The antitumor drug miltefosine (hexadecylphosphocholine, MIL) has recently been approved as the first oral agent for the treatment of visceral leishmaniasis. Little is known about the mechanisms of action and uptake of MIL in either parasites or tumor cell lines. We have cloned a putative MIL transporter (LdMT) by functional rescue, using a Leishmania donovani-resistant line defective in the inward-directed translocation of both MIL and glycerophospholipids. LdMT is a novel P-type ATPase belonging to the partially characterized aminophospholipid translocase subfamily. Resistant parasites transfected with LdMT regain their sensitivity to MIL and edelfosine and the ability to normally take up [14C]MIL and fluorescent-labeled glycerophospholipids. Moreover, LdMT localizes to the plasma membrane, and its overexpression in Leishmania tarentolae, a species non-susceptible to MIL, significantly increases the uptake of [14C]MIL, strongly suggesting that this protein behaves as a true translocase. Finally, both LdMT-resistant alleles encompass single but distinct point mutations, each of which impairs transport function, explaining the resistant phenotype. These results demonstrate biochemically and genetically the direct involvement of LdMT in MIL and phospholipids translocation in Leishmania and describe for the first time a P-type ATPase involved in MIL uptake and potency in eukaryotic cells.

Leishmania donovani is a protozoan parasite that causes visceral leishmaniasis, an endemic disease of tropical and subtropical regions that is fatal if untreated (1). There are an estimated 500,000 cases per year in developing countries, half of them occurring in India (2). First-line treatment is based on pentavalent antimonials, but this is compromised by drug toxicity, high costs, and drug resistance (3). The growing incidence of visceral leishmaniasis among AIDS patients in the Mediterranean area emphasizes the need for optimal therapeutic management (4). The use of miltefosine (MIL) as the first effective oral drug against visceral leishmaniasis (5) and its recent approval for clinical use in India has provided some hope that we may now have an effective treatment (6). It is expected that MIL will play a key role in the control and possible eradication of visceral leishmaniasis (2), an Indian priority for the year 2010 (6). Thus, it is of particular importance to understand the mechanisms underlying the development of MIL resistance in Leishmania before its widespread clinical use.

MIL was first developed as an antitumoral drug, together with the alkyl-glycerophospholipid edelfosine and other related drugs. A direct correlation between drug uptake and cellular sensitivity has been found in different eukaryotic cells (7–10), but the mode of uptake of MIL and alkyl-glycerophospholipids is not well understood, and may vary depending on the cell type. We have described previously the biochemical characterization of the L. donovani MIL resistant line M-40 R. A 40-fold decrease in drug uptake was shown to be responsible for the resistant phenotype (10). The inability to normally take up MIL in the resistant line was the direct result of a defect in the inward translocation of the drug (the movement from the outer to the inner leaflet of the plasma membrane), because binding to the membrane, outward movement (efflux), drug metabolism, and bulk-phase endocytosis remained unaltered as compared with wild-type parasites. This resistant line is also defective in the inward-directed translocation of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino (NBD)-glycerophospholipid analogs across the plasma membrane, suggesting that MIL uptake and lipid translocation may share the same transporter (10). Reduced drug uptake has emerged as a common characteristic of drug-resistant trypanosomes, both in vitro and in vivo, enabling the molecular identification of drug transport systems (11, 12).

This paper reports the cloning of a new Leishmania P-type ATPase gene (LdMT, from Leishmania donovani putative Miltefosine Transporter) by functional rescue of the MIL-resistant line. LdMT belongs to the aminophospholipid translocase (APT) subfamily and localizes to the plasma membrane. LdMT mediates MIL and NBD-glycerophospholipids translocation across the plasma membrane in Leishmania parasites. New functional insights into LdMT activity strongly suggest that certain APTs behave as true phospholipid translocases. Further, we establish that single but distinct point mutations...
within the two alleles of the \textit{LdMT} locus are responsible for the resistant phenotype through inactivation of the protein. These results provide the first genetic and biochemical evidence of a protein directly involved in MIL uptake in eukaryotic cells and point to \textit{LdMT} as a gene possibly responsible for treatment failure in the future.

**EXPERIMENTAL PROCEDURES**

**Materials**—MIL was obtained from Zentaris (Frankfurt, Germany). Hexadecylphospho[1,2-ethylenc-14]choline (14C[MIL]) (1.33 MBq/mmol) was synthesized by Amersham Biosciences (Buckinghamshire, UK). 1-O-octadecyl-2-o-methyl-rac-glycerol-3-phosphocholine (edelfosin) was obtained from Calbiochem. 1-hexadecanoyl-sn-glycerol-3-phosphocholine, -phosphothanolamine, -phosphoserine (NBD-PC, -PE, -PS), and 6-[NBD-hexanoyl]phosphatidylcholine (NBD-SM) were obtained from Avanti Polar Lipids (Birmingham, AL). All other chemicals and reagents were of the highest quality available.

**Parasite Cell Culture and Cytotoxicity Assays**—The wild-type (MHOM/ET/67/HU3) and the MIL resistant M-40 R (13) lines of \textit{L. donovani} were cultivated in M-199 medium (Invitrogen) supplemented with 40 \textmu M HEPES (Sigma), 100 \textmu M adenosine (Sigma), hemin (0.2\% of a 250 \textmu M stock solution) (Sigma) and 10\% heat-inactivated fetal bovine serum (Invitrogen) at 28 °C. The \textit{Leishmania tarentolae} wild-type strain \textit{TcXXI} described previously (14) was cultured in the same medium. For determination of parasite sensitivity to MIL or edelfosin, 1 × 10\(^6\) cells were incubated for 48 h at different drug concentrations before determining cell proliferation as described previously (15).

**Transfection and Screening for MIL Sensitivity**—To isolate cosmids containing the MIL transporter gene, eight independent transfections were performed as described previously (16, 17). M-40 R stationary promastigotes were transfected with 10 \textmu g of DNA from a cosmid library of genomic DNA from the \textit{L. donovani} infantum strain LEM1317 cloned into the shuttle vector pCLHYG (16, 18). One day after transfection, the medium was supplemented with 40 \textmu g/ml of hygromycin B, and 24 h later parasites were plated onto 1% agar plates (Invitrogen) at 28 °C. The \textit{Leishmania tarentolae} wild-type strain \textit{TcXXI} described previously (14) was cultured in the same medium. For determination of parasite sensitivity to MIL or edelfosin, 1 × 10\(^6\) cells were incubated for 48 h at different drug concentrations before determining cell proliferation as described previously (15).

**Isolation of \textit{LdMT}, DNA Sequencing Analysis, and Construction of Expression Vectors**—To localize \textit{LdMT} within the 19D10 cosmid, restriction fragment length polymorphism was used to sequence the entire \textit{LdMT} open reading frame (ORF) in an ABI Prism 3100 DNA sequencer. For cloning and sequencing of the different \textit{LdMT} alleles from the wild-type and M-40 R lines, PCR amplification was accomplished with two different sets of primers using the high fidelity Triple Master polymerase from Eppendorf. P1 (5'-GTGACATTGAAGCTGGCCAGTACTATT and 3'-TGAGCTTCTAGGTTAGCTGTTTTAGACC) amplified only the coding region, whereas P3 (5'-GTAGACCTGTGGCCAGTACTATT and 3'-5' GTTACATTGAAGCTGGCCAGTACTATT) and P4 (5'-GTGACATTGAAGCTGGCCAGTACTATT and 3'-5' GTTACATTGAAGCTGGCCAGTACTATT) amplified the entire ORF, flanked by 714 and 685 bp of the untranslated 5' and 3' regions, respectively. BgII and XbaI restriction sites were added (underlined in sequence) for further retransfection. PCR amplified bands were subcloned into the pGEM-T vector (Promega) for sequencing. The selected alleles were also subcloned into the BamHI-XbaI sites of the pX expression vector.

To generate green fluorescent protein (GFP) fusions at the carboxyl terminus of \textit{LdMT} (LdMT-GFP), the ORF of the wild-type \textit{LdMT} gene without the STOP codon was amplified by PCR by using forward and reverse primers containing BglII and SmaI sites, respectively. After restriction digestion, the PCR product was subcloned in frame into the BamHI-EcoRV sites of the \textit{Leishmania} expression vector pXG-GFP+ (20).

**Immunoblotting and Fluorescence Localization**—To determine if MIL or edelfosin is transported into the \textit{Leishmania} expression vector pXG-GFP+ (20).

For localization of the \textit{LdMT}-GFP chimera, parasites were pelleted, washed three times in phosphate-buffered saline, attached to poly-l-lysine-coated coverslips, and fixed with 3% (w/v) paraformaldehyde for 20 min. Coverslips were rinsed with phosphate-buffered saline and mounted on slides with Vectashield (Vector Laboratories Inc.). Images were acquired with a TCS-SP confocal microscope (Leica) and processed with the Leica Confocal Software and Adobe Photoshop.

**Southern Analysis and Fluorescence-Activated Cell Sorting Analyses (FACS)**—Genomic DNA was purified from wild-type and M-40 R lines with the DNAzol reagent (Invitrogen). Restriction enzyme-digested DNA was hybridized to the \textit{LdMT} ORF following standard procedures (22). Total cell DNA was isolated with TRizol reagent (Invitrogen). Subsequently, 5 \mu g of total DNA was used to synthesize the first-strand cDNA with the 

**Functional Experiments**—\textit{L. tarentolae} TarII cells and \textit{L. donovani} wild-type and M-40 R cells were transfected with either pX-LdMT (a construct containing P1-P2 amplified wild-type genomic DNA in the pX vector) or pX-T420N (containing the P1-P2 T420N allele from M-40 R genomic DNA), pX-L568P (containing the L568P allele from M-40 R genomic DNA), or pX vector alone. The resulting transfected cells were selected in medium containing G418 until a concentration of 200 \mu g/ml was reached. At that point, the internalization of \textit{14C}MIL (1.33 MBq/ mmol) or fluorescent-labeled phospholipid analogs was measured as described previously (10). Briefly, 2 × 10\(^7\) promastigotes in culture medium were incubated with 0.09 \mu Ci/ml \textit{14C}MIL (2.5 \mu Ci) for 60 min at 28 °C. After washing with phosphate-buffered saline containing bovine serum albumin to allow for the removal of the drug fraction bound to the outer leaflet of the plasma membrane, followed by a second phosphate-buffered saline wash, both protein concentration and counts per minute were determined. With respect to NBD-phospholipid accumulation, 2 × 10\(^7\) cells were preincubated with 100 \mu M phenylmethylsulfonyl fluoride before the addition of NBD analogs from an ethanol stock solution. After 30 min at 28 °C, parasites were spun down, washed three times in phosphate-buffered saline, resuspended in bovine serum albumin, and the intracellular fluorescence of 10,000 gated cells was acquired using a FACScan flow cytometer (BD Biosciences).

**Screening for MIL Resistance in Mutagenized Parasites**—To localize \textit{LdMT} within the 19D10 cosmid, restriction fragment length polymorphism was used to sequence the entire \textit{LdMT} open reading frame (ORF) in an ABI Prism 3100 DNA sequencer. For cloning and sequencing of the different \textit{LdMT} alleles from the wild-type and M-40 R lines, PCR amplification was accomplished with two different sets of primers using the high fidelity Triple Master polymerase from Eppendorf. P1 (5'-GTGACATTGAAGCTGGCCAGTACTATT and 3'-TGAGCTTCTAGGTTAGCTGTTTTAGACC) amplified only the coding region, whereas P3 (5'-GTAGACCTGTGGCCAGTACTATT and 3'-5' GTTACATTGAAGCTGGCCAGTACTATT) and P4 (5'-GTGACATTGAAGCTGGCCAGTACTATT and 3'-5' GTTACATTGAAGCTGGCCAGTACTATT) amplified the entire ORF, flanked by 714 and 685 bp of the untranslated 5' and 3' regions, respectively. BgII and XbaI restriction sites were added (underlined in sequence) for further retransfection. PCR amplified bands were subcloned into the pGEM-T vector (Promega) for sequencing. The selected alleles were also subcloned into the BamHI-XbaI sites of the pX expression vector.

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**Immunoblotting and Fluorescence Localization**—To determine if MIL or edelfosin is transported into the \textit{Leishmania} expression vector pXG-GFP+ (20).

**Deduced Amino Acid Sequence of \textit{LdMT}**—Sequence analysis of \textit{LdMT} revealed 28\% amino acid identity and 47\% homology with the human ATPase II (23) and other members of the P-type ATPases subfamily, such as the yeast Drs2p (23\% identity). \textit{LdMT} is 96\% identical to the \textit{Leishmania major} putative ATP from chromosome 13. \textit{LdMT} shares all the P-type ATPase consensus sequences and the ATP subfamily-specific
motifs from both the yeast (24) and the mammalian homologues (25) (Fig. 3). It also contains 10 easily predictable hydrophobic transmembrane segments (TM) and a large cytosolic loop after the fourth TM containing the DKTGTLT phospho-drophobic transmembrane segments (TM) and a large cytosolic loop after the fourth TM containing the DKTGTLT phospho-
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Functional Characterization of LdMT—To establish the functional role of LdMT, the pX-LdMT construct was transfected into M-40 R L. donovani cells. We have established previously that NBD-glycerophospholipids and MIL are internalized in Leishmania parasites mainly by rapid transbilayer movement, a protein-mediated and energy-dependent process, independent of endocytosis (10, 28). Uptake assays using radiolabeled MIL or fluorescent-labeled NBD-phospholipid analogs demonstrated that LdMT was able to rescue inward translocation and thus to increase uptake of MIL (78-fold) (Fig. 4A), NBD-PC (35-fold), NBD-PE (14-fold), and NBD-PS (12-fold), but not NBD-SM in the M-40 R line (Fig. 4B). It also restored the normal sensitivity to MIL and edelfosine (Fig. 2). These results indicate that LdMT participates in the inward translocation machinery, which distinguishes sphingolipids from glycerolipids and recognizes only the latter as substrates for translocation across the plasma membrane, together with the non-glycerol based alkylphosphocholine MIL. This feature makes LdMT the phospholipid translocase with the broadest substrate specificity so far studied.

One may still wonder whether LdMT is really a true translocase able to transport lipid analogs or just another member of the translocation machinery required for this activity. To gain further insight into this issue, we transfected wild-type L. donovani parasites with LdMT. Overexpression of LdMT increased the internalization of these phospholipid analogs (except NBD-SM) 2–5-fold as compared with control cells transfected with the empty vector (Fig. 4). A concomitant 2–3-fold increase was seen in sensitivity to MIL and edelfosine (Fig. 2). The increase in phospholipid uptake, both in wild-type and M-40 R parasites, correlated with the G418 selective pressure that controls plasmid copy number and thus the level of LdMT overexpression, as shown by Western blotting with a functional LdMT-GFP chimera (Fig. 5 and data not shown). These data further suggest that LdMT is not only involved in the translocation machinery, but that it is likely to be the direct MIL and glycerophospholipid transporter.

If LdMT were a true translocase, it should confer its biochemical activity to any other cell type lacking this function, as far as the system contains the elements required for proper functioning of the pump. The lizard parasite Leishmania tarentolae shows a natural resistance to alkylphosphocholines, with sensitivities one order of magnitude higher than those of the human parasites L. donovani, Leishmania tropica, Leishmania mexicana, or Leishmania panamensis (personal observations). We then tested MIL accumulation in L. tarentolae and found that this was 16-fold lower than that of L. donovani wild-type cells (Fig. 4A). Therefore, L. tarentolae parasites constitute an optimal system to study LdMT function. Expression of LdMT into L. tarentolae increased MIL uptake 20-fold (Fig. 4A), with the concomitant gain of MIL sensitivity (data not shown). These results demonstrate that LdMT, at least inside the Leishmania genetic background, behaves as a true translocase of lipid analogs, being the key factor modulating MIL uptake and potency.

Cellular Localization of LdMT-GFP—To localize LdMT inside the cells, we constructed LdMT versions with a GFP tag at the carboxyl terminus. Tagging did not interfere with LdMT function, because M-40 R parasites expressing the chimera were able to rescue the transport-deficient phenotype (Fig. 5). Subcellular location of LdMT-GFP was monitored by using GFP fluorescence. As shown in Fig. 6, a significant portion of LdMT-GFP was localized to the plasma membrane but excluded from the flagellum. There was some labeling of the
flagellar pocket area, which may reflect a secondary location or just protein trafficking.

**Analysis of the LdMT Locus, Transcripts, and Allele Sequences in the M-40 R Line**—Genomic Southern blots (Fig. 7A) probed with the LdMT ORF revealed hybridizing fragments consistent with the map given for the cosmid in Fig. 1, indicating that LdMT is likely a single copy gene. The signal intensity and the restriction pattern in wild-type and M-40 R blots were similar, demonstrating that neither deletions nor rearrangement of the LdMT locus were responsible for the translocation-deficient phenotype on M-40 R cells. Reverse transcriptase-PCR experiments showed unique ethidium bromide-stained bands of the same intensity and expected size in both cell lines (Fig. 7B), indicating that loss of LdMT function in M-40 R cells is not caused by an alteration in RNA levels. To determine whether mutations within LdMT were responsible for the translocation-deficient and MIL-resistant phenotype, LdMT from the M-40 R cell line was cloned and sequenced, after its amplification from genomic DNA by PCR. M-40 R cells were compound heterozygotes at the LdMT locus containing two mutant alleles that encompassed single but distinct point mutations. One of the two LdMT-resistant alleles contained a Cys-1259° A mutation, which resulted in a T420N substitution inside the conserved, specific P-type phosphorylation motif DKT420GTLT (Fig. 3). The other allele contained a different single point mutation, Thr-2567° Cys, resulting in an L856P substitution in the large cytosolic loop before TM 5 (Fig. 3). These point mutations were confirmed by two additional independent PCRs from genomic DNA. Further, the presence of two independent alleles was confirmed by polymorphism analysis in the 5'UTR region of the gene (data not shown). M-40 R cells transfected with either the T420N or the L856P mutant alleles showed greatly reduced MIL uptake capabilities, similar to those observed in the M-40 R line, as opposed to the wild-type from the M-40 R cell line was cloned and sequenced, after its amplification from genomic DNA by PCR. M-40 R cells were compound heterozygotes at the LdMT locus containing two mutant alleles that encompassed single but distinct point mutations. One of the two LdMT-resistant alleles contained a Cys-1259° A mutation, which resulted in a T420N substitution inside the conserved, specific P-type phosphorylation motif DKT420GTLT (Fig. 3). The other allele contained a different single point mutation, Thr-2567° Cys, resulting in an L856P substitution in the large cytosolic loop before TM 5 (Fig. 3). These point mutations were confirmed by two additional independent PCRs from genomic DNA. Further, the presence of two independent alleles was confirmed by polymorphism analysis in the 5'UTR region of the gene (data not shown). M-40 R cells transfected with either the T420N or the L856P mutant alleles showed greatly reduced MIL uptake capabilities, similar to those observed in the M-40 R line, as opposed to the wild-type

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**Fig. 3. Alignment of human ATPase II and Leishmania LdMT polypeptides.** Amino acid sequence alignment was done with the ClustalW program (26). Asterisks indicate amino acid identity. Location of transmembrane segments (TM) as defined in human ATPase II (27) are highlighted from TM1 to TM10. Consensus characteristics of P-type ATPases are boxed in black. Aminophospholipid translocase subfamily specific motifs are boxed in gray (24, 25). The position of Thr-420 and Leu-856 in LdMT are marked by the lower arrowheads.
protein which is able to increase MIL uptake ~80-fold on the M-40 R line (Fig. 4A and data not shown). Moreover, the mutant alleles were unable to rescue the defective translocation of glycerophospholipids in the M-40 R line (data not shown). These latter results confirm that both independent mutations are sufficient to inactivate the putative transporter and that the molecular basis for the transport-deficient and drug-resistant phenotype consists of the acquisition of single but distinct point mutations within each of the two LdMT alleles present in the M-40 R line.

Phenotypic and Genotypic Analysis of Mutagenized Resistant Clones—After mild mutagenesis treatment, resistant clones were rapidly selected in semisolid medium. Six clones were selected for further analysis, and all of them were found to be defective in the internalization of glycerophospholipids and MIL (data not shown). Transfection with pX-LdMT was able to rescue this defect in all of the clones, suggesting that mutagenized clones bear the loss of LdMT function. Sequence analysis of one of those clones identified two different alleles, encompassing three (F414S + L780P + G824D) and four (L366P + L780P + G824D + F987L) point mutations each. M-40 R parasites transfected with these alleles also failed to recover the lipid transport defect, further demonstrating the genetic basis for this MIL resistance phenotype.

These experiments point to LdMT as the protein inactivated during the generation of MIL resistance in vitro, either by stepwise drug pressure selection (M-40 R) or after mutagenesis treatment, suggesting that LdMT is likely to suffer high selective pressure in the field.

DISCUSSION

LdMT encoding the L. donovani putative MIL transporter has been identified and cloned by rescuing the resistance phenotype of the M-40 R cell line. Biochemical characterization of this resistant line revealed a defect in the inward-directed
translocation across the plasma membrane of both MIL and glycerophospholipids, independently of their polar head group (10). The present work identifies LdMT as a new member of the P-type ATPase APT subfamily expressed at the plasma membrane. LdMT mediates the translocation of MIL and glycerophospholipids in Leishmania parasites, thus modulating the uptake and potency of alkylphosphocholine drugs. It also shows that the molecular basis for the drug-resistant phenotype consists of the acquisition of single but distinct point mutations within each of the two LdMT alleles present in the M-40 R line, leading to a loss of functionality.

LdMT is clearly a member of the third subfamily of P-type ATPases, enzymes proposed to function as APTs (29) and currently represented by over a dozen genes in the human genome (25), 5 genes in Saccharomyces cerevisiae (24), 12 genes in Arabidopsis (30), and many others identified from protozoa to mammals. However, the biological functions remain elusive for the majority of them. APTase II has been proposed as the protein likely responsible for the translocation of aminophospholipids in mammalian cromaffin granules (27, 31). The five yeast members have been characterized recently (32, 33), and Dnf1p and Dnf2p were found to be involved in phospholipid translocation and maintenance of the plasma membrane asymmetry (33).

All mammalian APT activities described so far have shown specificity for aminophospholipids, phosphatidylserine being a better substrate than phosphatidylethanolamine (29). Thus, a striking difference between previously described mammalian APT and the LdMT-dependent translocation activity is that the latter has an increased range of substrates. Indeed, LdMT is able to promote the translocation of not only aminophospholipids but also glycerophosphocholine molecules such as NBD-PC or edelfosine and the even more simple, non-glycerol based, alkylphosphocholine MIL. Thus, assuming a role for these proteins as the direct translocases and despite sequence homology (Fig. 3), the mammalian and parasitic APTs differ in their substrate specificity, which may explain in part the exquisite activity of MIL against Leishmania. Nevertheless, certain mammalian cell types and tumoral lines internalize glycerophosphocholine analogs and/or alkylphosphocholines (7, 9, 34, 35). It is tempting to suggest that some other members of the P-type ATPase APT subfamily differentially expressed in these cell types may be responsible for the phosphatidylcholine/MIL accumulation phenotype and thus be implicated in MIL potency in tumor cells. It will be challenging to identify those P-type ATPases and the pattern of tissue distribution in human beings.

Interestingly, the recently described Drs2p homologues, Dnf1p and Dnf2p, also localize to the plasma membrane and have been shown to be required for the rapid internalization of not only aminophospholipids but NBD-PC as well (33). Considering the amino acid identity between Dnf1p/Dnf2p and LdMT (21 and 20%, respectively), their cellular localization, and the phenotype exhibited when they are either deleted (33) or mutated (this work), it is possible to suggest that they are homologous proteins with similar functions. Moreover, the presence of some other distantly related P-type ATPases having a redundant function in the parasite, as occurs with Dnf1p and Dnf2p in yeast, cannot be ruled out. If this were the case, one would expect those proteins to be also inactivated in the M-40 R line. Another yeast protein, ROS3p/Lem3p, is required for the uptake of alkylphosphocholines NBD-PC and -PE but not -PS (36, 37). Considering the requirement of Dnf1p/Dnf2p in yeast or LdMT in Leishmania parasites for the uptake of all glycerophospholipids and alkylphosphocholines, ROS3p must be a protein somehow modulating the proper function of these P-type ATPases, but never a translocase.

Final proof of P-type APTs acting as direct phospholipid pumps will require reconstitution of purified proteins into chemically defined liposomes, an issue that we are currently approaching. Nevertheless, we propose that LdMT behaves as a true lipid translocase. Apart from localizing to the plasma membrane and mediating the translocation of glycerophospholipids and alkyl-phosphocholine in L. donovani, LdMT expression in L. tarentolae, a species naturally resistant to MIL, shifts cells sensitive to MIL and able to accumulate the drug in levels similar to those of other sensitive Leishmania species (Fig. 4A). This result constitutes the strongest evidence to date of a member of the P-type APT subfamily behaving as a real transporter.

The absence of detectable deletions or rearrangements at the LdMT locus in M-40 R cells and the presence of similar levels of LdMT mRNA in both mutant and wild-type lines suggested the presence of stable mutations of the gene in the M-40 R line leading to the resistance phenotype. The present PCR-based approach identified single but distinct point mutations in both alleles that are able to inactivate LdMT function. Thus, the gain of missense point mutations, and not loss of heterozygosity, had to occur during drug pressure selection. The T420N mutation is located within the phosphorylation motif DKT420GTLT. All P-type ATPases form an aspartyl-phosphate intermediate, the Asp residue being essential for their functionality (38). Interestingly, site-directed mutagenesis in the corresponding Thr residue of the Ca$^{2+}$-APTase of sarcoplasmic reticulum (another P-type ATPase) has shown it to be involved in the catalysis of ATP and the coordination of catalytic Mg$^{2+}$ (39). Thus, the Thr-420 position seems to be essential for LdMT activity, further highlighting the central role of this Thr residue in the phosphorylation site of P-type ATPases. The L856P substitution within the second LdMT allele is a position only partially conserved in other putative APTs. Considering its proximity to TM 5, misfolding of the protein could not be discarded. These inactivating point mutations highlight the convenience of this approach as a genetic tool for identifying residues within LdMT (both inside or outside conserved motifs) that are critical for its function and, ultimately, for identifying the residues responsible for substrate specificity.

To answer whether the MIL resistance phenotype found in the M-40 R line could be generalized to other resistant cases, we created Leishmania resistant lines by mutagenesis. These lines showed not only a similar phenotype, but also a similar genetic basis for the acquisition of resistance: inactivating point mutations within LdMT. This result further validates the previous findings, strongly suggesting that the generation of MIL-resistant clinical isolates bearing specific point mutations in LdMT is likely to occur.

In conclusion, we have found that the novel P-type ATPase LdMT mediates the translocation of MIL and glycerophospholipid analogs in Leishmania. The new functional insights into LdMT shown here strongly suggest that this subfamily of proteins behaves as true phospholipid translocases. Loss of function point mutations within LdMT confer MIL resistance in vitro, which may have a bearing on the future of MIL as a successful drug against leishmaniasis. Knowledge of the molecular basis of MIL resistance in vitro could anticipate strategies to prevent its appearance in vivo and to design second line drugs for drug-resistant cases.

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