTCNGGNGCNGCYTCRAAN-3'). The PCR mixture (50 µl) contained 1 µg of sheared *T. cruzi* genomic DNA, 120 pmol of each primer and 2.5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA, U.S.A.). PCR conditions were five cycles of 95 °C for 4 min, 95 °C for 1 min, 40 °C for 1 min and 72 °C for 1.5 min, then 35 cycles of 95 °C for 1 min, 45 °C for 1 min and 72 °C for 1.5 min, followed by 95 °C for 1 min and 72 °C

for 7 min in a PTC 100 Programmable Thermal Controller (MJ Research, Watertown, MA, U.S.A.). The PCR product of the expected size (522 bp) was isolated and cloned using a Topo TA Kit (Invitrogen, Carlsbad, CA, U.S.A.). Sequencing of cloned inserts was performed using the PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Wellesley, MA, U.S.A.) in an Applied Biosystems model 377 DNA automatic sequencer (Foster City, CA, U.S.A.).

PP2A gene was also isolated from a *T. cruzi* cDNA library constructed in vector pCMV-SPORT 6 (Gibco-BRL) using poly(A)⁺ mRNA from metacyclic trypomastigotes according to the manufacturer's instructions. The library was screened with a ³²P-labelled 522-bp PP2A amplicon described above, and the positive clones were purified and sequenced.

PP assay

PP2A activities of *T. cruzi* native or recombinant PP2A were determined by using a ³²P-labelled phosphorylase *a* assay system (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) according to the manufacturer's instructions. Cell extracts were prepared as described previously [12]. DH5- α *Escherichia coli* lysate samples were serially diluted 2-fold in 50 mM imidazole, pH 7.0, containing 2 mM MnCl₂, 1 mM EDTA, 2 mM dithiothreitol and 1% Triton X-100 prior to assay. One unit of phosphatase activity is defined as the amount that releases 1 nmol of [³²P]P_i from ³²P-labelled phosphorylase *a*/min. The concentrations of okadaic acid and calyculin A in the assay mixture ranged from 0 to 1 μ M.

RESULTS

Effect of okadaic acid on *T. cruzi* trypomastigote transformation

Trypomastigotes were incubated in the presence of different concentrations of genistein, staurosporine, herbimycin A, apigenin and okadaic acid, and their transformation into amastigotes assessed by light microscopy. Among the different kinase and PP inhibitors tested, only okadaic acid, a serine/threonine PP inhibitor, inhibited *T. cruzi* transformation. When the effect of different phosphatase inhibitors was evaluated at 10 μ M, only okadaic acid and calyculin A prevented transformation. On the other hand, tautomycin, a powerful inhibitor of PP1, blocked 50% of the transformation (Figure 1a). In order to investigate whether PP1 or PP2A were involved in transformation of trypomastigotes into amastigotes, we performed the transformation assay at pH 5.0 in the presence or absence of okadaic acid and tautomycin (Figure 1b). Even at low concentrations (0.1 μ M) okadaic acid had a profound effect on the transformation of trypomastigotes. In contrast, tautomycin at concentrations up to 10 μ M had moderate effect on parasite transformation. Moreover, 1-norokadaone, the cell-permeable inactive analogue of okadaic acid, did not affect transformation (Figure 1). In mammalian cells, okadaic acid is a potent PP2A inhibitor (the 50% inhibitory concentration, IC₅₀, is 2 nM), whereas higher concentrations are necessary for PP1 (IC₅₀, 60–200 nM) or PP2B (IC₅₀, 10 mM) inhibition. PP2C is unaffected by okadaic acid. In contrast, calyculin A inhibits both PP1 and PP2A, but not PP2B or PP2C, with high potency (IC₅₀, 0.5–1 nM). Tautomycin is a potent inhibitor of PP1 (IC₅₀, 1 mM) and 10-times-higher concentrations are necessary to inhibit PP2A (IC₅₀, 10 nM), whereas PP2B is weakly inhibited and PP2C is unaffected by this compound. These results suggest that PP2A-type enzymes are involved in parasite transformation. It should be stressed that during all incubations the parasites remained fully motile, indicating that okadaic acid is not toxic

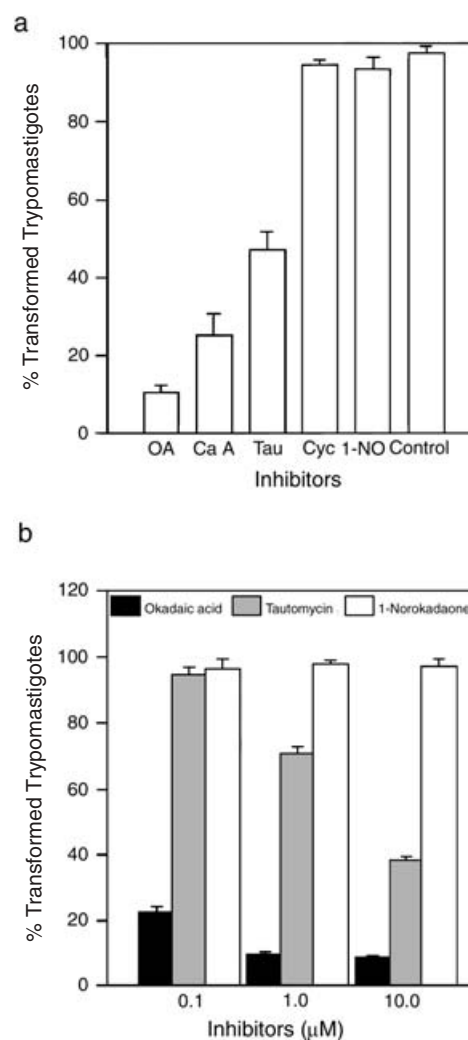


Figure 1 Effect of PP inhibitors on transformation of *T. cruzi* trypomastigotes into amastigotes

Parasites were incubated at 37 °C for 4 h in the transformation medium alone (Control) or containing 10 μ M of the following phosphatase inhibitors: okadaic acid (OA), calyculin A (CaA), tautomycin (Tau), cyclophosphamide (Cyc) or 1-norokadaone (1-NO) (a); or 0.1–10 μ M okadaic acid, tautomycin or 1-norokadaone (b). Transformation was scored in a double-blind fashion by light microscopy, and results expressed as means \pm S.D. from three independent experiments.

for the parasite. No effect or biological role had been described previously for PP2A in parasites.

The morphological changes induced by exposure of parasites to okadaic acid (0.1 μ M) were confirmed by scanning and transmission electron microscopy. Amastigotes derived from untreated trypomastigotes showed a characteristic slightly elongated form (Figure 2a) whereas okadaic acid-treated trypomastigotes, although not fully transformed, displayed an unusual morphology with posterior ends exhibiting considerable circularization (Figure 2b, arrows). Electron microscopy reveals that a typical amastigote structure was observed in untreated parasites (Figure 2c). However, okadaic acid treatment of trypomastigotes inhibited the reshaping of the kinetoplast and cells retained the flagellum and a characteristic basket-like appearance (Figure 2d). These results suggested that okadaic acid did not completely abolish all the steps required for transformation of trypomastigotes, but apparently arrested the process at an early stage.

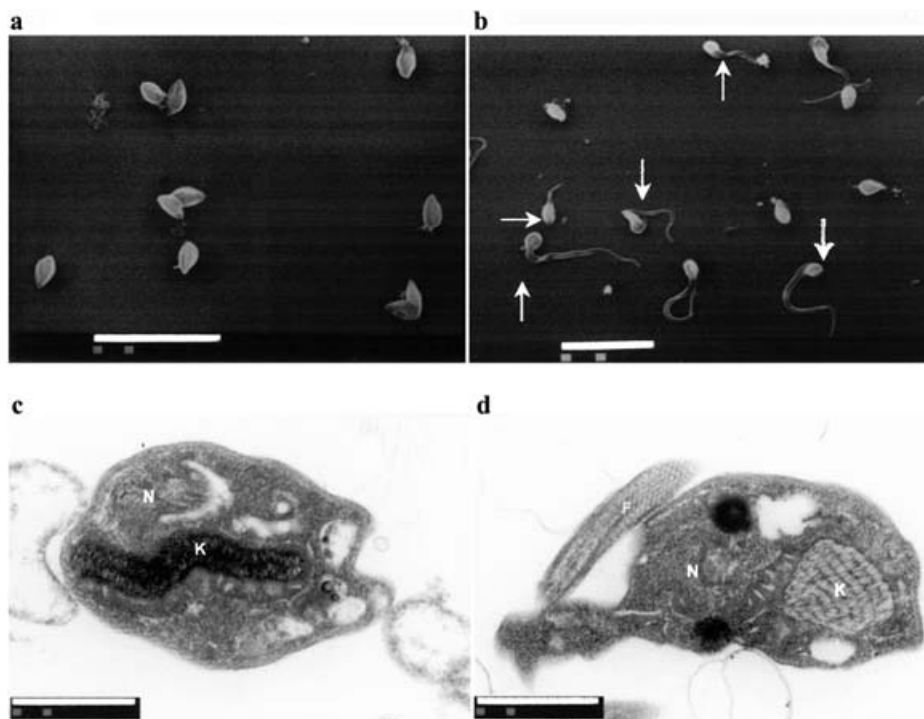


Figure 2 Scanning and transmission electron microscopic analysis of okadaic acid-treated and untreated *T. cruzi* after 4 h of incubation in DMEM at pH 5.0

Scanning electron microscopy of amastigotes obtained after differentiation of untreated trypomastigotes (**a**); okadaic-acid treated trypomastigotes that did not complete the transformation into amastigotes, showing rounded posterior ends (arrows; **b**). Transmission electron microscopy of amastigotes obtained from the transformation of untreated trypomastigotes, showed the characteristic amastigote rod-shaped kinetoplast (**c**), whereas treated trypomastigotes retained the round kinetoplast morphology (**d**). Scale bars: (**a**) and (**b**), 500 nm; (**c**) and (**d**), 10 μ m. N, nucleolus; F, flagellum; K, kinetoplast.

Cytoskeletal proteins of flagellar fraction are dephosphorylated during transformation

Figure 3(a) shows a Western blot of cytoskeletal proteins corresponding to the flagellar fraction stained with an anti-phosphoserine mAb. Although some proteins were phosphorylated at serine residues at pH 7.2, at least one polypeptide of around 60 kDa was phosphorylated soon after acidification. Furthermore, after 2 or 3 h of incubation at pH 5.0, polypeptides of 70 and 90 kDa were dephosphorylated. When experiments were performed in the presence of okadaic acid, all proteins remained phosphorylated, suggesting that phosphorylation and dephosphorylation processes occur during transformation and that serine/threonine phosphatases could be involved (Figure 3b). The inactive analogue of okadaic acid, 1-norokadaone, did not display any effect, as expected (results not shown).

Identification of okadaic acid target in *T. cruzi*

We used two approaches to identify the target of okadaic acid in *T. cruzi*. First, the native PP2A was isolated from *T. cruzi* epimastigotes by affinity chromatography on microcystin–Sephrose beads. This method is based on the observation that the cyanobacterial toxin microcystin covalently binds to Cys-273 on PP1 [28]. According to several reports, microcystin has been shown to be completely selective for the catalytic subunits of PP1 and PP2A. Furthermore, stringent column-washing conditions were employed throughout (1 M NaCl) to prevent non-specific ionic and hydrophobic interactions prior to elution. The eluted fractions were submitted to SDS/PAGE and four major bands of approx 34, 38, 45 and 55 kDa were identified (Figure 4). The major

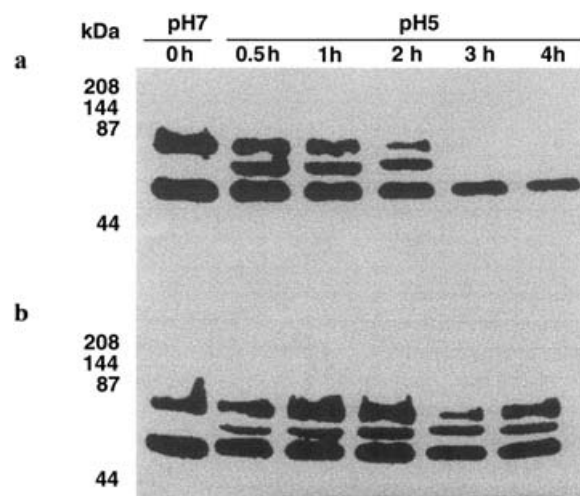


Figure 3 Phospho-immunoblot of *T. cruzi* trypomastigotes

Parasites were incubated in DMEM (pH 5.0) in the absence (**a**) or presence (**b**) of okadaic acid. Aliquots were collected at different time points between 0 and 4 h, lysed and submitted to SDS/PAGE. Proteins were transferred to PVDF membrane, incubated with a mAb against phosphoserine and developed by ECL. Molecular-mass standards are shown on the left.

bands were excised from the gel and subjected to MS using Q-TOF analysis. Four peptides (YGSVNVWR, QITQVYGFYDECLR, LFEAAPDDA and SHQLVMGYK) derived from the 34-kDa band shared high similarity with the catalytic domain of phosphoprotein phosphatase 2A (EC 3.1.3.16) of *Trypanosoma brucei*

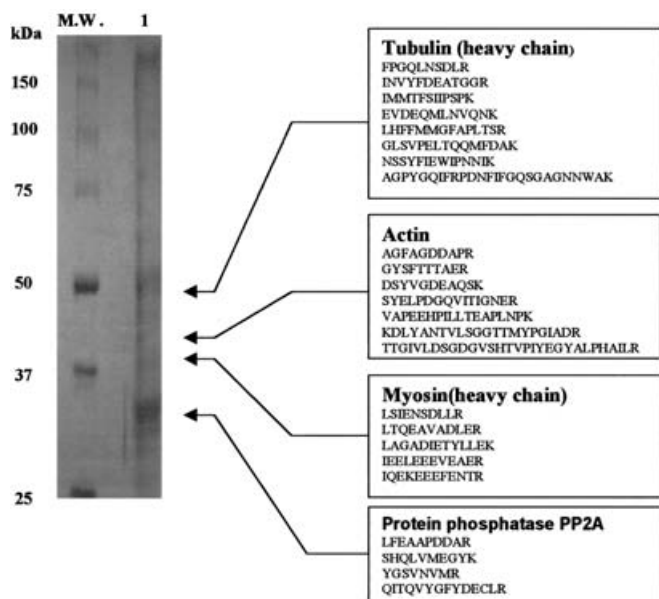


Figure 4 Purification of *T. cruzi* PP2A by microcystin-Sepharose affinity chromatography

Epimastigote extracts were incubated for 4 h at 4 °C with microcystin-Sepharose beads. Bound proteins were eluted with 3 M KSCN, submitted to SDS/PAGE and silver stained (lane 1). The major bands were identified by MS analysis. M.W., molecular-mass markers.

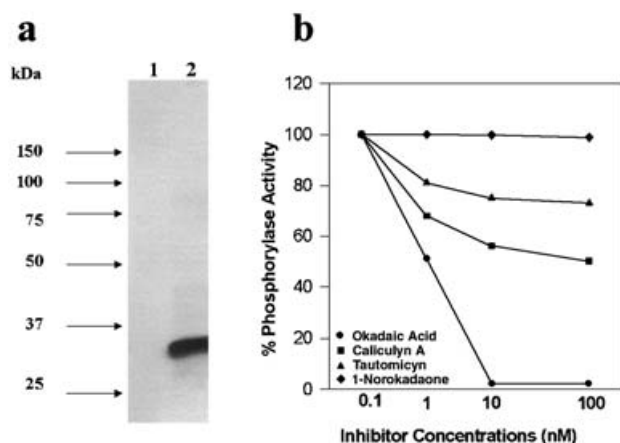


Figure 5 Phosphatase activity of native *T. cruzi* PP2A

(a) Affinity-purified TcPP2A was electrophoresed, blotted on to PVDF and revealed with an unrelated mAb 25A10 (lane 1) or a mAb raised against human PP2Ac (lane 2). (b) TcPP2A was immunoprecipitated with mAb against human PP2Ac and phosphatase activity against 32 P-labelled phosphorylase was assayed in the absence or presence of different concentrations of inhibitors and the inactive analogue 1-norokadaone. No phosphatase activity was detected when native enzyme was immunoprecipitated with an unrelated antibody.

[29]. The peptides obtained from the bands of 55, 45 and 43 kDa showed sequence similarity with α -tubulin, myosin and actin, respectively.

In order to demonstrate that mAb raised against human PP2Ac recognizes the *T. cruzi* PP2A (TcPP2A), a Western blot was performed. The affinity-purified enzyme was recognized by the mAb as a single band of 34 kDa, but not by the unrelated mAb 25A10 (Figure 5a). Secondly, PP2A activity was immunoprecipitated from the affinity-purified material using a

mAb raised against the catalytic region of human PP2A. Then immunoprecipitates were assayed for PP activity using 32 P-labelled phosphorylase *a* as a substrate. The PP2A activity was inhibited in a concentration-dependent manner by okadaic acid with an estimated IC_{50} of approx. 2 nM. The IC_{50} for inhibition of phosphatase activity by calyculin was approx. 100 nM. On the other hand, tautomycin only inhibited 20% of the phosphorylase *a* activity, and 1-norokadaone was completely inactive (Figure 5b).

Identification and characterization of genes encoding PP2A of *T. cruzi*

T. cruzi genomic fragments encoding PP2Ac were amplified by PCR using degenerate primers derived from the amino acid sequences (YGSVNVWR and LFEAAPD) of native PP obtained by MS. Amplification of genomic DNA with these primers gave rise to a 522-bp fragment, the size expected based on PP2A gene sequences from other organisms. The amplified fragment was cloned and the similarity of its amino acid sequence to catalytic subunits of PP2A was confirmed by BLASTN and BLASTP searches.

In order to isolate an entire copy of the PP2A gene, the 522-bp fragment was used as a probe to screen a trypanostigote cDNA library. Five clones were identified and one of them, named TcPP2A, was sequenced fully. The nucleotide sequence revealed an open reading frame starting at nucleotide 58 with a methionine codon and ending at nucleotide 966 with a TAG termination codon. The open reading frame encodes a 303-amino-acid polypeptide with a predicted molecular mass of 34.5 kDa, which is in agreement with the molecular masses of the native PP2A. The deduced amino acid sequence revealed that the consensus motifs VGDIH, GDYVDR, LRGNHE and SAPNYC, characteristic of serine/threonine PPs, are present in the catalytic region of the polypeptide coded by cDNA TcPP2A. In the same manner, the peptide sequences YGSVNVWR, QITQVYGFYDECLR, LFEAAPDDA and SHQLVMEGYK, obtained by MS, were also found in the peptide coded by PP2A cDNA. This finding strongly suggests that the product of the PP2A gene is the target of okadaic acid in the parasite. A BLASTP search [30] against sequences in the GenBank® database revealed a high identity (53–86%) with type 2A serine/threonine PPs from different species (Figure 6a). A high level of identity was observed with PP2A from *T. brucei* (86%) and *Arabidopsis thaliana* (61%), whereas *Homo sapiens*, *Drosophila melanogaster* and *Saccharomyces cerevisiae* displayed a lesser degree of identity (53–54%). A phylogenetic tree was constructed with seven PP2Ac (Figure 6b). The phenogram indicates the existence of two groups: one major group corresponding to many different eukaryotic organisms including human, and another group that comprises the PP2A of *T. brucei* and *T. cruzi*.

Genomic organization and transcription of PP2A genes

When genomic DNA was analysed by Southern blotting using the PP2A gene as a probe, a very simple hybridization profile was observed in each lane indicating the presence of a few copies of the PP2A gene (Figure 7a). Digestion with several enzymes (*Eco*RI, *Hind*III and *Sac*I) that do not cut within the PP2A gene produced two bands indicating the presence of two copies of this gene. Consistent with this, Southern-blot analysis of chromosomal DNA separated by pulsed-field gel electrophoresis (PFGE) revealed that PP2A genes were present in two chromosomal bands of 2.03 and 1.60 Mb (Figure 7b). These bands hybridized to the PP2A gene

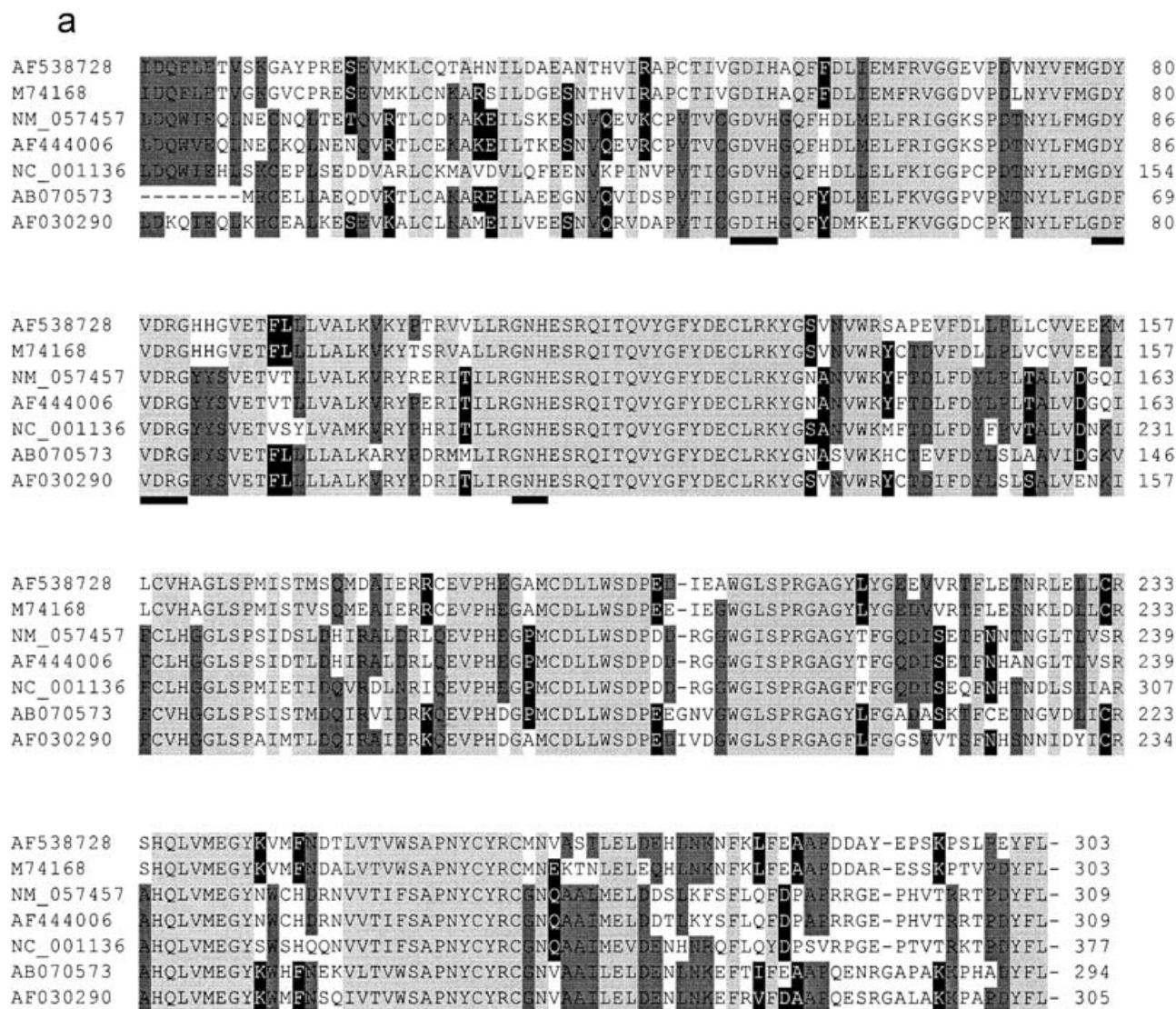


Figure 6 Sequence alignment of TcPP2A with different eukaryotic PP2A

(a) Comparison of *T. cruzi* PP2A (GenBank accession no. AY158228) with PPs from *T. brucei* (M74168), *A. thaliana* (AF030290), *C. elegans* (AB070503), *D. melanogaster* (NM_057457), *S. cerevisiae* (NC_001136) and *H. sapiens* (AF444006). Conserved residues are shaded in light grey (100% conservation), dark grey ($\geq 75\%$ conservation) and black ($\geq 50\%$ conservation) and no shading denotes residues with $< 50\%$ conservation. The four conserved motifs found in all members of the PPP family are underlined. (b) Sequence alignment and phenogram construction were performed using the Clustal method from LaserGene software. Numbers represent the percentage divergence.

probe with the same intensities, suggesting that they carry the same number of copies of the *PP2A* gene.

The steady-state level of *PP2A* transcripts in the *T. cruzi* developmental stages was determined by Northern-blot hybrid-

ization using the ^{32}P -labelled fragment of 522 bp as a probe. It hybridized with a major 2.1-kb mRNA that is present in all stages, suggesting that the *PP2A* gene is constitutively transcribed. Densitometric scanning analysis of the ethidium bromide-stained

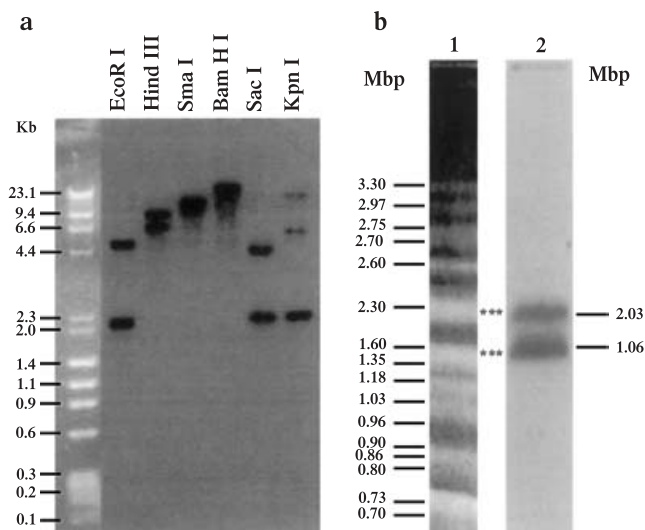


Figure 7 Genomic organization of *T. cruzi* PP2A genes

(a) Southern-blot hybridization of genomic DNA. Genomic DNA (4 μ g) was digested with the indicated restriction enzymes, electrophoresed in agarose gel, transferred to nylon membrane and hybridized to the *T. cruzi* PP2A gene probe. (b) Chromoblot hybridization. Chromosomal bands of epimastigotes were separated by PFGE, stained with ethidium bromide (lane 1), transferred to nylon membrane and hybridized with the same probe described above (lane 2). Standard size markers are in kb or Mb.

agarose gel and hybridization with the β -tubulin probe indicated that each lane carried equivalent amounts of RNA (results not shown).

DISCUSSION

PP2A is an important component of major regulatory pathways in higher eukaryotes, controlling a wide range of intracellular processes [5,6,14]. Although PP2A has been demonstrated in a broad range of organisms, including protozoan parasites like *T. brucei* [29] and *Plasmodium falciparum* [31], the enzyme had not yet been identified in *T. cruzi*. Even more, according to Orr et al. [12], no phosphoserine/threonine phosphatase gene other than the *PP1* gene had been amplified from *T. cruzi* genomic DNA when the universal PP1/PP2A/PP2B primers were used in PCR amplification. They suggested that PP2A-/PP2B-type phosphatases could not exist in this lower-eukaryotic protozoan. However, in this paper we provide conclusive evidence for a critical role for PP2A in the transformation of *T. cruzi*. Furthermore, the PP2A protein was identified and the corresponding gene was cloned and characterized.

The availability of highly specific inhibitors of PP1 and PP2A [32,33] has provided useful tools to investigate the role of PP2A in the transformation of *T. cruzi*. The pattern of sensitivity to these compounds suggests strongly that PP2A-type enzymes were involved in parasite transformation. In *T. brucei*, okadaic acid has been used to uncouple nucleus and kinetoplast segregation [34]. In the presence of this drug the kinetoplast DNA was able to replicate but segregation and formation of new organelles were inhibited. Moreover, flagellar duplication was incomplete and the organisms retained their elongated morphology. In contrast, *T. cruzi* trypomastigotes are not replicative forms, and consequently neither organelle segregation nor flagellar duplication occurs in this stage. However, the fact that okadaic acid-

treated trypanosomes did not appear to retain their shape after treatment suggests a common role for PP2A in trypanosome transformation.

Microcystin-Sepharose chromatography in association with MS analysis allowed us to identify a 34-kDa band corresponding to *T. cruzi* PP2A with three additional bands corresponding to myosin, actin and α -tubulin (Figure 4). According to previous reports using several cell types, microcystin was completely selective for the catalytic subunits of PP1 and PP2A. Therefore only these proteins and their associated regulatory subunits should be recovered by this approach [35,36]. However, we believe that the identification of proteins other than phosphatases was not fortuitous, because the contamination of the preparation with these proteins could be ruled out due to the high stringency of washing conditions employed before the elution of proteins specifically bound to the column. In fact, actin, myosin and α -tubulin have been identified previously as components of the *T. cruzi* cytoskeleton [37,38]. Then our findings strongly suggest that *T. cruzi* PP2A could be associated with the parasite cytoskeleton as has been shown in other cell types [39,40]. This possibility makes sense if we consider that, in mammalian cells, protein phosphorylation/dephosphorylation is an essential regulatory mechanism for maintaining the functionality of the cytoskeleton [41,42]. In another flagellate, it has been reported that PP1 and PP2A are located in distinct positions in the *Chlamydomonas* flagellar axoneme [43]. We could then speculate that PP2A participates in the transformation of *T. cruzi* regulating microtubule stability. Considering that okadaic acid-treated trypomastigotes maintained the elongated form and that its flagellum remained after incubation in DMEM at pH 5.0 for 4 h, we could imagine that transformation requires dephosphorylation of some unidentified protein(s). After dephosphorylation, the flagellar proteins are degraded and the parasite finally reaches the typical round form. The observations that flagellar proteins are phosphorylated and dephosphorylated during transformation, and that dephosphorylation is inhibited by okadaic acid but not by 1-norokadaone, come to support this hypothesis (Figure 3). To demonstrate this hypothesis, PP activity was co-immunoprecipitated with *T. cruzi* cytoskeletal proteins using mAbs raised against α -tubulin, actin and myosin. This activity was inhibited between 30 and 50% by okadaic acid but not by tautomycin or 1-norokadaone (results not shown). These results strongly suggest that PP2A among other PPs could be physically associated with the parasite cytoskeleton.

Although the *T. cruzi* PP2A enzyme was isolated and characterized, the PP2A target(s) and molecular mechanism where this signal component is involved remains elusive. A role for phosphorylation cascade in the transformation of trypomastigotes into amastigotes has been postulated [44]. In fact, calyculin A, a potent inhibitor of PP1A and PP2A, induced transformation of *T. cruzi* trypomastigotes into amastigotes at pH 7.5 [45]. According to these authors, an exposure of trypomastigotes to calyculin A concentrations as low as 1 nM for only 1–2 h was sufficient to induce transformation. However, in a different experiment, where trypomastigotes were pre-incubated with okadaic acid or calyculin A for 60–90 min at 37 °C in DMEM, pH 7.4, we were unable to detect any parasite transformation. The reasons for the differences from the observations reported by Grellier et al. [44] are not clear. Considering that these authors have worked with different *T. cruzi* strains, we could speculate that strain-dependent signal transduction pathways could be involved in *T. cruzi* transformation. On the other hand, activation of the inositol phosphate/diacylglycerol signalling cascade by acidic conditions could have an important role in the *T. cruzi* differentiation process [45].

The *T. cruzi* PP2A gene shares a high level of homology with *T. brucei* PP2A (86%). When *T. cruzi* PP2A was compared with the enzyme from different organisms, it was found that the level of identity ranged from 53 to 86% (Figure 6a). In addition, four conserved structural motifs from the catalytic domain, GDXHG, GDXVXRG, GNH and SAPNYC, found in all PPP proteins examined thus far [16,29,46], are also present in trypanosome phosphatases as well as in PP2A described here.

The amino acid sequence also reveals the presence of the LFEAAPDD motif that appears to be unique for trypanosomes, considering that is also present in *T. brucei* and appears less conserved or even absent in other species. On the other hand, the sequence YGSVNVW that is highly conserved in the PP2A catalytic site and absent in PP1 was present in *T. cruzi* PP2A (Figure 6a).

The C-terminal sequence PDYFL of the PP2Ac subunit is completely conserved among mammals, yeast, fruit flies and plants, suggesting that regulation of this enzyme activity by C-terminal methylation has been conserved throughout evolution. A methylation motif is also present in *T. cruzi* PP2A, but it contained aspartic instead of glutamic acid (Figure 6a). The significance of this modification is not clear but considering that glutamic and aspartic acids are negatively charged amino acids, we could speculate that the structural and functional features of the enzyme should remain intact.

The alignment and phenogram analyses showed that this enzyme shares extraordinary amino acid similarity with those from *T. brucei*, suggesting an evolutionary link via a common ancestor gene. On the other hand, the degree in sequence identity between trypanosomal and mammalian phosphatases is remarkable even considering the early divergence of the trypanosomatids from the main branch of the eukaryotic lineage. Thus it seems reasonable to believe that conserved domains between trypanosomal and higher-eukaryotic PP2Ac should be relevant from a functional point of view (Figure 6b).

Southern-blot hybridizations suggest that PP2A genes are present in at least two copies per genome (Figure 7a). PP2A genes were mapped in two chromosomal bands separated by PFGE in the G and CL strains, confirming the presence of two copies of the PP2A gene (Figure 7b). Gene cloning has revealed the existence of two PP2A isoforms in mammals [47], whereas in *Drosophila* [48] and *T. brucei* [29] a single PP2A gene was found.

Native PP2A was shown to catalyse the dephosphorylation of ³²P-labelled phosphorylase *a* and exhibited inhibitor sensitivities similar to those of its mammalian counterpart. Considering that the native protein has at least four amino acid motifs that are also present in the deduced amino acid sequence of the isolated gene, we can conclude that the actual target of okadaic acid during *T. cruzi* transformation is PP2A.

Taken together, our results are consistent with the presence of a PP2A in *T. cruzi* that is involved in parasite transformation. The enzyme displays high homology with *T. brucei* PP2A but less significant homology with the human enzyme. These findings open new possibilities for the rational design of chemotherapeutic agents to control Chagas' disease and *T. cruzi* PP2A could be considered as a new target, especially because of the differences from its human counterpart. This possibility becomes even more attractive in view of the fact that other signal components like protein kinases have already been proposed as drug targets in parasitic protozoa [49].

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REFERENCES

- Burleigh, B. A. and Andrews, N. W. (1995) The mechanisms of *Trypanosoma cruzi* invasion of mammalian cells. *Annu. Rev. Microbiol.* **49**, 175–200
- Tomlinson, S., Vanderkerckhove, F., Frevert, U. and Nussenzweig, V. (1995) The induction of *Trypanosoma cruzi* trypomastigote to amastigote transformation by low pH. *Parasitology* **110**, 547–54
- González, J., Ramalho-Pinto, F. J., Frevert, U., Ghiso, J., Tomlinson, S., Scharfstein, J., Corey, E. J. and Nussenzweig, V. (1996) Proteosome activity is required for the stage-specific transformation of protozoan parasite. *J. Exp. Med.* **184**, 1909–1918
- Parsons, M. and Ruben, L. (2000) Pathways involved in environmental sensing in trypanosomatids. *Parasitol. Today* **16**, 56–62
- Lechward, K., Awotunde, O. S., Swiatek, W. and Muszynska, G. (2001) Protein phosphatase 2A: variety of forms and diversity of functions. *Acta Biochim. Pol.* **48**, 921–933
- Cohen, P. (1992) Signal integration at the level of protein kinases, protein phosphatases and their substrates. *Trends Biochem.* **17**, 408–413
- Gonzales-Perdomo, M., Romero, P. and Goldenberg, S. (1988) Cyclic AMP and adenylate cyclase activators stimulate *Trypanosoma cruzi* differentiation. *Exp. Parasitol.* **66**, 205–212
- de Castro, S. L., Meirelles, M. N. and Oliveira, M. M. (1987) *Trypanosoma cruzi*: adrenergic modulation of cyclic AMP in proliferation and differentiation of amastigotes *in vitro*. *Exp. Parasitol.* **64**, 368–375
- Ulloa, R. M., Mesri, E., Esteve, M., Torres, H. N. and Tellez-Inon, M. T. (1988) Cyclic AMP-dependent protein kinase activity in *Trypanosoma cruzi*. *Biochem. J.* **255**, 319–326
- Gomez, M. L., Erijman, L., Arauzo, S., Torres, H. N. and Tellez-Inon, M. T. (1989) Protein kinase C in *Trypanosoma cruzi* epimastigote forms: partial purification and characterization. *Mol. Biochem. Parasitol.* **36**, 101–108
- Ogueta, S., Intosh, G. M. and Tellez-Inon, M. T. (1996) Regulation of Ca²⁺/calmodulin-dependent protein kinase from *Trypanosoma cruzi*. *Mol. Biochem. Parasitol.* **78**, 171–183
- Orr, G. A., Werner, C., Xu, J., Bennett, M., Weiss, L. M., Takvorkan, P., Tanowitz, H. H. and Wittner, M. (2000) Identification of novel serine/threonine protein phosphatases in *Trypanosoma cruzi*: a potential role in control of cytokinesis and morphology. *Infect. Immun.* **68**, 1350–135817
- Barton, G. F., Cohen, P. T. and Barford, D. (1994) Conservation analysis and structure prediction of the protein serine/threonine phosphatases. *Eur. J. Biochem.* **220**, 225–237
- Barford, D. (1996) Molecular mechanisms of the protein serine/threonine phosphatases. *Trends Biochem.* **21**, 407–412
- Cohen, P. (1989) The structure and regulation of protein phosphatases. *Annu. Rev. Biochem.* **58**, 453–508
- Janssens, V. and Goris, J. (2001) Protein phosphatase 2A: a highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem. J.* **353**, 417–439
- Mayer, R. E., Hendrix, P., Cron, P., Matthies, R., Stone, S. R., Goris, J., Merlevede, W., Hofsteenge, J. and Hemmings, B. A. (1991) Structure of the 55-kDa regulatory subunit of protein phosphatase 2A: evidence for a neuronal specific isoform. *Biochemistry* **30**, 3589–3597
- McCright, B. and Virshup, D. M. (1995) Identification of a new family of protein phosphatase 2A regulatory subunits. *J. Biol. Chem.* **270**, 26123–26128
- Hendrix, P., Mayer-Jaekel, R. E., Cron, P., Goris, J., Hofsteenge, J., Merlevede, W. and Hemmings, B. A. (1993) Structure and expression of a 72-kDa regulatory subunit of protein phosphatase 2A. Evidence for different size forms produced by alternate splicing. *J. Biol. Chem.* **268**, 15267–15276
- Yoshida, N. (1983) Surface antigens of metacyclic trypomastigotes of *Trypanosoma cruzi*. *Infect. Immun.* **40**, 836–839
- Collins, E. and Sim, A. T. (1998) Regulation of neuronal PP1 and PP2A during development. *Methods Mol. Biol.* **93**, 79–102
- Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**, 680–685
- Shevchenko, A., Wilm, M., Vorm, O. and Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. *Anal. Chem.* **68**, 850–858
- Ma, Y., Lu, Y., Zeng, H., Ron, D., Mo, W. and Neubert, T. A. (2001) Characterization of phosphopeptides from protein digests using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and nano-electrospray quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* **15**, 1693–1700

- 25 Altschul, S. F., Gish, W., Miller, W., Meyers, E. W. and Lipman, D. J. (1990) Basic local alignment search tool. *J. Mol. Biol.* **215**, 403–410
- 26 de Diego, J. L., Katz, J. M., Marshall, P., Gutierrez, B., Manning, J. E., Nussenzweig, V. and Gonzalez, J. (2001) The ubiquitin-proteasome pathway plays an essential role in proteolysis during *Trypanosoma cruzi* remodeling. *Biochemistry* **40**, 1053–1062
- 27 Cano, M. I., Gruber, A., Vazquez, M., Cortes, A., Levin, M. J., Gonzalez, A., Degraeve, W., Rondinelli, E., Zingales, B., Ramirez, J. L. et al. (1995) Molecular karyotype of clone CL Brener chosen for the *Trypanosoma cruzi* genome project. *Mol. Biochem. Parasitol.* **71**, 273–278
- 28 MacKintosh, R. W., Dalby, K. N., Campbell, D. G., Cohen, P. T., Cohen, P. and MacKintosh, C. (1995) The cyanobacterial toxin microcystin binds covalently to cysteine 273 on protein phosphatase 1. *FEBS Lett.* **371**, 236–240
- 29 Erondu, N. E. and Donelson, J. E. (1991) Characterization of trypanosome protein phosphatase 1 and 2A catalytic subunits. *Mol. Biochem. Parasitol.* **49**, 303–314
- 30 Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
- 31 Li, J. L. and Baker, D. A. (1997) Protein phosphatase, a putative type-2A protein phosphatase from the human malaria parasite *Plasmodium falciparum*. *Eur. J. Biochem.* **249**, 98–106
- 32 Hardie, D. G., Haystead, T. A. and Sim, A. T. (1991) Use of okadaic acid to inhibit protein phosphatases in intact cells. *Methods Enzymol.* **201**, 469–476
- 33 Favre, B., Turowski, P. and Hemmings, B. A. (1997) Differential inhibition and post translational modification of protein phosphatase 1 and 2A in MCF7 cells treated with calyculin A, okadaic acid, and tautomycin. *J. Biol. Chem.* **272**, 13856–13863
- 34 Das, A., Gale, M., Carter, V. and Parsons, M. (1994) The protein phosphatase inhibitor okadaic acid induces defects in cytokinesis and organelle segregation in *Trypanosoma brucei*. *J. Cell Sci.* **107**, 3477–3483
- 35 Moorhead, G., MacKintosh, R. W., Morrice, N., Gallagher, T. and MacKintosh, C. (1994) Purification of type 1 protein (serine/threonine) phosphatases by microcystin-Sepharose affinity chromatography. *FEBS Lett.* **356**, 46–50
- 36 Meek, S., Morrice, N. and MacKintosh, C. (1999) Microcystin affinity purification of plant protein phosphatases: PP1C, PP5 and a regulatory A-subunit of PP2A. *FEBS Lett.* **457**, 494–498
- 37 de Souza, W., Meza, I., Martinez-Palomo, A., Sabanero, M., Souto-Padron, T. and Meirelles, M. N. (1983) *Trypanosoma cruzi*: distribution of fluorescently labeled tubulin and actin in epimastigotes. *J. Parasitol.* **69**, 138–142
- 38 Oliveira, M. F., Bijovsky, A. T., Carvalho, T. U., de Souza, W., Alves, M. J. and Colli, W. (2001) A monoclonal antibody to *Trypanosoma cruzi* trypomastigotes recognizes a myosin tail epitope. *Parasitol. Res.* **87**, 1043–1049
- 39 Sontag, E., Nunbhakdi-Craig, V., Bloom, G. S. and Mumby, M. C. (1995) A novel pool of protein phosphatase 2A is associated with microtubules and is regulated during the cell cycle. *J. Cell. Biol.* **128**, 1131–1144
- 40 Hiraga, A. and Tamura, S. (2000) Protein phosphatase 2A is associated in an inactive state with microtubules through 2A1-specific interaction with tubulin. *Biochem. J.* **346**, 433–439
- 41 Eriksson, J. E., Brautigan, D. L., Vallee, R., Olmsted, J., Fujiki, H. and Goldman, R. D. (1992) Cytoskeletal integrity in interphase cells requires protein phosphatase activity. *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11093–11097
- 42 Inagaki, M., Matsuoka, Y., Isujimura, K., Ando, S., Tokui, T., Takahashi, T. and Inagaki, M. (1996) Dynamic property of intermediate filament: regulation by phosphorylation. *BioEssays* **18**, 481–487
- 43 Yang, P., Fox, L., Colbran, R. J. and Sale, W. S. (2000) Protein phosphatases PP1 and PP2A are located in distinct positions in the *Chlamydomonas* flagellar axoneme. *J. Cell Sci.* **113**, 91–102
- 44 Grellier, P., Blum, J., Santana, J., Byln, E., Mournay, E., Sinou, V., Teixeira, A. R. and Schrével, J. (1999) Involvement of calyculin A-sensitive phosphatase in the differentiation of *Trypanosoma cruzi* trypomastigotes to amastigotes. *Mol. Biochem. Parasitol.* **98**, 239–252
- 45 Furuya, T., Kashuba, C., Docampo, R. and Moreno, S. N. (2000) A novel phosphatidylinositol-phospholipase C of *Trypanosoma cruzi* that is lipid modified and activated during trypomastigote to amastigote differentiation. *J. Biol. Chem.* **275**, 6428–6438
- 46 Evers, R. and Cornelissen, A. W. (1990) The *Trypanosoma brucei* protein phosphatase gene: polycistronic transcription with the RNA polymerase II largest subunit gene. *Nucleic Acids Res.* **18**, 5089–5095
- 47 Ario, J., Woon, C. W., Brautigan, D. L., Miller, Jr, T. B. and Johnson, G. L. (1988) Human liver phosphatase 2A: cDNA and amino acid sequence of two catalytic subunit isotypes. *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4252–4256
- 48 Orgad, S., Brewis, N. D., Alpey, L., Axton, J. M., Dudai, Y. and Cohen, P. T. (1990) The structure of protein phosphatase 2A is as highly conserved as that of protein phosphatase 1. *FEBS Lett.* **275**, 44–48
- 49 Doering, C., Meijer, L. and Mottram, J. C. (2002) Protein kinases as drug targets in parasitic protozoa. *Trends Parasitol.* **18**, 366–371

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