Identification, characterization and subcellular localization of TcPDE1, a novel cAMP-specific phosphodiesterase from *Trypanosoma cruzi*

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Compartmentalization of cAMP phosphodiesterases plays a key role in the regulation of cAMP signalling in mammals. In the present paper, we report the characterization and subcellular localization of TcPDE1, the first cAMP-specific phosphodiesterase to be identified from *Trypanosoma cruzi*. *TcPDE1* is part of a small gene family and encodes a 929-amino-acid protein that can complement a heat-shock-sensitive yeast mutant deficient in phosphodiesterase genes. Recombinant TcPDE1 strongly associates with membranes and cannot be released with NaCl or sodium cholate, suggesting that it is an integral membrane protein. This enzyme is specific for cAMP and its activity is not affected by cGMP, Ca^{2+}, calmodulin or fenotiazinic inhibitors. TcPDE1 is sensitive to the phosphodiesterase inhibitor dipyridamole but is resistant to 3-isobutyl-1-methylxanthine, theophylline, rolipram and zaprinast. Papaverine, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride, and vinpocetine are poor inhibitors of this enzyme. Confocal laser scanning of *T. cruzi* epimastigotes showed that TcPDE1 is associated with the plasma membrane and concentrated in the flagellum of the parasite. The association of TcPDE1 with this organelle was confirmed by subcellular fractionation and cell-disruption treatments. The localization of this enzyme is a unique feature that distinguishes it from all the trypanosomatid phosphodiesterases described so far and indicates that compartmentalization of cAMP phosphodiesterases could also be important in these parasites.

Key words: cAMP, flagellum, membrane, phosphodiesterase, *Trypanosoma cruzi*.

INTRODUCTION

cAMP-specific phosphodiesterases (PDEs) catalyse the degradation of the second messenger cAMP. Over the last few years, it has been shown that these enzymes are not only responsible for the termination of the cAMP signal, but also have a pivotal role in the regulation of its biological action. In mammals, cAMP-PDEs are part of the cyclic nucleotide PDE superfamily. These proteins have been classified into 11 different families according to their sequence identity, biochemical and pharmacological properties, regulation and substrate specificity. The fact that each family is composed of one to four genes and many isoforms are generated by alternative splicing, shows the high complexity of this superfamily [1,2].

In trypanosomatids, cAMP has been implicated in the control of growth and differentiation [3–8]. Although many adenylate cyclases have been reported and characterized in these organisms [7–11], very little is known about cAMP-PDEs. The activity of these enzymes was described in *Trypanosoma cruzi* [12], *Trypanosoma brucei* [13], *Trypanosoma gambiense* [14], and the related organism *Leishmania mexicana* [15]. However, the genes that code for these proteins have only been identified in *T. brucei* [16–19]. In this parasite, two types of genes were reported, *TbPDE1* [16] and the *TbPDE2* family [17–19]. *TbPDE1* is a single-copy gene that is not related to the mammalian PDEs and is expressed at very low levels. *TbPDE2* is a small family of genes composed of at least five members that show a considerable similarity to all mammalian PDEs. *TbPDE1*-knockout experiments showed that the gene is not necessary for the survival of *T. brucei* in culture nor for the infection of tsetse flies [16]. On the contrary, compounds that inhibit TbPDE2A are lethal to *T. brucei* in culture [17] and RNAi (RNA interference) experiments indicated that TbPDE2C is an essential enzyme for this parasite [18], suggesting that the members of the TbPDE2 family are crucial for its survival.

It has been proposed that compartmentalization of the cAMP-related enzymes is important for the regulation and specificity of the cAMP signal [20–22]. In mammals, the differential subcellular distribution of cAMP-PDEs seems to have a key role in this process [20]. In fact, targeting of cAMP-PDEs to specific subcellular compartments has been reported for the mammalian PDE2, PDE3, PDE4 and PDE6 families [23–28]. Different isoforms of these families show distinct intracellular localizations depending on the cell type. They have been found in the cytoplasm, associated with the cell membrane, or even associated with the endoplasmic reticulum. The subcellular distribution of PDEs depends on the presence of hydrophobic stretches within their N-terminal domain, the interaction with anchoring proteins or the presence of carboxymethyl groups or lipids, added by post-translational modifications [23–28].

In trypanosomatids, compartmentalization of the cAMP signal could also be important. Specific localization of adenylate cyclases in the flagellum has been demonstrated in *T. brucei* [11] and suggested in *T. cruzi* [10]. However, the role of PDEs in

Abbreviations used: ADH, alcohol dehydrogenase; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DIC, differential interference contrast image; EHNA, erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride; GAF, cGMP-phosphodiesterases, adenylate cyclases and Fh1A; IBMX, 3-isobutyl-1-methylxanthine; NHP2, non-histone protein 2; NP-40, Nonidet P-40; PDE, phosphodiesterase; PFGE, pulse-field gel electrophoresis; PKA, protein kinase A.

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The nucleotide sequence reported for *Trypanosoma cruzi* phosphodiesterase PDE1 has been deposited in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY233464.

the compartmentalization of the cAMP signal has not yet been addressed. Except for a 5 % PDE activity associated with *L. mexicana* membranes [15], all trypanosomatid cAMP-PDEs were described as being soluble proteins [12–15,17–19].

In the present paper, we report the identification and characterization of the first cAMP-specific PDE from *T. cruzi*. The gene *TcPDE1* is part of a small gene family and encodes a highly conserved cAMP-PDE. Recombinant TcPDE1 expressed in yeast is specific for cAMP and strongly associates with membranes. It is interesting to note that, by using confocal laser scanning, we demonstrated that this enzyme is anchored to the plasma membrane of *T. cruzi* and is concentrated in the flagellum of the parasite. Taken together, these results suggest the presence of a small family of PDEs in *T. cruzi* and provide novel information about a possible compartmentalization of the cAMP signal in this parasite.

EXPERIMENTAL

Materials

All radiochemicals were from DuPont New England Nuclear (Boston, MA, U.S.A.). Bacto-tryptose, yeast nitrogen base and liver infusion were from Difco Laboratories (Detroit, MI, U.S.A.). Restriction endonucleases were from New England Biolabs (Beverly, MA, U.S.A.). Papaverine and theophylline were a gift from Dr Silvia Moreno (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina). All other reagents were purchased from Sigma. Anti-paraflagellar rod antibody was a gift from Dr Keith Gull (Sir William Dunn School of Pathology, University of Oxford, Oxford, U.K.) and anti-(arginine kinase) antibody was a gift from Dr Claudio Pereira (Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Argentina).

Cell cultures

T. cruzi epimastigote forms (CL Brenner strain) were cultured at 28 °C in LIT medium [5 g/l liver infusion, 5 g/l bacto-tryptose, 68 mM NaCl, 5.3 mM KCl, 22 mM Na_2PO_4, 0.2 % (w/v) glucose and 0.002 % (w/v) haemin] supplemented with 10 % (v/v) calf serum, 10 units/ml penicillin and 10 mg/l streptomycin. Cell viability was assessed by direct microscopic examination.

Saccharomyces cerevisiae strains PM943 (*MATa leu2-3*, *112 ura3-1 trp1-92 his3-11*, *15 ade2-1 can1-100 GAL SUC mal pde1::TRP1 pde2::URA3*) and J106 (*MATa leu2 his3 ura3 trp1 ade8 can1 pde1::URA3 pde2::HIS3*) were a gift from Dr Johan Thevelein (Laboratory of Molecular Cell Biology, Institute of Botany and Microbiology, Katholieke Universiteit Leuven, Leuven, Belgium) [29]. These strains were grown in YPD medium (2 % peptone, 1 % yeast extract and 2 % dextrose) at 30 °C. Transformants were selected in minimal medium containing 0.17 % yeast nitrogen base (without amino acids and ammonium sulphate), 0.5 % $(NH_4)_2SO_4$ and 2 % (w/v) glucose, supplemented with the corresponding amino acid mixture.

Cloning of *TcPDE1* gene

The *T. brucei TbPDE2B* PDE gene sequence (GenBank® accession number AF192755) was used to screen the *T. cruzi* sequence databases using the WU-Blast2 algorithm. Two oligonucleotides (5′-GTACGAAGGAACGATTGTGGCTG-3′ and 5′-CGAGTCCGGGTCCGAAAGAATT-3′) were designed from the three sequences identified and employed for PCR amplifications using *T. cruzi* genomic DNA. A band of expected size that showed significant sequence similarity to previously reported cAMP-PDEs was obtained. This fragment was used as a probe to screen a Lambda FIX II (Stratagene) genomic library from *T. cruzi* [31]. DNA from two positive phage clones was purified using the Qiagen Lambda MIDI kit and was sequenced.

Northern and Southern blot

Genomic DNA was purified as described by Pereira et al. [30]. Total RNA was prepared using the Total RNA Isolation reagent (TRIzol®, Gibco BRL). Northern and Southern blot analyses were performed as described by D'Angelo et al. [10]. The *TcPDE1* full-length probe was labelled with [α-^{32}P]dCTP using the Prime-a-Gene kit (Promega).

Fractionation of chromosomal bands by pulsed-field gel electrophoresis (PFGE)

PFGE was performed in a CHEF electrophoresis cell with a hexagonal electrode array. Agarose blocks containing about 10^7 epimastigote forms were prepared as described by Pereira et al. [30]. The separation of the chromosomal bands was carried out in 1.0 % Gel Seakem agarose (FMC BioProducts, Rockland, ME, U.S.A.) in 0.5 × TBE buffer. Electrophoretic conditions were as follows: 16 h, 6 V/cm pulsed each 60–90 s; 20 h, 3 V/cm pulsed each 200–500 s; and 24 h, 2.7 V/cm pulsed each 500–1200 s (at 14 °C). Following electrophoresis, the gel was stained with ethidium bromide (0.1 μg/ml) and photographed. Chromosomes from *S. cerevisiae* (Life Technologies) were used as molecular mass standards. The gel was incubated for 10 min with 0.25 M HCl and was washed twice with distilled water. DNA was denatured in a solution containing 0.4 M NaOH and 1 M NaCl, and was transferred and hybridized as described for Southern blots.

Complementation assay

The full-length *TcPDE1* gene was amplified by PCR using the oligonucleotides, 5′-AAGCTTATGGCGGAGACAGGCGGTCG-3′ and 5′-CCGCGGTCATATATACATCATTTATTTCCATCA-3′, and subcloned in the pADNS yeast expression vector [32] under the control of the pADH1 (alcohol dehydrogenase) promoter. The PM943 or J106 yeast strains were transformed with the empty vector, or with the vector carrying the *TcPDE1* gene, using the lithium acetate procedure [33]. Transformants were selected in minimal medium lacking leucine at 30 °C. For heat-shock complementation assays, transformants were grown in minimal medium to D_{600} of 1.8–2 and were incubated at 55 °C for a further 30 min. After treatment, cells were plated in YPD at different dilutions and incubated at 30 °C for two days.

Soluble extracts and membrane preparation

Yeast transformants were grown to a D_{600} of 0.8–1.5. Cells were resuspended in lysis buffer [20 mM Tris/HCl, pH 7.5, 5 % (v/v) glycerol, 1 mM dithiothreitol, 0.1 mM PMSF, 25 units/ml aprotinin and 0.5 mM tosyl-lysine chloromethyl ketone] and lysed by 10 cycles of 1 min vortex-mixing in the presence of glass beads (425–600 μm diameter) and cooling on ice. Cell debris was discarded by centrifugation at 2500 ***g*** and the supernatant was centrifuged further for 1 h at 100 000 ***g***. The pellet was resuspended in 20 mM Tris/HCl, pH 7.5, 5 % (v/v) glycerol and antiproteases, and used as membrane fraction.

For TcPDE1 membrane extraction, membranes were sequentially washed with lysis buffer containing 1 M NaCl, 0.1 or 0.5 % (w/v) sodium cholate, or 8 M urea for 30 min at 4 °C. After every wash, membranes were centrifuged for 40 min at 100 000 ***g*** and were washed with lysis buffer.

Subcellular fractionation was performed as described by Gomez et al. [34]. Briefly, *T. cruzi* epimastigote pellets were resuspended in a 0.25 M sucrose solution containing 5 mM KCl and

lysed by 10 cycles of freeze–thaw. Unbroken cells were discarded following centrifugation at 500 ***g*** for 10 min and the homogenate was subjected to six successive centrifugations at 1000 ***g*** for 15 min (flagella), 5000 ***g*** for 15 min (mitochondria), 11 000 ***g*** for 30 min (heavy lysosomal–glycosomal), 16 000 **g** for 30 min (plasma membrane), 30 000 ***g*** for 30 min (light lysosomal) and 105 000 ***g*** for 30 min (microsomal). All the fractions were washed and resuspended in the lysis buffer plus antiproteases.

cAMP-PDE assays

cAMP-PDE activity was determined as described by Téllez-Iñón et al. [12]. For the cGMP-PDE assay, the modifications described by Boudreau and Drummond [35] were introduced. In both cases, the reactions were performed in the presence of 20 mM Tris/HCl, pH 7.5, 5 mM Mg^{2+} and different concentrations of [^{3}H]cAMP or [^{3}H]cGMP. Incubations were carried out for 10 min at 30 °C in a total volume of 100 μl. For kinetic parameter determination, seven independent assays were performed with 10 μg of protein and 0.01–500 μM cAMP. The Ca^{2+}-calmodulin response was analysed as described [10]. For inhibition studies, assays were performed in the presence of 2 μg of proteins and 1 μM–1 mM of the corresponding inhibitors.

Expression of recombinant TcPDE1 in *Escherichia coli*

The full-length *TcPDE1* gene was amplified using the primers, 5′-GGATCCATGGCGGAGACAGGCGGTCG-3′ and 5′-AAGCTTTCATATATACATCATTTATTTCCATCA-3′, and was subcloned into the pMAL-c2 expression vector (New England Biolabs) in fusion with the maltose-binding protein. Expression of the recombinant protein was performed in the *BL21*(*DE3*)*pLysS* host {*E. coli B*, F^-, *dem*, *ompT*, *hsdS*, (rb^-, m_B^-), *gal*λ(*DE3*), [*pLysS*, *cam*^{r}]}. The fusion protein was purified using Amylose Resin (New England Biolabs) as described by the manufacturer.

Antibody preparation and Western blot analysis

TcPDE1 antiserum was obtained by using Balb/c mice immunized by intraperitoneal injection of 50 μg of recombinant protein plus Freund's adjuvant, followed by two more injections every 15 days with incomplete adjuvant. Mice were bled by exposing the ocular cavity. Antibodies were tested to determine titre and cross-reactivity using the recombinant protein and *T. cruzi* extracts. No cross-reactivity was observed at the dilutions used for Western blot analysis. Proteins were resolved by SDS/PAGE [36] on 8 % polyacrylamide gels and electrotransferred on to Hybond-C membranes (Amersham Biosciences). The membranes were blocked with 5 % (w/v) non-fat milk suspension in TBS/Tween 20. After an overnight incubation with a 1:1000 dilution of the mouse anti-TcPDE1 serum, detection was carried out by incubating with a 1:7000 dilution of a goat anti-mouse IgG labelled with peroxidase (KPL, Gaithersburg, MD, U.S.A.). The latter was developed with the ECL® (enhanced chemiluminescence) Plus™ Western Blotting Detection System (Amersham Biosciences).

Immunoflorescence microscopy

Epimastigote cells were harvested at (2–5) × 10^7 parasites/ml, washed with PBS and settled for 10 min on multiwell slides treated with VECTABOND™ Reagent (Vector Laboratories, Burlingame, CA, U.S.A.). The parasites were fixed for 10 min with 3.5 % (w/v) paraformaldehyde in PBS at room temperature (25 °C), washed with PBS and permeabilized with ice-cold methanol for 20 min. Cells were rehydrated for 10 min and blocked with 2 % (w/v) BSA in PBS/0.05 % (v/v) Tween 20 for 30 min before an overnight incubation with the primary anti-TcPDE1 antibody diluted 1:300. Parasites were washed three times with PBS/Tween 20 and reacted to Alexa Fluor® 488 Signal-Amplification Kit for mouse Antibodies (Molecular Probes, Eugene, OR, U.S.A.) following the manufacturer's instructions. After the final wash, 0.1 μg/ml of DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) in water was applied for 5 min. Cells were washed and mounted on coverslips using Prolong mounting media (Molecular Probes). A laser scanning confocal microscope (LSM 410 UV mounted on a Zeiss Axiovert 135 microscope) was used to obtain the fluorescence and differential interference contrast images (DICs). Excitation of Alexa Fluor® 488 was performed with the 488 nm line of an Ar/Kr Omnichrome laser. Excitation of DAPI was accomplished with a 351 nm/364 nm argon laser. When Z stacks were performed, the distance between slices was 0.5 μm.

Cell permeabilization of epimastigotes for cytoplasmic protein release was performed as described by Godsel and Engman [37]. The parasites were incubated with 100 mM Pipes, 1 % (v/v) Triton X-100, 1 mM NaCl and 25 mM Ca^{2+} for 3 min, and were washed with PBS before fixation. Cytoskeleton preparation was performed as described by Ersfeld and Gull [38]. Parasites were incubated with 100 mM Pipes, 0.5 % (v/v) Nonidet P-40 (NP-40), 2 mM EGTA and 1 mM $MgSO_4$ at 4 °C for 2 min, and were washed with PBS before fixation.

Sequence analysis

A *T. cruzi* database search was performed using WU-Blast2 (http://www.ebi.ac.uk/blast2/parasites.html). Sequence identity was analysed with the BlastP (http://www.ncbi.nlm.nih.gov/blast/index.html) and the ClustalW (http://www.ebi.ac.uk/clustalw/) programs, and protein domains were determined using SMART (http://smart.embl-heidelberg.de/) and PROSITE (http://us.expasy.org/prosite/). Hydrophobic analysis was performed with DAS (http://www.sbc.su.se/~miklos/DAS/), SPLIT (http://garlic.mefos.hr/split/), PRED-TMT (http://o2.db.uoa.gr/PRED-TMR/) and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html).

RESULTS

Cloning and characterization of *TcPDE1* gene

A computational search of *T. cruzi* databases using as query the *TbPDE2B* PDE gene from *T. brucei* resulted in the identification of three sequences (AZ050569, AZ050205 and AA952604). Two oligonucleotides designed from these sequences were used to amplify a *T. cruzi* DNA fragment (Figure 1A) that was used as a probe to screen a Lambda FIX II genomic library from this parasite [31]. Two of the clones identified contained a 2790 bp open reading frame coding for a 929-amino-acid polypeptide that was designated *TcPDE1* and submitted to the GenBank® database under accession number AY233464.

The predicted amino acid sequence presented a putative signal peptide (amino acids 1–22) followed by two GAF (cGMP-phosphodiesterases, adenylate cyclases and Fh1A) domains for cGMP binding (amino acids 237–387 and 410–560) and a highly conserved PDE catalytic domain (amino acids 669–840; Figure 1B). The presence of GAF domains has also been reported in mammalian PDEs PDE2, 5, 6, 10 and 11 [1,2] and also in some members of the *T. brucei* PDEs family TbPDE2 [17–19]. Apart from that, TcPDE1 showed a consensus motif for PKA (protein kinase A) serine phosphorylation (Lys-Arg-Lys-Ser) between amino acids 390–393, suggesting a possible regulatory mechanism by phosphorylation (Figure 1B). Using the Kyte

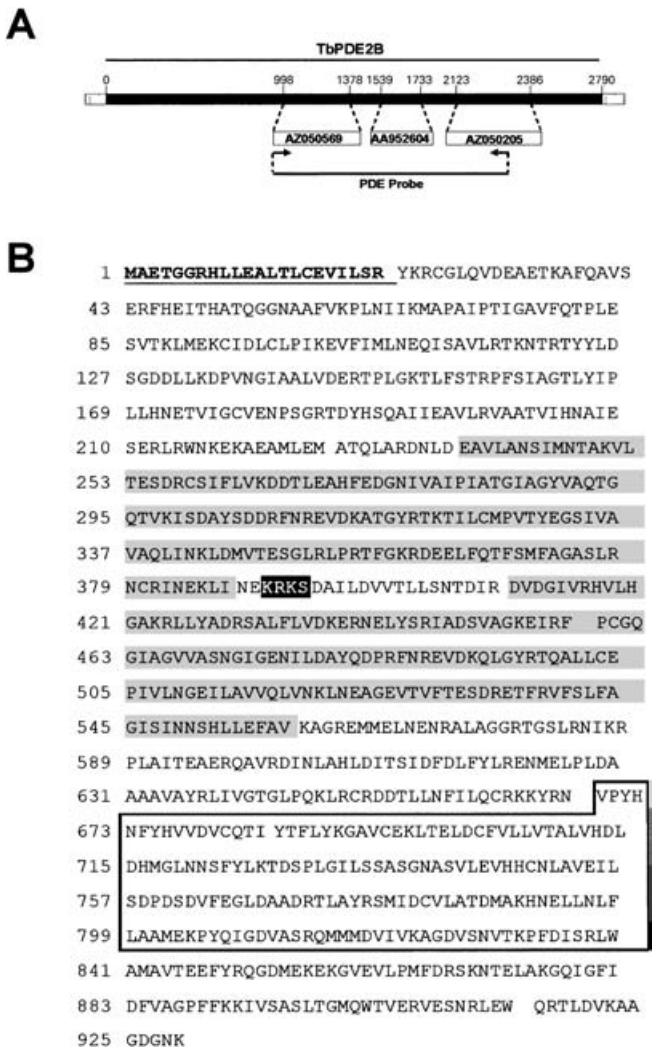

Figure 1 Cloning strategy and amino acid sequence of TcPDE1

(**A**) Diagram of the strategy used to amplify the probe used to a screen a Lambda FixII genomic library from *T. cruzi*. The three sequences identified in the *T. cruzi* database were aligned with *TbPDE2B* sequence used as input. Two oligonucleotides were designed from the sequences and used to amplify *T. cruzi* genomic DNA. (**B**) Amino acid sequence of TcPDE1. Bold underlined amino acids correspond to the putative signal peptide, grey highlighted letters indicate the GAF domains A and B, white letters highlighted in black correspond to the predicted PKA phosphorylation consensus, boxed letters show TcPDE1 catalytic domain.

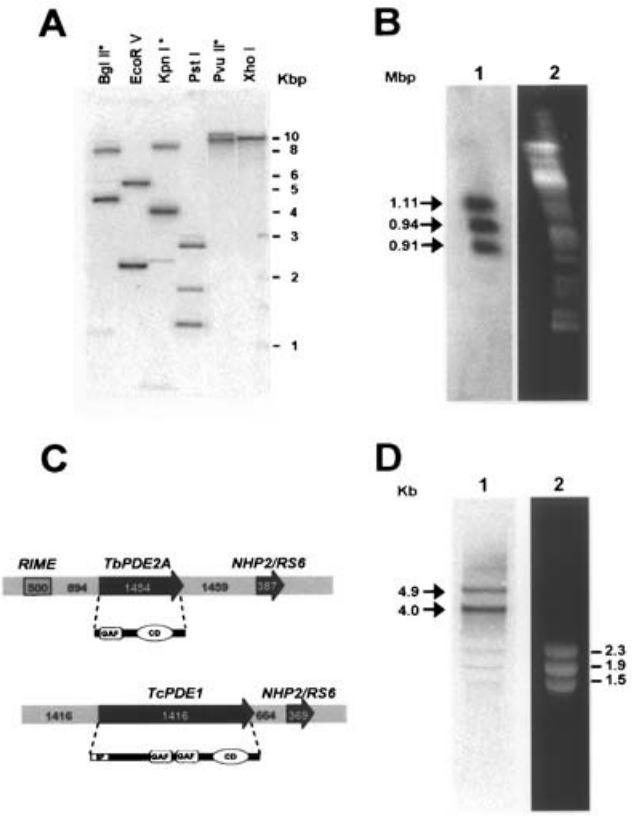

Figure 2 Genomic organization and expression of *TcPDE1*

(**A**) Southern blot. *T. cruzi* genomic DNA was digested with the endonucleases indicated, electrophoresed and transferred on to nylon membranes. Hybridization was performed with a specific probe representing the entire *TcPDE1* gene. Asterisks represent restriction enzymes that show more bands than expected by restriction mapping. (**B**) PFGE. *T. cruzi* chromosomes were prepared as described in the Experimental section, separated by PFGE, transferred and hybridized with the same probe used for Southern blot. Panel 1, ethidium bromide staining of *T. cruzi* chromosomes; panel 2, autoradiography of hybridized membranes. Arrows indicate the molecular mass of the bands recognized by the specific *TcPDE1* probe. (**C**) Scheme of the genomic organization of *TcPDE1* and *TbPDE2A* genes. Non-coding regions are shown in grey, coding regions are in black. Sequences were drawn to scale and numbers represent base pairs. (**D**) Northern blot. Total RNA from *T. cruzi* epimastigotes was electrophoresed in agarose-formaldehyde gels, transferred and hybridized. Panel 1, ethidium bromide staining of total RNA. rRNA molecular masses are indicated; panel 2, autoradiography of hybridized membranes. Arrows indicate the molecular mass of the bands recognized by the specific *TcPDE1* probe.

and Doolittle algorithm and other transmembrane prediction programs, no significant hydrophobic regions were detected in the TcPDE1 sequence except for the putative signal peptide.

Nucleotide sequence analysis showed that *TcPDE1* only presents significant identity (> 69 %) with *T. brucei TbPDE2* PDEs. In contrast, the amino acid sequence predicted from the open reading frame has also a considerable identity with all the mammalian PDE families (> 24 %), with the highest identity within the catalytic domain (27–33 %) and the GAF domains (14–42 %). These results include TcPDE1 in Class I PDEs.

Genomic organization and expression of *TcPDE1*

In Southern blot analysis using a specific probe representing the entire *TcPDE1* gene, three out of six restriction endonucleases (*Bgl*II, *Kpn*I and *Pvu*II) showed more bands than expected, indicating that *TcPDE1* is part of a small gene family in *T. cruzi* (Figure 2A). This was confirmed by database search (results not shown) and by PFGE assays, which showed that at least three chromosomic bands of about 0.915, 0.945 and 1.11 Mb are recognized by the *TcPDE1* probe (Figure 2B).

Surprisingly, it was found that *TcPDE1* has a very similar genomic organization to the *T. brucei* PDE gene *TbPDE2A* [17], with a gene encoding a putative NHP2 (non-histone protein 2) protein located downstream of the coding region (Figure 2C) [17]. NHP2 is a nuclear protein not related to the cAMP pathway that in mammals and yeast is associated with H/ACA small nucleolar RNAs (snoRNAs) [39,40]. The high homology of these PDE genes and the fact that they have the same genomic organization suggests a common evolutionary origin of these sequences.

Northern blot analysis of *T. cruzi* epimastigote total RNA revealed two bands; a stronger band of approx. 4 kb and another

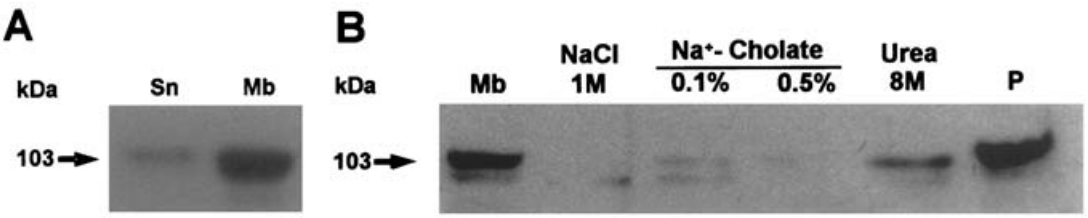

Figure 3 Membrane localization of recombinant TcPDE1

(**A**) The association of TcPDE1 with membranes was analysed by Western blot assays. Proteins of soluble or membrane extracts (40 μg) were resolved by SDS/PAGE (8 % gels), electrotransferred on to Hybond-C^{TM} membranes and revealed with TcPDE1-specific antiserum. Sb, soluble fraction; Mb, membrane fraction. (**B**) Yeast recombinant membranes were extracted to release TcPDE1 by sequential washes with 1 M NaCl, 0.1 or 0.5 % (w/v) sodium cholate, or 8 M urea. Mb, pre-extracted membranes; P, post-extracted pellet.

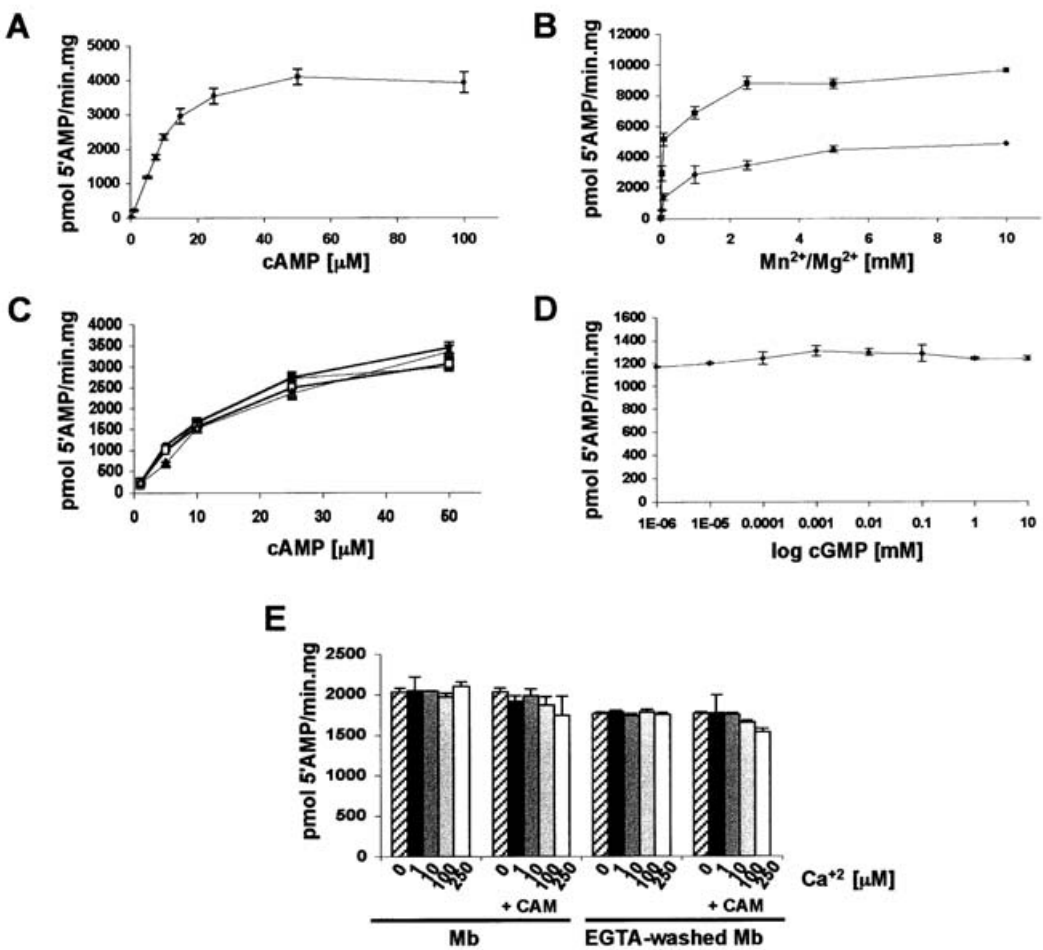

Figure 4 Biochemical characterization of recombinant TcPDE1

cAMP-PDE assays were performed with membranes purified from TcPDE1 recombinant yeasts using 2–10 μg of protein. (**A**) Substrate-dependence was analysed in the presence of 5 mM Mg^{2+}. (**B**) $MnCl_2$ (■) and $MgCl_2$ (●) -dependence was studied in the presence of 50 μM cAMP. (**C**) The effect of cGMP on TcPDE1 substrate affinity was evaluated in the absence (●) or presence of 10 nM (■), 1 μM (□) and 10 μM (△) cGMP. (**D**) cGMP competition assays were performed with 1 μM cAMP. (**E**) The effect of Ca^{2+} and calmodulin on TcPDE1 activity was tested in membranes (Mb), before and after an extensive washed with EGTA, in the absence or presence of 750 nM calmodulin (CAM) and 10 μM cAMP. One unit of calmodulin, as defined by the manufacturer, corresponds to 25 ng of protein. Assays represent one experiment representative of 3–5 independent experiments.

of approx. 4.9 kb, indicating that more than one isoform of this protein is expressed in this stage of the parasite (Figure 2D).

Complementation of yeast PDE-deficient strains

To analyse whether the *TcPDE1* gene encoded a functional cAMP-PDE, the sequence corresponding to the full-length gene was amplified by PCR and subcloned in the yeast expression vector pADNS under the ADH1 constitutive promoter. This construction was used to transform the PM943 and J106 yeast strains [29]. Both strains have the endogenous PDEs genes, PDE1 and PDE2, deleted and are sensitive to a heat shock at 55 °C. The heat-shock sensitivity of PDE-deficient yeast strains has been widely used as a tool for the identification and characterization of

many PDEs [41]. PM943 and J106 yeasts transformed with the *TcPDE1* gene were resistant to a 30 min heat shock at 55 °C. In contrast, the untransformed yeasts or the ones transformed with the empty vector were not able to rescue the sensitive phenotype (results not shown).

To characterize further the TcPDE1 enzyme, soluble and particulate extracts from PM943 recombinant yeasts were assayed for cAMP-PDE activity. It is interesting to note that the specific activity of TcPDE1 was higher in the membrane fraction (3139 ± 218 pmol of 5′-AMP/min per mg) than in the supernatant (837 ± 49 pmol of 5′-AMP/min per mg). The same results were obtained with J106 yeast extracts (results not shown).

Membrane localization of TcPDE1

The presence of TcPDE1 in membranes of the recombinant yeasts was studied by Western blot analysis. A band of the expected molecular mass (103 kDa) was detected in yeast extracts using a polyclonal antiserum raised against the recombinant TcPDE1 expressed in *E. coli*. The recognized band was concentrated in the particulate fraction (Figure 3A), confirming the membrane-association of the enzyme.

To investigate if TcPDE1 represented a peripheral or an integral membrane protein, membrane extracts from recombinant yeasts were subjected to sequential washes with 1 M NaCl, 0.1 or 0.5 % (w/v) sodium cholate, or 8 M urea for 30 min. As shown in Figure 3(B), small amounts of TcPDE1 (< 25 %) were only released with 8 M urea, indicating that the protein is strongly associated with the membrane fraction and suggesting that TcPDE1 represents an integral membrane protein.

Biochemical characterization of TcPDE1

The activity of TcPDE1 was characterized further in membranes of the recombinant yeasts. TcPDE1 showed a high affinity for cAMP with a K_m of 7.3 ± 0.9 μM (Figure 4A), confirming that the enzyme belongs to Class I PDEs, and its activity was higher in the presence of Mn^{2+} than Mg^{2+} as cofactor (Figure 4B). On the other hand, the enzyme was not able to hydrolyse cGMP at a broad range of concentrations (1 nM–10 mM; results not shown). This cyclic nucleotide had no effect either on the substrate affinity of TcPDE1 or on its cAMP-hydrolytic activity (Figures 4C and 4D). This indicates that TcPDE1 is a cAMP-specific PDE not regulated by cGMP.

The presence of a soluble Ca^{2+}-calmodulin-stimulated cAMP-PDE activity has been reported in *T. cruzi* [12]. However, the activity of TcPDE1 in yeast membranes was not stimulated by Ca^{2+} (1–250 μM) or by Ca^{2+}-calmodulin, even when the membranes were extensively washed with EGTA-containing buffers (Figure 4E) or when different concentrations of calmodulin (15–750 nM) and cAMP (1–50 μM) were used (results not shown). Furthermore, the calmodulin blockers chlorpromazine and fluperazine (1–250 μM) did not inhibit TcPDE1 activity (results not shown). This is in agreement with the fact that TcPDE1 lacks domains involved in the interaction with this complex.

Trypanosomatid PDEs show different sensitivity to specific inhibitors compared with their mammalian counterparts [17–19]. To test the effect of these compounds on TcPDE1 activity, cAMP-PDE assays were performed in the presence of different concentrations of inhibitors (1 μM–1 mM). As shown in Table 1, TcPDE1 was not significantly inhibited by IBMX (3-isobutyl-1-methylxanthine), theophylline or rolipram. Furthermore, papaverine, EHNA [erythro-9-(2-hydroxy-3-nonyl)-adenine hydrochloride] and vinpocetine were poor inhibitors of the enzyme

Table 1 Effect of PDE-specific inhibitors on TcPDE1

NS, non-selective; NI, no inhibition. Percentage inhibition represents maximum inhibition obtained. Assays were performed with 1 μM [^{3}H]cAMP. Results are means $\pm$ S.D., representative of four to six different experiments.

Inhibitor	Selectivity for PDE family	Mammalian IC_{50} (μM)	TcPDE1 IC_{50} (μM)	Inhibition (%)
Vinpocetine	1	20	134 ± 19	56 ± 5
EHNA	2	0.8	217 ± 4	68 ± 6
cGMP	3	–	NI	NI
Rolipram	4	2	> 500	31 ± 8
Zaprinast	5, 6, 9	0.45, 0.15, 35	> 500	NI
Dipyridamole	5, 6, 8, 10	0.9, 0.38, 4.5, 1.1	17 ± 4	79 ± 7
Papaverine	NS	5–25	111 ± 17	77 ± 1
IBMX	NS	2–50	> 1000	28 ± 5
Theophylline	NS	–	NI	NI

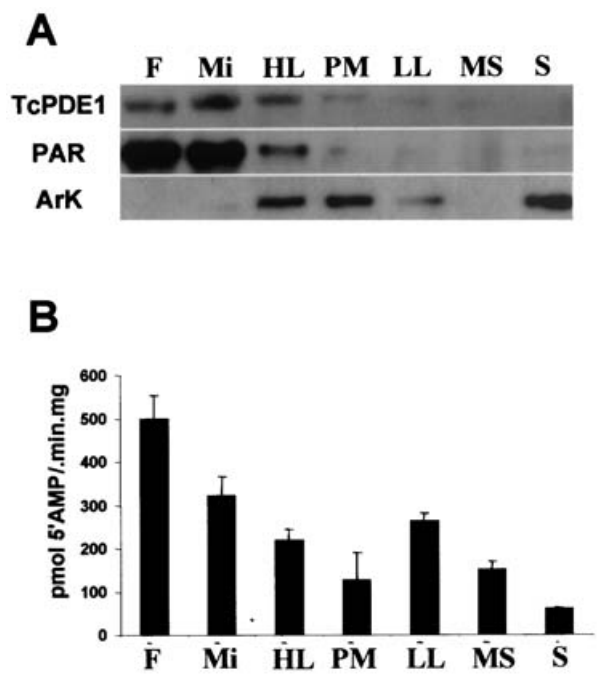

Figure 5 Subcellular fractionation of *T. cruzi* TcPDE1

(**A**) Total extracts prepared from *T. cruzi* epimastigotes were subjected to sequential centrifugation as described in the Experimental section. FL, flagella; Mi, mitochondria; HL, heavy lysosomal–glycosomal; PM, plasma membrane; LL, light lysosomal; MS, microsomal; S, supernatant. The membrane and soluble fractions were assayed by Western blotting using the specific antiserum against TcPDE1 (TcPDE1), a specific antibody against the flagellar protein paraflagellar rod (PAR), and an antiserum against the arginine kinase protein (ArK). (**B**) Enzymic activity was assayed in all the fractions using 5 mM $MgCl_2$ and 50 μM cAMP. Results shown represent one of four independent data sets.

(IC_{50}s of 111 ± 17, 217 ± 4 and 134 ± 19 μM respectively). In contrast, dipyridamole was a good inhibitor of TcPDE1 with an IC_{50} of 17 ± 4 μM.

Subcellular fractionation of TcPDE1 in *T. cruzi*

As described in the Introduction, some components of the cAMP pathway of trypanosomatids are located in the flagellum. To analyse if this was the case for TcPDE1, a subcellular fractionation of *T. cruzi* epimastigotes was performed. Parasites were lysed and subjected to successive centrifugations as described by Gómez

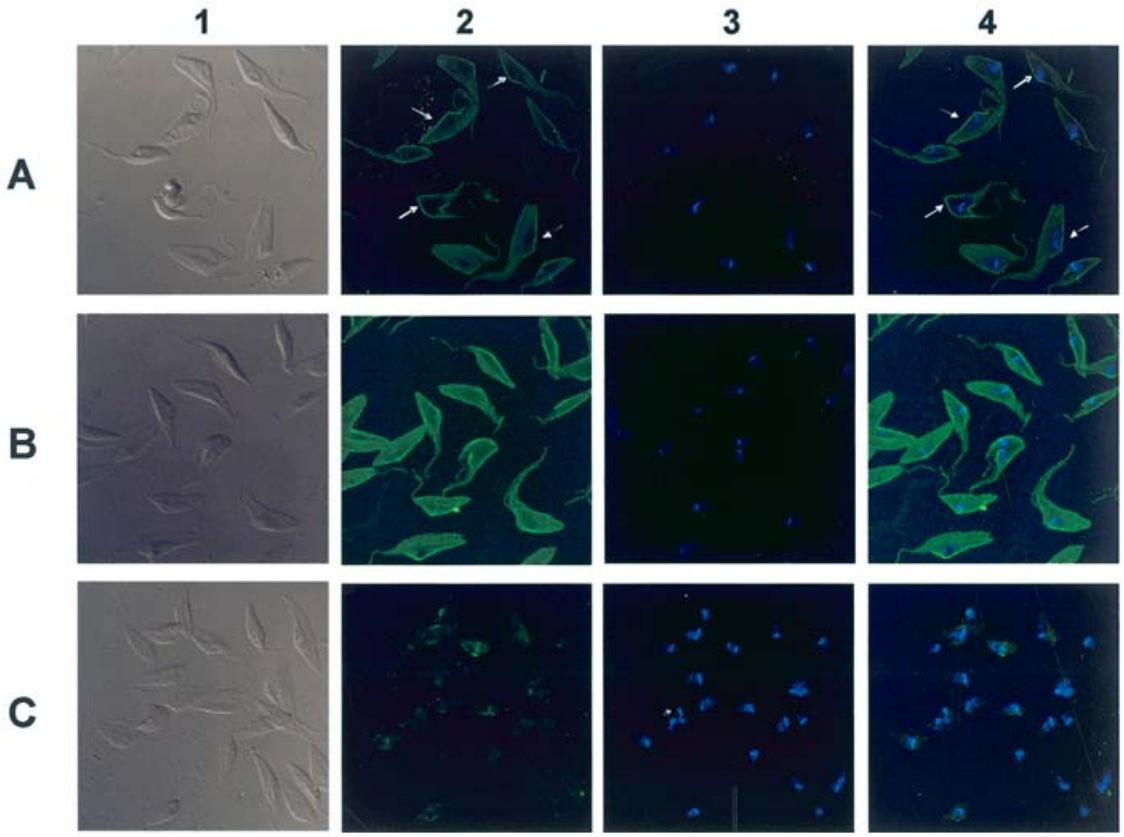

Figure 6 Immunolocalization of TcPDE1 in *T. cruzi* epimastigotes

T. cruzi epimastigotes were attached to multiwell slides, fixed, permeabilized and reacted to (**A**) TcPDE1 antibody, (**B**) anti-tubulin antibody, or (**C**) pre-immune serum. Images were collected with a confocal laser microscope. (**1**) DIC; (**2**) specific antibody; (**3**) DAPI, and (**4**) images (**2**) and (**3**) merged. Scale bar represents 5 μm. Arrows indicate the flagellar signal of TcPDE1.

et al. [34]. The different fractions were analysed by Western blot using the antiserum against TcPDE1, an antiserum against the paraflagellar rod, a flagellar protein [38], and an antiserum that recognizes the *T. cruzi* arginine kinase, a non-related protein [30]. Interestingly, TcPDE1 showed the same distribution as the paraflagellar rod protein (Figure 5A). In contrast, the arginine kinase protein was mainly localized in the soluble fraction and in some small membrane organelles as has been previously observed (Figure 5A; C. A. Pereira, G. A. Alonso, M. M. Flawiá and H. N. Torres, unpublished work). The cAMP-PDE activity measured in the fractions confirmed the observations made by Western blot analysis (Figure 5B).

Immunolocalization of TcPDE1 in *T. cruzi* detected by confocal laser scanning microscopy

Confocal laser scanning microscopy was used to characterize further the intracellular localization of TcPDE1 in epimastigotes of *T. cruzi*. Parasites were attached to multiwell slides, fixed and incubated with the polyclonal antiserum against TcPDE1. Anti-tubulin antibody was used as a permeabilization control and DAPI was used to determine the position of the nucleus. A strong signal associated with the plasma membrane was observed with the anti-TcPDE1 serum (Figure 6A). The signal was stronger in the flagellum (Figure 6A, arrows). On the other hand, when the anti-tubulin antibody was used, the signal was distributed all over the epimastigote cell (Figure 6B). No signal was detected when using the pre-immune serum (Figure 6C) or the secondary antibody alone (results not shown).

To confirm the association of TcPDE1 with the flagellum, the epimastigotes were subjected to two different treatments before fixation and incubation with the anti-TcPDE1 serum. In the first one, the cells were lightly permeabilized with a phosphate buffer containing 1 % (v/v) Triton X-100 to release cytoplasmic proteins [37]. As shown in Figures 7(A) and 7(B), after permeabilization, most of the signal was located in the flagellum. In the second treatment, the parasite cells were extracted with 0.5 % (v/v) NP-40 containing buffers to prepare cytoskeletons as described by Ersfeld and Gull [38]. In this case, a strong TcPDE1 signal also remained associated with the flagellum, as was observed with the paraflagellar rod protein used as a control (Figures 7C and 7D). These results indicate that TcPDE1 is localized in the plasma membrane of *T. cruzi* and concentrated in the flagellum.

DISCUSSION

We have cloned and characterized TcPDE1, the first cAMP-specific PDE to be identified from *Trypanosoma cruzi*. This enzyme is located in the parasite plasma membrane and is strongly associated with the flagellum, a unique feature described so far for the trypanosomatid PDEs.

It is interesting to note that the *TcPDE1* gene sequence shows a significant similarity only to previously described *T. brucei* cAMP-PDE genes; but the encoded protein shows also a considerable similarity to all mammalian PDE families. TcPDE1 presents a putative signal peptide, two GAF domains and a conserved catalytic domain. With this structure, TcPDE1 is

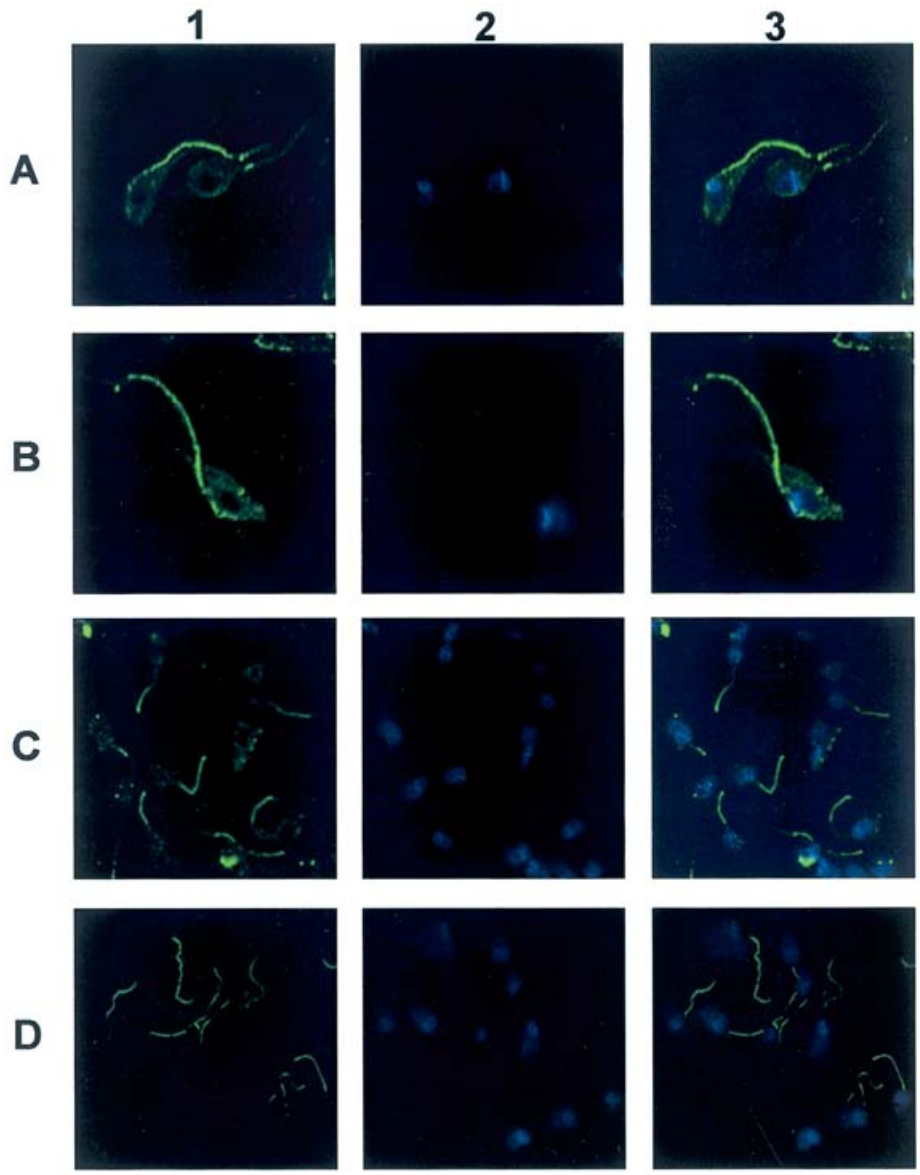

Figure 7 Association of TcPDE1 with the flagellar structure

Cells were pre-treated with 1 % (v/v) Triton X-100 (**A**) and (**B**) or with 0.5 % (v/v) NP-40 (**C**) and reacted to anti-TcPDE1 antibody. (**D**) Cells were pre-treated with 0.5 % (v/v) NP-40 and reacted to anti-(paraflagellar rod) antibody. (**1**) Specific antibody; (**2**) DAPI, and (**3**) images (**1**) and (**2**) merged.

closely related to the *T. brucei* PDEs TbPDE2B and TbPDE2C. On the other hand, the genomic organization of the *TcPDE1* is different from that of *TbPDE2B*, where two identical genes are arranged in tandem [19], and similar to that of *T. brucei TbPDE2A*, which shows the NHP2 gene located downstream from the coding sequence [17]. These data would indicate that *TcPDE1* and the *TbPDE2* family from *T. brucei* share a common evolutionary origin.

Southern blot and PFGE analysis demonstrated that *TcPDE1* is part of a small gene family from *T. cruzi*. The presence of different isoforms was also observed by Northern blots. This is consistent with previous reports made on *T. brucei* where two families of cAMP-PDEs have been identified [8,16–19].

Yeast strains deficient in PDE genes have been widely used for the identification and characterization of many cAMP PDEs [41]. This system allows the study of a unique PDE isoform independently of others that could be present in the cell. Complementation assays in yeast PDE-deficient strains, indicated that TcPDE1 is a functional PDE. In these cells, the enzyme is strongly associated with membranes, shows a high affinity for cAMP and is not able to hydrolyse cGMP. In addition, cGMP does not affect the affinity of TcPDE1 for its substrate or its cAMP-hydrolytic activity. On the other hand, yeast recombinant TcPDE1 shows 2–3-fold more activity in the presence of Mn^{2+} than Mg^{2+} and is not modulated by Ca^{2+}, calmodulin or by phenotiazinic inhibitors.

The use of PDE-specific inhibitors as therapeutic agents for the treatment of many diseases has long been known. In *T. brucei*, some of these compounds prevent cell proliferation in culture [17]. For this reason, the search for inhibitors that could block the activity of *T. cruzi* cAMP-PDE is of great importance because they could be used as a novel therapy for the treatment

of Chagas disease. The characterization of individual isoforms present in the parasite would be a requirement to assess this. Of the different compounds tested, only dipyridamole was a good inhibitor of TcPDE1. Papaverine, EHNA and vinpocetine were poor inhibitors and IBMX, theophylline and rolipram did not produce a significant inhibition.

As mentioned, TcPDE1 sequence shows the presence of two cGMP-binding domains (GAF). However, as occurs with some members of the *T. brucei* TbPDE2 family, cGMP does not affect its activity [17,19]. Several reports indicate that GAF domains may have important regulatory roles not related to cGMP binding. It has been demonstrated that these domains bind cAMP in *Anabaena* [42] and it has been proposed that they can also bind other molecules [1,2]. Interestingly, the GAF domains have recently been involved in the dimerization of mammalian PDE2A, PDE5 and PDE6 isoforms [43,44]. Further experiments will be necessary to determine the function and properties of these domains in the trypanosomatid PDEs.

The presence of different isoforms of cAMP-PDEs together with the large number of adenylate cyclases identified in trypanosomatids indicates that multiplicity of the cAMP-signalling components has a pivotal role in the regulation of the cAMP signal in these organisms. In mammals, the importance of isoform multiplicity in the cAMP pathway has been well-documented [20–22]. The different expression patterns and regulatory mechanisms presented by multiple isoforms enable the cell to generate different responses to a wide variety of extracellular signals. The fact that TcPDE1 is part of a multigene family and that it is not Ca^{2+}-calmodulin-regulated or soluble, as the activity previously described in *T. cruzi* [12], supports this postulation.

Compartmentalization of the cAMP signalling components is another important consequence of isoform multiplicity. Recent reports suggest that cAMP could act through local gradients or microdomains [22,45,46]. The spatio-temporal action of this second messenger enables the cell to produce a specific response without secondary undesired effects. Exclusive subcellular distribution of different isoforms and formation of signalling complexes is essential for the production of a local signal. In mammals, this is achieved mainly by the differential distribution of PKA and cAMP-PDEs. A-kinase anchoring proteins (AKAPs) anchor PKA close to its targets, allowing a rapid and specific response [47]. On the other hand, the subcellular distribution of cAMP-PDEs allows the production of local cAMP signals by limiting the diffusion of the second messenger to other locations in the cell. In trypanosomatids, the flagellar localization described for some adenylate cyclases [10,11] indicates that compartmentalization could also have an important role in these organisms. If this is so, different PDEs should be differentially located within the parasite. Supporting this hypothesis, TcPDE1 is located in the plasma membrane of *T. cruzi*, whereas other isoforms are present in the cytoplasm ([12]; the present study). Furthermore, TcPDE1 seems to be concentrated in the flagellum. These results, together with our previous report showing that TczAC adenylate cyclase from *T. cruzi* specifically interacts with the flagellar protein paraflagellar rod [10], indicate that the flagellum would have a crucial role in the cAMP signalling in this organism. The presence of cAMP PDEs and adenylate cyclases in this organelle could enable the parasite to generate a confined cAMP signal by locally synthesizing the second messenger and limiting its diffusion to other parts of the cell.

We thank Dr Johan Thevelein for yeast strain supplies, Dr Keith Gull for paraflagellar rod antibodies, Dr Silvia Moreno and Dr María Teresa Téllez-Iñón for drug supplies. We also thank Dr Marcela Raíces for critical reading of the manuscript. This work was supported by grants from the Universidad de Buenos Aires, the CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), the Agencia Nacional de Promoción Científica y Tecnológica, the Fundación Antorchas and the International Centre for Genetic Engineering and Biotechnology (Trieste, Italy). M. A. D. is a fellow of CONICET.

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Received 29 July 2003/6 October 2003; accepted 13 October 2003
Published as BJ Immediate Publication 13 October 2003, DOI 10.1042/BJ20031147