Second-site Suppression of a Nonfunctional Mutation within the *Leishmania donovani* Inosine-Guanosine Transporter*

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LdNT2 is a member of the equilibrative nucleoside transporter family, which possesses several conserved residues located mainly within transmembrane domains. One of these residues, Asp389 within LdNT2, was shown previously to be critical for transporter function without affecting ligand affinity or plasma membrane targeting. To further delineate the role of Asp389 in LdNT2 function, second-site suppressors of the Ldnt2-D389N null mutation were selected in yeast deficient in purine nucleoside transport and incapable of purine biosynthesis. A library of random mutants within the ldn2-D389N background was screened in yeast for restoration of growth on inosine. Twelve different clones were obtained, each containing secondary mutations enabling inosine transport. One mutation, N175I, occurred in four clones and conferred augmented inosine transport capability compared with LdNT2 in yeast. N175I was subsequently introduced into an *Ldn2*-D389N construct tagged with green fluorescent protein and transfected into a Δldnt1/Δldnt2 *Leishmania donovani* knockout. GFP-N175I/D389N significantly suppressed the D389N phenotype and targeted properly to the plasma membrane and flagellum. Most interestingly, N175I increased the inosine *Km* by 10-fold within the D389N background relative to wild type GFP-LdNT2. Additional substitutions introduced at Asn175 established that only large, nonpolar amino acids suppressed the D389N phenotype, indicating that suppression by Asn175 has a specific size and charge requirement. Because multiple suppressor mutations alleviate the constraint imparted by the D389N mutation, these data suggest that Asp389 is a conformationally sensitive residue. To impart spatial information to the clustering of second-site mutations, a three-dimensional model was constructed based upon members of the major facilitator superfamily using threading analysis. The model indicates that Asn175 and Asp389 lie in close proximity and that the second-site suppressor mutations cluster to one region of the transporter.

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*Leishmania donovani* is a protozoan parasite that causes visceral leishmaniasis, a disease that is invariably fatal if untreated. The genus is digenetic, existing as the extracellular promastigote in the phlebotomine sandfly vector and as the intracellular amastigote within the phagolysosome of macrophages in the infected mammalian host. The current arsenal of drugs employed to treat leishmaniasis is far from ideal, and drug efficacy is compromised by both toxicity and therapeutic failure. The need for additional drugs is therefore acute.

Rational approaches to drug development dictate exploitation of novel targets within the parasite. Perhaps the most striking metabolic disparity between protozoan parasites and their mammalian hosts is the inability of the former to synthesize purine nucleotides *de novo* (1). Thus, purine acquisition from the host is an indispensable nutritional function for all protozoan parasites, and many unique purine salvage enzymes have been identified and characterized (1). The initial component of purine uptake involves the translocation of purines into the parasite, a process that is mediated by nucleoside and nucleobase transporters.

Nucleoside permeation into *L. donovani* is mediated by two high affinity transporters with nonoverlapping ligand specificity, LdNT1 and LdNT2, both of which are members of the equilibrative nucleoside transporter (ENT) family. LdNT1 transports adenosine and pyrimidine nucleosides, whereas LdNT2 is selective for inosine and guanosine (2, 3). Essentially nothing is known about the permeation mechanism of the ENTs, and only a few amino acids that govern ligand recognition have been identified. Conserved Gly residues within transmembrane domains (TM) 4 and 5 of human ENT1 (hENT1) and TM5 of LdNT1 have been shown to be critical for transport function and ligand selectivity (4, 5), and a Leu within TM2 of hENT1 has also been revealed as a ligand specificity determinant (6). Additional studies have supported a role for TMs 3–6 (7) and Met33 within TM1 of hENT1 in the binding of dipyrindamole and dilazep (8). Dipyrindamole and dilazep are potent inhibitors of hENT1 but do not significantly affect the majority of parasite nucleoside transporters (9).

Multisequence alignments of ENT family members reveal a number of conserved or “signature” residues, located predominantly in predicted TMs (10). The most striking among these signatures are two charged residues, an Asp and an Arg, that are located within TM8. The evolutionary conservation of these charged residues within a TM implies their significance in transporter structure or function. Site-directed mutagenesis studies revealed that although Asp389 mutants of LdNT2 are produced and localize normally to the cell surface, they are...
severely compromised in function. Thus, Asp^{389} is essential for both inosine and guanosine permeation supporting a functional role of this residue in nucleoside translocation (11).

To elucidate further the role of Asp^{389} in the translocation mechanism of LdNT2, a screen for second-site suppressors was implemented using the nonfunctional ldnt2-D389N mutant. The screen was performed with ade2 Saccharomyces cerevisiae that are mutationally deficient in purine biosynthesis and naturally incapable of purine nucleoside transport, although they maintain the capacity to transport purine nucleobases and can therefore survive with exogenous adenine (6). A number of second-site suppressor mutations were identified. However, one mutation, N175I, occurred with considerable frequency and was subsequently shown to significantly suppress the D389N phenotype in yeast and Leishmania. The distribution of mutations throughout the TMs 1–8 underscores the premise that Asp^{389} is a conformationally sensitive residue, because diverse mutations suppress the D389N null phenotype. Finally, a tertiary topology prediction of LdNT2 is presented, a model that is supported by previously reported biochemical and genetic data. This model indicates that the second-site suppressor mutations cluster in one region of the transporter and, perhaps most significantly, that Asn^{175} and Asp^{389} are located proximal to each other.

**EXPERIMENTAL PROCEDURES**

*Strains and Culture Methods*—The S. cerevisiae strain YPH499 (Mat a ura3-52 lys2-801 ade2-101 trpl-1Δ his3 Δ200 leu2Δ3) was constructed by Sikorski and Hieter (12). Rich (YPD) and minimal (SC) medium plates were prepared as described, and standard methods were used for genetic manipulation of yeast (13). All Escherichia coli transformations were performed in the DH5â–a strain (Invitrogen) by using usual methodologies (14). L. donovani promastigotes were cultured at 26°C in DME-Medium (Invitrogen) as described (15). The construction and phenotypic characterization of the Δldnt1/Δldnt2 L. donovani null mutant in which both copies of LdNT1 and LdNT2 were eliminated by targeted gene replacement followed by loss-of-heterozygosity will be described elsewhere. The Δldnt1/Δldnt2 strain was cultured continuously in 50 μg/ml hygromycin (Roche Applied Science) and 50 μg/ml phleomycin (Research Products International, Mount Prospect, IL) for which the selective markers hygromycin and phleomycin are toxic. Transport activity was measured by using the membrane-permeable, ATP-dependent fluorescent dye 3,3′-di(2-tetramethylrhodamine)ethylammonium bromide (TTCM-AM) according to previously published methods (16). The concentration of 100 μM was added to induce transporter expression, and cells were grown to mid-log phase (A_{600}~1). For each time point, 5.0 ml of culture at an A_{600}~1 was sedimented by centrifugation and resuspended in 100 μl of yeast nitrogen base medium supplemented with 0.5% galactose and 2% glucose. Transport rate was then assayed at 10 μM [3H]inosine (0.2 Ci/mmol) (Moravek Biochemicals, Inc., Brea, CA).

Nucleoside transport measurements in L. donovani promastigotes were performed by the oil-stop method described previously (20). Transport was measured using 10 μM [3H]inosine (0.2 Ci/mmol) and 10 μM [3H]guanosine (0.15 Ci/mmol), and kinetic data were obtained by measuring the amount of [3H]inosine and [3H]guanosine permeation at concentrations of 1 and 5 μM for the N175I mutant and 1 and 50 μM for the N175I/D389N mutant. [3H]Inosine (19.5 Ci/mmol) and [3H]guanosine (15 Ci/mmol) were purchased from Moravek Biochemicals, Inc. Transport rates were calculated by linear regression analysis and kinetic parameters determined by the method of Hanes (21).

Introduction of Site-directed Mutations into ldnt2—Mutations within LdNT2 and D389N-ldnt2 were introduced by the QuickChangeâ–© method described above. Mutations were inserted within the LdNT2 open reading frame in the previously described pXG-GFP-2′-ldnt2 construct (11), confirmed by nucleotide sequencing, and the wild type and mutant constructs were then transfected into the Δldnt1/Δldnt2 cell line using standard electroporation parameters (22).

**Integral Membrane Protein Preparations**—Crude membrane fractions were prepared as reported previously (11). The pattern of expression of mutant ldnt2 proteins in L. donovani was determined by immunoblotting (11).

**Cell Surface Labeling**—Cell surface biotinylation was performed as described (11), with the exception that the beads were ImmunoPureâ–© immobilized streptavidin beads (Pierce). For analysis, all samples were fractionated on 10% SDS-PAGE gels by electrophoresis and, biotinylated green fluorescent protein (GFP)-tagged LdNT2 protein was detected by immunoblotting using mouse anti-GFP (living colors A.V. monoclonal) and rabbit anti-mouse (Amersham and Pharmacia) antibodies. The intensity of each band corresponding to GFP-tagged transporter was assessed by the UN-
Suppression of Loss-of-Function Mutation in LdNT2

Isolation of Suppressor Mutants—Previous data have shown that the D389N mutation in LdNT2 abolishes both inosine and guanosine transport in parasites and imply that this residue is part of a generalized ENT translocation mechanism (11). To examine further the role of Asn175 in LdNT2 function, a screen for second-site suppressor mutations was developed for ldnt2-D389N. This screen was implemented in the YPH499 strain of L. donovani. These lines were designated Δldnt1/Δldnt2 (GFP-N175D389N) and Δldnt1/Δldnt2 (GFP-N175I, Δldnt1/Δldnt2 (GFP-D389N) cells incorporated inosine and guanosine at levels comparable to the wild type Δldnt1/Δldnt2 (GFP-LdNT2) transfectant, as expected, the Δldnt1/Δldnt2 (GFP-D389N) line did not take up inosine as the sole purine source, whereas YPH499 cells were able to transport inosine, and 6 (shaded gray in Table II) exhibited a transport capacity significantly greater than YPH499 cells transformed with wild type LdNT2. Because of

| Clone no. | Mutations           |
|----------|---------------------|
| 1        | N175I               |
| 2        | N175I, I345V        |
| 3        | N175I, L123Q        |
| 4        | N175I, D205G, W339R |
| 5        | Y213C               |
| 6        | Y213C, R339Q        |
| 7        | D159N, I220T        |
| 8        | P52H, 1209V         |
| 9        | V124G, I218T, W339R |
| 10       | N84S, I118P, F214C  |
| 11       | S50T                |
| 12       | I327T, G365V, M378T |

RESULTS

Isolation of Suppressor Mutants—Previous data have shown that the D389N mutation in LdNT2 abolishes both inosine and guanosine transport in parasites and imply that this residue is part of a generalized ENT translocation mechanism (11). To examine further the role of Asn175 in LdNT2 function, a screen for second-site suppressor mutations was developed for ldnt2-D389N. This screen was implemented in the YPH499 strain of S. cerevisiae, which is incapable of both purine biosynthesis and purine nucleoside transport. In initial experiments, YPH499 cells transformed with pBS424-Cu-LdNT2 were shown to be capable of forming colonies on plates containing 100 μM inosine as the sole purine source, whereas YPH499 cells transformed with pBS424-Cu-ldnt2-D389N did not grow even after 7 days of incubation (data not shown). A library of random mutants within ldnt2-D389N was generated as described under “Experimental Procedures,” and a total of 5 × 10^6 transformants was screened for growth on inosine. A total of 24 colonies were obtained after 5 days on inosine plates; however, plasmids from only 16 clones restored the ability of growth on inosine following retransformation in YPH499 cells. Two had reverted back to Asn175 from the D389N mutation, and three were identical, each containing a single N175I second-site mutation. Of the 12 unique clones (Table I), three harbored single mutations that suppressed the D389N phenotype (S50T, Y213C, and N175I), whereas the remaining nine contained either two or three secondary mutations, as well as the starting D389N alteration. These “suppressor” mutations spanned TMs 1–8, excluding TM5 (Fig. 1 and Table I).

Transport Capability of the Second-site Suppressor Clones—To verify the transport phenotype of each positive clone that grew on inosine, transport measurements were conducted at 10 μM [3H]inosine (Fig. 2). As shown in Fig. 2, all 12 clones that suppressed the D389N growth phenotype were capable of transporting inosine, and 6 (shaded gray in Fig. 2) exhibited a transport capacity significantly greater than YPH499 cells transformed with wild type LdNT2. Because of the frequency of N175I within the 12 clones and the robust transport capacity that it conferred to the transformants, it was chosen for further investigation.

Creation and Evaluation of ldnt2 Mutants in Leishmania—To ensure that the suppression of the D389N mutation could be reproduced in other expression systems, the N175I mutation was inserted into both pXG-GFP2−/−ldnt2-D389N and pXG-GFP2−/−LdNT2, and the resulting constructs were transfected into nucleoside transport-deficient Δldnt1/Δldnt2 L. donovani. These lines were designated Δldnt1/Δldnt2 (GFP-N175D389N) and Δldnt1/Δldnt2 (GFP-N175I, Δldnt1/Δldnt2 (GFP-D389N) cells were also generated as positive and negative controls, respectively. To verify the function of the selected second-site suppressor mutations in intact parasites, each transfectant was evaluated for both [3H]inosine and [3H]guanosine transport at a concentration of 10 μM, a ligand concentration determined previously to be saturating for LdNT2 transport (2). Whereas the Δldnt1/Δldnt2 (GFP-N175D389N) and Δldnt1/Δldnt2 (GFP-N175I) cells incorporated inosine and guanosine at levels comparable with the wild type Δldnt1/Δldnt2 (GFP-LdNT2) transfectant, as expected, the Δldnt1/Δldnt2 (GFP-D389N) line did not take up nucleosides (Fig. 3).

Kinetics of the Asn175 Mutants—To determine whether the mutation at Asn175 affected the kinetic parameters of ldnt2, the rates of inosine transport were measured in the Δldnt1/Δldnt2 (GFP-N175I) and Δldnt1/Δldnt2 (GFP-N175D389N) lines (Fig. 4A and Table II), close to the 1.3 ± 0.6 μM value determined previously for wild type GFP-LdNT2 (11). Most interestingly, the Km values obtained for [3H]inosine transport in Δldnt1/Δldnt2 (GFP-N175I) was 0.4 ± 0.2 μM for GFP-Ldnt2-N175I (Fig. 4A and Table II), close to the 1.3 ± 0.6 μM value determined previously for wild type GFP-LdNT2 (11).

Cell Surface Targeting of GFP-ldnt2—To determine whether the mutant ldnt2 proteins expressed and targeted properly in Δldnt1/Δldnt2 L. donovani, GFP fluorescence was measured in Δldnt1/Δldnt2 (GFP-LdNT2, Δldnt1/Δldnt2 (GFP-N175I), Δldnt1/Δldnt2 (GFP-N175D389N), and Δldnt1/Δldnt2 (GFP-D389N). Direct fluorescence measurements indicated that the wild type LdNT2 and mutant ldnt2-N175I, ldnt2-D389N, and ldnt2-D389N alleles are all robustly expressed, and their encoded proteins localize to the parasite plasma membrane and flagellum in the Δldnt1/Δldnt2 knockout (Fig. 5). However, the relative amounts of transporter on the cell surface for the four L. donovani transfectant lines could not be directly assessed by this approach.

To compare the levels of LdNT2/ldnt2 protein at the plasma membrane and flagellum among the transfectants, the GFP-LdNT2/GFP-ldnt2 on the cell surface was quantitated using a membrane-impermeable biotin probe (Fig. 6). Equal loading of
the cell surface biotinylation membrane fractions was verified by probing with an independent antibody to MIT. Comparable amounts of GFP-LdNT2/GFP-ldnt2 were detected at the cell surface in all transfectants except H9004 ldnt1/H9004 ldnt2[GFP-N175I] in which surface membrane ldnt2 protein was 50% of the LdNT2 level observed in the H9004 ldnt1/H9004 ldnt2[GFP-LdNT2] line.

As expected, no cell surface biotinylation was observed in H9004 ldnt1/H9004 ldnt2[GFP] parasites. The results of the biotinylation experiments were mirrored by parallel analyses of fractionated integral membrane proteins from each of the GFP transfec-
tants (Fig. 6). These analyses confirmed the reduced level of ldnt2 protein in the H9004 ldnt1/H9004 ldnt2[GFP-N175I] parasites.

Substitutions at Asn175—To assess the range of secondary mutations at Asn175 that would suppress the D389N phenotype, a series of mutations were created at Asn 175 within pRS424-Cu-ldnt2-D389N, and the mutant alleles were trans-
formed into yeast and screened for growth on 100 μM inosine. Of the substitutions introduced (Gly, Ala, Ser, Thr, Gln, Asp, Lys, Phe, Val, or Leu) at Asn175, only the branched chain amino acids, Val and Leu, as well as Ile, at residue 175 enabled growth on inosine plates (Table III).

Threading Analyses and Tertiary Topology Prediction—To
Spatially orient the second-site suppressor mutants, a tertiary topology model of LdNT2 was constructed. A suitable template for constructing the ENT model was ascertained by threading analyses, which exploit web-based algorithms to identify existing macromolecular crystal structures that exhibit similarity at the secondary structure level to secondary topology profiles of ENTs without the necessity of significant sequence identity (25, 26). Three independent threading algorithms (mGenThreadder, 3DPSMM, and ModWeb) all selected a major facilitator superfamily (MFS) member as a template upon which to construct an ENT model. Alignment of selected members of the ENT family with the identified MFS members, despite low primary sequence homology, showed good agreement in their secondary topologies (see Supplemental Material). The three-dimensional model predicted by ModWeb was considered the best because it was the most consistent with the secondary topology of the ENTs. Specifically, ModWeb identified the crystal structure of the E. coli glycerol 3-phosphate transporter, an MFS member, with a high probability (expected exponential value of $^{13}$). In this structure, the helix packing for LdNT2 was similar to that of MFS members, excluding, of course, TM12 of the latter (Fig. 7A). Furthermore, all the residues on various ENTs that have been identified through biochemical and genetic data to be either solvent-accessible (27, 28) or important ligand selectivity determinants (4–8) were oriented toward the putative ligand-binding pore (Fig. 7B). Finally, the majority of the second-site suppressor mutations obtained in the screen are localized on TMs that cluster in the tertiary topology model (Fig. 7B).

**DISCUSSION**

All ENT family members that have been functionally characterized to date contain a conserved DXXXR pentapeptide within TMS (10). Site-directed mutants of Asp$^{389}$ within LdNT2 cripple transport capability, which cannot be ascribed to changes in ligand affinity or protein expression at the parasite plasma membrane and flagellum (11). Thus, Asp$^{389}$ is a key component of the LdNT2 translocation mechanism. Because structural information on ENTs is limited, a forward genetic strategy was exploited to select for second-site suppressor mutations within LdNT2 that would further our understanding of the role that Asp$^{389}$ plays in nucleoside transport. Second-site suppression strategies have proven useful in the elucidation of the functional role of cryptic first-site mutations within a variety of membrane proteins, revealing information about both tertiary structure and critical functional domains.
FIG. 7. Tertiary topology prediction. A, the schematic of the predicted helical arrangement is shown with the residues that have been found to be critical in ligand binding (4–8) highlighted in magenta. These include Met33 and Leu92 within hENT1 (6, 8), Gly179 and Gly183 predicted helical arrangement is shown with the residues that have been

(29–31). In this study, second-site mutations that suppress the D389N null-transport phenotype were selected in S. cerevisiae purine auxotrophs by virtue of their ability to confer growth on inosine. A diverse number of mutations that occurred throughout the first 8 TMs (except TM5) and their interconnecting loops were identified (Fig. 1). Of the 16 positive clones, 2 were revertants to the wild type allele. No other substitution at position 389 was obtained in our screen, substantiating our previous observation that Asp389 is a crucial signature residue in the ENT permeation mechanism.

Because Asp389 is a negatively charged residue within a TM helix, we conjectured that it might participate in an electrostatic interaction with a positively charged counterpart within the LdNT2 structure. The only alteration of a positive charge among the suppressors was R393Q that appeared in context of TM5 relative to TM8. The predilection for Ile mutations, as suppressor mutations at position 175 implies a local volume compensation that might shift the position of TM5 relative to TM8. The predilection for Ile mutations, rather than genetic alterations to Val and Leu, at position 175 portended potential interactions between residues that were not interact within LdNT2 (Fig. 4 and Table II). In contrast, N175I in the context of D389N did not alter ligand selectivity, because no uptake of adenosine and uridine was detectable (data not shown). The only amino acid substitutions at Asn175 able to suppress the D389N phenotype were the branched chain amino acids, Ile, Val, and Leu (Table III). This preference for large nonpolar aliphatic residues as suppressor mutations at position 175 implies a local volume compensation that might shift the position of TM5 relative to TM8. The predilection for Ile mutations, rather than genetic alterations to Val and Leu, at position 175 in the suppressor screen (Table I), would be expected from the higher number of nucleotide changes required for the Val and Leu missense mutations to occur. Most interestingly, the Ile, Val, and Leu substitutions at Asn175 are all predicted to shift the local secondary structure probability from loop to helix according to the Protein Sequence Analysis server (bmerc-www.biu.ac.il), suggesting that perhaps these mutations cause an extension of one helical turn in TM5. No local secondary structure shifts were predicted for the D389N mutation by using this algorithm.

The diversity of second-site mutations obtained in the screen implied that the D389N mutation likely confers a constraint that is alleviated by other mutations within the ldnt2 structure. The second-site mutations grouped into three regions of LdNT2: TMs 1–3, TM6, and the COOH cusp of the large intracellular loop between TMs 6 and 7 (Fig. 1). Mutations within the second cluster were found in almost 50% of the suppressor mutants. The third clustering in the highly divergent intracellular loop between TMs 6 and 7 suggests that this loop may also contribute to the conformational changes that accompany translocation (Fig. 1). A clustering of second-site suppressor mutations was also observed with the lactose permease, which portended potential interactions between residues that were later confirmed when the lactose permease crystal structure was solved (31, 33). The lack of a crystal structure for any ENT family member is obviously an impediment to interpreting the significance of the clustering of the second-site suppressor mutants within the first 8 TMs of the D389N ldnt2 null mutant.

In the absence of structural information on ENTs, a tertiary structure prediction for all ENTs was computationally gener-
ated by threading analysis in order to provide a structural framework in which to inspect the D389N and suppressor mutations. Threading analyses utilize algorithms that generate a consensus secondary structure profile for a protein family, which is then used to search available protein structure data bases to identify suitable templates upon which a three-dimensional model can be constructed. Such threading analyses have become more reliable recently because of the accuracy of the more sophisticated secondary structure algorithms (25). Although the tertiary topology prediction was constructed by the ModWeb algorithm, which elected to build a tertiary model for the ENT family upon the template of the structurally resolved glycerol 3-phosphate transporter, it is notable that all three independent threading analyses identified MFS members as suitable templates. Most interestingly, Baldwin et al. (9) have previously hypothesized that the overall three-dimensional structures of ENTs would be similar to those of the human glucose transporters, which are members of the MFS. Crystal structures of MFS members encompass 12 TMs with cytosolic NH2 and COOH termini and a large intracellular loop between TMs 6–7. ENTs are similar to MFS members in their predicted membrane topology accommodating a large loop between TMs 6 and 7, although they are conjectured to have 11 TMs with an extracellular COOH terminus (34). Although the exclusion of TM12 in the ENT prediction from the MFS template is a concern, an extensive mutagenesis study of TM12 of the lactose permease, an MFS member, indicates that residues in this domain do not participate in ligand binding or translocation (35). The validity of the predicted helix packing in the ENT model (Fig. 7A) is supported by the location of key residues within TMs 1, 2, 4, and 5, all of which map toward the putative aqueous pore in the model, that have been identified by structure-function studies to be critical for ligand selectivity or to be solvent-accessible (4–6, 8, 27, 28, 36). Specifically these include the following: Met33 and Leu92 within hENT1, which have been implicated in the interaction with the vasodilators, dipyridamole and dilazep (Met33 and Ref. 8), as well as with the ligands, inosine, guanosine, S-nitrobenzyl-4-thioinosine and dilazep (Leu92 and Ref. 6); Gly179 and Gly183 within hENT1 (5) and hNT1 (4), respectively, that are important determinants for uridine transport as well as for dilazep interaction in the case of Gly179; Asp389 and Arg393 within TM8 of LdNT2, which constitute a conserved signature motif (DXXXR) within the majority of ENTs (10) and are important determinants for transporter function and targeting (11); Cys337 in LdNT1, which when mutated to a bulkier residue results in a transporter with impaired transport capability (4); residues mapped to the solvent-accessible face of TM5 in LdNT1 (27); and an exofacial Cys140 in rENT1 (28). Because of the low sequence identity (<14%) between the MFS and ENTs and the inherent difficulties in modeling unstructured loop domains, this ENT structural prediction will, of course, require extensive computational refinement and experimental validation to evolve. When the second-site mutations that suppress the D389N null phenotype are mapped onto this model, they cluster across TMs 1, 3, and 6, and all map to one region of the protein, suggesting that alterations in the tertiary structure in this part of the protein are able to suppress the deleterious effects of the D389N mutation (Fig. 7B). Thus, we have developed a second-site suppressor screen in yeast for LdNT2, an ENT family member. This strategy, which has proven instrumental in probing the transport mechanism of the lactose permease, provides a useful approach for future structure-function studies of members of this ENT family. The second-site suppressor data confirm our previous hypothesis that Asp389 induces or stabilizes a conformation of the transporter that is vital to the LdNT2 permeation mechanism (11). Furthermore, we have generated a preliminary tertiary prediction for the ENT family that can serve as a framework in which to understand the structural role of specific residues and domains in nucleoside translocation and/or recognition.

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Second-site Suppression of a Nonfunctional Mutation within the *Leishmania donovani* Inosine-Guanosine Transporter

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