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The flagellar adenylate kinases of *Trypanosoma cruzi*

María de los Milagros Camara[1], León A. Bouvier[1], Mariana R. Miranda[2] and Claudio A. Pereira[2,*]

[1]Instituto de Investigaciones Biotecnológicas (IIB-INTECH-UNSAM), (1650) Buenos Aires, Argentina and [2]Laboratorio de Biología Molecular de Trypanosoma cruzi (LBMTC), Instituto de Investigaciones Médicas Alfredo Lanari, Consejo Nacional de Investigaciones Científicas y Técnicas and Universidad de Buenos Aires, (1427) Buenos Aires, Argentina

*Corresponding author: IDIM, Combatientes de Malvinas 3150, (1427) Bs. As., Argentina. Tel: 5411-4514-8701; Fax: 5411-4523-8947; E-mail: cpereira@retina.ar

One sentence summary: Two novel adenylate kinase isoforms were found in the Trypanosoma cruzi flagellum.
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ABSTRACT

Adenylate kinases (ADK) are key enzymes involved in cell energy management. Trypanosomatids present the highest number of variants in a single cell in comparison with the rest of the living organisms. In this work, we characterized two flagellar ADKs from *Trypanosoma cruzi*, called TcADK1 and TcADK4, which are also located in the cell cytosol. Interestingly, TcADK1 presents a stage-specific expression. This variant was detected in epimastigotes cells, and was completely absent in trypomastigotes and amastigotes, while TcADK4 is present in the major life cycle stages of *T. cruzi*. Both variants are also regulated, in opposite ways, along the parasite growth curve suggesting that their expression depends on the intra- and extracellular conditions. Both, TcADK1 and TcADK4 present N-terminal extension that could be responsible for their subcellular localization. The presence of ADK variants in the flagellum would be critical for the provision of energy in a process of high ATP consumption such as cell motility.

Key words: *Trypanosoma cruzi*; *Trypanosoma brucei*; adenylate kinase; flagellum; phosphotransferase; Chagas' disease

INTRODUCTION

Trypanosomatids are responsible for important veterinary infections and severe human diseases. In Africa, *Trypanosoma brucei* causes sleeping sickness or African trypanosomiasis, while *T. cruzi* is the ethiological agent of Chagas' disease (Barrett *et al.*, 2003). This parasite presents a complex life cycle which involves major morphological and gene expression modifications as they encounter different environments. Due to the extremely variable nutrient availability conditions, they need a coordinated regulatory response in order to maintain ATP homeostasis.

Adenylate kinases (ADKs, ATP:AMP phosphotransferases, EC 2.7.4.3) are enzymes widely distributed in organisms from bacteria to higher eukaryotes involved in cellular energetics (Noda 1973). They catalyze the interconversion of constituents of the adenine nucleotide pool, duplicating the ATP energetic potential. Most organisms express one to three ADK variants while the presence of an expanded ADK family seems to be a common feature of trypanosomatids; *T. brucei* has seven variants targeted to different subcellular structures, such as flagellum, glycosome, mitochondrion and cytosol (Pullen *et al.*, 2004; Ginger *et al.*, 2005). Furthermore according to genomic data *Leishmania major* presents six putative variants as well as *T. cruzi* whose six variants are targeted to different predicted subcellular compartments (Bouvier *et al.*, 2006; Milagros Camara Mde *et al.*, 2012). In addition, a seventh ADK variant was recently characterized in *T. cruzi* located in the cell nucleus and would be responsible for ribosomal RNA processing (Camara Mde *et al.*, 2013).

Trypanosoma brucei presents three flagellar ADKs which present N-terminal flagellar localization signals. The TbADKE

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variant was found inside the axoneme whereas TbADKA and TbADKB in the extra-axonemal structure called paraflagellar rod (PFR) (Pullen *et al.*, 2004). *Trypanosoma cruzi* presents orthologs of the *T. brucei* axonemal variant but only one of the PFR variants (TbADKB).

The main objective of this study was to determine if the *T. cruzi* ADKs containing atypical N-terminal extensions localize in the parasite flagellum. Our results confirmed that *T. cruzi* ADK variants TcADK1 and TcADK4 are located in different structures of the flagellum. In addition, both variants presented stage-specific expression.

MATERIALS AND METHODS

Parasite culture

Epimastigotes of the CL Brener strain were cultured at 28°C in plastic flasks (25 cm^2), containing 5 ml of LIT medium (started with 10^6 cells per milliliter) supplemented with 10% fetal calf serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Camargo 1964). The parasites were subcultured with passages each 7 days. Vero monolayers were grown in MEM supplemented with 10% fetal calf serum at 37°C. During infection, fetal calf serum concentration was reduced to 2%. The trypomastigotes, released from the cultured cells around the 5th–6th day after infection, were washed three times in MEM before used for further experiments.

Production of recombinant proteins and enzyme activity assays

TcADK1 and TcADK4 genes (TriTrypDB accession numbers: TcCLB.503479.30 and TcCLB.507057.20, respectively) were obtained by PCR amplification using genomic *T. cruzi* DNA as template. Obtained PCR products were cloned into the pRSET A expression vector (Invitrogen, Carlsbad, CA). Expression of the recombinant ADK was performed in *E. coli* strain BL21(DE3)pLysS (Invitrogen, Carlsbad, CA). Recombinant proteins were purified by affinity chromatography using a Ni^{2+-} agarose resin (Qiagen, Maryland, USA). For enzyme activity assays, a sample of 50 μg of recombinant protein was added to the reaction mixture (100 mM Tris-HCl buffer pH 7.5, 20 mM glucose, 5 mM $MgCl_2$, 100 mM KCl, 2 mM dithiothreitol, 1 mM NADP+, 5 units per ml hexokinase and 2 units per ml glucose-6-phosphate dehydrogenase) in a cuvette to give a final volume of 0.5 ml. After 5 min at 35°C, the reaction was started by the addition of a small volume of ADP to a final concentration of 10 mM.

Antibodies

TcADK1 and TcADK4 antisera were obtained from BALB/c mice immunized with three doses of recombinant protein (10 μg) during 2 months. For Western blot analysis, anti-TcADK1, anti-TcADK4 and anti-PAR2 antibodies were diluted 1:2000. Anti-PAR2 antibody was kindly provided by Prof. Maria Julia Manso Alves (University of Sao Paulo, Brazil). Antibody specificity was tested by Western blot using recombinant TcADK1 and TcADK4 (see Supporting Information).

Flagellar preparation

Flagellar purification was performed as previously described (Saborio *et al.*, 1989). Briefly, 10^9 epimastigotes were collected and resuspended in a solution of NP-40 1% (v/v). Parasites were lysed by freezing and thawing and centrifuged at 1500 xg. The obtained supernatant (S1) was conserved and the pellet was extracted using the same protocol. After centrifugation, the second supernatant was obtained (S2) and the pellet was resuspended in 0.1% NP-40, 1 M NaCl. After centrifugation, the obtained pellet contains the cell debris and the supernatant was then centrifuged at 12 000 xg. The supernatant (S3) was conserved and the pellet was treated using the same extraction procedure so obtaining the supernatant (S3) and the pellet containing the purified flagella (F).

Immunofluorescence

Freshly grown trypanosome samples were washed twice in phosphate buffered saline (PBS). Cells were left to settle for 30 min at room temperature onto poly-L-lysine coated coverslips, and parasites were fixed at room temperature for 20 min with 4% formaldehyde in PBS, followed by a cold methanol treatment for 5 min. Afterwards, all the samples were treated with anti-TcADK1 or anti-TcADK4 (1:200) for 1 h followed by secondary antibody incubation anti-mouse for 1 h. Slides were mounted using Vectashield with DAPI (Vector Laboratories). Cells were observed in an Olympus BX60 fluorescence microscope. Images were recorded with an Olympus XM10 camera. Images were analyzed with MBF ImageJ for microscopy bundle.

RESULTS

A subgroup of ADK presents unusual N-terminal extensions

A bioinformatic approach was performed in the TriTryp database (http://tritrypdb.org/) followed by sequence and synteny analysis allowing us to identify two putative flagellar *T. cruzi* ADKs. As Fig. 1A shows, TcADK1 and TcADK4 and its orthologs in *T. brucei* (TbADKB) and *L. major* present amino acid identities between 32% and 60%. All these ADK variants present an N-terminal extension of 55–58 amino acids related to the canonical variant (TcADK5). Furthermore, *T. cruzi* N-terminal extension shares 47% identity with *T. brucei* and 36% with *L. major* ones (Fig. 1B). Even though we performed an extensive analysis, we were not able to identify a *T. cruzi* equivalent for the other *T. brucei* paraflagellar (PFR) variant (TbADKA), being exclusively found in the genome of *T. brucei* (Pullen *et al.*, 2004). Remarkably, in the human genome the two-domain ADK variant 8 (HsADK8, GenBank ID: NP˙689785.1) presents a similar N-terminal extension to TcADK1 and TcAKD4 (Fig. 1C).

In order to validate TcADK1 and TcADK4 as *bona fide* ADK, the genes were cloned, expressed in *E. coli*, and the recombinant proteins were purified. Enzyme activity assays were performed revealing that TcADK1 as well as TcADK4 was able to transform ADP to ATP (Fig. 1D). In addition, their ADK activity could be inhibited in the presence of the ADK-specific inhibitor P1, P5-bis(5`-adenosyl) pentaphosphate (AP5A), confirming the enzyme's identity (Nageswara Rao and Cohn 1977). The estimated IC_{50} values for AP5A were in the range 20–40 μM for both ADK variants. These results are in accordance with previously published data (Ginger *et al.*, 2005).

TcADK1 and TcADK4 are located in the cell cytosol and flagellum

We next focused our study on the subcellular localization of TcADK1 and TcADK4 in epimastigotes cells. We performed a flagellar purification protocol followed by Western blot assays (Fig. 2A). Successive detergent and salt extractions using the flagellar marker (PFR) revealed that both ADK variants were

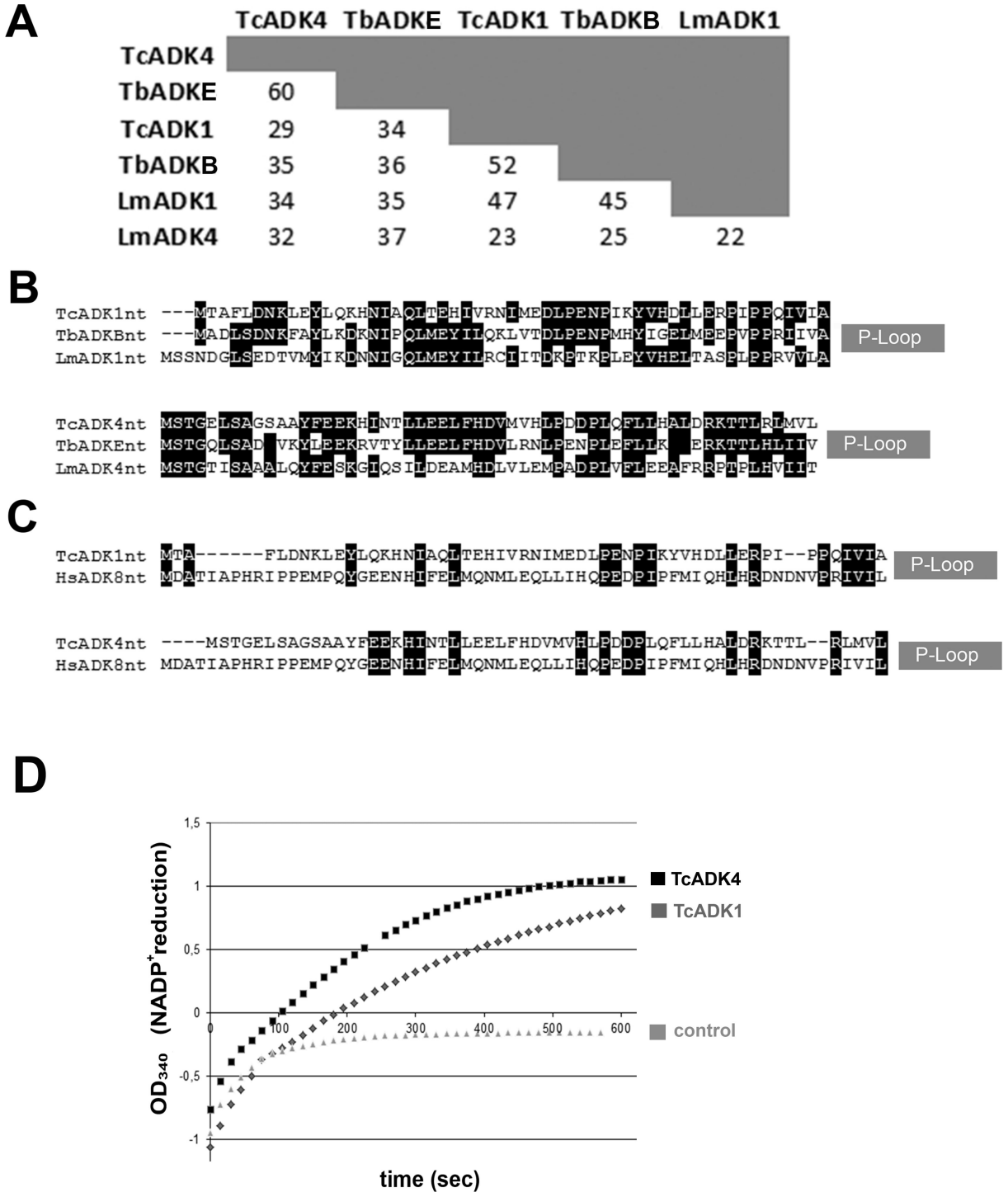

Figure 1. Sequence analysis and validation of trypanosomatids' ADK1 and ADK4. (**A**) Amino acid identities between ADK from *T. cruzi* (TcADK1 and TcADK4), *T. brucei* (TbADKB and TbADKE) and *L. major* (LmADK1 and LmADK4). (**B**) Sequence alignment of the N-terminal extension of TcADK1 and TcADK4 orthologs in *T. brucei* (TbADKB and TbADKE) and *L. major* (LmADK1 and LmADK4) or (**C**) the human adenylate kinase (HsADK8). The P-loop (GxPGxGK) indicates the fragment that interacts with the phosphoryl groups of ATP. Black boxes indicate identical residues. (**D**) TcADK1 and TcADK4 were evaluated for ADK activity by ATP synthesis from ADP as described under the section 'Materials and Methods'. The graphic shows the reduction of NADP+ (increase in absorbance at 340 nm) as a function of time. Controls were performed using a reaction mixture without ADP.

present in the crude flagellar fraction (lane Fl). In addition, both ADK variants were also detected in the soluble fraction (supernatant of the first detergent extraction, lane S1) and cell debris (300 xg pellet, lane NP). However, the proportion of TcADK4 detected in flagella was higher than the one observed in TcADK1 samples, which is mainly solubilized in the first detergent extraction. Results obtained by enzyme activity assays are in agreement with those obtained by Western blot (data not shown). The different proportions of both variants located in flagellum and cytosol may be a

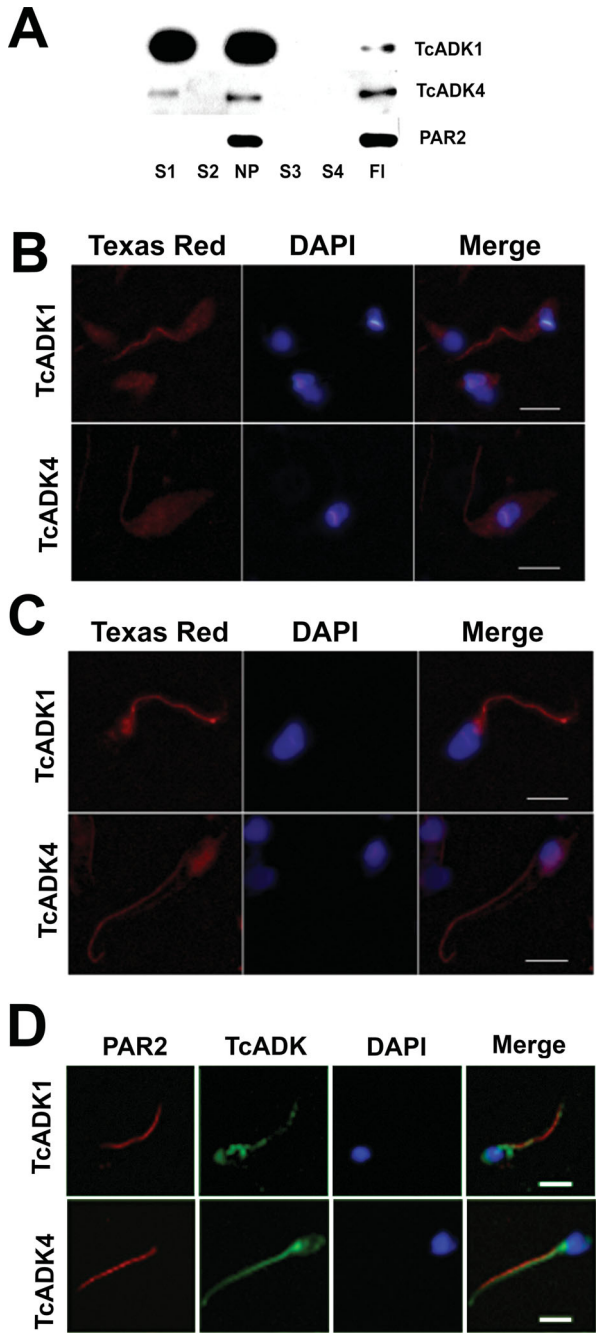

Figure 2. TcADK1 and TcADK4 localization by subcellular fractionation and immunofluorescence analysis. (**A**) Western blot using samples obtained from successive extraction steps, as follows: (S1) supernatant of a protein extraction with 1% (w/w) Nonidet P40; (S2) supernatant of a re-extraction of the pellet using the same treatment; (NP) pellet 300 xg of sample S2 pellet, extracted with 1 M NaCl; (S3) supernatant of sample S2 after a centrifugation at 12 000 xg; (S4) supernatant of a re-extraction of pellet from sample S3 and crude flagella (Fl). Western blot analyses were performed using mouse polyclonal anti-TcADK1, anti-TcADK4 and anti-paraflagellar rod protein 2 (PAR2) antibodies. (**B**) and (**C**) TcADK1 and TcADK4 localization by indirect immunofluorescence using anti-TcADK1 and anti-TcADK4 antibodies. *Trypanosoma cruzi* epimastigotes were attached to multiwell slides, fixed and reacted to the specific antibodies. TcADK antibodies coupled to the red-fluorescent dye (Texas Red), DAPI, and the merged images (Merge) are shown in the indicated columns. (B) Fluorescence images of parasites without treatment. (C) Fluorescence images of parasites permeabilized with detergent treatment. (**D**) Co-localization of TcADK1 (upper panel) and TcADK4 (lower panel) with paraflagellar rod protein 2 (PAR2). White bars in the left corner of the merged images represent 5 mm.

consequence of dissimilar retention strengths to the intraflagellar structures.

Flagellar localization was also confirmed by indirect immunofluorescence using anti-TcADK1 and anti-TcADK4 antibodies. Figure 2B showed the fluorescence images of parasites without treatment, showing a location of TcADKs in both flagellum and cytoplasm. In Fig. 2C, the samples were permeabilized with detergent to confirm the flagellar location by removing the soluble fraction of the enzymes. To further characterize the subflagellar localization of both ADK variants, co-localization analyses were carried out using anti-ADKs and anti-PAR2 antibodies. TcADK4 was found in a structure, parallel to the PFR, which is probably the axoneme since TcADK1 was located only in discrete foci along the flagellum (Fig. 2D).

TcADK1 and TcADK4 are regulated along the parasite life cycle

The large number of ADK variants raises the question whether flagellar ADK variants may present differential localizations or they could be differentially expressed along the parasite life cycle. In order to test if TcADK1 and TcADK4 expression is regulated along the parasite's life cycle, Western blot analyses using samples from the major *T. cruzi* stages were performed. As Fig. 3A shows, a band corresponding to TcADK1 (predicted MW: 29.4 kDa) was detected in epimastigotes cells, and was completely absent in bloodstream trypomastigotes or amastigotes samples. These data constitute strong evidence of the stage-specific expression of this ADK variant. On the other hand, Western blot analysis of TcADK4 (predicted MW: 29.6 kDa) revealed that this variant is present in all *T. cruzi* life cycle stages (Fig. 3A). However, in trypomastigotes a duplex band, which duplicates the predicted molecular weight of TcADK4, could be observed. These bands were also present in amastigotes, in addition to a 40 kD signal. The observed results suggest the presence of post-translational modifications or a strong association between TcADK4 monomers that cannot be dissociated with the treatment of the samples. However, by bioinformatic analysis no post-translational modifications in TcADK4 sequence could be predicted.

Finally, we studied the expression of both proteins along the epimastigotes growth curve where the environmental conditions mimic the epimastigotes passage through the insect host. Western blot analyses were performed using parasite samples obtained from days 1st to the 7th of the epimastigotes' growth curve. As Fig. 3B shows, TcADK1 increases continuously, reaching maximum values during the stationary phase, while TcADK4 presents an opposite pattern decreasing along the growth curve. These results indicate that these variants are under a tight regulation that could be due to the different environments and energy demands that the parasite affronts.

DISCUSSION

About 100 million years of divergence between African and American trypanosomes brought on the variations between theirs vectors, hosts and life cycles. Adaptation to different environments and morphologies requires changes in their metabolic pathways (Stevens *et al.*, 1999). In this work, we identified two flagellar ADKs which present unusual N-terminal extensions that could be responsible for their subcellular localization. TcADK4 seems to be located all along the axonemal structure while TcADK1 was found in foci distributed along

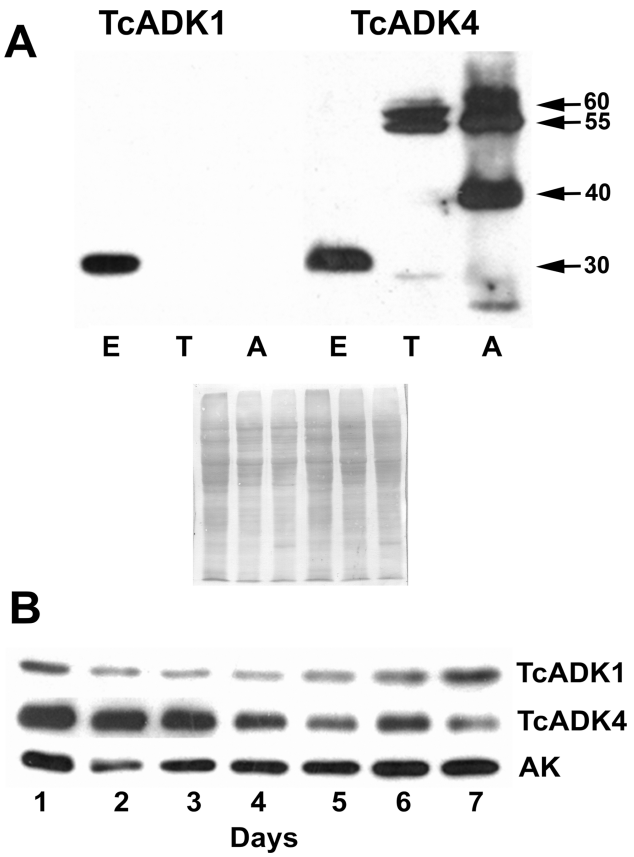

Figure 3. TcADK1 expression in different stages of *T. cruzi* life cycle and culture growth phases. (**A**) Western blot analyses were performed using a mouse polyclonal anti-TcADK1 and anti-TcADK4 antibodies and samples from *T. cruzi* epimastigotes (E), bloodstream trypomastigotes (T) and amastigotes (A). Samples were obtained from 4×10^6 parasites. Total protein staining was used as loading control (lower panel). (**B**) Western blot of epimastigotes samples obtained from different days of culture (1–7). Arrow indicates the cell passage cycle of culture. Samples were obtained from 4×10^6 parasites. Arginine kinase (AK) was used as loading control.

the flagellum and close to the basal body. This location differs from the *T. brucei* ortholog (TbADKB) which was present only in the PFR.

In addition, both enzyme variants present stage-specific expression. The presence of TcADK1 only in epimastigotes stage and TcADK4 in all life cycle stages indicates the requirement of an additional ADK in the parasite insect stage. Interestingly, TcADK1 seems to be necessary when the energy sources are scarce since it reaches the maximum levels under these conditions. On the contrary, TcADK4 presents maximum amounts when nutritional resources are abundant. This variation might be in response to extracellular conditions; for example, insufficient nutrient availability, as occurs mainly in the insect stages, induces the expression of TcADK1 whilst TcADK4 is operative mainly in the mammalian stages when the carbon sources are rich and constants. The stage specificity of TcADK1 seems to be due a translational or post-translational regulation since previous studies demonstrated that TcADK1 mRNA is present not only in epimastigotes but also in trypomastigotes (Bouvier *et al.*, 2006).

The constraints on the diffusion of adenine nucleotides make ADK systems critical in the flagellar motility process since they can rapidly synthesize ATP in the same microenvironment that ATPases produced ADP, thus duplicating its energy potential.

SUPPLEMENTARY DATA

Supplementary data is available at FEMSLE online.

ACKNOWLEDGEMENTS

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Conflict of interest statement. None declared.

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