Functional Analysis of an Inosine-Guanosine Transporter from *Leishmania donovani*: The Role of Conserved Residues, Aspartate 389 and Arginine 393

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**Running Title:** Functional Analysis of the LdNT2 Nucleoside Transporter

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ABSTRACT

Equilibrative nucleoside transporters encompass two conserved, charged residues that occur within predicted transmembrane domain 8. To assess the role of these 'signature' residues in transporter function, the Asp$^{389}$ and Arg$^{393}$ residues within the LdNT2 nucleoside transporter from *Leishmania donovani* were mutated and the resultant phenotypes evaluated after transfection into Δldnt2 parasites. Whereas an R393K mutant retained transporter activity similar to that of wild type LdNT2, the R393L, D389E, and D389N mutations resulted in dramatic losses of transport capability. Tagging the wild type and mutant ldnt2 proteins with green fluorescent protein demonstrated that the D389N and D389E mutants targeted properly to the parasite cell surface and flagellum, whereas the expression of R393L at the cell surface was profoundly compromised.

To test whether Asp$^{389}$ and Arg$^{393}$ interact, a series of mutants was generated, D389R/R$^{393}$, D$^{389}$/R393D, and D389R/R393D, within the green fluorescent protein-tagged LdNT2 construct. Although all of these ldnt2 mutants were transport deficient, D389R/R393D localized properly to the plasma membrane, while neither D389R/R$^{393}$ nor D$^{389}$/R393D could be detected. Moreover, a transport-incompetent D389N/R393N double ldnt2 mutant also localized to the parasite membrane, whereas a D389L/R393L ldnt2 mutant did not, suggesting that an interaction between residues 389 and 393 may be involved in LdNT2 membrane targeting. These studies establish genetically that Asp$^{389}$ is critical for optimal transporter function and that a positively charged or polar residue at Arg$^{393}$ is essential for proper expression of LdNT2 at the plasma membrane.
INTRODUCTION

*Leishmania donovani* is the causative agent of visceral leishmaniasis, a disease that is invariably fatal if untreated. These protozoan parasites exhibit a digenetic lifecycle, existing as extracellular, flagellated promastigotes within the sandfly vector, and as intracellular amastigotes within the phagolysosome of macrophages and reticuloendothelial cells of the mammalian host. Since there is no immediate prospect of an antileishmanial vaccine, and current drug regimens are complicated by toxicity and curative failure, the need for new drugs to treat leishmaniasis is acute. Rational therapeutic approaches for antiparasitic drug development require the exploitation of fundamental biochemical differences between parasite and host. Perhaps the most striking metabolic discrepancy between parasites and their mammalian hosts is the disparate mechanisms by which they generate purine nucleotides. Whereas mammalian cells synthesize purine nucleotides from amino acids and 1-carbon compounds, all of the protozoan parasites studied to date are incapable of *de novo* synthesis of the purine ring (1). Thus, purine acquisition from the host is an indispensable nutritional function for which each genus of protozoan parasite has evolved a unique complement of purine salvage enzymes. The initial step in purine salvage involves the translocation of host purines across the parasite surface membranes, a process that is mediated by nucleoside and nucleobase transporters.

Nucleoside permeation into *L. donovani* is mediated by two high affinity transporters with non-overlapping ligand specificity, LdNT1 and LdNT2. LdNT1 transports adenosine and pyrimidine nucleosides, whereas LdNT2 is selective for inosine and guanosine (2,3). Mutants genetically deficient in either LdNT1 or LdNT2 activity have been created by negative selection, and the genes coding for these permeases were subsequently cloned from a cosmid library by functional rescue of nucleoside transport-deficient *Leishmania* (2,3). Based on their primary structures and predicted membrane topologies, LdNT1 and LdNT2 belong to the equilibrative nucleoside transporter1 (ENT) family first described in mammals (4).

Numerous putative members of the ENT family have subsequently been characterized (5) or unveiled within available genome databases (6). However, the elucidation of the functional

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1 The abbreviations used are: ENT, equilibrative nucleoside transporter; TM, transmembrane; GFP, green fluorescent protein; FoB, formycin B; RT, room temperature; PBS, phosphate buffered saline; MIT, myo-inositol transporter; IFB, immunofluorescence buffer; PBS-gs, phosphate buffered saline with 2% goat serum.
determinants within the ENT family that govern ligand translocation and specificity are still largely unknown. Glycine residues within TM5 of hENT1 and LdNT1 have emerged as important determinants of uridine transport (7). However, by far the majority of analyses focused on the interactions between mammalian ENTs and their specific inhibitors, and thus, do not easily extrapolate to other members of the ENT family. These studies support a role for transmembrane (TM) domains 3-6 within hENT1 in the binding of the potent inhibitor 4-nitrobenzylthioinosine (8), while Met^{33} within TM1 of hENT1 has emerged as a determinant of dipyridamole and dilazep sensitivity (9). The elucidation of functional determinants for ENT-mediated transport is hindered by the overall diversity that members of the ENT family exhibit for ligand selectivity and affinity (5). Although ENT members exhibit strikingly low amino acid sequence identities (5), multiple sequence alignments of ENT family members from both parasite and mammalian cells reveal a number of conserved or “signature” residues predominantly located in predicted TM spanning regions (5,6). The most striking of these residues are Asp and Arg, two charged residues that reside in predicted TM8 (see Fig. 1). The preservation of these residues and their topological prediction implies a role in the translocation mechanism. Using the LdNT2 inosine-guanosine transporter as a paradigm for all ENT family members, we describe studies on the role of these two conserved charged residues within LdNT2, Asp^{389} and Arg^{393}. These investigations demonstrate that Asp^{389} is critical in the translocation mechanism and imply a structural role for both Asp^{389} and Arg^{393}. 
EXPERIMENTAL PROCEDURES

Cell Culture - *L. donovani* was cultured at 26 °C in DME-L medium (Invitrogen Life Technologies, Carlsbad, CA) as described (10). The construction and phenotypic characterization of the null mutant Δldnt2 in which both wild type *LdNT2* copies have been eliminated by targeted gene replacement and loss-of-heterozygosity will be described elsewhere. The *Δldnt2* strain was cultured continuously in 50 µg/ml hygromycin (Roche Pharmaceuticals, Nutley, NJ) for which the selective marker, hygromycin phosphotransferase, used in the gene replacement strategy confers resistance. Cell lines generated by transfection of *Δldnt2* with plasmids pALTNEO (11) and pXG-GFP+2’ (12) were selected and maintained in 100 µg/ml G418 (BioWhittaker, Inc., Walkersville, MD), as well as 50 µg/ml hygromycin.

Generation and Expression of Site-directed Mutants - Mutations in *LdNT2* were generated by the QuikChange® site-directed mutagenesis protocol, a polymerase chain reaction-based mutagenesis strategy (Stratagene, La Jolla, CA). Mutations were inserted within the *LdNT2* open reading frame that had been ligated into the pALTNEO leishmanial expression plasmid (11). Mutations were confirmed by sequencing and the mutant constructs transfected into the *Δldnt2* cell line using standard electroporation conditions (13). *Δldnt2* cells were also transfected with pALTNEO-*LdNT2* (wild type *LdNT2*). The wild type *LdNT2* open reading frame was subsequently cloned into the BamHI site of pXG-GFP+2’ (12), a leishmanial expression plasmid that tags proteins at the NH2-terminus with the green fluorescent protein (GFP) and the mutants recreated using the QuikChange® method with the same mutagenic primers as above. All strains transfected with pXG-GFP+2’ constructs were obtained by standard electroporation protocols as for the pALTNEO transfectants.

Formycin B Growth Curves - The *Δldnt2* parental knockout and its transfectants were tested for their sensitivity to formycin B (FoB) (Sigma Chemical Co., St. Louis, MO), an inosine isomer that is a potent inhibitor of *L. donovani* growth (14). Parasites were incubated with various concentrations of FoB and enumerated after ~6 days on a Coulter Counter Model ZM. The EC50 value reported is the effective concentration of FoB that inhibits growth by 50%.

Transport Assays - Nucleoside transport measurements were performed by a previously described oil-stop method (15). Time courses were generated at 10 µM [3H]inosine (0.38 Ci/mmol) and 10 µM [3H]guanosine (0.06 Ci/mmol) and kinetic data obtained by measuring rates of inosine transport at concentrations between 0.3 nM and 10 µM. [3H]inosine (38.4
Ci/mmol) and [3H]guanosine (5.5 Ci/mmol) were purchased from Moravek Biochemicals, Inc. (Brea, CA). Rates of uptake were calculated by linear regression analysis, and kinetic parameters were determined by the method of Hanes (16).

**Integral Membrane Protein Preparations - Leishmania** at a density of 1 X 10^7 parasites/ml were pelleted at 2000 X g, resuspended in 1.0 ml of double deionized water in which a Complete™ Mini, EDTA free protease inhibitor cocktail tablet (Roche Pharmaceuticals) had been dissolved, and lysed with one round of freezing at −70 °C and thawing at room temperature (RT). The lysates were then sedimented in a microcentrifuge for 2 min at RT and the pellets resuspended on ice in 1.0 ml of 100 mM NaHCO₃, pH 11.0. After an additional 30 min of sedimentation in the microcentrifuge at 4 °C, the pellet was resuspended in 2X Laemmli buffer (BioRad, Hercules, CA) supplemented with 8 M urea and stored at -20 °C until further use.

**Cell Surface Labeling** - 1 X 10⁸ cells were washed with phosphate buffered saline (PBS) and incubated on ice with 1 mg/ml EZ-Link Sulfo-NHS-LC-Biotin (Pierce Biotechnologies, Rockford, IL) in PBS for 2 h. The biotinylation reaction was quenched by washing the cells three times with 50 mM glycine made up in PBS. Cells were incubated on ice for 30 min in 100µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% NP40, and 10% glycerol). The cell lysate was pelleted in a microcentrifuge, after which the supernatant was removed to a new microcentrifuge tube and incubated for 1 h at RT with 20 µl of packed streptavidin beads (Invitrogen Life Technologies) prewashed in lysis buffer. After the 1 h incubation the beads were washed two times with 1.0 ml of lysis buffer and resuspended in 20 µl of 2X Laemmli buffer supplemented with 8 M urea. All samples were fractionated by SDS polyacrylamide gel electrophoresis on a 10 % slab gel using standard conditions (17) and biotinylated GFP-tagged LdNT2 protein detected by immunoblotting with a GFP monoclonal antibody (see below). To quantitate the amount of mutant and wild type GFP-tagged LdNT2 protein labeled at the cell surface, Western blot films were scanned using a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA) and the intensity of each band corresponding to GFP-tagged transporter assessed by the Molecular Analyst Software version 2.1.2 (Bio-Rad). Background signal was subtracted for each sample and the percentage of cell surface expression calculated for each mutant GFP-tagged transporter relative to wild type GFP-tagged LdNT2.

**Immunoblotting** - Protein samples were fractionated by SDS polyacrylamide gel electrophoresis on a 10 % slab gel using standard conditions (17). Immunoblotting was
performed as described (18). A mouse GFP antibody (Living colors A.v. monoclonal JL-8, BD Biosciences, Palo Alto, CA) was used at a 1:1,000 dilution in PBS containing 0.01 % Tween 20 (Sigma Chemical Co.) and 5 % nonfat dry milk. Secondary antibody (goat anti-mouse conjugated to alkaline phosphatase, Jackson Immunoresearch, Westgrove, PA) was used at a 1:10,000 dilution in PBS containing 0.01 % Tween 20 (Sigma Chemical Co.) and 5 % nonfat dry milk and signal detected by the CDP-Star chemiluminescence detection system (Roche Pharmaceuticals). A rabbit antibody to the *L. donovani* myo-inositol transporter (MIT) (19) was used at a 1:1,000 dilution in PBS containing 0.01 % Tween 20 (Sigma Chemical Co.) and 5 % nonfat dry milk as a control to normalize protein loading onto each lane of the slab gel. Goat anti-rabbit conjugated to horseradish peroxidase (Jackson Immunoresearch) was used at 1:10,000 to detect anti-myoinositol transporter signal.

**Fluorescence Microscopy** – Lab-Tek® II Chambered Coverglass slides (Fisher Scientific, Pittsburgh, PA) were coated with a 1:10 dilution of poly-L-lysine (Sigma Chemical Co.) for 15 min. The chambers were rinsed with double deionized water to remove excess poly-L-lysine, allowed to dry for 1 h at 60 °C, and then cooled to RT. 1 X 10⁷ *Leishmania* promastigotes were resuspended in 1.0 ml PBS, pipetted into the coverslip chamber, and allowed to attach for 15 min. Chambers were rinsed once with PBS and then overlaid with 500 µl PBS. Images were acquired by Aurelie Snyder of the OHSU-MMI Research Core Facility at this institution (http://www.ohsu.edu/core) with the Applied Precision Deltavision® image restoration system. Deconvolution was performed using the iterative constrained algorithm of Sedat and Agard (20) and additional image processing performed on an SGI Octane workstation.

**Immunofluorescence Microscopy (Live cells)** – 1 X 10⁷ *Leishmania* promastigotes were washed twice with 1.0 ml of PBS and resuspended in 30 µl of immunofluorescence buffer (IFB) consisting of PBS, 1 % glucose, 10 % fetal calf serum. Cells were incubated on ice for 30 min with occasional mixing with either 50 µl IFB containing mouse anti-GFP (Living colors A.v. monoclonal JL-8, BD Biosciences) diluted 1:100 or 50 µl IFB containing rabbit anti-tubulin (ICN Pharmaceuticals, Inc. Costa Mesa, CA) diluted 1:10. Cells were washed twice with 1.0 ml of ice-cold IFB at 2000 X g and incubated on ice for 30 min with occasional mixing with either 30 µl of goat anti-mouse or goat anti-rabbit conjugated to Rhodamine Red (ICN Pharmaceuticals, Inc.) and both diluted to 1:150 in IFB. Finally, cells were washed twice with 1.0 ml of ice-cold IFB at 2000 X g, resuspended in 250 µl of ice-cold IFB, and then overlaid on Lab-Tek® II
Chambered Coverglass slides (Fisher Scientific) coated with poly-L-lysine and processed for imaging as described in the previous section.

Immunofluorescence Microscopy (Permeabilized cells) – 1 X 10^7 Leishmania promastigotes were washed twice in 1.0 ml of PBS and resuspended in 500 µl of fixative containing 4 % paraformaldehyde and 0.1 % glutaraldehyde made up in PBS. Cells in fixative were incubated for 30 min at RT after which time they were overlaid on Lab-Tek® II Chambered Coverglass slides (Fisher Scientific) coated with poly-L-lysine. Attached cells were washed twice with PBS containing 2% goat serum (PBS-gs) (Sigma Chemical Co.) and permeabilized with 500 µl of PBS-gs containing 0.1% Triton-X-100 (Roche Pharmaceuticals) for 30 min at RT. At this time all subsequent steps were performed in the dark to prevent the bleaching of GFP within the permeabilized cells. Cells were incubated for 1 h at RT with either 250 µl of mouse anti-GFP (Living colors A.v. monoclonal JL-8, BD Biosciences) diluted 1:100 in PBS-gs or 250 µl of rabbit anti-tubulin (ICN Pharmaceuticals Inc.) diluted 1:10 in PBS-gs. Attached cells were washed twice with 1.0 ml of PBS-gs and then incubated at RT for 1 h with either 250 µl of goat anti-mouse or goat anti-rabbit antibodies conjugated to Rhodamine Red (ICN Pharmaceuticals, Inc.) diluted 1:150 in PBS-gs. After this final incubation the cells were washed twice with 1.0 ml of PBS-gs and then overlaid with 500 µl of PBS for imaging. The images were acquired as described previously above.
RESULTS

Creation and Growth Phenotype of ldnt2 Mutants - Multiple sequence alignments have revealed the presence of ~16 conserved amino acids residues among functionally characterized ENT family members from mammalian and parasitic organisms (5). Two of these, Asp\textsuperscript{389} and Arg\textsuperscript{393} in LdNT2, are charged, and according to the hidden Markov topological algorithm (21) are located within predicted TM8 (Fig. 1). To assess the functional roles of these two charged residues in ligand permeation, the mutations D389E, D389N, R393K, and R393L were inserted into the LdNT2 gene within the expression construct pALTNEO-LdNT2 and the mutant constructs transfected into ∆ldnt2 L. donovani to create ∆ldnt2[D389E], ∆ldnt2[D389N], ∆ldnt2[R393K], and ∆ldnt2[R393L], respectively. A wild type LdNT2 transfectant, ∆ldnt2[LdNT2], was also created as a control. The ∆ldnt2 cell line was obtained by deleting both copies of the LdNT2 gene from wild type L. donovani and is, consequently, deficient in inosine-guanosine transport.

Each of these transfectant lines was initially evaluated for sensitivity to FoB, a cytotoxic inosine analog to which the nucleoside transport-deficient parental ∆ldnt2 strain is highly resistant (Table I). The transfectants harboring wild type or mutated copies of LdNT2 varied greatly in their growth susceptibility to the drug. Whereas, the EC\textsubscript{50} value of FoB that inhibited ∆ldnt2 cell growth was ~8.3 \(\mu\)M, transfection and amplification of an episomal copy of wild type LdNT2 within ∆ldnt2 (∆ldnt2[LdNT2]) increased the sensitivity of the null mutant to FoB by 3 orders of magnitude (EC\textsubscript{50} value = 7.3 nM). This nanomolar level of sensitivity to FoB is comparable to that previously reported for wild type L. donovani (2). Three of the four mutant transfectant lines, ∆ldnt2[D389E], ∆ldnt2[R393K], and ∆ldnt2[R393L], also exhibited varying degrees of sensitivity to FoB compared to the parental recipient knockout strain but not to the extent of the ∆ldnt2[LdNT2] wild type transfectant. The only exception, ∆ldnt2[D389N], exhibited an EC\textsubscript{50} value of 5.7 \(\mu\)M, a value close to that of the parental ∆ldnt2 line (Table I).

Transport Capabilities of the ldnt2 Mutants - The altered sensitivities of transfectant lines harboring Asp\textsuperscript{389} and Arg\textsuperscript{393} mutations toward FoB implied that LdNT2 transport capacity was impaired. To determine the effects of these mutations on transport directly, each of the transfectants was evaluated for [\textsuperscript{3}H]inosine and [\textsuperscript{3}H]guanosine uptake at a saturating ligand concentration for LdNT2 (10 \(\mu\)M) (2). The ability of the transfectant cell lines to take up both 6-
oxypurine nucleosides was directly proportional to the sensitivity of the strain to FoB (Fig. 2, Panels A and B). Whereas \( \Delta ldnt2[R393K] \) cells incorporated both inosine and guanosine at a rate comparable to the wild type \( \Delta ldnt2[LdNT2] \) transfectant, the capacities of the \( \Delta ldnt2[D389E] \) and \( \Delta ldnt2[R393L] \) lines to take up both ligands was less than 10% of the wild type transfectant (Fig. 2). The D389N mutation was even more incapacitating, since the \( \Delta ldnt2[D389N] \) line, like the \( \Delta ldnt2 \) knockout, exhibited no measurable \(^3\text{H}\)inosine or \(^3\text{H}\)guanosine uptake capability under these parameters. Because these radiolabel incorporation assays were carried out over a 4 min interval, uptake measurements were also performed over shorter intervals to assess impaired transport capabilities more accurately and to minimize metabolic contributions to the uptake measurements. The results of rapid transport assays (see Experimental Procedures), where the uptake of 10 \( \mu \text{M} \), \(^3\text{H}\)inosine or \(^3\text{H}\)guanosine was measured over intervals up to 10 s, correlated well with the results of the longer 4 min uptake assays (Fig. 2, Panels C and D).

To determine whether these transport-incapacitating mutations affected the affinity of the LdNT2 protein for ligand, transport assays were performed at varying \(^3\text{H}\)inosine concentrations over time intervals where uptake was linear. \( K_m \) values of 0.4 \( \pm \) 0.19 \( \mu \text{M} \), 2.5 \( \pm \) 1.2 \( \mu \text{M} \), 1.8 \( \pm \) 1.1 \( \mu \text{M} \), and 2.0 \( \pm \) 1.6 \( \mu \text{M} \) were calculated by Hanes analysis for \( \Delta ldnt2[LdNT2] \), \( \Delta ldnt2[R393K] \), \( \Delta ldnt2[R393L] \) and \( \Delta ldnt2[D389E] \), respectively (data not shown). The lack of transport by \( \Delta ldnt2[D389N] \) cells obviously precluded an accurate \( K_m \) determination.

**Cell surface targeting of GFP-LdNT2 and GFP-ldnt2** - Since the ligand affinities of the mutant transporters were apparently not altered in comparison to the wild type transporter, to determine whether the impaired transport capacity of the \( ldnt2 \) transfectants affected proper targeting or expression of the transporter both wild type and all of the mutant transporters were tagged with GFP at the NH\(_2\)-terminus and the corresponding genes transfected into the \( \Delta ldnt2 \) background. The wild type transfectant, \( \Delta ldnt2[\text{GFP-LdNT2}] \), exhibited an EC\(_{50}\) value of 7.9 \( \pm \) 0.8 nM for FoB, a value virtually identical to that observed for \( \Delta ldnt2[LdNT2] \) (Table I). Moreover, \( \Delta ldnt2[\text{GFP-LdNT2}] \) parasites also displayed robust \(^3\text{H}\)inosine uptake with a \( K_m \) value of 1.3 \( \pm \) 0.6 \( \mu \text{M} \) (n=3) (Fig. 3 and Table II), an inosine affinity slightly less than that observed with the \( \Delta ldnt2[LdNT2] \) transfectant described above. Localization studies using direct GFP fluorescence and deconvolution microscopy demonstrated that GFP-LdNT2 was robustly
synthesized and targeted to both the plasma membrane and flagellum (Fig. 4, Panel A). A diffuse and relatively weak cytosolic GFP fluorescence was observed with Δldnt2 parasites transfected with the empty pXG-GFP+2' vector alone (Fig. 4, Panel B).

The transport capabilities of the GFP-tagged wild type and mutant transporters were similar to those observed for the untagged transporters. The Δldnt2[GFP-R393K], Δldnt2[GFP-R393L], and Δldnt2[GFP-D389E] lines transported [3H]inosine at 21%, 6%, and 6% of the rate of Δldnt2[GFP-LdNT2] parasites, respectively (values based upon the observed Vmax reported in Table II), whereas the Δldnt2[GFP-D389N] transfectant showed no [3H]inosine uptake capability (Table II). Km values for [3H]inosine transport were equivalent among all the transport-competent transfectants (Fig. 3 and Table II). All of the mutant ldnt2 transporters localized to the parasite plasma membrane, except GFP-R393L, which predominantly localized to the flagellar membrane (Fig. 4, Panels C-F). Additionally, a rod-like formation was observed in the Δldnt2[GFP-R393L] mutant (Fig. 4, Panel D). A similar structure, a novel multivesicular tubular compartment, was previously described in Leishmania parasites where the cell surface trafficking of membrane proteins was impaired (22,23).

Although GFP fluorescence data suggested that nearly all of the mutant transporters (with the exception of GFP-R393L) are produced and targeted to the cell surface, similar to GFP-LdNT2, the relative level of cell surface protein expression could not be assessed by this approach. Thus, to ascertain the amount of LdNT2 protein at the cell surface the GFP-tagged transporters were quantitated using a membrane-impermeable biotin probe (Fig. 5). Significant levels of LdNT2 were detected at the cell surface in all lines except Δldnt2[GFP-R393L] in which ldnt2 protein at the cell surface was ~7% of the LdNT2 levels observed in the Δldnt2[GFP-LdNT2] line. As expected, no cell surface biotinylation was observed for Δldnt2[GFP] parasites which contained no GFP-tagged transporter (Fig. 5). The results of the biotinylation experiments were bolstered by parallel analyses of fractionated integral membrane proteins from each of the GFP transfectants probed with a GFP antibody (Fig. 5). These analyses confirmed the reduced level of ldnt2 expression in the Δldnt2[GFP-R393L] cells. Equal loading of the integral membrane fractions was verified by probing with an independent antibody to the leishmanial MIT (19).
The overall transport capacities of the mutant nucleoside transporters could, therefore, be readjusted based on the relative levels of LdNT2/ldnt2 at the cell surface in each of the transfectants (Table II). The adjusted specific activities of the Arg^{393} mutants, ldnt2-R393K and ldnt2-R393L, were, therefore, 36% and 79% of that of LdNT2, whereas the two Asp^{389} mutants, ldnt2-D389E and ldnt2-D389N, were severely crippled in their permease function.

**Mutational analysis of Asp^{389} and Arg^{393} interactions** - The location and proximity of Asp^{389} and Arg^{393} within the predicted helical TM8 structure implies that these charged residues are likely situated on the same side of the helix and conceivably interact. Therefore, a series of mutants was generated in which either Asp^{389} or Arg^{393} were substituted for the opposite residue. Thus, D389R and R393D single mutations and a D389R/R393D double mutation were constructed within the pXG-GFP+2'-LdNT2 plasmid and each construct transfected into the ∆ldnt2 cell line. Growth curves in the presence of increasing concentrations of FoB revealed that ∆ldnt2 cells expressing each of these mutations were at least 3 orders of magnitude less sensitive to FoB compared to ∆ldnt2[GFP-LdNT2] parasites (Table I). Furthermore, inosine and guanosine transport measurements indicated that ∆ldnt2[GFP-D389R], ∆ldnt2[GFP-R393D], and ∆ldnt2[GFP-D389R/R393D] cells were all transport incompetent, equivalent to ∆ldnt2 cells producing GFP alone, whereas ∆ldnt2[GFP-LdNT2] cells expressing GFP-tagged LdNT2 exhibited robust transport capability of both 6-oxypurine nucleosides (Fig. 6, panel A). To assess whether the severely impaired transport capability of the mutants could be ascribed to transporter mislocalization, total integral membrane proteins from each of the transfectants were isolated and subjected to western blotting using a GFP monoclonal antibody for detection. As revealed in Fig. 6B, ldnt2 was found within the membrane fraction of ∆ldnt2[GFP-D389R/R393D] parasites but not in ∆ldnt2[GFP-D389R] or ∆ldnt2[GFP-R393D] cells. Direct fluorescence of GFP using deconvolution microscopy confirmed the plasma membrane localization of ldnt2 in ∆ldnt2[GFP-D389R/R393D] cells, whereas minimal diffuse fluorescence was observed for the ∆ldnt2[GFP-D389R] and ∆ldnt2[GFP-R393D] transfectants (Fig. 6, panel C).

Subsequently, two additional double mutants were created in which both Asp^{389} and Arg^{393} were replaced with either a polar (Asn) or hydrophobic (Leu) residue within the pXG-GFP+2'-LdNT2 vector. Only the ∆ldnt2[GFP-D389N/R393N] transfectant was expressed and localized to the parasite plasma membrane (Fig. 6, panels B and C), however, as expected, both
the Δldnt2[GFP-D389N/R393N] and Δldnt2[GFP-D389L/R393L] transfectants were functionally impaired, similar to the Δldnt2 cell line (Table I and Figure 6, panel A).

Orientation of the NH$_2$-Terminus of LdNT2 – The ability to express a functional GFP-tagged LdNT2 also provided a vehicle to solve the NH$_2$-terminal topology of the transporter. Indirect immunofluorescence analysis of Δldnt2[GFP-LdNT2] using a GFP antibody with permeabilized and non-permeabilized parasites indicated that the NH$_2$-terminus of GFP-LdNT2 is located on the cytoplasmic face of the parasite membrane (Fig. 7). Non-permeabilized parasites did not react with the GFP antibody or with control tubulin antisera, whereas both GFP and tubulin could be detected with their respective antibodies after permeabilization. This cytoplasmic orientation of the NH$_2$-terminus of LdNT2 is consistent with the recently solved gross topology determined for the human hENT1 (24), a protein with which LdNT2 shares ~25% sequence identity (2,5).
DISCUSSION

Multisequence alignments of all functionally characterized ENTs from phylogenetically disparate eukaryotes, from protozoa to human, reveal the presence of a 'signature' motif comprised of two charged amino acids separated by 3 nonconserved residues within predicted TM8 (Fig. 1 and Ref. 5). Specifically, this motif, D-x-x-x-R, is found in both nucleoside transporters of \textit{L. donovani} and all mammalian and parasite ENTs characterized to date. It should be noted, however, that a number of ENT sequences containing the 'signature' motif D-x-x-x-K have recently emerged from various genome sequencing databases (6), but these proteins have yet to be functionally characterized. To dissect the functional roles of these charged residues in a model transporter, site-directed mutants were generated in Asp$^{389}$ and Arg$^{393}$ of LdNT2, a \textit{L. donovani} ENT that exhibits a strict and unusual ligand specificity for 6-oxypurine nucleosides. The data demonstrate that even conservative mutations introduced at Asp$^{389}$ crippled LdNT2 inosine and guanosine transport activity (Fig. 2 and Table II). However, this diminished transport capacity cannot be ascribed to decreased ligand affinity (where it can be measured, i.e., for the D389E mutant) or to a reduced amount of protein at the parasite plasma membrane (Table II and Fig. 4). Thus, it appears that Asp$^{389}$ is a key residue within the translocation process. Negatively charged residues in TM domains of several different transporters are known to play a direct role in ligand recognition, particularly for positively charged ligands such as choline or protons (25). However, since the D389E mutant exhibited wild type affinity for inosine, it is unlikely that Asp$^{389}$ functions primarily to bind to the uncharged nucleoside ligand. Moreover, these residues are conserved among all functionally characterized ENT family members, which display a wide variation in ligand specificities (nucleoside and/or nucleobase) and affinities (5). Thus, Asp$^{389}$ must play another role in the translocation mechanism, likely by inducing or stabilizing a conformation of the transporter that is vital to the permeation mechanism. Although these studies are too preliminary to speculate further on the role of this conserved residue in the permeation mechanism, positive genetic screens to select for second site suppressors of the D389N mutation may reveal a plausible mechanism by which Asp$^{389}$ participates in transport.

Conversely, biochemical characterization of the Arg$^{393}$ mutants has suggested that this positively charged residue is important for the stability and localization of the transporter rather
than being central to ligand translocation or recognition. The introduction of a Lys at Arg\textsuperscript{393} results in a functional transporter with properties similar to the wild type transporter (Fig. 2 and Table II). It is intriguing that ENT-like sequences from several eukaryotes have been deposited in GenBank (6) that possess a Lys at this position, although it should be noted that their ability to translocate nucleosides and nucleobases has not been experimentally demonstrated. The introduction of a Leu, however, at Arg\textsuperscript{393} drastically reduces transport capability to a level similar to that observed with the D389E mutant (Fig. 2). Unlike the D389E mutant, however, the low transport capacity of the R393L mutant can be imputed to dramatically reduced amounts of transporter at the cell surface (Fig. 4 and Table II). Accounting for the observation that GFP-R393L cell surface levels in Δldnt2[GFP-R393L] parasites were only ~7% of the LdNT2 amounts in the Δldnt2[GFP-LdNT2] transfectant, adjustment of the kinetic parameters revealed that the R393L ldnt2 mutant possessed kinetic properties similar to those of the wild type LdNT2 protein (Table II). Intriguingly, most of the GFP-R393L ldnt2 that is produced in the Δldnt2[GFP-R393L] transfectants is targeted to the parasite flagellar membrane (Fig. 4), as well as to the flagellar pocket and possibly the multivesicular tubule (22,23). In contrast, GFP-LdNT2 and GFP-R393K are found chiefly at the parasite plasma membrane and flagellar pocket (Fig. 4). Such differences in localization were unexpected and are, as yet, inexplicable. Currently, the mechanisms of protein segregation between the flagellar and plasma membranes are unknown. Regardless, the limited amount of mutant transporter in the Δldnt2[R393L] strain favors the flagellar compartment, and whether this is due to altered interactions with protein sorting machinery or to the distinct (or unique) composition of the flagellar compartment (26,27) remains to be determined.

Given the proximity of Asp\textsuperscript{389} and Arg\textsuperscript{393} within TM8 and that helical wheel predictions suggest that both residues lie on the same side of the helix (D-x-x-x-R), it seemed plausible that these two residues participate in either a charge stabilizing or hydrogen bond interaction. To test this conjecture, both Asp\textsuperscript{389} and Arg\textsuperscript{393} were mutated to the opposite charge to destabilize these potential interactions. As demonstrated in Fig. 6, neither GFP-D389R nor GFP-R393D could be detected by immunoblotting of the integral membrane fraction and only diffuse cytosolic staining equivalent to that observed with GFP alone could be detected by direct fluorescence (Fig. 6). Surprisingly, the D389R/R389D double mutant appeared to be synthesized and present in normal
amounts in Alidlnt2[GFP-D389R/R393D] transfectants. Furthermore, the D389R/R389D protein was appropriately targeted to the plasma membrane (Fig. 6). The D389R/R393D ldnt2 protein, as expected from the Asp\(^{389}\) alteration, was nonfunctional (Fig. 6). These data reinforce the concept that Asp\(^{389}\) is absolutely critical for proficient transporter function but also signify a possible interaction between Asp\(^{389}\) and Arg\(^{393}\) in the maintenance of LdNT2 structural integrity and membrane targeting. It is not known whether the nature of this interaction is in the form of an intrahelical salt bridge, as found for the *E. coli* glucose-6-phosphate carrier (28), and the melibiose carrier (29), or a hydrogen bond interaction between the Asp\(^{389}\) and Arg\(^{393}\). Support for a hydrogen bond interaction is bolstered by the fact that a D389N/R393N double mutant, although transport incompetent, is expressed and targeted to the plasma membrane, whereas a D389L/R393L double mutant is neither stably produced nor properly targeted (Fig. 7). Since the double Asn mutant has the propensity to form hydrogen bonds, whereas the double Leu mutant is unable to form such an interaction yet would be expected to favor the formation of an \(\alpha\) helix (30), this suggests that an interaction between these two charged residues maybe key for the stable production and proper localization of LdNT2.

In addition to providing evidence for the role of conserved, charged TM residues in the translocation mechanism, these studies demonstrate for the first time that GFP-tagged LdNT2 is localized to both the flagellar and pellicular plasma membranes, as well as to the flagellar pocket (Fig. 4A) and displays a cytoplasmic N-terminal topology (Fig. 8) consistent with that observed for the human hENT1 (24). The only other parasite nucleoside transporters that have been localized are LdNT1 (31) and PfNT1 (32), the *Plasmodium falciparum* nucleoside transporter. Although the location of LdNT2 still needs to be confirmed using antisera against LdNT2 protein to demonstrate conclusively that the cellular environment of the transporter is not influenced by the presence of a large fluorescent tag, it is noteworthy that LdNT1 does not show flagellar targeting when a similar GFP targeting construct is used to monitor its overproduction and localization (31).

In conclusion, these studies support a role for Asp\(^{389}\) and Arg\(^{393}\) in LdNT2 function, cellular maintenance, and targeting. Previous studies using chimeras between the human hENT1 and hENT2 implicate TM-spanning domains 3-6 in inhibitor binding, and mutational dissection of hENT1 (7,8,33) and LdNT1 signify a role for TMs 5 and 7 in ligand translocation and
specificity (31). Our results with LdNT2 suggest that conserved residues within TM8 also contribute to the ENT translocation mechanism. Moreover, they establish genetically that intrahelical interactions within TM8 are important determinants of transporter stability. Finally, the conservation of these amino acids within the ENT family suggests that these residues are likely crucial in a generalized ENT translocation mechanism.

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FIG. 1. **Multiple sequence alignment of predicted TM8 of ENT family members.** hENT1 (AAF02777), hENT2 (Q14542), hENT3 (AAK00958), LdNT1 (AAC32597), LdNT2 (AAF74264), LmaNT3 (CAC33972), TbAT1 (AAD45278), TbNT2 (AAF04490), PfNT1 (CAD52595), and TgAT (AAF03247), were aligned by the method of Feng and Doolittle (34). Amino acids identical among all aligned TM8 domains are **shaded black**, and highly conserved amino acids are **shaded gray**.

FIG. 2. **Functional characterization of Asp^{389} and Arg^{393} ldnt2 mutants.** Panels A and B, ∆ldnt2 parasites expressing ∆ldnt2[LdNT2] (●), ∆ldnt2[R393K] (■), ∆ldnt2[R393L] (□), ∆ldnt2[D389E] (▲), ∆ldnt2[D389N] (Δ) were tested for uptake of 10 µM [³H]inosine or 10 µM [³H]guanosine over 4 min. The ∆ldnt2 cell line (○) served as a negative control. Panels C and D, These experiments were performed essentially as Panels A and B with the exception that the initial rate of uptake was measured over 10 s. Results are expressed as mean ± standard deviation (n = 2).

FIG. 3. **Asp^{389} and Arg^{393} mutant transporter kinetics.** Uptake of [³H]inosine by ∆ldnt2 cells expressing ∆ldnt2[GFP-LdNT2] (●), ∆ldnt2[GFP-R393K] (■), ∆ldnt2[GFP-R393L] (□), ∆ldnt2[GFP-D389E] (▲) was determined over 10 s for GFP-LdNT2 and GFP-R393K or over 1 min for GFP-R393L and GFP-D389E. The experiments were performed at a range of [³H]inosine concentrations (0.3 - 10µM) and the rate of uptake was determined for each concentration by linear regression analysis. The results represented as a Hanes analysis (16) are expressed as inosine(µM)/rate of uptake (pmol/s/10⁸ cells) as a function of inosine (µM). The calculated kinetic parameters are reported in Table II.

FIG. 4. **Deconvolution microscopy of ∆ldnt2 transfectants.** Live parasites were prepared for deconvolution microscopy as described in the Experimental Procedures. Deconvolved fluorescent images of ∆ldnt2 parasites overexpressing GFP-LdNT2 (Panel A), GFP (Panel B), GFP-R393K (Panel C), GFP-R393L (Panel D), GFP-D389E (Panel E), GFP-D389N (Panel F). Each fluorescent image is accompanied by a phase contrast of the field of view shown for the
respective GFP illumination.

**FIG. 5.** **Cell surface expression of wild type and mutant ldnt2 in Δldnt2 transfectants.** Panel A, Live parasites were subjected to cell surface biotinylation, lysis, immunoprecipitation, and Western analysis using a GFP antibody as described in the Experimental Procedures. Panel B, Detection of GFP in integral membrane protein fractions prepared from GFP-\(LdNT2\) and GFP-\(ldnt2\) mutants using a GFP antibody. Panel C, Detection of an endogenous transporter, MIT, used as a control to demonstrate equal loading of lanes in the integral membrane western analysis.

**FIG. 6.** **Characterization of D389R/R\(^{393}\), D\(^{389}/R393D\), D389R/R393D GFP-ldnt2 mutants.** Panel A, Uptake of 10 µM \(^{3}H\)inosine (shaded black) and 10 µM \(^{3}H\)guanosine (shaded gray) by GFP-\(LdNT2\) and GFP-\(ldnt2\) mutants over a 1 min interval. Results are expressed as mean ± standard deviation (\(n = 3\)). Panel B, Detection of GFP in integral membrane protein fractions using a GFP antibody. A MIT antibody was used as a control for equal loading of lanes. Panel C, Deconvolution microscopy of GFP-D389R, GFP-R393D, and GFP-D389R/R393D as described in the Experimental Procedures. I, Δldnt2[GFP-LdNT2]; II, Δldnt2[GFP-D389R]; III, Δldnt2[GFP-R393D]; IV, Δldnt2[GFP-D389R/R393D]; V, Δldnt2[GFP-D389N/R393N]; VI, Δldnt2[GFP-D389L/R393L]; VII, Δldnt2[GFP].

**FIG. 7.** **Topology of the NH\(_{2}\)-Terminus of GFP-LdNT2.** Direct and indirect fluorescence deconvolution microscopy in both membrane-intact and permeabilized parasites. GFP direct fluorescence is shown in the left hand panels, whereas indirect immunofluorescence with tubulin and GFP antibodies is depicted in the right hand panels. Methods are according to the Experimental Procedures.
TABLE I

Sensitivity to Formycin B

Δldnt2 and its transfectants were incubated with varying concentrations of FoB and the parasites enumerated after 6 days to determine the EC₅₀ value, which is the effective concentration of drug that inhibits parasite growth by 50%. All analyses were performed three times or more with the exception of Δldnt2[GFP-D389N/R393N] and Δldnt2[GFP-D389L/R393L].

| Cell Line            | EC₅₀ (µM)     |
|----------------------|---------------|
| Δldnt2               | 8.3 ± 0.9     |
| Δldnt2[LdNT2]        | 0.0073 ± 0.0017 |
| Δldnt2[R393K]        | 0.19 ± 0.04   |
| Δldnt2[R393L]        | 0.6 ± 0.1     |
| Δldnt2[D389E]        | 0.71 ± 0.15   |
| Δldnt2[D389N]        | 5.7 ± 1.4     |
| Δldnt2[D389R]        | 5.7 ± 0.9     |
| Δldnt2[R393D]        | 7.3 ± 0.9     |
| Δldnt2[D389R/R393D]  | 6.3 ± 1.3     |
| Δldnt2[GFP-LdNT2]    | 0.0079 ± 0.0008 |
| Δldnt2[GFP-D389N/R393N] | 6.0         |
| Δldnt2[GFP-D389L/R393L] | 7.5         |
TABLE II

Kinetic parameters of GFP-LdNT2 and Asp389 and Arg393 GFP-lldnt2 mutants.

The kinetic parameters, \( V_{\text{max}} \) and \( K_m \), were calculated according to the Hanes method (16). The relative expression was determined by densitometry on data obtained from western analysis of the cell surface biotinylation experiments detailed under Experimental Procedures. The results are expressed as mean ± standard deviation \((n = 3)\).

| Cell Line         | \( V_{\text{max}} \) (pmol/min/10^8 cells) | % Expression | Adjusted \( V_{\text{max}} \) | \( K_m \) (\( \mu M \)) |
|-------------------|------------------------------------------|--------------|-------------------------------|--------------------------|
| \( \Delta ldn2 \)[GFP-LdNT2] | 14 ± 5.3                                   | 100          | 14                            | 1.3 ± 0.6                |
| \( \Delta ldn2 \)[GFP-R393K]    | 3 ± 0.8                                    | 67 ± 25      | 5                             | 1.7 ± 0.3                |
| \( \Delta ldn2 \)[GFP-R393L]    | 0.8 ± 0.3                                  | 7.1 ± 0.7    | 11                            | 1.6 ± 0.4                |
| \( \Delta ldn2 \)[GFP-D389E]    | 0.8 ± 0.4                                  | 120 ± 37     | 0.7                           | 1.1 ± 0.5                |
| \( \Delta ldn2 \)[GFP-D389N]    | N.D.*                                     | 140 ± 20     | N.D.*                         | N.D.*                    |

N.D., not detectable.
| Protein | Sequence |
|---------|----------|
| hENT1   | 323 T W E R Y F I P V S C F L T F N I F D W L G R . . . . . . . S L T A . 349 |
| hENT2   | 323 K W S Q F F N P I C C F L L F N I M D W L G R . . . . . . . S L T S . 349 |
| hENT3   | 341 W T K F F I P L T T F L L Y N F A D L C G R . . . . . . . Q L T A . 367 |
| LdNT1   | 361 W F S T I A V F I . . . . . . . F N V F D V L G R F S . . . . . . . P S L K L . 385 |
| LdNT2   | 375 G W Y M T I I V T L . . . . . . . F H A G D F V A R . . . . . . . V L L . 396 |
| LmNT3   | 377 W . . . . F A T I A I L L Y H C G D A T G R . . . . . . . W L S S . 398 |
| TbAT1   | 337 G W Y L T I A A L . . . . . . . F N L G D F L S R . . . . . . . L C L . 358 |
| TbNT2   | 337 F W Y F P V A I A M . . . . . . . F N L G D F L S R . . . . . . . L V L . 358 |
| PfNT1   | 273 D Y N V T I I V G M . . . . . . . F Q V F D F L S R Y P P N . . . . . . . L T H I K . 300 |
| TgAT    | 332 V N N H F I I M F G . . . . . . . V F A L G D V T G R F F P D L S Q F S P K . 362 |
FIG. 2

[Graphs showing data on inosine and guanosine uptake over time]
FIG. 3

![Graph showing the relationship between inosine concentration and rate of uptake for different GFP variants.](http://www.jbc.org/)

- **GFP-LdNT2**
- **GFP-R393K**
- **GFP-R393L**
- **GFP-D389E**
FIG. 5

A
Anti-GFP

B

C
Anti-MIT

GFP-LdNT2  GFP-R393K  GFP-R393L  GFP-D389E  GFP-D389N  GFP

Surface Biotinylation

Integral Membrane Fractionation
Functional analysis of an inosine-guanosine transporter from Leishmania donovani: The role of conserved residues, aspartate 389 and arginine 393
Shirin Arastu-Kapur, Ethan Ford, Buddy Ullman and Nicola S. Carter

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