Identification and Characterization of a Polyamine Permease from the Protozoan Parasite Leishmania major*

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The proteins that mediate polyamine translocation into eukaryotic cells have not been identified at the molecular level. To define the polyamine transport pathways in eukaryotic cells we have cloned a gene, LmPOT1, that encodes a polyamine transporter from the protozoan pathogen, Leishmania major. Sequence analysis of LmPOT1 predicted an unusual 803-residue polytopic protein with 9–12 transmembrane domains. Expression of LmPOT1 cRNA in Xenopus laevis oocytes revealed LmPOT1 to be a high affinity transporter for both putrescine and spermidine, whereas expression of LmPOT1 in Trypanosoma brucei stimulated putrescine uptake that was sensitive to inhibition by pentamidine and proton ionophores. Immunoblot analysis of LmPOT1 revealed LmPOT1 to be expressed predominantly in the insect vector form of L. major, and immunofluorescence demonstrated that LmPOT1 was localized predominantly to the parasite plasma membrane. To our knowledge this is the first molecular identification and characterization of a cell surface polyamine transporter in eukaryotic cells.

Polymamines are ubiquitous aliphatic polycations that play vital roles in key cellular processes such as growth, differentiation, and macromolecular biosynthesis (1). The polyamine content of cells originates from both de novo synthesis (2) as well transport of exogenous polyamines (3). Extensive molecular, biochemical, and structural studies have been performed on the polyamine biosynthetic pathway in a multitude of organisms ranging from simple prokaryotes to human (4). In contrast, there is essentially no information at the molecular level on the mechanisms by which polyamines are translocated across the plasma membrane of eukaryotic cells. Three polyamine permease genes have been identified in Escherichia coli, two of which encode members of the ATP binding cassette superfamily, whereas the third, PotE, codes for a protein in the amino acid/polyamine/organicocation (APC) superfamily (3, 5, 6). However, the only eukaryotic polyamine transporters that have been identified to date at the molecular level are those from Saccharomyces cerevisiae, and these are intracellular transporters that localize to the yeast vacuole (7, 8). These yeast vacuolar transporters are members of the major facilitator superfamily and share very limited homology with their prokaryotic counterparts. Because polyamine uptake is enhanced in tumor cells, and extracellular polyamines can bypass the cellular effects of polyamine anti-metabolites (9), the therapeutically germane nature of cell surface polyamine transporters has stimulated considerable interest in their identification and characterization.

The polyamine pathway has been successfully targeted with drugs in protozoan parasites. d,L-a-Difluoroethylornithine, an irreversible inactivator of ornithine decarboxylase, the first enzyme in the polyamine biosynthesis pathway, can eliminate Trypanosoma brucei infections in both mice (10) and humans with late stage African sleeping sickness (11). Another compound, 5’-[[Z]-4-amino-2-butenyl]methylamino]-5-deoxadenosine (MDL73811), a potin inhibitor of S-adenosylmethionine decarboxylase, is effective in eradicating T. brucei infections in mice (12). Polyamine anti-metabolites such as d,L-a-difluoroethylornithine are also active against a variety of other parasite genera, including Plasmodium, Giardia, and Leishmania (13–15).

Leishmania, the etiologic agent of a spectrum of devastating and often lethal diseases, is a genus of protozoan parasite that is phylogenetically similar to T. brucei. The parasite is digenetic, existing as the extracellular promastigote in the phagotomine sandfly vector and as the amastigote sequestered within the phagolysosome of macrophages of the infected mammalian host. Leishmania have been particularly valuable in investigations characterizing the polyamine biosynthesis pathway of parasites. All of the genes of the polyamine pathway, ornithine decarboxylase (ODC), S-adenosylmethionine decarboxylase (ADOMETDC), and spermidine synthase (SPDSYN), have been cloned from Leishmania donovani, and the creation of acde, sadometdc, and spdsyn knock-out lines by targeted gene replacement has demonstrated the essential role of each of these genes in L. donovani proliferation and illuminated significant dissimilarities between the polyamine pathways of Leishmania and the mammalian host (16–18).

Despite the plethora of molecular and biochemical studies on the polyamine pathway in L. donovani and other parasites (16–18), little is known about the avenues by which extracellular polyamines are translocated across the parasite cell surface. Leishmania promastigotes and amastigotes are both capable of scavenging exogenous polyamines (19, 20), and biochemical studies in intact parasites have distinguished two transporters, one specific for putrescine and one for spermidine (20). It has also been suggested that pentamidine, a diamidine drug used in the treatment of leishmaniiasis, might enter the parasite via a polyamine transporter (21). Thus far, investiga-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/ EBI Data Bank with accession number(s) AY727900.

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The abbreviations used are: APC, amino acid/polyamine/organicocation; PBS, phosphate-buffered saline; ORF, open reading frame; TM, transmembrane domain.
tions into the pathways by which parasites, and for that matter any eukaryote, transport polyamines from the extracellular environment, have defied molecular analysis.

We now report the molecular identification and characterization of LmPOT1, a polyamine transporter from *Leishmania major*. LmPOT1 is a member of the APC superfamily and recognizes both putrescine and spermidine with relatively high affinity. LmPOT1 does not appear to transport amino acids and is inhibited by proton uncouplers, suggesting that the permease may be a proton symporter, and by pentamidine, an antileishmanial drug. Moreover, LmPOT1 localizes predominantly to the leishmanial plasma membrane. To our knowledge, LmPOT1 is the first cell surface polyamine transporter to be recognized in eukaryotic cells.

**MATERIALS AND METHODS**

Chromosomal and Reagents—[2-3H(N)]putrescine (31 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA), [1,4-3H(C)putrescine (110 mCi/mmol), [14C]spermidine (23.5 mCi/mmol), and [14C]spermine (113 mCi/mmol) were obtained from PerkinElmer Life Sciences, [3H]putrescine, [3H]spermidine, and [3H]spermine were from Amersham Biosciences (Piscataway, NJ). X. laevis isothionate (98 Ci/mmol) was a gift from Dr. Michael Barrett (Glasgow University). Oligonucleotides were acquired from Invitrogen, and restriction endonucleases were from New England Biolabs, Inc. (Beverly, MA). The *Xenopus laevis* oocyte pOG-1 (21) and *T. brucei* pH3D09 (22) expression vectors were furnished by Drs. Susan Amara and George Cross, respectively. *X. laevis* were obtained from *Xenopus* Express (Plant City, FL), and rabbit antibodies against LmPOT1 using a synthetic peptide corresponding to amino acids 71–89 of LmPOT1 as immunogen were raised at Bio-synthesis Inc. (Lewisville, TX). All other chemicals were of the highest grade commercially available.

**Parasite Cell Culture—** *L. major* (Friedlin VI) promastigotes were grown in BSA-BS-1 medium supplemented with 20% heat-inactivated fetal bovine serum. Amastigotes were obtained by infection of J747.1 macrophages that were acquired from the American Type Culture Collection (Manassas, VA). Macrophages were cultured at 37 °C in RPMI1640 medium with 10% fetal bovine serum and infected with stationary phase *L. major* promastigotes at a ratio of 20 parasites per macrophage. After overnight infection, macrophages were washed 3 times with phosphate-buffered saline (PBS) containing 2 mM EDTA to remove residual promastigotes, and fresh growth medium was added for an additional 48 h. The macrophage monolayer was then washed 3 times with PBS-EDTA buffer and detached by scraping, and the cells were lysed by repeated passage through a 30-gauge needle. Amastigotes were then purified by Percoll density gradient centrifugation using standard protocols (24). *T. brucei* procyclies were grown at 26 °C in SDM-79 medium with 10% heat-inactivated fetal bovine serum.

**Cloning of LmPOT1—** The LmPOT1 open reading frame (ORF) was identified in the *L. major* genome sequencing data base (accession number ATY727900) using several mammalian amino acid transporters as query sequences. The LmPOT1 ORF was amplified using the PCR from *L. major* genomic DNA with the Advantage® 2 HF 2 PCR kit (Clontech, Palo Alto, CA) and the PCR product ligated into both the PCR® 2.1-TOPO® (Invitrogen) and pHDr909 (25) vectors. For insertion into PCR® 2.1-TOPO®, the sense primer was 5'-CTGAAAAAGCTTCATCATGACGATGATCGGGCCTC-3', and the antisense primer was 5'-GCGGGGCTTACGAGAGCGCGGCACCC-3' (restriction sites are underlined, and the initiation and termination codons are in boldface). The LmPOT1 ORF was then excised and ligated into pOG-1 to create pOG-1-LmPOT1. To insert LmPOT1 into pHDr909, the sense primer was 5'-CTGAAAAAGCTTCATCATGACGATGATCGGGCCTC-3', and the antisense primer was 5'-GCGGGGAGCTTACGAGAGCGCGGCACCC-3' (HindIII sites are underlined, and start and termination codons are in boldface). The resultant plasmid was designated pHDr909-LmPOT1. The fidelity of the PCR-amplified LmPOT1 inserts was confirmed by automated DNA sequencing.

**Translation—** *T. brucei* procyclies were transfected with 5–10 μg of NotI-linearized pHDr909-LmPOT1 in Cytomix buffer (25) using a Bio-Rad Gene Pulser electroporator set at 1350 V and 25 microfarads. After electroporation, cells were diluted into 5 ml of *T. brucei* growth medium, and transfectants were selected in 50 μg/ml hygromycin. The resultant transfectant was designated TUB:HD9309-LmPOT1 according to standard genetic nomenclature for *T. brucei*. A control line, TUB:HD9309, transfected with pHDr909 alone, was also generated.

**Transport Assays in *T. brucei*—** *T. brucei* procyclies were harvested in mid-log phase and washed 3 times in PBS supplemented with 10 mM glucose (PBS-glucose). 1 × 10⁷ cells in 100 μl were added to an equal volume of PBS-glucose containing radiolabeled ligand and inhibitors, as indicated. Uptake experiments were terminated by a modified oil-stop technique (27) in which parasites were centrifuged through a 100-μl dibutyl phthalate cushion. The cell pellets were flash-frozen in liquid N₂, and incorporated radioactivity was quantitated by liquid scintillation.

**Immunoblot Analysis—** *L. major* promastigotes and amastigotes were washed twice in PBS and lysed in hypertonic buffer consisting of 96 mM HEPS, 500 mM sucrose, 400 mM potassium acetate, 1 mM EDTA, pH 7.5. Parasite proteins were fractionated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane as described (28). Membranes were probed with the LmPOT1 antibody that had been purified over an agarose column to which the peptide immunogen was attached. Antigen-specific rabbit antibodies (Pierce) were used as a positive control. The primary antibodies were detected with horseradish peroxidase-labeled goat anti-rabbit secondary antibody (Pierce).

**Immunofluorescence—** *L. major* promastigotes were affixed to coverslips with 2% paraformaldehyde for 30 min at room temperature. Parasites were then washed 3 times in PBS and kept intact or permeabilized for 5 min in 0.2% Triton X-100, 50 μg/ml pepsin. After 3 additional washes with PBS, parasites were incubated with affinity-purified LmPOT1 antibody (1 μg/ml) in PBS containing 1% rabbit serum and 1% goat serum at room temperature with gentle shaking for 1 h. After rinsing the coverslips in PBS, LmPOT1 antibodies were detected with anti-rabbit secondary antibodies conjugated to Alexa Fluor-568 (Molecular Probes, Eugene, OR). Coverslips were rinsed three times in wash buffer and mounted onto slides, and images were obtained using the Applied Precision Deltavision® image restoration system. Deconvolution was performed using the iterative constrained algorithm of Agard et al. (29), and additional image processing was accomplished on an SGI Octane work station located in the OHSU Core Imaging Facility in the Molecular Microbiology and Immunology Department.

**RESULTS**

**Isolation of LmPOT1—** Because amino acids and some organoculture transporters are members of the APC superfamily (30) and their ligands share obvious structural similarities with polyamines, a search was initiated for novel APC transporters by screening the *L. major* data base with several mammalian amino acid transporters as query sequences. The data base from the *L. major* genome project encompassed a multiplicity of APC transporter genes, one of which, LmPOT1, predicted an unusual primary structure (see below). LmPOT1 was then cloned by PCR from genomic DNA using primers from the annotated sequence. Southern blot analysis of *L. major* genomic DNA revealed LmPOT1 to be a single copy gene in the *L. major* genome (data not shown), consistent with the deposited sequence surrounding the LmPOT1 locus on *L. major* chromosome 14. Translation of the LmPOT1 sequence revealed a 2409-bp ORF that encoded an 803-amino acid APC superfamily member (Fig. 1A). The sequence of the LmPOT1 clone was identical to its data base counterpart. Of the 10 distinct transport families within the APC superfamily, LmPOT1 encompassed much of the evolutionarily conserved signature sequence (boldface and underlined in Fig. 1A) of the L-type amino acid transporter family (30). A putative calmodulin binding

**LmPOT1 Expression in Xenopus Oocytes—** *X. laevis* oocytes were isolated and collagenase-treated as described (26). pOG-1-LmPOT1 was linearized with XbaI, and capped cRNA was synthesized using the mMessage mMachine™^®^ kit (Ambion, Inc., Austin, TX). Stage V-VI oocytes were injected with 20 ng of cRNA 1 day after harvest and incubated for 3 days at 16 °C in ND-96 buffer (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 5 mM HEPS, pH 7.5) supplemented with 2.5 mM sodium pyruvate, 50 μg/ml gentamicin, and 3% feto bovine serum. Uptake of [³H]putrescine, [³H]spermidine, and [³H]spermine, and [³H]agmatine was measured at room temperature in LmPOT1 cRNA-injected and H₂O-injected control oocytes. After incubation with radiolabel, oocytes were washed 3 times with ice-cold ND-96 and lysed with 200 μl of 1% Triton X-100, and radiolabels were quantitated by liquid scintillation.

**Materials and Methods**

**Transfection—** *T. brucei* Transfection—

**Chemicals and Reagents—**

**Polyamine Transport in Leishmania**

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motif is also detected within LmPOT1 (*italics* in Fig. 1A).

Various topological algorithms, *e.g.* PHD2.1 (31), SOSUI (32), Hidden Markov (33, 34), predicted a polytopic protein between 9 or 12 transmembrane domains (TMs) flanked by 90–100 and 200–300 amino acid hydrophilic NH$_2$-terminal and COOH-terminal extensions, respectively (TMs predicted by PHD 2.1 are depicted in Fig. 1A). Pair-wise alignments between LmPOT1 and members of the APC superfamily from phylogenetically divergent organisms revealed significant homology over the region encompassed by the TMs with amino acid identities ranging from 20 to 30%. No homology was detected to the hydrophilic extensions. Each of these extensions was in turn used as a query sequence to screen the NCBI database. This analysis revealed only one homolog, a gene in *Trypanosoma cruzi* (gi44490054), a parasite evolutionarily related to *Leishmania*. LmPOT1 also exhibited limited homology with the known bacterial and yeast polyamine transporters. Pair-wise alignments of LmPOT1 with the *E. coli* putrescine transporter PotE (35) and the *S. cerevisiae* vacuolar polyamine transporter TPO2 (7) revealed amino acid identities of 10.8 and 11.7%, respectively. Of note, LmPOT1 exhibited significant homology with a region of PotE that has been implicated in putrescine recognition (36), including two conserved residues critical for ligand binding (Fig. 1B).

**Functional Expression of LmPOT1 in X. laevis Oocytes**—To determine LmPOT1 ligand specificity, LmPOT1 cRNA was expressed in stage V-VI *Xenopus* oocytes, a heterologous expression system that exhibits negligible endogenous transport capabilities for many ligands. Oocytes injected with LmPOT1 cRNA transported 10$^{6}$/H$^{9}$262 M [14C]putrescine and 10$^{6}$/H$^{9}$262 M [14C]spermidine 10-fold more effectively than water-injected control oocytes (Fig. 2A). In contrast, no significant increases in [14C]spermine, [14C]arginine, [3H]agmatine, or [3H]pentamidine uptake were detected in oocytes expressing LmPOT1 cRNA compared with controls (data not shown). To determine the kinetic parameters for LmPOT1, polyamine transport was measured over a range of ligand concentrations (Fig. 2B). LmPOT1 exhibited saturable kinetics with both polyamine ligands, and apparent K$_{m}$ values of 6.7 ± 1.4 and 14.3 ± 0.9 $\mu$M and V$_{max}$ values of 0.11 ± 0.01 and 0.064 ± 0.004 nmol/h/oocyte were calculated for putrescine and spermidine, respectively.

**Functional Expression of LmPOT1 in T. brucei**—To confirm the biochemical properties of LmPOT1 in a second heterologous system, the LmPOT1 ORF was cloned into the tubulin locus of *Trypanosoma brucei*.
\[\text{inhibited} \ 3\text{H} \text{putrescine uptake by 39 and 64\%, respectively}\]
\[\text{as expected, excess putrescine abolished the presence of a 250-fold excess of a variety of structurally related compounds.}\]
\[\text{to Leishmania that exhibits very low rates of endogenous putrescine uptake. A control parasite line transfectected with the empty vector was also created. Western blotting of extracts from the TUB::HD309-LmPOT1 and TUB::HD309 control parasites using the LmPOT1 antisera revealed the presence of two polypeptides unique to the parasites in which LmPOT1 was integrated into the tubulin locus (Fig. 3A). These bands corresponded to masses of \(\sim90\) and \(\sim180\) kDa, roughly equivalent to what would be expected for LmPOT1 monomers and dimers, respectively. The TUB::HD309-LmPOT1 transfectant exhibited a robust uptake capability for putrescine over the TUB::HD309 control parasites in which putrescine uptake was negligible even after 3 min (Fig. 3B, inset). Putrescine transport into TUB::HD309-LmPOT1 cells was shown to be saturable, with an apparent \(K_m\) value of 10.2 ± 3.2 \(\mu\)M and a \(V_{max}\) value of 0.061 ± 0.002 nmol/min/10\(^6\) cells (Fig. 3B).

To assess ligand specificity of LmPOT1, [\(^3\)H]putrescine uptake into TUB::HD309-LmPOT1 parasites was measured in the presence of a 250-fold excess of a variety of structurally related compounds. As expected, excess putrescine abolished uptake of the radiolabel, whereas spermidine and spermine inhibited [\(^3\)H]putrescine uptake by 39 and 64\%, respectively (Fig. 4A). A battery of charged and polar amino acids did not appreciably interfere with polyamine incorporation into the T. brucei transfectant. Interestingly, pentamidine, a diamidine that is used in the treatment of both African trypanosomiasis and leishmaniasis, obliterated [\(^3\)H]putrescine uptake, whereas agmatine, the decarboxylated product of arginine, inhibited putrescine incorporation by 85\% (Fig. 4A). However, TUB::HD309-LmPOT1 cells did not exhibit augmented transport capability of radioactive pentamidine or agmatine compared with TUB::HD309 control parasites (data not shown).

Because the transport of many ligands into Leishmania is mediated by ion symporters, the effects of ionophores on putrescine uptake into the TUB::HD309-LmPOT1 cells were evaluated (Fig. 4B). Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and dicyclohexylcarbodiimide (DCCD), both proton ionophores, monensin, a Na\(^+\) ionophore, and A23187, a Ca\(^2+\) ionophore, all significantly inhibited putrescine uptake into the TUB::HD309-LmPOT1 transfectants in a dose-dependent fashion, whereas valinomycin, a K\(^+\) ionophore, had no significant inhibitory effect (Fig. 4B). LmPOT1 was also assessed as a function of extracellular pH ranging from 5 to 9 and found to be optimal \(-pH\ 7.0\) (data not shown).

The Na\(^+\) dependence of LmPOT1 was also examined in the TUB::HD309-LmPOT1 transfectant. Surprisingly, replacement of NaCl with an isotonic sucrose actually increased the ability of TUB::HD309-LmPOT1 parasites to take up putrescine, whereas substitution of NaCl with either choline chloride or LiCl diminished putrescine uptake 63 and 79\%, respectively (Fig. 5A). Taken together, the data suggest that Na\(^+\), choline, and Li\(^+\) actually interfere with putrescine transport. This conjecture was tested by measuring putrescine uptake into TUB::HD309-LmPOT1 T. brucei under isoosmolar assay conditions in which NaCl and sucrose were varied. The data demonstrated that putrescine uptake into TUB::HD309-LmPOT1 cells was indirectly proportional to the NaCl concentration (Fig. 5B).
To verify the location and stage-specific expression of LmPOT1, antibodies directed against an LmPOT1 peptide were raised in rabbits. These antibodies recognized a band of Mr/H11011 kDa in an L. major promastigote extract but failed to generate any signal with an amastigote lysate (Fig. 6A). The expected size of LmPOT1 is 90 kDa. The presence of a signal at 180 kDa is observed intermittently in some blots (see Fig. 3A), possibly implying that LmPOT1 may exist as a dimer (data not shown). The localization of LmPOT1 was then determined by deconvolution microscopy. Immunofluorescence analysis of LmPOT1 stained with Alexa fluor-conjugated secondary antibodies revealed the presence of the polyamine transporter on the cell surface and flagellum of both permeabilized and non-permeabilized L. major promastigote (Fig. 6B). No LmPOT1 protein could be detected in L. major amastigotes.

**DISCUSSION**

This study reports the first identification and functional characterization of a gene encoding a surface membrane polyamine transporter in eukaryotes. The study of eukaryotic polyamine transporters in eukaryotes has previously proven refractory to molecular dissection because of the low level of homology among the previously characterized prokaryotic and yeast vacuolar transport proteins. LmPOT1 was identified from a L. major genome data base screen using APC members as query sequences. The original presumption that polyamine transporters might be members of the APC family (the basis for the original data base screen for LmPOT1) reflected the observations that known prokaryotic and yeast vacuolar transporters are major facilitator superfamily members. LmPOT1 was originally selected among several APC genes in the L. major data base because of its unusual length and its hydrophilic NH2- and COOH-terminal extensions. Of the 175 APC members from phylogenetically diverse organisms that were examined by Jack et al. (30), only one, SSy1 Sce from S. cerevisiae, is larger than LmPOT1, and the vast majority contain 400–600 amino acids. Secondary structure algorithms predicted 9–12 TMs for LmPOT1, consistent with expected topologies for other APC superfamily members, especially L-type amino acid transporters (30). Essentially all of the sequence conservation between LmPOT1 and APC permeases is found within the TMs. The hydrophilic termini of LmPOT1 did not exhibit significant homology to any deposited sequences in the eukaryotic or prokaryotic databases, except a putative homolog in T. cruzi. The level of LmPOT1 homology to amino acid permeases was also significantly greater than that exhibited between LmPOT1 and the E. coli PotE or S. cerevisiae TPO4 vacuolar polyamine transporters. Interestingly, how-

**FIG. 4.** Effectors of Putrescine Transport by LmPOT1. A, a substrate specificity profile for LmPOT1 was determined in TUB::HD309-LmPOT1 T. brucei using 10 μM putrescine and 2.5 mM concentrations of structurally similar compounds. Results represent the percent of putrescine uptake against a no-inhibitor control (NI). Each value is the mean ± S.D. of three independent experiments. B, the influence of ionophores monensin and valinomycin on putrescine uptake were also evaluated in TUB::HD309-LmPOT1 T. brucei. Cells were preincubated for 10 min at room temperature in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP) dicyclohexylcarbodi- imide (DCCD), Ca2+ ionophore (A23187), valinomycin, or monensin at the concentrations indicated before the assessment of putrescine uptake at 10 μM. Results represent the percent of putrescine uptake against a control without ionophore (NI). Each value is the mean ± S.D. of three independent experiments.

**FIG. 5.** Effect of extracellular Na+ on putrescine uptake. A, [3H]putrescine uptake by TUB::HD309-LmPOT1 T. brucei was measured in PBS glucose in which NaCl was isosmotically replaced with choline chloride, LiCl, or sucrose. B, [3H]putrescine uptake by TUB::HD309-LmPOT1 T. brucei was measured in PBS glucose with different concentrations of NaCl. The isosmolarity was maintained constant by the addition of sucrose. Each point is the mean ± S.D. of three independent experiments.
however, a pairwise alignment between LmPOT1 and PotE indicated that LmPOT1 accommodated two of the three residues, Trp$^{201}$ and Gln$^{207}$, that have been experimentally demonstrated to be involved in the interaction of PotE with putrescine (36). LmPOT1 also contains a hydrophobic residue (Met$^{335}$), whose counterpart in PotE (Val$^{248}$) is necessary for optimal transport function (37).

Functional analysis of LmPOT1 in X. laevis oocytes revealed LmPOT1 to be a high affinity transporter for both putrescine and spermidine, with a calculated $K_m$ value for putrescine similar to that obtained with intact Leishmania mexicana and L. donovani (20). LmPOT1 did not mediate the uptake of spermine, a polyamine that is not synthesized by Leishmania (18), as well as arginine, agmatine, or pentadecylamine. The ability of LmPOT1 to transport putrescine was confirmed in TUB::HD309-LmPOT1 T. brucei promastigotes (36). LmPOT1 also contains a hydrophobic residue (Met$^{335}$), whose counterpart in PotE (Val$^{248}$) is necessary for optimal transport function (37).

Inhibition studies of putrescine uptake into the TUB::HD309-LmPOT1 transformant implied a narrow ligand specificity of LmPOT1 for polyamines, since an assortment of amino acids at a 250-fold molar excess did not diminish $[^3]H$putrescine uptake. Agmatine, the decarboxylation product of arginine, did not inhibt $[^3]H$putrescine uptake, however, and a 250-fold surfeit of pentadecylamine, a drug used for the treatment of both leishmaniasis and African trypanosomiasis obliterated $[^3]H$putrescine uptake by TUB::HD309-LmPOT1 parasites, although the drug itself is not transported through LmPOT1. These data parallel results with intact Leishmania in which polyamine transport is potently inhibited by pentadecylamine yet pentadecylamine uptake is not affected by excess polyamines (20, 38). Although pentadecylamine transport has not been meticulously investigated in Leishmania (21, 38), at least three biochemically distinct pentamidine transporters have been identified in T. brucei (39).

The ability of LmPOT1 to mediate the transport of polyamines into oocytes and T. brucei implies that the polypeptide is targeted to the cell surface in the two heterologous expression systems. This parallels direct observations in Leishmania promastigotes, where LmPOT1 expression appears to be restricted to the parasite cell surface and flagellar compartments. Interestingly, the protein was not detected in amastigotes, an intracellular milieu that is likely rich in polyamines. The stage specificity of LmPOT1 expression might not be surprising in view of the observation that LmPOT1 activity is reduced at pH $\sim 5.0$, the reported pH of the phagolysosome (40) in which the amastigote resides. This would suggest that additional polyamine transporters must be available to the amastigote, a hypothesis that is substantiated by biochemical studies with L. mexicana and L. donovani amastigotes (20).

Putrescine transport via LmPOT1 was inhibited in a dose-dependent fashion by the proton ionophores FCCP and dicyclohexylcarbodiimide. Because Leishmania promastigotes maintain a large proton electrochemical gradient across their cell surface with a resting potential $\sim -100$ mV (41), it can be reasonably inferred that this genus often takes advantage of this proton gradient to steer nutrient uptake into the parasite. Examples of proton-driven leishmanial transporters include the myo-inositol, adenosine-pyrimidine nucleoside, and inosine-guanosine transporters from L. donovani (42, 43). Whether LmPOT1 is a proton/polyamine co-transporter remains to be elucidated but can be conjectured from the pharmacologic evidence. In contrast, many mammalian active transporters are Na$^+$-dependent (44–46). The ability of LmPOT1 to take up putrescine in Na$^+$-free medium, however, establishes that LmPOT1-mediated transport is Na$^+$-independent. Although Na$^+$ is not required as a ligand for putrescine transport by LmPOT1, the cation is likely an important element in the maintenance of the membrane potential. Thus, inhibition of LmPOT1-mediated transport by monensin could be ascribed to a disruption of the Na$^+$ gradient across the plasma membrane. Interestingly, Na$^+$ as well as Li$^+$ and choline$^+$ appeared to exert an inhibitory effect upon LmPOT1 (Fig. 5). Inhibition of polyamine transport by Na$^+$, Li$^+$, and choline$^+$ has also been observed with mammalian cells (47). Ca$^{2+}$ is also known to affect polyamine transport into eukaryotes, although the effects are disparate. Ca$^{2+}$ stimulates polyamine uptake in human fibroblasts (48) and breast cancer cells (49), whereas the divalent cation inhibits polyamine transport into embryonic mesenchymal cells (49) and Neurospora crassa (50). Because the calcium ionophore A23687 inhibited putrescine uptake into TUB::HD309-LmPOT1 T. brucei, it is possible that putrescine transport via LmPOT1 is Ca$^{2+}$-dependent. This notion is reinforced by the prediction of a putative calmodulin binding motif within the LmPOT1 sequence (see Fig. 1).

The overall contribution of LmPOT1 to polyamine transport in Leishmania remains to be elucidated. To determine whether LmPOT1 is an essential protein and whether other polyamine transporters exist in the parasite is being addressed by targeted gene replacement strategies in which each LmPOT1 copy will be replaced with a drug resistance cassette. Although one might conjecture that polyamine uptake into Leishmania is not an essential process, the homolog in T. cruzi, if functionally validated, may very well have an essential function. T. cruzi lacks both arginase and ornithine decarboxylase activities and are, therefore, auxotrophic for polyamines (51, 52). Thus, polyamine transport from the external milieu is an obligate nutritional function for T. cruzi. The availability of LmPOT1 and expression systems for functional validation of polyamine up-
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