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Molecular and functional characterization of a spermidine transporter (*Tc*PAT12) from *Trypanosoma cruzi*

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Abstract

Trypanosoma cruzi, the etiological agent of Chagas' disease, is the only eukaryotic cell which lacks the ability to synthesize polyamines de novo. In this work, we describe for the first time the molecular and biochemical properties of a high-affinity spermidine transporter from *T. cruzi*. The transporter gene *TcPAT12* was functionally expressed in *Xenopus laevis* oocytes, showing high levels of spermidine uptake. Similar apparent affinity constants for spermidine uptake were obtained when comparing *T. cruzi* epimastigotes and heterologous expressed *Tc*PAT12 in *X. laevis*. In addition, *Tc*PAT12 also transports putrescine and the amino acid L-arginine at lower rates than spermidine.

Keywords: *Trypanosoma cruzi*; Polyamines; Spermidine; Putrescine; Arginine; Permease; Chagas' disease

Trypanosoma cruzi is the etiological agent of Chagas' disease, a zoonosis affecting about 18 million people in the Americas. One of the common features of parasitic organisms is the replacement along evolution of biosynthetic anabolic pathways by transport systems. Transporters can be considered as cellular environmental sensors, responding to the presence of substrate molecules in extracellular media, and making them available for physiological processes. This is particularly relevant in organisms such as trypanosomes that endure broad range variations in their environment. However, trypanosomatid transporters have been poorly studied as possible drug targets in spite of their potential already demonstrated for *Trypanosoma* spp. and *Leishmania* spp. [1–3]. In fact, the uptake of trypanocidal drugs used for *Trypanosoma brucei* therapy such as the melamine phenyl arsenical melarsoprol and diamidines occurs through adenosine transporters [4,5].

Polyamines are polycationic compounds that play a critical role as regulators of cell growth and differentiation [6]. 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 trypanosomatid protozoa [7]. Polyamines could be obtained by synthesis de novo from ornithine and in some cases from arginine (via agmatine), or transported from the extracellular medium. In contrast with other protozoa, *T. cruzi* is auxotrophic for polyamines because of their inability to synthesize putrescine due to the lack of both, arginine decarboxylase (ADC) and ornithine decarboxylase (ODC) [8,9]. Therefore, the intracellular availability of polyamines in *T. cruzi* depends exclusively on transport processes. Polyamine transport and metabolism constitutes an appropriate target for chemotherapeutic strategies against parasitic diseases ever since Bacchi and co-workers reported that blocking ornithine decarboxylase enzymatic activity using α-difluoromethylornithine (DFMO) was able to cure

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acute infections of mice with *Trypanosoma brucei brucei* [10,11]. The same drug has also been successfully used in humans against sleeping sickness caused by *Trypanosoma brucei gambiense* [12]. A high-affinity diamine transport system has been biochemically characterized in *T. cruzi* epimastigotes [13,14]. This transporter showed a high specificity for the diamines putrescine and cadaverine and a low specificity for the polyamines spermidine and spermine. Recently, a high-affinity polyamine permease from *Leishmania major* was cloned and functionally characterized [15]. This was the first report of a eukaryotic polyamine permease studied at a molecular level.

In the present work, we describe the molecular and functional properties of a *T. cruzi* high-affinity spermidine permease, which constitutes a potential therapeutic target due to lack of de novo polyamine biosynthetic pathways in this parasite.

Materials and methods

Plasmid constructions. A DNA fragment containing the complete open-reading frame of the *T. cruzi* putative amino acid transporter 12 gene (*TcPAT12*, GenBank Accession No. AY526253) [16] was amplified by polymerase chain reaction (PCR) using genomic DNA from the CL Brener strain as template and the following primers: 5′-CCCGGG ATGAATCCCGGTGGTGAATC-3′ (forward primer containing the *Xma*I restriction site) and 5′-TCTAGAGTTTACGTGTGGGCATTT GC-3′ (reverse primer containing the *Xba*I restriction site). The 1900 bp amplification product was cloned into a pGEMHE plasmid (pGEM-*TcPAT12*) [17] which was kindly provided by Dr. Belen Elgoyhen (INGEBI, Argentina). This plasmid contains the 5′ and 3′ untranslated regions (UTRs) of the *Xenopus laevis* β-globin gene to increase expression in *X. laevis* oocytes. The final construct was confirmed by DNA sequencing analysis using an ABI 377 automated DNA sequencer.

In vitro cRNA synthesis. PGEM-*TcPAT12* was linearized by digestion with *Nhe*I, purified and resuspended in RNAse-free water. In vitro transcriptions were performed in a 30-μl reaction volume using 0.2 μg DNA template, 40 U T7 RNA polymerase (Ambion Inc.), 2 mM m7GpppG cap structure analog, 0.8 mM GTP, and 2 mM UTP, CTP, and ATP. The transcription reaction was incubated at 37 °C for 2 h. DNA template was removed from the reaction mix by DNAse I treatment. The obtained cRNA was purified using the RNeasy Mini Kit (Qiagen Inc.) and quantified, as described elsewhere [18].

TcPAT12 expression in X. laevis oocytes. Xenopus laevis oocytes isolation and handling were performed as previously described [19]. Selected defolliculated oocytes were microinjected with 45 nl (5–20 ng) *TcPAT12* cRNA, linearized or supercoiled plasmid DNA or DNAase/RNAse-free water as controls, using a semi-automatic injector. Oocytes were maintained at 16 °C in Barth's solution pH 7.4 (88 mM NaCl, 2.4 mM $NaHCO_3$, 1 mM KCl, 0.33 mM $Ca(NO_3)_2$, 0.41 mM $CaCl_2$, 0.82 mM, $MgSO_4$, 5 mM Tris/HCl) supplemented with 50 μg/ml gentamicin, 100 U/ml penicillin, and 100 mg/l streptomycin.

Uptake assays in X. laevis oocytes. Uptake of [^{14}C]-polyamine, L-[2,3-^{3}H]-arginine, and [^{14}C-U]-D-glucose was assayed at the indicated times in 24-well plates by incubating 10 oocytes per well in Barth's solution, pH 5.5, containing different compound concentrations. Oocytes were washed quickly three times in cold Barth's solution containing a 2 mM concentration of the corresponding unlabeled substrate (stop solution) and counted for radioactivity in UltimaGold XR liquid scintillation cocktail (Packard Instrument Co.). Kinetic parameters were determined by fitting the data to the Michaelis–Menten equation [20]. Competition assays were performed by incubation of the oocytes with the transport mixture and a 10-fold excess of the competing molecules (1 mM). All assays were performed at least in triplicate and data are representative of at least three independent experiments.

Uptake assays in T. cruzi. Aliquots of CL Brener epimastigote cells (10^7 parasites) grown in LIT medium [21] were centrifuged at 8000*g* for 30 s and washed once with phosphate-buffered saline (PBS). Cells were then resuspended in 0.2 ml PBS containing different concentrations of [^{14}C]-spermidine. Following incubation for 5 min at 28 °C, cells were centrifuged at 8000*g* for 30 s and washed twice with 1 ml of ice-cold PBS containing 1 mM spermidine. Pellets were then resuspended and counted for radioactivity in UltimaGold XR liquid scintillation cocktail. Non-specific transport and carryover were measured in transport mixtures containing 1 mM spermidine. Assays were run at least by triplicate. Cell viability was assessed by direct microscopic examination.

Results

Cloning of TcPAT12 and sequence analysis

*Tc*PAT12 is the most divergent member, in terms of amino acid sequence, of the previously identified family of *T. cruzi* "Putative Amino Acid Transporters" [16]. According to the "Transport Classification Database" (TCDB, http://www.tcdb.org/), *TcPAT12* belongs to the "Amino Acid-Polyamine-Organocation" (APC, TC 2.A.3) transporter family. *TcPAT12* was first classified as an amino acid transporter because the predicted primary structure contains two conserved domains from amino acid permeases (pfam00324.11, residues 76–412; and COG0531.1 residues 50–477). In silico topology analysis tools predicted 9 to 12 transmembrane helices. The "TMpred" program (http://www.ch.embnet.org/) showed a strongly preferred model with a total of 9 transmembrane helix, 50 intracellular residues corresponding to the N-terminus, and 153 extracellular residues from the C-terminus (Fig. 1). Two copies of *TcPAT12* were found in the *T. cruzi* genome database (GeneDB, http://www.genedb.org/) the 1884 bp (627 amino acid) herein characterized, and a 1842 bp copy (98% nucleotide identity) which codifies for an identical polypeptide 14 amino acids shorter. The slight difference between the copies is probably due to the hybrid nature of the CL Brener *T. cruzi* strain [22]. *Tc*PAT12 showed a 55.3% amino acid identity with the previously described *L. major* polyamine transporter *Lm*POT1 (GenBank Accession No. AY727900), with most of the sequence differences in the C-terminus. Lower identities were found with bacterial amino acid and polyamine permeases, i.e., 13.4% amino acid identity with the *Escherichia coli* polyamine permease POT E (GenBank Accession No. NP_415219). In order to functionally express *Tc*PAT12 in *X. laevis* oocytes, the full-length coding sequence was amplified by PCR and cloned into the pGEMHE plasmid, that contains the 5′ and 3′ untranslated regions (UTRs) of a *Xenopus* β-globin gene [17] as described under Materials and methods.

Expression of TcPAT12 in X. laevis oocytes

To ascertain the functional properties of *Tc*PAT12, in vitro synthesized cRNA was microinjected in *Xenopus* oocytes and expression was followed by spermidine uptake

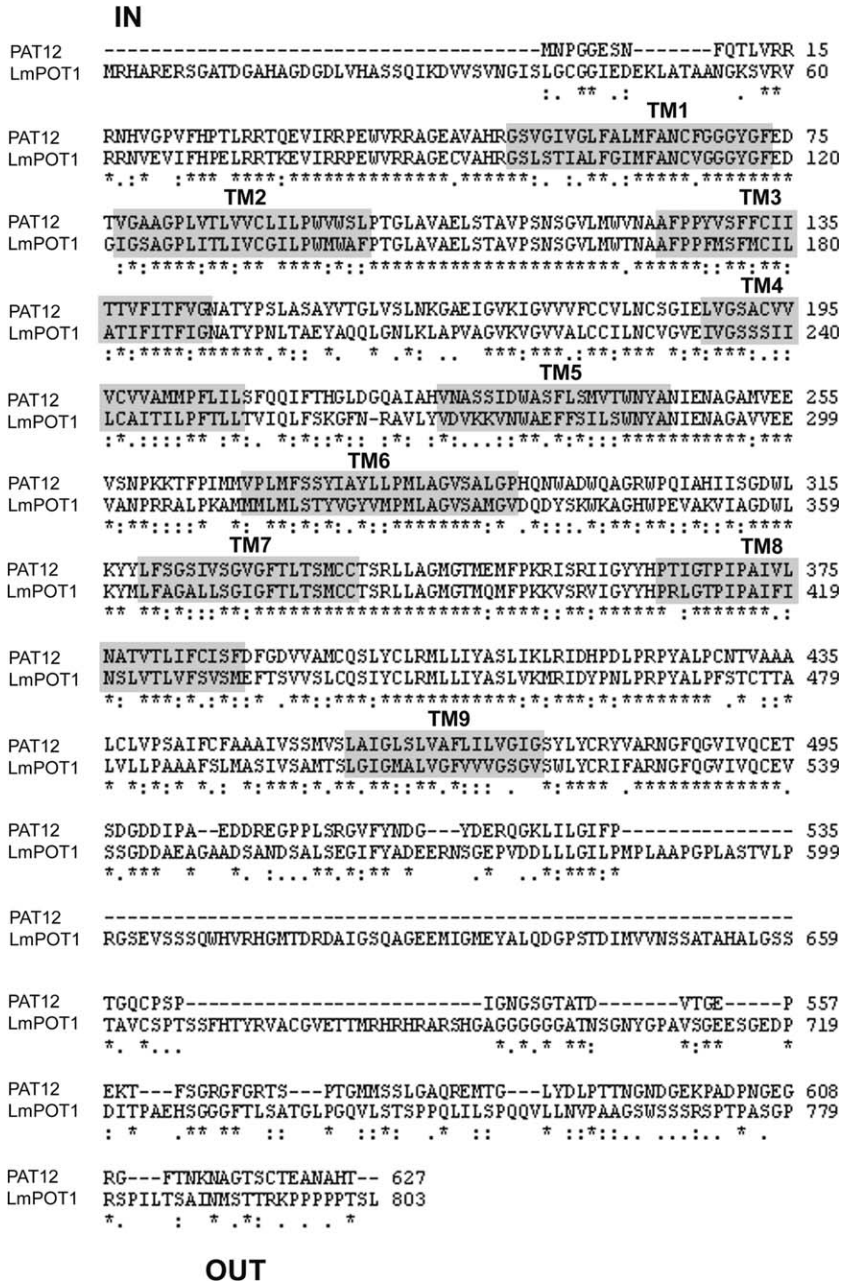

Fig. 1. Sequence alignment of polyamine transporters from *T. cruzi* and *L. major*. Amino acid sequences from *Tc*PAT12 (GenBank Accession No. AY526253) and *Lm*POT1 (GenBank Accession No. AY727900) were aligned using the Clustal W algorithm. Gray boxes indicate the nine predicted transmembrane domains (TM1 to TM9). IN and OUT indicate the predicted localization of the N- and C- termini, inside or outside the cell, respectively. Consensus symbols: ''*'', identical residues; '':'', conserved substitutions; ''.'', semi-conserved substitutions.

assays at 24, 48, and 72 hours post-injection. Oocytes microinjected with water, supercoiled, and linearized pGEM-*TcPAT12* DNA were used as controls. The uptake of radiolabeled spermidine was analyzed at a substrate concentration of 100 μM during 30 min. The optimal time after microinjection for spermidine uptake assays was determined to be 72 h. Surprisingly, oocytes microinjected with pGEM-*TcPAT12* (linearized or supercoiled) showed more than 10-fold increase in spermidine uptake compared with those microinjected with *TcPAT12* cRNA (Fig. 2A). To confirm whether the pGEMHE plasmid is transcriptionally active in *X. laevis* oocytes, a pGEM-Luciferase plasmid was used as expression control. Significant luciferase activity was detected only in pGEM-Luciferase microinjected oocytes at 48 h post microinjection (Fig. 2B). The presence of a cryptic eukaryotic RNA Polymerase II promoter in the pGEMHE plasmid was also analyzed using different prediction programs (FPROM, TSSG, TSSW, and http://www.softberry.com/). Interestingly, all algorithms predict the presence of a putative TATA-box promoter located 512 bp upstream the *TcPAT12* translation start point.

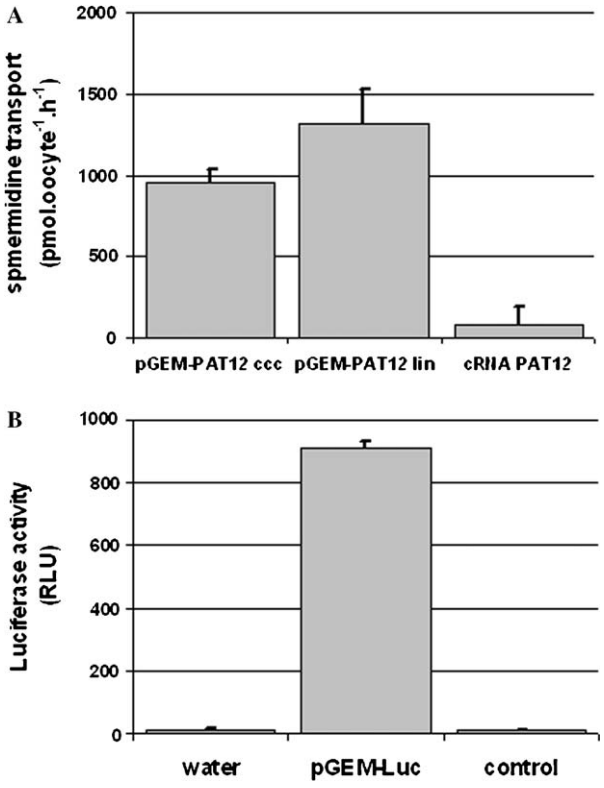

Fig. 2. Spermidine uptake in TcPAT12 microinjected *X. laevis* oocytes. (A) Spermidine uptake was measured during 30 min in oocytes microinjected with 15 ng of supercoiled (ccc), linearized (lin) pGEM-*TcPAT12* plasmid, and cRNA synthesized in vitro from *TcPAT12*. Background uptake measured with water microinjected oocytes was subtracted from all values. (B) Luciferase activity (RLU: relative light units) measured in oocytes microinjected with 5 ng pGEM-Luciferase (Luc), water or without microinjection (control).

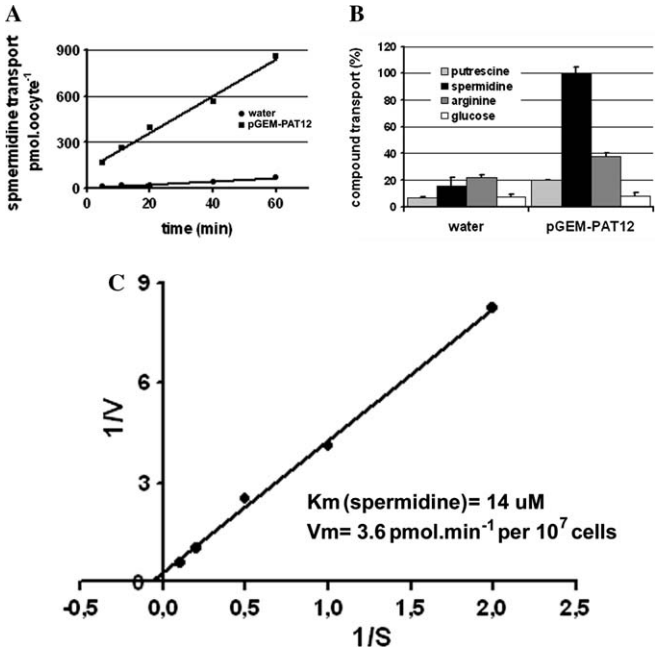

Fig. 3. Biochemical properties of polyamine uptake. (A) Time course of spermidine uptake in oocytes microinjected with pGEM-*TcPAT12*. (B) Comparison of spermidine, putrescine, arginine, and glucose uptake in oocytes microinjected with pGEM-*TcPAT12* or water. Values are expressed as percentages of the spermidine uptake in oocytes microinjected with pGEM-*TcPAT12*. (C) Kinetics of spermidine transport in *T. cruzi* epimastigotes. Initial rates of spermidine transport (V_0) were measured as a function of spermidine concentration in the range 0.5–20 μM. Maximum velocity and Michaelis–Menten constant (K_m) were 3.6 pmol min^{-1} per 10^7 cells, and 14 μM, respectively, calculated using a Lineweaver–Burk plot.

Biochemical properties of TcPAT12

Although the obtained uptake rates varied between the different assays, oocytes expressing *Tc*PAT12 exhibit a linear uptake of spermidine during the first 60 min, 12-fold higher than water or pGEM-Luciferase microinjected cells (about 720 pmol h^{-1} per oocyte; Fig. 3A). Putrescine and the amino acid L-arginine were also taken up by *Tc*PAT12 expressing oocytes at a 6.7- and 5.4-fold lower rates than spermidine, respectively (Fig. 3B). No significant differences on uptake rates of the unrelated compound [^{14}C-U]-D-glucose were observed in *Tc*PAT12 expressing oocytes when comparing with water microinjected oocytes. The apparent affinity of *Tc*PAT12 for spermidine was determined; *Tc*PAT12-mediated uptake of spermidine showed saturable kinetics that followed the Michaelis–Menten equation. Using Lineweaver–Burk plots, the apparent K_m value was in the range 14–26 μM (data not shown). To define the substrate specificity of *Tc*PAT12, competition assays were performed using [^{14}C]-spermidine in the presence of different compounds. Spermidine transport was only inhibited by ornithine (39.5% inhibition) at a 10-fold molar excess but not by agmatine or unrelated compounds (data not shown).

Spermidine apparent K_m and maximum velocity (V_{max}) corresponding to the uptake process were also determined in *T. cruzi* epimastigotes. The uptake rate was dependent on spermidine concentration and showed saturable kinetics. Maximum velocity and the apparent K_m values were 3.6 pmol min^{-1} per 10^7 cells, and 14 μM, respectively (Fig. 3C).

Discussion

Polyamines are essential compounds for parasite growth, replication, and stage differentiation. In the specific case of *T. cruzi*, polyamine transport is critical because it is the only trypanosomatid organism lacking the ability to synthesize putrescine. Consequently, polyamines must be obtained from the extracellular medium exclusively through transport processes. The first characterization at a molecular level of a eukaryotic (*L. major*) polyamine transporter has been recently reported [15]. We were now able to identify a member of a previously described *T. cruzi* putative amino acid transporter (TcPAT) family [16] as a candidate for a polyamine permease. When *Tc*PAT12 was expressed in *X. laevis* oocytes, a high-affinity spermidine uptake was detected with similar kinetic parameters to those measured in *T. cruzi* epimastigotes and some

values for other polyamine transporters previously reported. Both K_m values corresponding to spermidine uptake in oocytes or epimastigotes parasites were calculated around 14 μM, of the same order as for *L. major* *Lm*POT1 (14.3 μM) [15] or putrescine uptake biochemically characterized by different groups in *T. cruzi* (2 μM and 5.7 μM, for Dm28c and MHOM/BR/78/Silvio strains, respectively), *Leishmania mexicana* (10.7 μM), and *Crithidia fasciculata* (66 μM) [13,14]. A permease from *Saccharomyces cerevisiae* named AGP2 was first described as an amino acid (L-carnitine) transporter, and recently as a high-affinity polyamine transporter [23]. Similarly, TcPAT12 also showed a reduced transport activity for putrescine and L-arginine; however, the physiological relevance of such processes is still unknown. On the other hand, it is important to point out some technical features which probably allowed the successful expression of *T. cruzi* membrane proteins in *X. laevis* oocytes. Using a bacterial plasmid not designed for eukaryotic protein expression (pGEMHE) probably gives rise to low-level of mRNA transcription, which might be a critical condition for the functional expression of toxic gene products such as *Tc*PAT12. To ascertain whether *Tc*PAT12 is the only way for polyamine intake in *T. cruzi* will require further investigation.

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