Structural model of a putrescine-cadaverine permease from Trypanosoma cruzi predicts residues vital for transport and ligand binding

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The TcPOT1.1 gene from Trypanosoma cruzi encodes a high affinity putrescine-cadaverine transporter belonging to the APC (amino acid/polyamine/organocation) transporter superfamily. No experimental three-dimensional structure exists for any eukaryotic member of the APC family, and thus the structural determinants critical for function of these permeases are unknown. To elucidate the key residues involved in putrescine translocation and recognition by this APC family member, a homology model of TcPOT1.1 was constructed on the basis of the atomic co-ordinates of the Escherichia coli AdiC arginine/agmatine antiporter crystal structure. The TcPOT1.1 homology model consisted of 12 transmembrane helices with the first ten helices organized in two V-shaped antiparallel domains with discontinuities in the helical structures of transmembrane spans 1 and 6. The model suggests that Trp361 and a Glu347–Arg405 salt bridge participate in a gating system and that Asn205, Tyr248 and Tyr400 contribute to the putrescine-binding pocket. To test the validity of the model, 26 site-directed mutants were created and tested for their ability to transport putrescine and to localize to the parasite cell surface. These results support the robustness of the TcPOT1.1 homology model and reveal the importance of specific aromatic residues in the TcPOT1.1 putrescine-binding pocket.

Key words: homology modelling, parasite, polyamine, putrescine, transport, Trypanosoma cruzi.

INTRODUCTION

Trypanosoma cruzi is the aetiologic agent of Chagas’ disease, a devastating disease of parasitic origin for which new drugs and new drug targets are acutely needed. In theory, a selective drug for Chagas’ or any parasitic disease should target determinants in the parasite that are absent in or substantially discrepant from those in the mammalian host. One metabolic pathway in T. cruzi that has triggered therapeutic interest is that for the acquisition of polyamines, ubiquitous aliphatic polycations that function as key regulators of cell growth, development and cell differentiation in all eukaryotic cells. Whereas most organisms are capable of synthesizing polyamines de novo, T. cruzi lacks the biochemical machinery to synthesize putrescine from amino acids and consequently must scavenge diamines (putrescine and cadaverine) and/or polyamines (spermidine) from the host to survive and proliferate [1,2]. Thus diamine and polyamine permeases play essential nutritional functions in T. cruzi.

Although the polyamine biosynthetic pathway in both prokaryotes and eukaryotes has been extensively studied, few polyamine transporters have been identified at the molecular level, particularly in eukaryotic cell systems. To date, polyamine transporters have been identified in bacteria, yeasts and protozoan parasites and their primary structures reveal that most are members of the APC (amino acid/polyamine/organocation) transporter superfamily [3]. In fact, several of these polyamine permeases also recognize basic amino acids, including the PotE putrescine/ornithine and CadB cadaverine/lysine antiporters from Escherichia coli [4–6]. For a considerable period of time the only polyamine transporters that had been identified in eukaryotes were polyamine excretion proteins or intracellular polyamine permeases in Saccharomyces cerevisiae [7,8]. However, several cell surface polyamine transporters have now been described from S. cerevisiae, each of which exhibits overlapping ligand specificities with amino acids, S-adenosylmethionine or urea [9], and a putrescine-spermidine transporter that localizes to the plasma membrane was identified in Leishmania major [10], a protozoan parasite that is phylogenetically related to T. cruzi. To date, no polyamine transporters have been functionally identified in mammalian systems.

Several groups have described and characterized robust putrescine and spermidine transport activities in cultured epimastigotes of T. cruzi, but only recently were the parasite transporters that are the molecular effectors of the putrescine transport activity, TcPOT1.1 and TcPOT1.2, identified at the molecular level [11]. TcPOT1.1 and TcPOT1.2, both members of the APC superfamily, are saturable high-affinity diamine permeases that transport putrescine and cadaverine, but do not recognize spermidine, spermine or structurally related amino acids. Further, the TcPOT1.1 and TcPOT1.2 genes are allelic variants present in the hybrid T. cruzi CL Brener strain that was originally sequenced as part of the T. cruzi genome sequencing initiative [11]. Interestingly, the activities of both allelic proteins and the subcellular environments in which they are localized are profoundly influenced by the presence or absence of ligand in the culture medium [11].

There is a dearth of high resolution structural information on polytopic transporter proteins from any eukaryote. Indeed, no crystal structures exist for any eukaryotic APC permease. In order to initiate a dissection of the TcPOT1.1 permeation pathway, computational strategies were implemented to construct a homology model of TcPOT1.1 using the crystal structure of AdiC, a prokaryotic antiporter that is also a member of the APC superfamily and imports arginine and exports agmatine in its...
arginine-bound form [12]. This homology model enabled both the mapping of residues participating in the TcPOT1.1 permeation pathway and highlighted amino acids forming the putative binding pocket. The model was then experimentally tested by reverse genetic strategies, and tcpot1.1 mutants were evaluated for their ability to transport putrescine as well as for proper targeting of the mutated transporters to the cell surface of the parasite. Together, these findings forge the first mechanistic understanding of a diamine transporter.

EXPERIMENTAL

Chemicals and reagents

[2,3-3H]Putrescine dihydrochloride (60 Ci/mmol) was purchased from Moravek Biochemicals. Oligonucleotides were acquired from Integrated DNA Technologies. Coral tree lectin was obtained through Sigma–Aldrich. The T. cruzi pTEX-GFP expression plasmid, a vector in which the GFP (green fluorescent protein) gene was ligated into the pTEX shuttle vector [13], was provided by Professor Roberto Docampo (University of Georgia, Athens, GA, U.S.A.). All other reagents were of the highest grade commercially available.

Culture methods

Epimastigotes of the genome project CL Brener T. cruzi reference strain (http://tcruzidb.org/) [14] were cultured at 28°C in autoclaved LIT (liver-infused tryptose) medium [15]. LIT medium is composed of beef liver infusion, tryptone and hemin, and the medium was then supplemented with 10% FBS (fetal bovine serum) to propagate the epimastigotes. Transfectants were routinely maintained in FBS-supplemented LIT medium to which 200 μg/ml Geneticin (G418) was added.

Homology modelling

The HHpred server (http://toolkit.tuebingen.mpg.de/hhpred) was used to identify the arginine/agmatine antiporter (AdiC) from E. coli (PDB code 3L1L [12]) as the most fitting template for the three-dimensional prediction of the structure of TcPOT1.1 [16,17]. Two homology models of the TcPOT1.1 structure were built using the structure of AdiC. The first model was constructed using the YASARA [18] and WHAT IF [19] twinset, whereas a second homology model was built by the automated mode of the SWISS-MODEL server [20,21]. The latter performed an independent search for the most fitting structural template and also selected the E. coli AdiC arginine/agmatine antiporter structure [12].

Site-directed mutagenesis of TcPOT1.1

Primers were designed to mutate residues within the TcPOT1.1 open reading frame of the pTEX-TcPOT1.1::GFP expression vector [11]. The QuikChange® II XL site-directed mutagenesis method (Stratagene) was employed to incorporate the point mutations. The presence of the desired mutations was then confirmed by automated DNA sequencing using the DNA sequencing services offered by Retrogen and by the DNA Sequencing Core Facility of the Vollum Institute at the Oregon Health and Science University. Wild-type and mutant DNA constructs within the pTEX-GFP vector are designated TcPOT1.1 and tcpot1.1 respectively throughout the present paper in accordance with the generally accepted genetic nomenclature for trypanosomatids [22]. Specific mutations within tcpot1.1 are indicated by a suffix, e.g. tcpot1.1E247Q.

T. cruzi transfections

Exponentially growing Trypanosoma cruzi epimastigotes were washed in HBS buffer [21 mM Hepes, 137 mM NaCl, 5 mM KCl, 0.7 mM Na2HPO4, and 6 mM glucose (pH 7.5)] and resuspended at a cell density of 1×10^6 cells/ml. Each transfection was conducted in a 2-mm gap cuvette using 400 μl of parasite suspension and 50–80 μg of one of the mutant tcpot1.1 plasmid DNAs. Electroporations were conducted using a Bio-Rad Laboratories Gene Pulser set at 1500 V and 50 μF [23]. Immediately after transfection, parasites were placed in 5 ml of LIT medium supplemented with 10% FBS. G418 was added to the cultures at 24 h after electroporation at a dilution of 50 μg/ml, a concentration that was gradually increased to 200 μg/ml. The wild-type T. cruzi TcPOT1.1 transfectant has been reported previously [11].

Transport assays

T. cruzi transfectants expressing either TcPOT1.1, one of the tcpot1.1 mutant constructs or GFP alone were harvested from mid-exponential phase cultures propagated in the FBS-supplemented LIT medium. Parasites were then washed three times in PBS-glucose (PBS supplemented with 10 mM glucose). The transport measurements were performed in Eppendorf tubes using a previously described rapid oil-stop technique [24]. Briefly, 1×10^7 cells suspended in 100 μl of PBS-glucose were added to an equal volume of PBS-glucose containing 200 nM [2,3-3H]putrescine dihydrochloride (0.64 Ci/mmol) layered on top of 100 μl of a chemically inert bromodecane cushion. Parasite exposure to the exogenous radiolabelled ligand was terminated by centrifugation of the cells through the bromodecane layer in a Centrifuge 5415D (Eppendorf) at 16000 g for 1 min at 25°C. The sedimented cell pellets in the Eppendorf tubes were flash frozen in liquid nitrogen and excised into a scintillation vial. The radioactivity in the frozen cells was then quantified by liquid scintillation spectrometry on a Beckman LS6500 scintillation counter and the data processed using the GrafFit software package (Erithacus Software).

Immunofluorescence microscopy

Poly-L-lysine-treated coverglass chambers were incubated for 15 min at room temperature (25°C) with 0.5 mg/ml coral tree (Erythrina cristagalli) lectin, a lectin that binds to glycoproteins on the parasite cell surface and facilitates parasite immobilization [25]. The coral tree lectin was removed and the glass chambers allowed to dry in a laminar flow hood. Parasites from the same T. cruzi cultures employed for the transport studies were collected by centrifugation 8000 g for 5 min at 25°C and washed twice in PBS. The cells were then allowed to attach to the coverglass chambers for 15 min, after which unattached epimastigotes were removed by rinsing the chambers with PBS. GFP fluorescence from TcPOT1.1 or the mutant tcpot1.1 was measured by excitation at 488 nm and collection at 507 nm. Cellular fluorescence was detected on a Zeiss Axiovert 200M microscope and the images were captured on an AxioCam MRm camera and then processed using Axiovision Release 4.6. software.

Membrane preparation

T. cruzi transfectants strains expressing either TcPOT1.1–GFP or one of the mutant tcpot1.1–GFP constructs (1×10^6 cells per sample) were washed twice in PBS at 4°C and lysed in 0.1 M KH2PO4, 10% glycerol, 5 mM EDTA, 1 mM PMSF, 50 μM E-64 and Complete Mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of buffer; Roche Diagnostics). Cell lysates were
sonicated on ice for 30 s and centrifuged at 4 °C at 16000 g for 15 min. The cell supernatants were collected, transferred into TL-100 microcentrifuge tubes and spun for 45 min at 80000 g at 4 °C using a TLA-100.2 rotor. After removal of the supernatants, the pellets were resuspended in 100 μl of PBS, 2% SDS, 1 mM PMSF, 50 μM E-64 and Complete Mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of buffer). The protein concentration of each sample was measured using the DC™ Protein Assay (Bio-Rad Laboratories) and membrane preparations were stored at −80 °C in 30 μl aliquots.

Western blot analysis
A sample volume corresponding to 50 μg of proteins was mixed with 2× Laemmli buffer, boiled for 5 min and separated by SDS/PAGE (10% gels). The proteins were transferred onto to a PVDF membrane using a Mini Trans-Blot® electrophoretic transfer cell apparatus (Bio-Rad Laboratories), the membrane blocked with 5% non-fat dried milk in PBS and then probed overnight at 4 °C with a mouse monoclonal anti-GFP antibody (Clontech Laboratories) at a dilution of 1:1000. After three 10 min washes with PBS containing 1% Tween 20, the blot was incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (Thermo Fisher Scientific) at a 1:10000 dilution. Following three washes in PBS containing 1% Tween 20, proteins were visualized using the Western Lightning™ Chemiluminescence Reagent Plus kit (PerkinElmer). Mouse monoclonal anti-β-tubulin antibody (Calbiochem) at a 1:2000 dilution was used to detect parasite tubulin as a loading control. Densitometry analysis of the GFP and tubulin Western blots was performed using the AlphaEaseFC™ Software (Alpha Innotech) and sample loading differences were corrected using tubulin as a control.

RESULTS
Homology modelling of TcPOT1.1 to AdiC
A three-dimensional model of TcPOT1.1 was constructed to the experimentally determined crystal structure of the arginine-bound AdiC E. coli arginine/agamatine antiporter that was solved to 3.0 Å (1 Å = 0.1 nm) resolution [12]. AdiC was identified as the best-fitting template by HHpred, a server based on the pairwise alignments of hidden Markov models, with an E-value of 1.4×10−45. A pairwise alignment of TcPOT1.1 and AdiC displayed 18% identity at the amino acid level (Supplementary Figure S1 at http://www.biochemj.org/bj/452/bj4520424add.htm). Using AdiC as a template, two three-dimensional molecular models were generated, one using the YASARA twinset (Figure 1) and the second by the automated mode of SWISS-MODEL (Figure 2 and Supplementary Figure S2 at http://www.biochemj.org/bj/452/bj4520423add.htm). The YASARA model encompassed 12 TM (transmembrane) domains spanning between amino acids 42 and 476 (Figure 1), whereas the model obtained by the automated mode of SWISS-MODEL predicted ten TM domains between amino acids 53 and 439 of TcPOT1.1. However, despite a lack of prediction of the last two TM domains, the orientation of some key residues (i.e. Glu247), as predicted by SWISS-MODEL, better suits the experimental data collected in the present study. However, the positions of the residues in the two models were similar. Therefore the homology model predicted by SWISS-MODEL was chosen as the structural template for functional tests by reverse genetic strategies.

The transporter path of the TcPOT1.1 model is formed by TM1, TM3, TM6, TM8 and TM10 with TM1 and TM6 possessing a region of helical discontinuity, and the C- and N-termini of the transporter located on the cytoplasmic interface (Figure 1). The arginine-bound AdiC structure revealed three gates, spatially distributed along the transporter permeation path, which occlude the transporter-bound substrate from the exo- and endo-facial sides of the membrane [12]. The AdiC exofacial gate is defined by Trp241 (TM6) at that position (Figure 2A). The middle gate is defined by Trp203 in the AdiC crystal structure, and the corresponding amino acid is Ser334 (TM8) in the TcPOT1.1 model (Figure 2A). The third AdiC gate, the one projected to be on the cytoplasmic interface, consists of three amino acids, Tyr244, Glu208 and Tyr403, and these residues are supplanted in the TcPOT1.1 model with Thr217 (TM3), Glu247 (TM6) and Met404 (TM10) (Figure 2B). The TcPOT1.1 model suggests an alternative third gate involving Glu247 (TM6) and Arg299 (TM10) in a salt-bridge interaction (Figure 2B).

A comparison of the residues forming the AdiC arginine-binding pocket with the amino acids forming the putative putrescine-binding pocket in TcPOT1.1 revealed disparities that are not unexpected given the structural differences between the ligands of the two transporters (Table 1). The crystal structure of AdiC reveals that arginine carboxyl moiety forms hydrogen bonds with Ser289 in TM1 and this interaction is lost in the TcPOT1.1 model with a Gly496 at that position (Figure 2C). The α-amino group of arginine forms hydrogen bonds with the backbone carbonyl oxygens of Ile239, Trp240 and Ile245 in the prokaryotic antiporter and these are Cys462 (TM1), Trp411 (TM6) and Ala244 (TM6) respectively in the parasite transporter (Figure 2D). Residues in the AdiC ligand-binding pocket that interact with the guanidinium group of arginine are only partially conserved in the TcPOT1.1 model: Asn241 and Ser357 in AdiC, which form hydrogen bonds with the guanidinium group of arginine, are Asn443 and Cys396 respectively in the TcPOT1.1 model (Figure 2E). However, the π-cation interaction of the guanidinium moiety with Trp203 in AdiC is lost in TcPOT1.1, which has Ser334 in the corresponding position (Figure 2A).

The homology model of the TcPOT1.1 transporter predicts additional amino acids in the diamine-binding pocket of the polyamine permease that are not conserved in the AdiC structure. These include Asn245 in TM6 of TcPOT1.1, which appears capable of accepting hydrogen bonds from one of the two amino moieties of putrescine, as well as two tyrosine residues, Tyr148 (TM3) and Tyr403 (TM10), that protrude into the TcPOT1.1 transporter pore (Figures 2F and 2G) and that could form hydrogen-bond interactions with the primary amino groups or van der Waals interactions with the backbone of putrescine respectively. Similarly, the TcPOT1.1 model suggests that Phe330, which corresponds to Ser289 in AdiC, could also stabilize the putrescine backbone (Figure 2G). The residues implicated in ligand binding in the TcPOT1.1 model are summarized in Figure 2(H).

Transporter function in T. cruzi transfectants expressing the tcpot1.1 mutants
Scrutiny of the TM folding and polyamine permeation cavity predicted by the TcPOT1.1 homology model enabled the identification of amino acid residues whose genetic modification would be predicted to alter TcPOT1.1 function by virtue of their localization within the permeation path. Site-directed mutagenesis was therefore implemented to evaluate the contribution of residues that are either conjectured to participate in the TcPOT1.1 gating system (Trp241, Glu247 and Arg493) or in the TcPOT1.1 putative...
Figure 1  Homology model of TcPOT1.1

Ribbon diagrams of a homology model of TcPOT1.1 were constructed using YASARA. (A) A side view of the model with the extracellular side up. (B) A top view of the model from the extracellular side. (C) Superimposition of TcPOT1.1 comparative model (coloured) with arginine-bound AdiC structure (PDB code 3L1L; grey). (D) A view of the TcPOT1.1 model discontinuous TM1 and TM6 and of the proposed putrescine binding pocket. The RMSD (root mean square deviation) of the alpha carbons between the TcPOT1.1 model obtained from YASARA with the structure of arginine-bound AdiC (PDB code 3L1L) is 3.84Å. The Figures were generated using PyMOL (http://www.pymol.org).

Table 1  Comparison of residues forming the ligand-binding pocket in AdiC and TcPOT1.1

<table>
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<th>AdiC</th>
<th>TcPOT1.1 model</th>
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<td>α-Amino group</td>
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<td>Side chain (hydrophobic)</td>
<td>Ala244</td>
<td>Conserved (hydrophobic)</td>
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binding pocket (Asn\(^{245}\), Tyr\(^{148}\), Tyr\(^{400}\) and Phe\(^{330}\)). After the mutations were verified by DNA sequencing, mutant tcpot1.1 constructs were individually transfected into wild-type \textit{T. cruzi} epimastigotes. The level of expression of each of the tcpot1.1 mutants was measured by Western blot analysis using purified cell membranes (Figure 3), and the capabilities of the transfectants to transport putrescine were normalized with respect to expression of the wild-type TcPOT1.1 \textit{T. cruzi} transfectant. The transporter rates of each mutant cell lines were then normalized relative to the wild-type TcPOT1.1 expression level (Figure 4). The mutant transporters in the tcpot1.1 constructs could not be detected by Western blot analysis. This lack of expression of these mutant tcpot1.1 transfectants can most likely be ascribed to an intrinsic structural instability of the mutated transporter.

Mutations involving residues within the putative TcPOT1.1 gating system were analysed for their ability to transport putrescine. The predicted exofacial gating residue, Trp\(^{241}\), had the mutant variations W241F, W241A and W241L. The putrescine-transport capability of the tcpot1.1/W241L mutant was similar to the wild-type, whereas putrescine transport was abolished for the mutant tcpot1.1/W241A and tcpot1.1/W241L transporters (Figure 4). Among the residues envisaged to participate in the endofacial gating system, Glu\(^{247}\) and Arg\(^{403}\) were mutated and their ability to transport putrescine was analysed. The mutations E247A and E247Q reduced putrescine transport by more than 80\%, whereas the E247K and E247R mutations triggered a complete loss in TcPOT1.1-mediated putrescine transport in the \textit{T. cruzi} transfectants. However, the E247D transfectant transported putrescine at a rate equal to or faster than that of the wild-type TcPOT1.1 (Figure 4). Among the mutations introduced at Glu247 and Arg403, the tcpot1.1/E247R and tcpot1.1/R403A mutants were not produced to a detectable level (Figures 3 and 4).

In an attempt to assess the importance of a putative salt bridge in the TcPOT1.1 gating mechanism, point mutations were made at both Glu247 and Arg403 to recreate the salt bridge by charge reversal at both sites. Two separate double mutants, R403E/E247K and R403E/E247R, were constructed at these two sites; both were expressed, but exhibited a complete putrescine-transport deficit.

Mutations were also introduced at positions occupied by residues (Asn\(^{245}\), Tyr\(^{148}\), Tyr\(^{400}\) and Phe\(^{330}\)) predicted to reside within the putative putrescine-binding pocket of TcPOT1.1 that were not conserved in the AdiC ligand-binding pocket. Among
these residues, Tyr\textsuperscript{148} was substituted by a phenylalanine or a threonine and the mutated transporter retained \~70\% of their putrescine-transport capacities. However, the Y148A mutation caused a drastic increase in putrescine-transport capability compared with the wild-type TcPOT1.1 (Figure 4). The same three substitutions at Tyr\textsuperscript{400}, another residue predicted to be within the putrescine-binding pocket, resulted in a loss of \~60–70\% putrescine-transport capacity for the Y400F and Y400T mutations respectively, and a putrescine-transport capability superior to the wild-type for the Y400A mutation. However, the introduction of a double mutation, tcpot1.1\textsuperscript{Y148A/Y400A} and tcpot1.1\textsuperscript{Y148T/Y400T}, led to a complete loss in transporter function (Figure 4). TcPOT1.1 Asn\textsuperscript{245} was also highlighted in the model as a non-conserved residue within the presumed putrescine-binding pocket, and the introduction of a N245A, N245D or N245Q substitution at that position diminished putrescine-transport capability to \~65\%, \~60\% or \~40\% respectively of the TcPOT1.1-mediated transport rates (Figure 4).
The importance of Phe330 to TcPOT1.1 function was tested via the construction of F330A and F330W mutants. Although the F330A tcpot1.1 mutant was not expressed and therefore non-functional, the more conservative F330W mutant also displayed a dramatic reduction in putrescine-transport capability. The TcPOT1.1 model indicates that the side chains of Phe330 to Tyr348 and Tyr400 might directly interact with putrescine in the transporter-binding pocket (Figures 2F–2H). To test this, mutations were introduced into TcPOT1.1 at these sites and evaluated for their abilities to affect ligand-binding affinity (Table 2). The $K_m$ values of the tcpot1.1Y148A, tcpot1.1Y148F, tcpot1.1N245A, tcpot1.1N245Q, tcpot1.1Y400A and tcpot1.1Y400F transfectants for putrescine were deduced from Michaelis–Menten analysis and revealed no significant $K_m$ value changes instigated by the N245Q, Y148F, Y400A and Y400F mutations, a 2-fold decrease in $K_m$ value for the tcpot1.1N245A transfectant and a ~6-fold increase in $K_m$ value for the tcpot1.1Y148A transfectant line (Table 2).

The kinetics of $[^{3}H]$putrescine transport into T. cruzi expressing TcPOT1.1, tcpot1.1Y148A, tcpot1.1Y148F, tcpot1.1N245A, tcpot1.1Y400A and tcpot1.1Y400F

<table>
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<th>TcPOT1.1 variants</th>
<th>$K_m$ (nM)</th>
<th>$V_{max}$ (pmol/s/10^8 cells)</th>
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<tbody>
<tr>
<td>TcPOT1.1</td>
<td>829 ± 157</td>
<td>86 ± 17</td>
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<td>tcpot1.1Y148A</td>
<td>5998 ± 757</td>
<td>165 ± 46</td>
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<td>910 ± 100</td>
<td>109 ± 22</td>
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<td>tcpot1.1N245A</td>
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<td>tcpot1.1N245Q</td>
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<td>tcpot1.1Y400A</td>
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<tr>
<td>tcpot1.1Y400F</td>
<td>1093 ± 244</td>
<td>28 ± 10</td>
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Table 2 Kinetics of putrescine transport into T. cruzi expressing TcPOT1.1, tcpot1.1Y148A, tcpot1.1Y148F, tcpot1.1N245A, tcpot1.1Y400A and tcpot1.1Y400F

The kinetics of $[^{3}H]$putrescine transport into T. cruzi epimastigotes was measured as a function of putrescine concentration. The putrescine concentrations ranged from 96 nM to 3 μM. Transport measurements on parasites expressing GFP alone served as controls for endogenous transport, and control rates were subtracted from experimental rates. The average transport values displayed are of three independent experiments and were not corrected for TcPOT1.1 variants differential expression levels. The mutants with the largest change in $K_m$ value compared with the wild-type are shown in bold.

DISCUSSION

In the last decade, an increasing number of three-dimensional structures of secondary active transporters have been solved by X-ray crystallography and, despite a lack of sequence similarities, these transporters have revealed unanticipated overall fold similarities [26]. The first ten TM of AdiC (APC superfamily), LeuT [NSS (neurotransmitter/sodium symporter) family], CaiT [BCCT (betaine/choline/carnitine) transporter family], Mhp1 [NCS1 (neuronal calcium sensor 1) family] are all organized in two anti-parallel domains of five TMs each, with the first TM within each of these domains, TM1 and TM6, harbouring a discontinuous helical region. Interestingly, these unwound regions are critical for transporter function, as they create a polar microenvironment favouring interactions of the transporter with ligands and co-transported ions [27,28]. Beyond these overall structural similarities, however, diversity arises from the residues forming the transporter ligand-binding pocket. Owing to the unique specificity of TcPOT1.1 toward diamines, a homology...
Figure 5 Localization of TcPOT1.1 and tcpot1.1 transfectants by fluorescence microscopy

Intact parasites were grown in LIT medium supplemented with 10% FBS. All constructs harbouring a C-terminal GFP fusion were visualized as described in the Experimental section. Each fluorescent image is accompanied by a phase-contrast representation of the corresponding GFP image.

model of the transporter was constructed to provide a structural layout from which the amino acid residues that form the permeation path or are involved in the transporter binding pocket could be mapped.

This model has also provided insights into the putative gating mechanism employed by TcPOT1.1. In the alternate access model of transport [26], a system of gates occludes the transporter binding pocket from one side of the membrane, whereas the carrier is open to the other side. The AdiC gating system consists of a possible three-gate stratum that is oriented proximal, middle and distal from the periplasm and defined by residues Trp202, Trp293 and Glu208, Tyr93 and Tyr365 [12]. Homology modelling of TcPOT1.1 to arginine-bound AdiC predicted that the first proximal gate formed by AdiC Trp202 is conserved (Trp241), and site-directed mutagenesis confirmed that an aromatic residue at that position is crucial to conserve transporter function. Gao et al. [12] further demonstrated that TM6a rotates ~40° upon arginine binding bringing AdiC Trp202 into the pore to interact with the ligand and subsequently preventing arginine release into the periplasm. The AdiC middle gate is defined by Trp293 located within TM8 and lies at the bottom of the ligand-binding pocket when AdiC is in the arginine-bound conformation and interacts with its guanidinium moiety [12]. The Trp203 counterpart in the TcPOT1.1 homology model is the non-conserved Ser34, and the lack of conservation of this large aromatic amino acid in TcPOT1.1 is unsurprising since putrescine lacks the guanidinium group of the amino acid. In the vicinity of AdiC Trp203 two residues, Asn198 and Ser357, have been shown to be important for proper positioning of the guanidinium group over Trp203 [29]; these two residues are partially conserved in the TcPOT1.1 model and are Asn145 and Cys396 respectively. Among the residues involved in the AdiC distal gate, Glu208, Tyr93 and Tyr365, only Glu247 was conserved in the TcPOT1.1 model. Thus an alternative gating system involving Glu247 and Arg403 in a salt-bridge interaction was proposed and evaluated. The importance of Glu247 and Arg403 in maintenance of the transporter structure is supported by the inherent structural instability of many of the single tcpot1.1 Glu247 and Arg403 mutants. This stability was recovered when mutations were inserted in both positions by charge reversal. However, this charge reversal did not lead to recovery of transporter function and suggests the importance of the orientation of the salt bridge for TcPOT1.1 function (Figure 4). A similar gating system based on a salt-bridge formation between
Glu52 and Arg138 has been found in the E. coli OmpA (outer membrane protein A) ion channel [30]. Interestingly, the OmpA Glu52–Arg138 salt bridge is stabilized by aromatic amino acids and in particular by π–cation formation between Arg138 and Phe40, and a similar π–cation interaction can be inferred from the TcPOT1.1 model between Arg403 and Tyr400 [30]. Glu247 also appears to play an important role in TcPOT1.1 function, since only the most conservative mutation, E247D, conserved putrescine-transport capability.

The ligand-binding pocket in both the AdiC crystal structure and the TcPOT1.1 model is localized below the first gate and is formed by TM1, TM3, TM6, TM8 and TM10. Owing to ligand specificity differences between the prokaryotic antiporter and the T. cruzi permease, the discrepancy in residues that comprise the binding pocket is unsurprising. Polyamines do not possess the α-carboxylate group found in amino acids, and a polyamine-specific transporter such as TcPOT1.1 shows a lack of conservation of the residues whose side chain is engaged in a hydrogen bond at that position. The TcPOT1.1 counterpart of the Gly25–Ser26–Gly27 motif found in AdiC (in which the side of the serine residue forms a hydrogen bond with the amino acid carboxyl group) is Gly64–Gly66–Gly67 in the TcPOT1.1 homology model. The most prominent discrepancies between the putative binding pocket of the TcPOT1.1 model and that of AdiC are the presence of Tyr148 and Tyr400 in the diamine transporter, which are Met104 and Thr148 in AdiC. Tyr148 and Tyr400 appear to lie within hydrogen-bonding distance to putrescine fitted into the TcPOT1.1 model, but it can also be reasonably conjectured that these residues form van der Waals interactions with the aliphatic backbone of the diamine. A comparable distribution of aromatic amino acids has been observed in the binding pocket of PotF, the periplasmic component of the E. coli putrescine-transporter complex PotF, PotG, PotH and PotI [31]. The structure of PotF, the component of the E. coli putrescine uptake complex that binds putrescine [32], has been solved at 2.2 Å [31]. Of these two aromatic residues in TcPOT1.1, the most interesting was Tyr400, since a Y148A mutation decreased the transporter affinity for putrescine ~6-fold, whereas the Y148F mutation had no significant impact, revealing a direct role of this aromatic residue in putrescine recognition.

In the absence of experimental structural information, modelling of proteins to high resolution crystal structures of homologous proteins provides an instructive avenue for envisioning the structural features of a protein that contribute to its function. The TcPOT1.1 homology model suggests a transporter with a similar architecture to the three-dimensional structure of AdiC and other secondary active transporters. The molecular characterizations of individual residues forming the polyamine-binding pocket revealed the importance of aromatic amino acids, and in particular, of tyrosine residues, in ligand binding, as well as a putative salt bridge as an alternative gating system on the cytoplasmic side of the transporter. These findings are significant because of the conjectured importance of TcPOT1.1 to the basic nutrition and survival of T. cruzi and because of their relevance to other eukaryotic polyamine transporters.

**AUTHOR CONTRIBUTION**

Radika Soysa performed the transport assays and the Western blot analysis; Marie-Pierre Hasne generated the mutants with a contribution from Jacqueline Poston; Marie-Pierre Hasne collected the microscopy images; Hanka Venselaar generated the TcPOT1.1 model using YASARA and Marie-Pierre Hasne generated the TcPOT1.1 model using SWISS-MODEL; Buddy Ullman contributed to the data analysis and the writing of the paper; Marie-Pierre Hasne designed the research and wrote the paper.

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**REFERENCES**


SUPPLEMENTARY ONLINE DATA

Structural model of a putrescine-cadaverine permease from *Trypanosoma cruzi* predicts residues vital for transport and ligand binding

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Figure S1  Pairwise alignment of TcPOT1.1 and AdiC

TcPOT1.1 and AdiC pairwise alignment used by SWISS-MODEL and presented in a CLUSTALW format [1]. Identical amino acids are indicated by black background and conservative amino acid substitutions by a grey background. The predicted TM domains within TcPOT1.1 are represented by grey boxes below the sequence; boxes of darker grey indicate TM domains predicted to form the transporter pore. Numbers below the TcPOT1.1 primary structure reveal the positions of amino acids that have been mutated in the present study.

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Figure S2  Alternative structural model of TcPOT1.1 based on homology modelling with AdiC using SWISS-MODEL

The TcPOT1.1 model that was obtained using the automatic alignment mode of SWISS-MODEL is displayed. (A and B) Side views of a ribbon representation of the automated model with the numbers indicating the TM domains. The Figures were generated using PyMOL. The root mean square deviation of the alpha carbons between the SWISS-MODEL TcPOT1.1 model and the structure of arginine-bound AdiC (PDB code 3L1L) is of 2.49 Å.

REFERENCE


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