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Structural Model of a Putrescine-Cadaverine Permease from *Trypanosoma cruzi* Predicts Residues Vital for Transport and Ligand Binding

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Abstract

The *TcPOT1.1* gene from *Trypanosoma cruzi* encodes a high affinity putrescine-cadaverine transporter belonging to the amino acid, polyamine, organocation (APC) 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 amino acid residues involved in putrescine translocation and recognition by this APC family member, a homology model of TcPOT1.1 was constructed based upon the atomic coordinates of the *E. coli* AdiC arginine-agmatine antiporter crystal structure. The TcPOT1.1 homology model consisted of 12 transmembrane helices with the first 10 helices organized in two V-shaped antiparallel domains with discontinuities in the helical structures of transmembrane spans 1 and 6. The model suggests that residues Trp²⁴¹, and a Glu²⁴⁷-Arg⁴⁰³ salt bridge participate in a gating system and that residues Asn²⁴⁵, Tyr¹⁴⁸ 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.

Keywords

Polyamines; putrescine; transport; parasites; *Trypanosoma cruzi*; homology modeling

INTRODUCTION

Trypanosoma cruzi is the etiologic 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

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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 manuscript; Marie-Pierre Hasne designed the research and wrote the manuscript.

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, cadaverine) and/or polyamines (spermidine) from the host to survive and proliferate [1, 2]. Thus, diamine and polyamine permeases play essential nutritional functions for *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 amino acid/polyamine/organocation (APC) 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]. Until recently, 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 recently determined 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 Moravsek Biochemicals, Inc. (Brea, CA). Oligonucleotides were acquired from Integrated DNA Technologies (Coralville, IA). Coral tree lectin was obtained through Sigma-Aldrich (St. Louis, MO). The *T. cruzi* pTEX-GFP expression plasmid, a vector in which the green fluorescent protein (GFP) gene was ligated into the pTEX shuttle vector [13], was generously provided by Dr. Roberto Docampo (University of Georgia, Athens GA). All other reagents were of the highest grade commercially available.

Culture methods

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

Homology modeling

The HHpred server (<http://toolkit.tuebingen.mpg.de/hhpred>) was used to identify the arginine/agmatine antiporter (AdiC) from *E. coli* (PDB file 3L1L [12]) as the most fitting template for the three dimensional prediction of TcPOT1.1 structure [16, 17]. Two homology models of the TcPOT1.1 structure were built using the structure of AdiC. A first model was constructed using the YASARA [18] & WHAT IF [19] Twinset, while 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 QuickChange® II XL site directed mutagenesis method (Stratagene, La Jolla, CA) 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 Inc. (San Diego, CA) and by the DNA Sequencing Core Facility located in the Vollum Institute at the Oregon Health & Science University. Wild type and mutant DNA constructs within the pTEX-GFP vector are designated *TcPOT1.1* and *tcpot1.1*, respectively, throughout this manuscript 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 21 mM HEPES, 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄, and 6 mM glucose, pH 7.5 (HBS buffer) and resuspended at a cell density of 1×10^8 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 Gene Pulser set at 1500 volts and 50 microfarads [23]. Immediately after transfection, parasites were placed in 5 ml of LIT medium supplemented with 10% FBS. G418 was added to the cultures 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 phosphate buffered saline supplemented with 10 mM glucose (PBS-glucose). Transport measurements were performed in Eppendorf tubes using a previously described rapid oil-stop technique [24]. Briefly, 1×10^7 cells suspended in 100 μ l 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 μ l of a chemically inert bromododecane cushion. Parasite exposure to the exogenous radiolabelled ligand was terminated by centrifugation of the cells through the bromododecane layer in a Centrifuge 5415D (Eppendorf North America, Hauppauge, NY) at $16,000 \times g$ for 1 min. 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 GraFit software package (Erithacus Software Limited, Horley, U.K.).

Immunofluorescence microscopy

Poly-L-lysine-treated cover glass chambers were incubated for 15 min at room temperature 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 and washed twice in PBS. The cells were then allowed to attach to the cover glass chambers for 15 min, after which unattached epimastigotes were removed by rinsing the chambers with PBS. GFP fluorescence from TcPOT1.1 or mutant *tcpot1.1* was measured after excitation at 488 nm and collection at 507 nm. Cellular fluorescence was detected on a Zeiss Axiovert 200M microscope, and images were captured on an AxioCam MRm camera and 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 (1×10^8 cells per sample) were washed twice in PBS at 4°C and lysed in 0.1 M KH₂PO₄, 10% glycerol, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride, 50 μ M E-64 and cOmplete Mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of buffer) (Roche Diagnostics, Indianapolis, IN). Cell lysates were sonicated on ice for 30 s and centrifuged at 4°C at $16,000 \times g$ for 15 min. The cell supernatants were collected, transferred to TL-100 microcentrifuge tubes and spun for 45 min at 262,000 g at 4°C in a TLA-100.2 rotor. After removal of the supernatants, the pellets were resuspended in 100 μ L of PBS, 2% sodium dodecyl sulfate, 1 mM phenylmethylsulfonyl fluoride, 50 μ M E-64 and cOmplete Mini EDTA-free protease inhibitor cocktail tablet (1 tablet/10 ml of buffer) (Roche Diagnostics, Indianapolis, IN). The protein concentration of each sample was measured using the DC Protein Assay (Bio-Rad, Hercules, CA) and membrane preparations were stored at -80°C in 30 μ l aliquots.

Western blot analysis

A sample volume corresponding to 50 μ g of proteins was mixed with 2X Laemmli buffer, boiled for 5 min, and separated by SDS-PAGE (10%). The proteins were transferred onto a polyvinylidene difluoride membrane using a Mini Trans-Blot[®] electrophoretic transfer cell apparatus (Bio-Rad, Hercules, CA), the membrane blocked with 5% milk in PBS, and probed overnight at 4°C with a mouse monoclonal anti-GFP antibody (Clontech

Laboratories, Mountain View, CA) 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, Waltham, MA) 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, Waltham, MA). Mouse monoclonal anti- α -tubulin antibody (Calbiochem, San Diego, CA) 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 Corporation, Johannesburg, South Africa), and sample loading differences were corrected using tubulin as a control.

RESULTS

Homology modeling 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-agmatine antiporter that was solved to 3.0 Å resolution [12]. AdiC was identified as the best fitting template by HHpred (<http://toolkit.tuebingen.mpg.de/hhpred>), a server based on the pairwise alignments of hidden Markov models, with an expected-value (E-value) of 1.4×10^{-45} . A pairwise alignment of TcPOT1.1 and AdiC displayed 18% identity at the amino acid level (Supplemental Figure S1). Using AdiC as a template, two three-dimensional molecular models were generated, one by the YASARA twinset (Figure 1) and the second by the automated mode of SWISS-MODEL (Supplemental Figure S2 and Figure 2). The YASARA model encompassed 12 TM domains spanning between amino acids 42–476 (Figure 1), while the model obtained by the automated mode of SWISS-MODEL predicted 10 transmembrane (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 this 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 endofacial sides of the membrane [12]. AdiC exofacial gate is defined by Trp²⁰² and is conserved in the TcPOT1.1 model with a Trp²⁴¹ (TM6) at that position (Figure 2A). The middle gate is defined by Trp²⁹³ 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 — Tyr⁹³, Glu²⁰⁸ and Tyr³⁶⁵ — and these residues are supplanted in the TcPOT1.1 model by Thr¹³⁷ (TM3), Glu²⁴⁷ (TM6), and Met⁴⁰⁴ (TM10) (Figure 2B). The TcPOT1.1 model suggests an alternative third gate involving Glu²⁴⁷ (TM6) and Arg⁴⁰³ (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 Ser²⁶ in TM1 and this interaction is lost in the TcPOT1.1 model with a Gly⁶⁹ at that position (Figure 2C). The α -amino group of arginine forms hydrogen bonds with the backbone carbonyl oxygens of Ile²³, Trp²⁰² and Ile²⁰⁵ in the prokaryotic

antiporter and these residues are Cys⁶⁶ (TM1), Trp²⁴¹ (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: residues Asn¹⁰¹ and Ser³⁵⁷ in AdiC, which form hydrogen bonds with the guanidinium group of arginine, are respectively Asn¹⁴⁵ and Cys³⁹⁶ in the TcPOT1.1 model (Figure 2E). However, the π -cation interaction of the guanidinium moiety with Trp²⁹³ 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 Tyr residues, Tyr¹⁴⁸ (TM3) and Tyr⁴⁰⁰ (TM10), that protrude into the TcPOT1.1 transporter pore (Figure 2F and G) 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 2H.

Transporter function in *T. cruzi* transfectants expressing *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, Arg⁴⁰³) or in the TcPOT1.1 putative binding pocket (Asn²⁴⁵, Tyr¹⁴⁸, Tyr⁴⁰⁰, Phe³³⁰). After mutations were verified by DNA sequencing, mutant *tcpot1.1* constructs were individually transfected into wild type *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* *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*W241L, E247R, R403A and F330A transfectants 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 analyzed for their ability to transport putrescine. The predicted exofacial gating residue, W241, was mutated to W241F, W241A and W241L. The putrescine transport capability of the *tcpot1.1*W241F mutant was similar to wild type, while putrescine transport was obliterated for the mutant *tcpot1.1*W241A and *tcpot1.1*W241L transporters (Figure 4). Among residues envisaged to participate in the endofacial gating system, E247 and R403 were mutagenized and their ability to transport putrescine analyzed. Mutations E247A, E247Q reduced putrescine transport by more than 80% while E247K, or E247R mutations triggered a complete loss of TcPOT1.1-mediated putrescine transport in *T. cruzi* transfectants. However, the E247D transfectant transported putrescine at a rate equal or superior of that of the wild type TcPOT1.1 (Figure 4). Mutations R403A, R403E or R403Q exhibited a >80% loss of putrescine transport competence compared to TcPOT1.1 (Figure 4). It is important to note that, among the mutations introduced at E247 and R403 positions, 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 inserted 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 (Asn²⁴⁵, Tyr¹⁴⁸, Tyr⁴⁰⁰, Phe³³⁰) 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, Tyr¹⁴⁸ was substituted by a Phe or a Thr, and the mutated transporter retained ~70% of their putrescine transport capacities. However, the Y148A mutation caused a drastic increase in putrescine transport capability compared to wild type TcPOT1.1 (Figure 4). The same three substitutions inserted at Tyr⁴⁰⁰, 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 wild type for the Y400A mutation. However, the introduction of a double mutation, *tcpot1.IY148A/Y400A* and *tcpot1.IY148T/Y400T*, led to a complete loss of transporter function (Figure 4). TcPOT1.1 Asn²⁴⁵ was also highlighted in the model as a non-conserved residue within the presumptive putrescine binding pocket, and the introduction of a N245A, N245D or N245Q substitutions at that position diminished putrescine transport capability to ~65%, ~60%, and ~40%, respectively of TcPOT1.1-mediated transport rates (Figure 4). The importance of Phe³³⁰ to TcPOT1.1 function was tested via the construction of F330A and F330W mutations. Although the F330A *tcpot1.1* mutant was not expressed and therefore nonfunctional, the more conservative F330W mutant also displayed a dramatic reduction in putrescine transport capability.

The TcPOT1.1 model indicates that Asn²⁴⁵, Tyr¹⁴⁸, and Tyr⁴⁰⁰ side chains might directly interact with putrescine in the transporter binding pocket (Figure 2F–H). To test this conjecture, 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 *tcpot1.IY148A*, *tcpot1.IY148F*, *tcpot1.IN245A*, *tcpot1.IN245Q*, *tcpot1.IY400A* and *tcpot1.IY400F* transfectants for putrescine were deduced from Michaelis-Menten analysis and revealed no significant K_m changes instigated by the N245Q, Y148F, Y400A and Y400F mutations, a two-fold decrease in K_m value for the *tcpot1.IN245A* transfectant and a ~6-fold increase in K_m value for the *tcpot1.IY148A* transfectant line (Table 2).

Cellular localization of *tcpot1.1* mutants

To assess whether the mutationally triggered loss of putrescine transport capability in the *tcpot1.1* transfectants was due to loss of function of the mutated transporter or to mislocalization, the subcellular context of each of the GFP-tagged mutant transporters was determined by direct fluorescence. Previous studies have established that TcPOT1.1 transport capability is not affected by GFP tagging at the COOH terminus [11]. These measurements indicated that none of the visualized mutant *tcpot1.1* transporters exhibited an aberrant intracellular distribution and that all of the mutant transporters localized, like wild type TcPOT1.1 [11], to the parasite cell surface (either plasma membrane or contractile vacuole) (Figure 5). Noteworthy, however, *tcpot1.IW241L*, E247R, R403A and F330A were not detected by direct fluorescence (Figure 5). The inability to detect these mutant *tcpot1.1* transporters by direct fluorescence is consistent with the western blot data shown in Figure 3 and indicative of a structural instability of these mutated transporters.

DISCUSSION

In the last decade, an increasing number of three-dimensional structures of secondary active transporter has been solved by x-ray crystallography, and despite a lack of sequence similarities, these transporters have revealed unanticipated overall fold similarities [26]. The first 10 TMs of AdiC (APC superfamily), LeuT (**Neurotransmitter:Sodium Symporter** (NSS) family), CaiT (betaine/choline/carnitine (BCCT) *transporter* family), Mhp1 (Neuronal Calcium Sensor 1 (NCS1) family) are all organized in two anti-parallel domains of 5 TMs each, with the first TM within each of these domains, TM1 and TM6, harboring a discontinuous helical region. Interestingly, these unwound regions are critical for transporter function, as they create a polar microenvironment favoring 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. Because of the unique specificity of TcPOT1.1 toward diamines, a homology 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 while 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 the three residues Glu²⁰⁸, Tyr⁹³ Tyr³⁶⁵, respectively [12]. Homology modeling of TcPOT1.1 to arginine-bound AdiC predicted that the first proximal gate formed by AdiC Trp²⁰² is conserved (Trp²⁴¹), and site directed mutagenesis at that position confirmed that an aromatic residue at that position is crucial to conserve transporter function. Gao *et al.* 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 [12]. 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 Trp²⁹³ counterpart in the TcPOT1.1 homology model is the non-conserved Ser³³⁴, 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 Trp²⁹³, two residues Asn¹⁰¹ and Ser³⁵⁷, have been shown to be important for proper positioning of the guanidinium group over Trp²⁹³[29]; these two residues are partially conserved in the TcPOT1.1 model and are respectively Asn¹⁴⁵ and Cys³⁹⁶. 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 tspot1.1 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 ion channel [30]. Interestingly, this 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. Because of 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 like 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 Ser residue forms a hydrogen bond with the amino acid carboxyl group) is Gly⁶⁸-Gly⁶⁹-Gly⁷⁰ 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 Thr³⁶¹ in AdiC, respectively. The Tyr¹⁴⁸ and Tyr⁴⁰⁰ residues 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, PotI [31]. The structure of PotF, the component of the *E. coli* putrescine uptake complex that binds putrescine [32], has been solved to 2.2 Å [31]. Of these two aromatic residues in TcPOT1.1, the most interesting was Tyr¹⁴⁸, since a Y148A mutation decreased the transporter affinity for putrescine ~6-fold, while a Y148F mutation had no significant impact, revealing a direct role of this aromatic residue in putrescine recognition.

In the absence of experimental structural information, modeling 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used are

APC	amino acid/polyamine/organocation superfamily
GFP	green fluorescent protein
LIT	liver-infused tryptose
FBS	fetal bovine serum

G418	Geneticin
PBS	phosphate buffered saline
TM	transmembrane helices
RMSD	root mean square deviation

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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) Superposition of TcPOT1.1 comparative model (rainbow) with Arg-bound AdiC structure (PDB: 3L1L) (gray). (D) A view of the TcPOT1.1 model discontinuous TM1 and TM6 and of the proposed putrescine binding pocket. The root mean square deviation (RMSD) of the alpha carbons between the TcPOT1.1 model obtained from YASARA with the structure of Arg-bound AdiC (pdb: 3L1L) is 3.84Å. Figures were generated using PyMol.

Gating system

Binding pocket

Figure 2. Comparative analysis of the location of key residues in the TcPOT1.1 homology model generated by SWISS-MODEL with counterparts from the structurally determined AdiC, and PotF proteins

(A) Trp²⁴¹ and Trp²⁹³ in AdiC are shown superimposed with their counterparts in the TcPOT1.1 model. (B) Overlay of TcPOT1.1 model and AdiC showing differences among key residues of the distal gate of the transporters. (C–G) Comparison of residues involved in the predicted TcPOT1.1 binding pocket. (H) Residues predicted to be involved in putrescine recognition in the TcPOT1.1 model. In all panels the TcPOT1.1 homology model is depicted in rainbow colors and the AdiC counterpart is shown in gray.

Figure 3. Western blot analysis of TcPOT1::GFP and tcpot1.1::GFP in *T. cruzi* transfectants
GFP expression from TcPOT1.1::GFP and tcpot1.1::GFP transfectant membrane preparations (~50 µg of protein per lane) was detected by Western blot analysis using a GFP mouse monoclonal antibody. **(A)** Western blot analysis of *T. cruzi* cell lines overexpressing TcPOT1.1::GFP (TcPOT1.1) and tcpot1.1::GFP (W241A, W241F, W241L...). WT, denotes a control using membrane preparation from wild type untransfected *T. cruzi* demonstrating anti-GFP antibody specificity. α -tubulin is shown below each blot as a loading control. Data are shown as one representative experiment performed at least in duplicate for every mutant. **(B)** Quantification of TcPOT1::GFP and tcpot1.1::GFP expression levels by densitometry analysis corrected for sample loading difference using tubulin as control. Data were obtained from Western blots performed from two biological replicates for every mutant.

Figure 4. Putrescine transport by *tcpot1.1* mutants

The abilities of *T. cruzi* parasites transfected with *TcPOT1.1* or a *tcpot1.1* mutant construct to transport putrescine is shown. All constructs encoded GFP fused to the COOH-terminus of the *TcPOT1.1* or *tcpot1.1* open reading frame. Transport measurements on *T. cruzi* epimastigotes expressing GFP alone served as controls for endogenous transport, and transport rates from the controls were subtracted from experimental rates. Transport rates were measured at a concentration of 200 nM putrescine over a 10 s time course. The relative amounts of *tcpot1.1* expression are illustrated by grey circles and were ascertained by two independent western blot analyses and normalized to TcPOT1.1 expression (Figure 3). The data shown are the results of three independent measurements and are presented as a percentage of transport in a *T. cruzi* line transfected with wild type *TcPOT1.1*. The absolute rate of putrescine uptake by *T. cruzi* transfected with wild type TcPOT1.1 was determined to be 2.0 ± 0.5 pmol/s/ 10^8 cells (n=9).

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 harboring a COOH-terminal GFP fusion were visualized as described in “Experimental Procedures.” Each fluorescent image is accompanied by a phase contrast representation of the corresponding GFP image.

Table 1

Comparison of residues forming the ligand binding pocket in AdiC and TePOT1.1

Arginine	AdiC		TePOT1.1 model	
	Residues	interactions	Residues	interactions
α -amino group	I23	Backbone	C66	✓ conserved
	W202	Backbone	W241	✓ conserved
	I205	Backbone	A244	✓ conserved
α -carboxylate group	S26	Side chain	G69	× not conserved
	G27	Backbone	G70	✓ conserved
Guanidinium group	W293	Side chain (π -cation)	S334	× not conserved
	S357	Side chain	C396	× not conserved
Nitrogen atoms of guanidinium group	N101	Side chain	N145	✓ conserved
	A96	Backbone	I140	✓ conserved
	C97	Backbone	T141	✓ conserved
Aliphatic chain	M104	Side chain	Y148	✓ conserved
	W202	Side chain	W241	✓ conserved
	I205	Side chain (Hydrophobic)	A244	✓ conserved (Hydrophobic)
				Aliphatic chain

Table 2
**Kinetics of putrescine transport into *T. cruzi* expressing TcPOT1.1, *tcpot1.1Y148A*,
tcpot1.1Y148F *tcpot1.1N245A*, *tcpot1.1N245Q*, *tcpot1.1Y400A* and *tcpot1.1Y400F***

The kinetics of [³H]putrescine transport into *T. cruzi* epimastigotes was measured as a function of putrescine concentration. Putrescine concentrations ranged from 96 nM to 3 μ M. 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.

TcPOT1.1 variants	K _m (nM)	V _{max} (pmol/s/1×10 ⁸ cells)
TcPOT1.1	829 ± 157	86 ± 17
<i>tcpot1.1 Y148A</i>	5998 ± 757	165 ± 46
<i>tcpot1.1 Y148F</i>	910 ± 100	109 ± 22
<i>tcpot1.1 N245A</i>	334 ± 143	12 ± 4
<i>tcpot1.1 N245Q</i>	1505 ± 163	24 ± 6
<i>tcpot1.1 Y400A</i>	1388 ± 546	41 ± 10
<i>tcpot1.1 Y400F</i>	1093 ± 244	28 ± 10