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Trypanosoma cruzi Polyamine Transporter: Its Role on Parasite Growth and Survival Under Stress Conditions

Chantal Reigada[1] · Melisa Sayé[1] · Edward Valera Vera[1] · Darío Balcazar[2] · Laura Fraccaroli[2] · Carolina Carrillo[2] · Mariana R. Miranda[1] · Claudio A. Pereira[1]

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Abstract *Trypanosoma cruzi* is the etiological agent of Chagas disease, a major health problem in Latin America. Polyamines are polycationic compounds that play a critical role as regulators of cell growth and differentiation. In contrast with other protozoa, *T. cruzi* is auxotrophic for polyamines because of its inability to synthesize putrescine due to the lack of both, arginine and ornithine decarboxylase; therefore, the intracellular availability of polyamines depends exclusively on transport processes. In this work, the polyamine transporter TcPAT12 was overexpressed in *T. cruzi* epimastigotes demonstrating that growth rates at different concentrations of polyamines strongly depend on the regulation of the polyamine transport. In addition, parasites overexpressing TcPAT12 showed a highly increased resistance to hydrogen peroxide and the trypanocidal drugs nifurtimox and benznidazole, which act by oxidative stress and interfering the synthesis of polyamine derivatives, respectively. Finally, the presence of putative polyamine transporters was analyzed in *T. cruzi*, *Trypanosoma brucei*, and *Leishmania major* genomes identifying 3–6 genes in these trypanosomatids.

Keywords *Trypanosoma cruzi* · Polyamine transporter · Chagas disease

Introduction

American trypanosomiasis or Chagas disease is a major health and economic problem in Latin America and its causative agent is the hemoflagellate *Trypanosoma cruzi* (Chagas 1909; Rassi et al. 2010). According to the World Health Organization, about 6–7 million people are estimated to be infected worldwide, mostly in Latin America where Chagas disease is endemic (http://www.who.int/mediacentre/factsheets/fs340/). In addition, the chronicity of the pathology implies great health expenditures, due to the disability associated with the chronic state of this infection, which induce heart failure as main disabling condition (Rassi et al. 2010). Along the life cycle, *T. cruzi* is exposed to different environments in the gut of the insect vectors, in the bloodstream of the mammalian hosts, and also within different cell types (Barrett et al. 2003). The availability of nutrients in these dissimilar milieus determines the need for complex metabolic adaptations. Unicellular parasitic organisms support a strong selective pressure to replace biosynthetic pathways by transport systems. Uptake of nutrients from the extracellular medium is inexpensive in terms of energy economy compared to the metabolic synthesis of these compounds. According to the increase in the biological complexity, there is a tendency to reduce the number of transporter genes per megabase of genome. Data obtained from "The Transporter Classification Database" (TCDB: http://www.tcdb.org/) reveals that *T. cruzi* has 11, *Drosophila melanogaster* 5, and *Homo sapiens* 0.3 transporter genes per megabase of genome. A clear case of metabolism—transport

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✉ Claudio A. Pereira
cpereira@retina.ar

[1] Laboratorio de Parasitología Molecular, Instituto de Investigaciones Médicas "Alfredo Lanari", Universidad de Buenos Aires and CONICET, Av. Combatientes de Malvinas 3150, 1427 Buenos Aires, Argentina

[2] Instituto de Ciencias y Tecnología "Dr. César Milstein", CONICET, Buenos Aires, Argentina

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replacement is the supply of polyamines in *T. cruzi*. Polyamines are polycationic compounds that play a critical role as regulators of cell growth and differentiation (Igarashi and Kashiwagi 2000). In trypanosomes, polyamines are involved in crucial cellular processes including the synthesis of the antioxidant compound trypanothione (bis-glutathionyl spermidine) which has been found exclusively in these protozoa (Fairlamb et al. 1985). Polyamines could be obtained by synthesis de novo from ornithine and in some cases from arginine, or transported from the extracellular medium (Colotti and Ilari 2011). In contrast with other protozoan parasites, *T. cruzi* is auxotrophic for polyamines because of its inability to synthesize putrescine due to the lack of both, arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) (Carrillo et al. 1999, 2003). Therefore, the intracellular availability of polyamines in *T. cruzi* depends exclusively on transport processes. The only amino acid transporter family identified in kinetoplastids is the "Amino Acid/Auxin Permeases" (AAAP; TC 2.A.18), one of the major transporter families of the "Amino acid/polyamine/organocation" (APC) superfamily (Bouvier et al. 2004; Young et al. 1999). Until now, only one polyamine transporter was identified in *T. cruzi*. Carrillo et al. (2006) described the functionality of the spermidine transporter TcPAT12 in a *Xenopus laevis* model and sometime later Hasne et al. (2010) characterized an identical protein in *T. cruzi* as a putrescine transporter.

Considering the importance of polyamines in *T. cruzi*, in this work, we analyzed some properties of polyamine transporters, in particular TcPAT12, and the relationship between TcPAT12 activity, parasites growth, and resistance to oxidative stress and trypanocidal drugs.

Materials and Methods

Cell Cultures

Epimastigotes of the Y strain were cultured at 28 °C in plastic flasks (25 cm^2), containing 5 ml of BHT (brain–heart infusion-tryptose) medium (started with 5×10^6 cells per milliliter) supplemented with 10 % fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 20 μg ml^{-1} hemin (Camargo 1964). For parasite survival analysis, the semi-defined SDM-79 medium (Brun and Schonenberger 1979) containing 10 % fetal calf serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 7.5 μg ml^{-1} hemin alone or supplemented with 0.2 mM putrescine was used. It is noteworthy that low levels of polyamines in the media are from the serum supplement (Magnes et al. 2014). Cells were counted using a hemocytometer.

Transport and Drug Resistance Assays

Aliquots of epimastigote cultures (10^7 parasites) were centrifuged at $8000 \times g$ for 30 s and washed once with phosphate-buffered saline (PBS). Cells were resuspended in 0.1 ml PBS and then added 0.1 ml of the transport mixture containing 100 μM (^{3}H)- putrescine, spermidine, or arginine (PerkinElmer's NEN® Radiochemicals; 0.4 μCi). Following incubation at 28 °C, reaction was stopped by adding 1 ml of ice-cold PBS. Cells were centrifuged as indicated above and washed twice with ice-cold PBS. Cell pellets were resuspended in 0.2 ml of water and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co., Meridien CT, USA) (Cupello et al. 2011; Pereira et al. 1999). Cell viability was assessed by direct microscopic examination. Non-specific uptake and carry over were measured in transport mixture at T_0, or incubated at 4 °C. Treatments with hydrogen peroxide and trypanocidal drugs (benznidazole and nifurtimox) were performed incubating 1.3×10^6 cells ml^{-1} in 24-well plates for 24 h at the indicated concentrations of each drug.

Plasmid Constructions and Parasite Tranfection

The polyamine transporter TcPAT12 (GeneDB: TcCLB.504213.110) was amplified using genomic *T. cruzi* DNA as template and the following primers: 5′-GAATTCATG AATCCCGGTGGTG-3′ (forward) and 5′-CTCGAGGTC GACGGTATCGATAAG-3′ (reverse). Amplification product was subcloned employing the EcoRI and XhoI restriction enzyme sites present in the pTREX (Vazquez and Levin 1999) expression plasmid. The same plasmid with the green fluorescence protein (GFP) gene and non-transfected parasites were used as controls. Constructions were transfected into *T. cruzi* epimastigotes as follows. 10^8 parasites grown at 28 °C in BHT medium were harvested by centrifugation, washed with PBS, and resuspended in 0.35 ml of electroporation buffer (PBS containing 0.5 mM $MgCl_2$ and 0.1 mM $CaCl_2$). This cell suspension was mixed with 50 μg of plasmid DNA in 0.2 cm gap cuvettes (Bio-Rad Laboratories). The parasites were electroporated using a single pulse of 400 V, 500 μF with a time constant of about 5 ms (Pereira et al. 2003).

Semi-quantitative RT-PCR

Expression of TcPAT12 was determined in *T. cruzi* epimastigotes transfected with pTREX-TcPAT12 and controls. Genomic DNA and cDNA were obtained from pTREX-TcPAT12 and control *T. cruzi* epimastigotes (0.1–100 ng). Genomic DNA samples were evaluated by PCR using the above mentioned primers for TcPAT12 and

controls. RT-PCR assays were performed using cDNA obtained from both populations of *T. cruzi* epimastigotes according to the manufacturer instructions (M-MLV Reverse Transcriptase, Promega). PCR products were separated in agarose gels stained and quantified using ImageJ software (http://www.imagej.nih.gov/). The results were expressed as arbitrary units (AU) and normalized to 18S rRNA. Data shown represents the mean from 3 independent experiments. Statistical analysis was performed using Student *t* test (**$p < 0.01$).

Bioinformatic Analysis

Sequence analysis were performed using different resources: ''ClustalX'' (ftp://ftp-igbmc.u-strasbg.fr/pub/ClustalX/) (Aiyar 2000) for global sequence alignments and phenogram construction, ''TreeView'' (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html) (Page 1996) for phenogram constructions, ''Vector NTI-Suite 10′' package (InforMax) for sequence analysis, and ''BLAST'' (http://www.blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990) for local sequence alignment. Finally, for synteny analysis and motifs identification, the TriTryp database (http://www.tritrypdb.org/tritrypdb/) and the "Multiple EM for Motif Elicitation" (MEME) algorithm (Bailey et al. 2006) were employed.

Statistics and Data Analysis

All the experiments were made in triplicates and results presented here are representative of three independent assays. For all experiments, the statistical significance was established at $p < 0.05$. IC_{50} values were obtained from nonlinear regressions to dose–response logistic functions. IC_{50} curves were compared using extra sum-of-squares F test. All statistical analyses were performed using GraphPad Prism 6 software.

Results

Characterization of a TcPAT12 Overexpressing Parasite Model

The physiological roles of TcPAT12 were studied using a transgenic model of *T. cruzi* epimastigotes overexpressing the transporter gene (pTREX-TcPAT12). To validate the functionality of the pTREX-TcPAT12 parasites, different transport assays were performed using putrescine, spermidine, and arginine as substrates, accordingly to previous data (Carrillo et al. 2006) (Fig. 1a). The obtained pTREX-TcPAT12 parasites presented transport rates for putrescine, spermidine, and arginine of 1067 % (5.95 pmol min^{-1}),

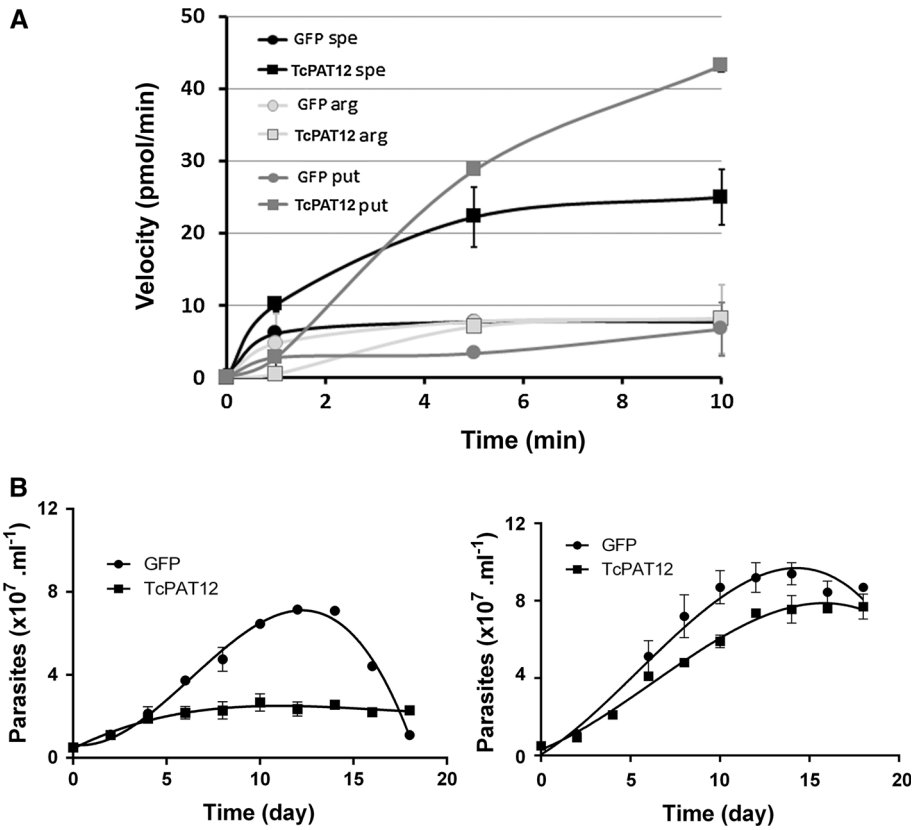

Fig. 1 Overexpression of TcPAT12 and parasite growth curves in low and high concentrations of polyamines. **a** Time course representation of the spermidine (*black line*), putrescine (*dark gray*), and arginine (*light gray*) transport rates in pTREX-TcPAT12 (*square*) or pTREX-GFP (*circle*) parasites. **b** Epimastigote growth curves in low concentrations of polyamines (*left panel*) or in the presence (*right panel*) of 0.2 mM putrescine. *Square symbols* indicate pTREX-TcPAT12 and *circle symbols* pTREX-GFP parasites

233 % (4.03 pmol min^{-1}), and 33 % (0.69 pmol min^{-1}) higher than GFP controls (pTREX-GFP; 0.51, 1.21, and 0.52 pmol min^{-1}, respectively). These results confirmed that TcPAT12 is a putrescine/spermidine transporter almost lacking the capacity to uptake arginine. In addition, affinities for both substrates, putrescine and spermidine, were compared between pTREX-TcPAT12 and control parasites (Supplementary Fig. 1). The results show that pTREX-TcPAT12 parasites have a slight but significant decrease in affinities for both substrates (K_m put = 18.3 μM and K_m spe = 30.3 μM) when compared to controls (K_m put = 10.8 μM and K_m spe = 14.9 μM). Finally, to further validate the TcPAT12 overexpression model, semi-quantitative RT-PCR using genomic and cDNA from controls and pTREX-TcPAT12 parasites was performed. After data normalization to 18S rRNA, pTREX-TcPAT12 presented an increase of 2.3-fold in the transporter cDNA than the control parasites (Supplementary Fig. 2).

Parasites Survival in Media with Low Levels of Polyamines

To test if parasites are able to sustain the growth conditions regulating the polyamine transport rate according to the extracellular availability of such compounds, we took advantage of the transgenic pTREX-TcPAT12 cells. These parasites have a constitutive overexpression of the polyamine permease with high levels of transport activity, as stated above. Therefore, epimastigote forms were grown in a medium with low levels of polyamines, provided by the serum supplement (Magnes et al. 2014), and the parasites growth was assessed during 18 days. As Fig. 1b (left panel) shows, pTREX-GFP parasites had standard growth kinetics with a maximum growth rate between days 1–10, after that parasites had a short stationary phase followed by a declination phase. On the contrary, pTREX-TcPAT12 parasites had a very short slow-replication phase between days 1–4 followed by a prolonged stationary phase. In both cases, after the stationary phase, the parasites died probably due to depletion of polyamines in the medium. When the same experiment was performed in the presence of 0.2 mM putrescine, both parasites, pTREX-TcPAT12 and pTREX-GFP, showed a very similar growth curve in SDM-79 medium (Fig. 1b, right panel). As reference, a standard growth curve of pTREX-GFP controls in rich BHT medium was shown in Supplementary Fig. 3.

Overexpression of TcPAT12 and Resistance to Stress

Polyamines are key molecules in abiotic and biotic stress responses in different organisms (Alcazar and Tiburcio 2014). To study the relationship between polyamine transport rates and stress resistance, parasites pTREX-TcPAT12 were incubated with hydrogen peroxide and trypanocidal drugs. Under hydrogen peroxide treatments in the range 0–150 μM, the concentrations that reduced the parasites growth by half (IC_{50}) were significantly different (p value = 0,0064), 96.8 μM and 16 μM, for pTREX-TcPAT12 cells and the pTREX-GFP controls, respectively

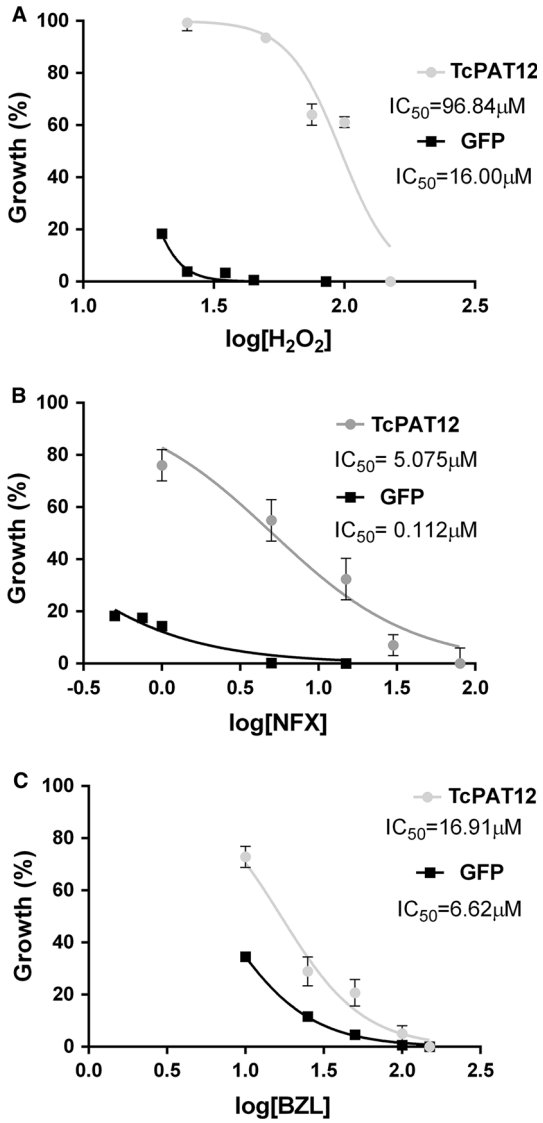

Fig. 2 Effect of TcPAT12 overexpression in oxidative stress and drug resistance. About 1.3 × 10^6 parasites ml^{-1} (pTREX-TcPAT12 cells: PAT12, *gray line*; and pTREX-GFP controls: GFP, *black line*) were incubated with hydrogen peroxide at different concentrations from 0 to 150 μM and counted 24 h after treatment (**a**). The same parasites' groups used at point **a** were incubated with nifurtimox (**b**) and benznidazole (**c**) and at different concentrations 0–80 or 0–150 μM, respectively. Cells were counted 24 h after treatment. IC_{50} values were calculated using nonlinear regressions to dose–response logistic functions. IC_{50} curves were compared using extra sum-of-squares *F* test. All statistical analyses were performed using GraphPad Prism 6 software. Experiments were made in triplicates and results presented here are representative of three independent assays

Table 1 Putative polyamines transporters genes in the *T. cruzi* genome

TcPT	Esmeraldo-like	Non-Esmeraldo-like	AA	TMs	*T. brucei*	*L. major*
TcPAT12	TcCLB.504213.110	TcCLB.506985.40	613	12	−	+
TcPT-2	TcCLB.509551.20	−	521	13	+	+
TcPT-3	TcCLB.506773.90	TcCLB.508799.120	503	10	+	+
TcPT-4	−	TcCLB.506833.70	548	12	+	−
TcPT-5	TcCLB.509167.40	TcCLB.506831.20	716	10	−	−
TcPT-6	−	TcCLB.509733.160	594	10	+	−

Table summarizes the main feature of the six putative polyamine transporters (column 1). The TriTryp identification number of each gene and it allele (Esmeraldo-like and Non-Esmeraldo-like), the amino acid number (column 4), and transmembrane spans (column 5) were indicated. Columns 6 and 7 show the presence (+) or absence (−) of orthologs in *T. brucei* and *L. major*

(Fig. 2a). In addition, pTREX-TcPAT12 cells were treated with the trypanocidal drugs benznidazole (0–150 μM) and nifurtimox (0–80 μM). Both treatments presented significant differences (p value 0.0001) in IC_{50} values for pTREX-TcPAT12 when compared to control pTREX-GFP parasites. IC_{50} for nifurtimox were 5.08 and 0.11 μM for pTREX-TcPAT12 and pTREX-GFP cells, respectively (Fig. 2b). IC_{50} for benznidazole were 16.91 and 6.62 μM, for pTREX-TcPAT12 and pTREX-GFP cells, respectively (Fig. 2c). These results demonstrate that overexpression of TcPAT12 increases resistance to both trypanocidal drugs.

Other Putative Polyamine Transporters in Trypanosomatids

Finally, the diversity and redundancy of polyamine transporters in trypanosomatid genomes were analyzed. A database screening was performed using the information of the TriTryp database followed by bioinformatics sequence analysis. Using the data of the *T. cruzi* genome and "bait" amino acid sequences corresponding to bacterial, plant, and the *T. cruzi* transporter TcPAT12 as positive control, five new putative polyamine transporters were identified. Table 1 summarizes the main features of *T. cruzi* putative polyamine transporters. The overall amino acid identity was 20 % and the consensus positions 46 %. All putative permeases have 503–716 amino acids long with 10–13 transmembrane spans. The results showed that all trypanosomatid species analyzed present putative polyamine transporter genes, including *T. brucei* (4), *T. cruzi* (6), and *Leishmania major* (3). *T. cruzi* presents one *bona fide* and five putative polyamine transporters named TcPAT12, and TcPT-2 to 6, respectively. Interestingly, *T. brucei* and *Leishmania major* which are capable of *de novo* polyamine synthesis possess 2 or 3 putative transporter genes less than *T. cruzi*, respectively (Table 1). Using the "Multiple EM for Motif Elicitation" (MEME) algorithm, a "fingerprint" sequence of 16 amino acids located in an intracellular loop between transmembrane spans 8–11 was found to be present in all putative polyamine transporters identified. However, the biological relevance of this sequence requires further investigation (Fig. 3a, b). Finally, a phenogram was

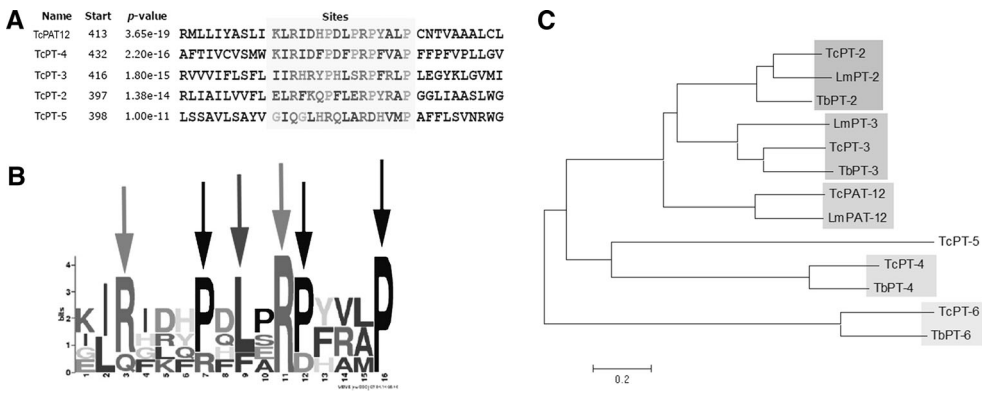

Fig. 3 Common sequence motifs and clustering of the putative polyamine transporters. A "Multiple EM for Motif Elicitation" (MEME) analysis (Bailey et al. 2006) was performed in order to identified common "fingerprint" motifs between the six putative polyamine transporters. **a** Identification of the 16 amino acid length fingerprint motif. **b** Logos graphic indicating the most frequent residues in each position of the fingerprint motif (*arrows*). **c** The evolutionary relationships of putative polyamine transporters were inferred by the neighbor-joining method using the software MEGA6 (Tamura et al. 2013). The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree

constructed based on the amino acid sequence identities. As Fig. 3c shows, the orthologs in the three kinetoplastid species were grouped, and only the groups containing PT-2 and PT-3 belong to the same cluster.

Discussion

Polyamine transport and metabolism are key pathways to design specific inhibitors with tripanocidal effects. This is the case of difluoromethyl ornithine (DFMO), an ornithine analog that blocks the polyamine biosynthesis being strongly toxic for the parasites prototroph for this route such as *T. brucei* and *Leishmania* (Bacchi et al. 1979, 1980; Bachrach et al. 1979). In the specific case of *T. cruzi*, which is auxotroph for the synthesis of polyamines *de novo*, the transport systems are the only way to obtain such molecules, essential for the synthesis of the antioxidant trypanothione, between other functions (Fairlamb et al. 1985). For these reasons, the identification and functional characterization of polyamine transporters, especially in *T. cruzi*, are relevant in terms of drug design for application in Chagas disease. In this work, we confirmed through the overexpression of TcPAT12 in a *T. cruzi* homologous model that it is a putrescine/spermidine transporter. Moreover, the results suggest that, despite the low levels of polyamine, provided by serum supplement of the culture media, these are enough to sustain the control parasites growth. In the case of pTREX-TcPAT12 cells, the unregulated polyamine transport probably depletes the extracellular medium of polyamines interrupting the parasites growth. Since both transgenic models have similar growth kinetics when cultured in polyamine rich medium, we could discard a toxic effect due to an increased intracellular concentration of polyamines and associate the low growth curve of pTREX-TcPAT12 parasites with the transport deregulation. In addition, an increased polyamine transport improves the parasites resistance to oxidative stress generated using hydrogen peroxide or trypanocidal drugs such as nifurtimox and benznidazole. An intriguing issue is the fact that, even though the antioxidant trypanothione has an important role in benznidazole resistance, pTREX-TcPAT12 cells have a much higher resistance to nifurtimox ($IC_{50,TcPAT12}/IC_{50,TcGFP} = 45.3$ and 2,6 for nifurtimox and benznidazole, respectively). Benznidazole produces a decrease over the redox active thiols including trypanothione (Trochine et al. 2014) and nifurtimox generates superoxide and nitro anion radicals, acting through induction of oxidative stress in reactions catalyzed by type II nitroreductases (Hall et al. 2011). Considering the differences between both action mechanisms, the increased nifurtimox resistance in TcPAT12 could be a direct, trypanothione-independent effect of an increased polyamine concentration. The observed differences in affinities between the parasites overexpressing TcPAT12 and controls were of the same magnitude for both substrates. The increase in K_m values for putrescine and spermidine (about twofold) in pTREX-TcPAT12 parasites could be explained by slight differences in the transporters folding and assembly in the plasma membrane.

Although in this study the functionality of the putative transporters PT-2/6 was not confirmed, the presence of a single permease for these key molecules is improbable due the existence of additional molecules that carry out a redundant but essential function.

Considering that *T. cruzi* is exposed to different polyamine concentrations during its life cycle, a tightly regulation, to maximize the use of extracellular polyamines, is needed for parasite survival.

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