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Phospholipid Translocation and Miltefosine Potency Require Both *L. donovani* Miltefosine Transporter and the New Protein LdRos3 in *Leishmania* Parasites*[S]

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F. Javier Pérez-Victoria[1], María P. Sánchez-Cañete[1,2], Santiago Castanys[3], and Francisco Gamarro[3,4]

From the Instituto de Parasitología y Biomedicina "López-Neyra," Consejo Superior de Investigaciones Científicas, Avda. del Conocimiento s/n 18100 Armilla, Granada, Spain

The antitumor drug miltefosine has been recently approved as the first oral drug active against visceral leishmaniasis. We have previously identified the *L. donovani* miltefosine transporter (LdMT) as a P-type ATPase involved in phospholipid translocation at the plasma membrane of *Leishmania* parasites. Here we show that this protein is essential but not sufficient for the phospholipid translocation activity and, thus, for the potency of the drug. Based on recent findings in yeast, we have identified the putative β subunit of LdMT, named LdRos3, as another protein factor required for the translocation activity. LdRos3 belongs to the CDC50/Lem3 family, proposed as likely β subunits for P_4-ATPases. The phenotype of LdRos3-defective parasites was identical to that of the *LdMT*−/−, including a defect in the uptake of 7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino)-phosphatidylserine, generally considered as not affected in Lem3p-deficient yeast. Both LdMT and LdRos3 normally localized to the plasma membrane but were retained inside the endoplasmic reticulum in the absence of the other protein or when inactivating point mutations were introduced in LdMT. Modulating the expression levels of either protein independently, we show that any one of them could behave as the protein limiting the level of flippase activity. Thus, LdMT and LdRos3 seem to form part of the same translocation machinery that determines flippase activity and miltefosine sensitivity in *Leishmania*, further supporting the consideration of CDC50/Lem3 proteins as β subunits required for the normal functioning of P_4-ATPases.

Different *Leishmania* spp. constitute the etiological agents of leishmaniasis, a tropical disease with different clinical manifestations varying from cutaneous self-healing lesions to visceral disease, fatal if left untreated (1). From the estimated 500,000 new cases per year of visceral leishmaniasis or kala azar, more than 90% occur in endemic areas of India, Bangladesh, Brazil, and Sudan due to the *Leishmania donovani* species complex (2). These endemic areas constitute foci of anthroponotic transmission of the parasite, which increases the chances for the fast spreading of drug-resistant parasites once these have been generated (3). Indeed, the classical leishmanicidal treatment, based on pentavalent antimonials, is no longer active for more than 60% of the clinical cases in the Bihar area (4). This dramatic situation has called for the urgent development of new therapeutic agents against leishmaniasis (5). Among these, miltefosine (MLF)[5] is the first oral drug active against both visceral (6) and cutaneous (7) leishmaniasis. Phase IV clinical trials are about to finish in India, and depending on the outcome, the Indian government may consider MLF as the first-line drug to treat kala azar (8, 9). Consequently, studies on the mechanisms of MLF resistance and their generation are deeply important for the appropriate long-term use of this drug.

Chemically, MLF as well as the related ether-lipids edelfosine and perifosine is a short-chain phospholipid derivative that resembles lysophosphatidylcholine. We and others have clearly demonstrated that all these analogs are primarily taken up by a specific protein-dependent translocation step across the plasma membrane (PM) in both *Leishmania* parasites (10) and yeast (11, 12). This uptake activity occurs not only for MLF but also for fluorescent analogs of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS) as well and receives different names such as flippase activity, translocase activity, or more accurately, inward-directed translocation activity across the PM. Inactivation of this activity causes MLF resistance in both organisms (12, 13). Thus, MLF uptake serves as an easy method for determining the flippase activity across the PM. Even though the identity of this activity remains to be established, strong candidates such as the leishmanial LdMT and yeast Dnf1 and Dnf2 are members of the P4 subfamily of P-type

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1.

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[1] These authors contributed equally to this work.

[2] Recipient of a fellowship from the Ministerio de Educación y Cultura.

[3] Senior investigators in this study.

[4] To whom correspondence should be addressed. Tel.: 34-958-181667; Fax: 34-958-181632; E-mail: gamarro@ipb.csic.es.

[5] The abbreviations used are: MLF, miltefosine or hexadecylphosphocholine; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; ORF, open reading frame; GFP, green fluorescent protein; TM, transmembrane segment; LdRos3, *L. donovani* β subunit for LdMT; *LdRos3*, the gene encoding LdRos3; LdMT, *L. donovani* miltefosine transporter; PM, plasma membrane; ER, endoplasmic reticulum.

Supplemental Material can be found at: http://www.jbc.org/cgi/content/full/M605214200/DC1

ATPases (here referred to as P_4-ATPases) (13, 14). Interestingly, P_4-ATPases are also involved in vesicle-mediated protein sorting (for a review, see Ref. 15), and mutations in some human P_4-ATPases are known to produce inherited diseases (16, 17). How these processes and diseases are related to the likely phospholipid translocation activity of P_4-ATPases is still unknown.

Of the more than 200 identified members of the P-type ATPase family (18), only animal Na,K- and H,K-ATPase isozymes and the bacterial Kdp-ATPase are known to contain, in addition to the catalytic α subunit, one or two subunits, respectively, which are obligatory for the enzymes function (19). Nevertheless, certain yeast P-type ATPases phospholipid translocases seem to depend on proteins of the Lem3/CDC50 family (20). Lem3p-Dnf1p colocalizes to the PM, and CDC50p-Drs2p colocalizes to the trans-Golgi network; both pairs can co-immunoprecipitate, and the phenotypes, when either the ATPase or the Lem3/CDC50 protein are absent, are strikingly similar (20) but not absolutely identical (12, 14, 20, 21). It has been suggested that Lem3p and CDC50p may constitute specific β subunits for P_4-ATPases (15, 20). Further data supporting these observations in a different system is needed.

In this report we identify and characterize the *L. donovani* LdRos3 protein as the functional homolog of Lem3p/Ros3p. Similarly to LdMT, LdRos3 is required for the translocation of phospholipids from the PM, including MLF and 7-nitrobenz-2-oxa-1,3-diazol-4-yl-amino (NBD)-PS. Thus, LdRos3 defective parasites are highly resistant to MLF. Both proteins depend on each other for their proper localization at the PM. We propose that Lem3/CDC50 protein family should be considered as the specific β subunits for P_4-ATPases.

EXPERIMENTAL PROCEDURES

Chemical Compounds—MLF (hexadecylphosphocholine) was from Zentaris (Frankfurt, Germany). Hexadecylphospho[1,2-*ethylene*-^{14}C]choline ([^{14}C]MLF) (1,33 MBq/mmol) was synthesized by Amersham Biosciences. NBD-hexanoyl-1-hexadecanoyl-*sn*-glycero-3-phosphocholine, -phosphoethanolamine, -phosphoserine (NBD-PC, -PE, and –PS, respectively) and 6-[NBD-hexanoyl]sphingosylphosphocholine were from Avanti Polar Lipids (Birmingham, AL). All other chemicals and reagents were of the highest quality available.

Strains and Culture Conditions—Promastigote forms of wild-type *L. donovani* (MHOM/ET/67/HU3), the MLF-resistant line (M-40 R) described previously (22), and another MLF-resistant line (M-1M), which was created by treating 4×10^7 wild-type parasites with 50 μM MLF in 5 ml until growth was seen in the culture (at around day 35), were maintained at 28 °C in the M-199 medium (Invitrogen) supplemented with 40 mM HEPES (Sigma), 100 μM adenosine (Sigma), hemin (0.2% of a 2 μg/ml stock solution; Sigma), and 10% heat-inactivated fetal bovine serum (Invitrogen). For determination of parasite sensitivity to MLF, 1×10^6 cells were incubated for 72 h at different drug concentrations before determining cell proliferation by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay (13).

Isolation of LdRos3, DNA-sequencing Analysis, and Construction of Plasmids—Putative members of the Lem3/CDC50 gene family were searched in the different *Leishmania* genome network gene data bases using the GeneDB ominiBLAST server against the yeast Lem3 gene. We found three different genes in the *Leishmania infantum* genome (systematic GeneDB sequences LinJ35.3500, LinJ09.1080, and LinJ32.1040, respectively). For cloning and sequencing of the different *L. donovani* orthologue alleles of LinJ32.1040 (*LdRos3*) from the wild-type and M-1M lines, PCR amplification was accomplished with two different sets of primers using the high fidelity Triple Master polymerase from Eppendorf. P1 (5′-GCCCCGGGTCATGGCGCCTCTACCCCCT) and P2 (5′GCGGATCCCTACTCTGTATAGACTGGCATGATCA) amplified only the coding region, whereas P3 (5′-AACGCAGACGCTTCGTGATGG) and P4 (5′-TAAGGCTGTCATCAACGTATG) amplified the entire ORF flanked by 1314 and 1119 bp of the untranslated 5′ and 3′ regions, respectively. XmaI and BamHI restriction sites were added (underlined in the sequence) for further cloning. PCR amplified bands were subcloned into the pGEM-T vector (Promega) and sequenced on an ABI Prism 3100 DNA sequencer. The selected alleles were also subcloned into the XmaI-BamHI sites of the pXGHYG and pXG expression vectors (23).

To generate green fluorescent protein (GFP) fusions at the carboxyl terminus of LdRos3 (LdRos3-GFP), the wild-type *LdRos3* ORF without the STOP codon was amplified by PCR using forward and reverse primers containing XmaI and EcoRV sites, respectively. After restriction digestion, the ORF was subcloned in frame into the XmaI-EcoRV sites of the *Leishmania* expression vector pXG-′GFP+ (23).

Generation of LdMT and LdRos3 Null Mutants—For targeted gene replacement of the *L. donovani LdMT* gene, a targeting DNA fragment was constructed in which the *hyg* gene, conferring resistance to hygromycin B, was flanked at the 5′ end by a 1.3-kilobase *LdMT* region containing the initiation codon and at the 3′ end by a 1.4-kilobase *LdMT* region containing the stop codon (Fig. 1*A*). To ensure high level expression in promastigotes, the 5′-untranslated region (400 bp) of the *L. major dhfr-ts* gene was inserted directly before the initiation codon of the *hyg* gene. The different fragments were amplified by PCR from genomic DNA (primers KO1 GTGGTACCGACTCCTCAACTCCTTATATTG, and KO2 GTAAGCTTATTAGATCTCTCGCCCGTCCAGGTTA for the 5′ region; KO3 CACCCGGGAAGAGGTTCTTCTGGAAC and KO4 GTAAGCTTAAGATGATGAGCATCATCGTCG for the 3′ region) or the cLHYG vector (KO5 GTAGATCTACCACTTTCTGCCTTCTG and KO6 GGAAGCTTCTATTCCTTTGCCCTCGGACG) for the resistance cassette), subcloned into pGEM-T vector (Promega), and assembled in this vector. As for the *LdRos3* replacement, a similar strategy was used (Fig. 4*B*). Primers KO7 (AACGCAGACGCTTCGTGATGG) and KO8 (GCATTTAAATTTCGAGTGTGGCTTAGGGGGT) amplified a 5′-untranslated region of 1.2 kilobases. Primers KO9 (GCATTTAAATTCTAGATCATGGCAGTGACACTTCT) and KO10 (TAAGGCTGTCATCAACGTATG) amplified a 3′-untranslated region of 1.4 kilobases. Both fragments were cloned adjacent to the *hyg* cassette using SwaI and XbaI restriction sites.

Log phase *L. donovani* promastigotes were transfected with 7 μg of the linearized DNA targeting constructions by electroporation as previously described (13). In the first round of *LdMT* and *LdRos3* targeting, electroporated promastigotes were incubated in 5 ml of drug-free culture medium for 1 day, after which the parasites were centrifuged (1500 × *g*, 5 min), resuspended in 900 μl of media, and plated onto 3 plates of semi-solid culture medium containing 30 μg/ml hygromycin B. In the second round of gene targeting, loss of heterozygosity was promoted by increasing hygromycin B concentration up to 400 μg/ml in 96-well plates. Wells grown at such concentrations were tested against MLF sensitivity, and those resistant to 40 μM MLF were expanded and further analyzed by Southern blot.

Southern Analysis—Genomic DNA was purified from wild-type, M-1M, *LdMT*−/+, *LdMT*−/−, *LdRos3*−/+, and *LdRos3*−/− lines with the DNAzol reagent (Invitrogen). Restriction enzyme-digested DNA was hybridized to either *LdMT* and *LdRos3* ORFs or the 5′-untranslated region of *LdRos3* following standard procedures (24).

Cell Transfection—*L. donovani* parasites were transfected and selected for G418 or hygromycin B resistance as previously described (13). For double transfection experiments, parasites were first transfected with a single plasmid and selected at a concentration of 100 μg/ml concentrations of either G418 or hygromycin B. Then a second transfection was set, selecting with the minimum concentration of the new selective drug and maintaining the drug pressure for the previous plasmid. Increasing expression levels of the protein driven by the second plasmid were achieved by increasing the concentration of the selective drug in a stepwise manner, as stated in text.

Functional Experiments—The internalization of [^{14}C]MLF (1.33 MBq/mmol) or fluorescent-labeled phospholipid analogs was measured as described previously (10).

Fluorescence Microscopy, Immunofluorescence, and Immunoblotting Experiments—For localization of the different LdMT-GFP and LdRos3-GFP chimeras, live parasites were pelleted, washed three times in phosphate-buffered saline, and attached to poly-L-lysine-coated coverslips, and images were acquired with an epifluorescent microscope Zeiss Axiophot (Germany). Images were captured with a SPOT camera (Diagnostic Instrument, Inc.) and analyzed using Adobe Photoshop 5.5 software. To differentiate the PM from intracellular organelles, parasites were incubated in hypotonic buffer (5 mM Tris-HCl, pH 7.4, plus 100 μM phenylmethylsulfonyl fluoride) for 30 min on ice and directly mounted on coverslips. *Leishmania* parasites possess a subpellicular microtubule cytoskeleton attached to the cytoplasmic face of their PM, which allows the generation of ghost cells maintaining the normal cellular morphology. Surface membranes possessing such subpellicular microtubules were obtained from hypotonic-treated parasites by Dounce homogenization for 5 min on ice (25).

For immunofluorescence studies, parasites were fixed in suspension with 3% paraformaldehyde in phosphate-buffered saline (PBS), attached to poly-L-lysine-coated slides, permeabilized with methanol, blocked with 1% bovine serum albumin in PBS, and incubated with an anti-BiP polyclonal antibody raised in rabbits (kindly provided by Jay Bangs (26)) at a 1:300 dilution. Antibody binding was visualized by treatment with Texas Red-conjugated anti-rabbit IgG antibodies diluted at 1:1000 (Sigma). Cells were mounted on 90% glycerol and observed microscopically using either a Texas Red or a fluorescein isothiocyanate filter set.

Immunoblots of comparable amounts of proteins were performed with a polyclonal anti-GFP antibody (1:5000) (Molecular Probes) and a horseradish peroxidase-conjugated secondary goat anti-rabbit (1:5000) IgG (Dako), as described previously (13).

RESULTS

LdMT Is Required but Not Sufficient for the Inward-directed Translocation Activity of Phospholipids and MLF across the PM—In a previous report we demonstrated that LdMT promotes the translocation of MLF and NBD-phospholipids in *Leishmania* (13), similarly to what happens in yeast with Dnf1p and Dnf2p (14). To answer whether LdMT is the only flippase expressed at the PM of *Leishmania* parasites or whether there is any other protein with similar functions, we have obtained *L. donovani* parasites in which both alleles of the *LdMT* gene have been sequentially deleted (Fig. 1*A*). Proper targeting of the constructions was tested by a Southern blot of genomic DNA (Fig. 1*B*). Heterozygous parasites for *LdMT* showed levels of MLF internalization that were half those for the wild-type line (Fig. 1*C*). Their sensitivity to MLF were decreased 2-fold with respect to wild-type parasites (Table 1), demonstrating a direct correlation between drug uptake and cellular sensitivity, as previously suggested (13). *LdMT* null mutant (*LdMT*−/−) parasites were defective in the uptake of MLF, with internalization values 50-fold lower than wild-type parasites (Fig. 1*C*). Similarly, *LdMT*−/− parasites were deficient in the internalization of fluorescent NBD-PC, -PE, and -PS (Table 2). Transfection of *Leishmania LdMT*−/− with episomal plasmids bearing *LdMT* (translating either LdMT or the LdMT-GFP chimera) completely recovered MLF uptake (Fig. 1*C*) and sensitivity as well as NBD-PC, -PE, and -PS internalization (Table 2). Thus, it can be concluded that LdMT is the only flippase expressed at the PM of promastigotes, being essential for the uptake and potency of MLF.

We have previously described the generation of experimental MLF-resistant parasites by either drug pressure selection (22) or by mutagenesis followed by selection against high drug concentrations (13). In both cases the generation of inactivating point mutations inside *LdMT* was responsible for the resistance phenotype, and thus, this phenotype could be rescued by transfection with a wild-type *LdMT* gene (13). Following a different approach, we have created a new experimental resistant line, named M-1M (see "Experimental Procedures"). Its resistance phenotype was strikingly similar to those previously described (Table 1), including a defective internalization of MLF (Fig. 1*C*) and NBD-PC, -PE, and -PS (Table 2). Nevertheless, transfection with the *LdMT* gene was unable to rescue the defective phenotype (Fig. 1*C* and Table 2), indicating that different mutations in another gene(s) may be present in this strain. To answer whether LdMT is expressed and targeted to its proper cellular localization, we transfected M-1M and *LdMT*−/− parasites with the pXG′-LdMTGFP plasmid.

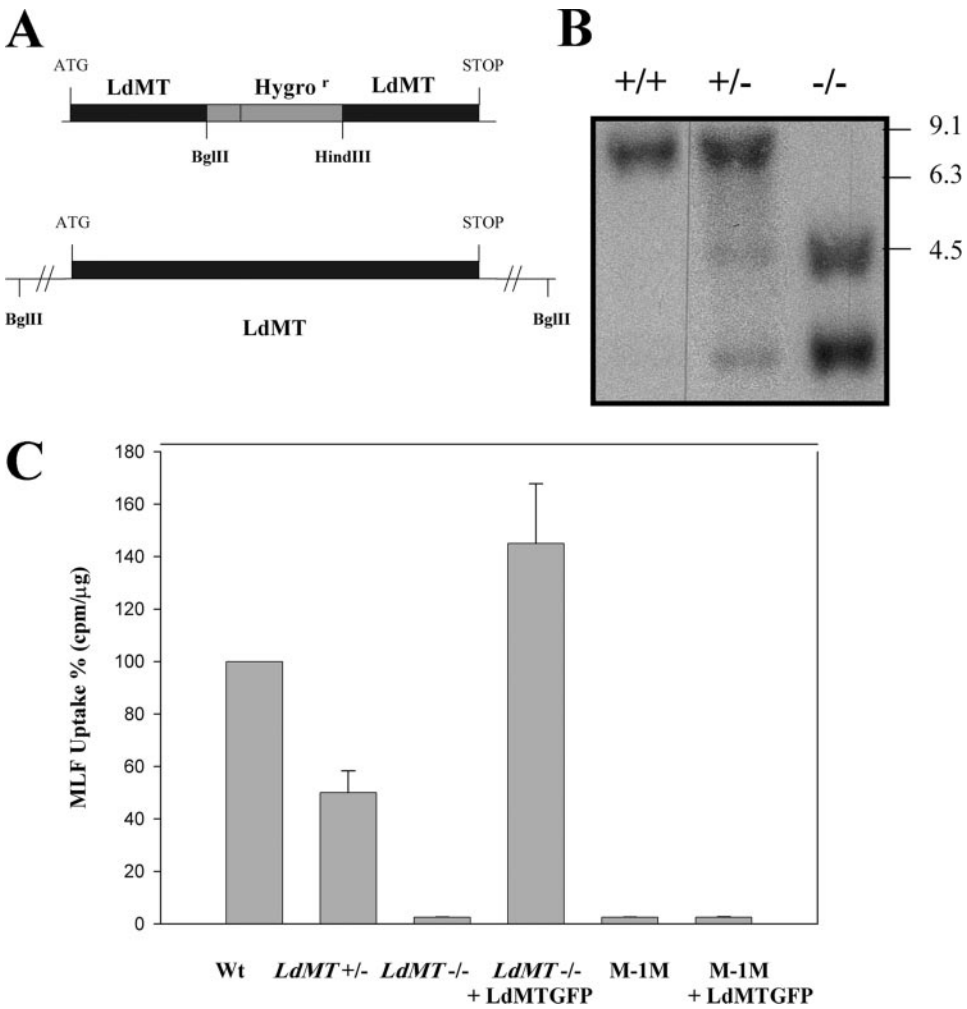

FIGURE 1. **LdMT is essential but not sufficient for the uptake and potency of MLF.** *A*, construction of a disruption cassette for *LdMT*. Restriction enzymes within the cassette and the wild-type *LdMT* locus are shown. *B*, Southern blot of genomic DNA from wild-type parasites (+/+), heterozygous *LdMT*+/− (+/−), and null mutants *LdMT*−/− (−/−), which were double-digested with BglII and HindIII restriction enzymes and hybridized with a labeled *LdMT* ORF probe. Molecular markers are shown in the *right*. *C*, [^{14}C]MLF uptake profile for different *L. donovani* strains used in these studies. *Bars* represent the mean from three independent experiments ± S.D. and are expressed as the percentage uptake relative to control wild-type (*Wt*) cells; 100% corresponds to 380 cpm/μg of protein.

TABLE 1

MLF sensitivity and resistance indexes (RI) in different *Leishmania* strains

MLF growth inhibition was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide colorimetric assay as stated under "Experimental Procedures." Results are the mean ± S.D. of at least two independent experiments.

Strains	IC_{50}	RI
	μM	
Wild type	6.2 ± 3.5	1
LdMT+/−	12.1 ± 4.4	1.9 ± 0.3
LdMT−/−	81.0 ± 25.7	13.7 ± 2.6
M-1M	77.2 ± 15.3	13.1 ± 2.2
LdRos3+/−	10.3 ± 5.0	1.7 ± 0.3
LdRos3−/−	84.2 ± 17.5	14.2 ± 2.7

This construction was able to rescue the knock-out parasites but not the M-1M line (Fig. 1*C*). Western blots of total cell lysates showed similar profiles between both parasite lines, suggesting that the protein is produced normally (data not shown). LdMT-GFP has to reach the PM to account for its flippase activity. Nevertheless, clear labeling of PM in intact parasites is hampered by the overexpression of the protein and/or its intracellular routing. Incubation of promastigotes in hypotonic buffer permitted the easy discrimination between the LdMT-GFP pool expressed at the PM and the intracellular one, as seen in *LdMT*−/− parasites expressing LdMT-GFP (Fig. 2*A*). M-1M parasites were found defective in the proper targeting of LdMT-GFP to the PM, as observed in parasites under hypotonic shock or in PM fragments prepared from these hypotonic buffer-treated parasites (Fig. 2*B*). The big intracellular structures positive for LdMT-GFP after hypotonic shock most likely represent endoplasmic reticulum (ER)-derived structures. Indeed, LdMT-GFP colocalized with the ER resident protein BiP, suggesting that the ATPase is unable to exit the ER in M-1M parasites (Fig. 3*A*). These results indicate that even though LdMT is likely to be the MLF and phospholipid transporter of *Leishmania* parasites present at the PM, it is not sufficient to promote lipid transport by itself, *i.e.* it requires another cellular factor(s) to exit the ER, reach the PM, and be able to exert its function.

Identification of LdRos3 by Functional Complementation of M-1M Parasites—MLF and NBD-glycerophospholipids uptake in yeast depend not only on the expression of the P-type ATPases Dnf1p or Dnf2p but also on the presence of Lem3p (also named Ros3p) (12, 21). Lem3p is a member of the CDC50-Lem3 protein family with homologs present in all Eukaryotae. Recently, Saito *et al.* have demonstrated the Lem3p requirement in the normal localization of Dnf1p at the PM (20). With this background, we started to study putative Lem3 homologs present in the *Leishmania* genome, identifying three genes belonging to this family. We amplified them by PCR from *L. donovani* genomic DNA, cloned them into episomal multicopy expression vectors, and tested whether any one of them was able to rescue the MLF resistance and uptake-deficient phenotype in the M-1M line. Indeed, only *LdRos3* (GenBank^{TM} accession number DQ205096) was able to partially rescue the MLF uptake phenotype in the M-1M line (see below), suggesting that a defect inside this gene may be responsible for the deficient flippase activity in these parasites.

LdRos3 shows a 25% identity and 51% similarity with yeast Lem3p (see Fig. S1 for an alignment of the two proteins). It contains the two transmembrane (TM) segments near the NH_2

TABLE 2

Percentage of accumulation of NBD-labeled phospholipids of different *Leishmania* strains relative to wild-type cells

The accumulation of NBD-phospholipids (5 μM for NBD-PC, -PE, and - sphingomyeline (SM); 15 μM for NBD-PS) was measured by flow cytometry as stated under "Experimental Procedures." The intrinsic fluorescence of the GFP chimera was subtracted where appropriate. Results are the mean ± S.D. of at least three independent experiments (the relative fluorescence intensity for the distinct NBD derivatives in the wild-type line is shown in parentheses).

Strains	NBD fluorescence			
	NBD-PC	NBD-PE	NBD-PS	NBD-SM
	% of Wild-type			
Wild type	100 (127)	100 (115)	100 (20)	100 (12)
LdMT−/−	12.0 ± 4.4	18.5 ± 2.3	30.2 ± 14.8	98.2 ± 10.4
LdMT−/− + LdMT	147.1 ± 25.7	124.4 ± 12.4	210.2 ± 64.4	104.6 ± 22.4
M-1M	9.8 ± 6.3	14.8 ± 6.4	33.2 ± 12.4	105.2 ± 32.4
M-1M + LdMT	9.4 ± 3.7	16.3 ± 5.0	38.4 ± 5.6	91.6 ± 26.6
M-1M + LdRos3	32.2 ± 6.5	41.1 ± 10.9	52.0 ± 41.4	102.5 ± 23.4
M-1M + LdMT +LdRos3-GFP	95.5 ± 36.0	113.0 ± 33.5	148.2 ± 48.1	95.3 ± 30.8

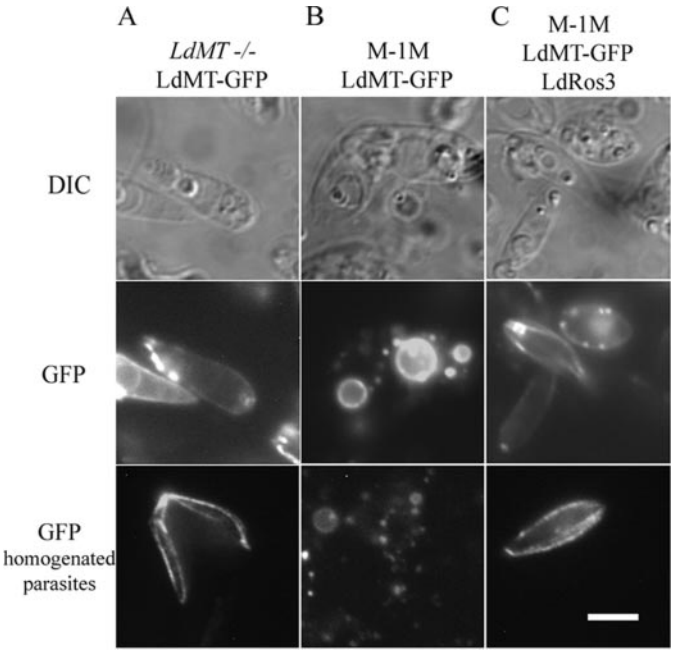

FIGURE 2. **The localization of LdMT-GFP at the parasite PM depends on the presence of LdRos3.** *A*, *LdMT−/−* parasites transfected with LdMT-GFP. *B*, M-1M parasites transfected with LdMT-GFP. *C*, M-1M parasites double-transfected with LdMT-GFP and LdRos3. *Upper panels*, Nomarski differential interference contrast images (*DIC*) of hypotonic buffer-treated parasites. *Intermediate panels*, fluorescence images showing localization of LdMT-GFP in the corresponding parasites. *Lower panels*, fluorescence images of Dounce-homogenized parasites. LdMT-GFP is located in PM fragments (*A* and *C*) or in intracellular organelles (*B*). *Bar*, 5 μm.

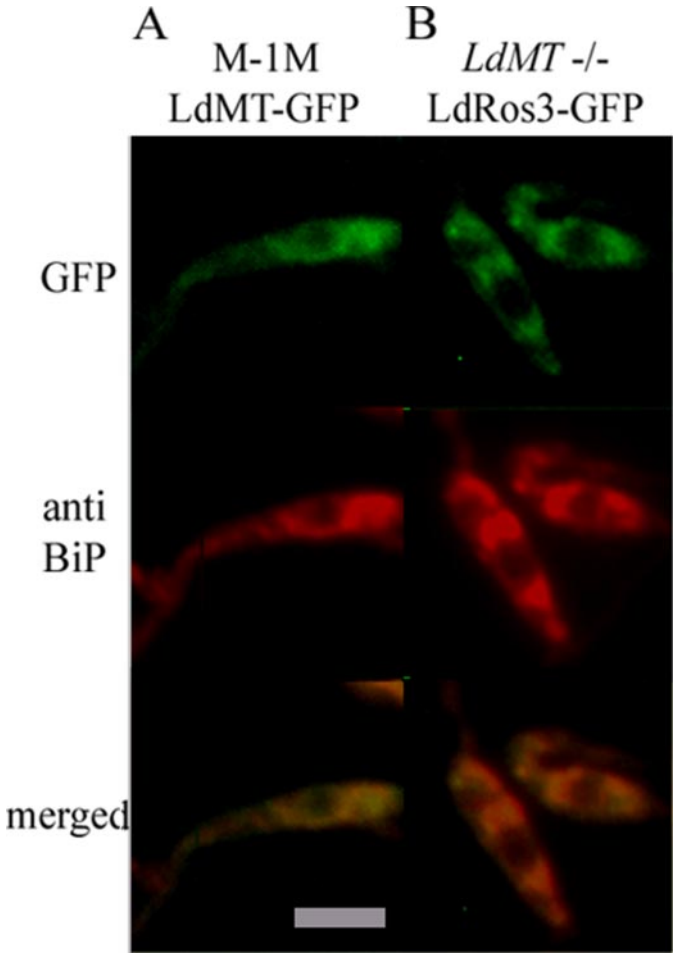

FIGURE 3. **LdMT-GFP and LdRos3-GFP are retained in intracellular organelles that colocalize with the ER resident protein BiP in drug-resistant parasites.** M-1M parasites stably expressing LdMT-GFP (*A*) or *LdMT−/−* parasites expressing LdRos3-GFP (*B*) were immunostained with an anti-BiP antibody followed by a secondary Texas Red-conjugated antibody (*red*). Epifluorescence of the GFP chimeras are shown in *green*. Obtained images were merged to demonstrate the coincidence of the two signal patterns. *Bar*, 5 μm.

and COOH termini as described for the members of the CDC50-Lem3 protein family. Remarkably, this protein family does not contain any known functional amino acid sequence motif (27).

To study whether LdRos3 is required for the normal localization of LdMT, M-1M parasites expressing LdMT-GFP were cotransfected with LdRos3. As expected, the double transfection not only rescued MLF uptake but properly targeted LdMT-GFP to the parasite PM (Fig. 2*C*).

Genetic Characterization of M-1M Parasites and Generation of LdRos3 Knock-out Parasites—Different PCR amplification reactions of the *LdRos3* locus and further sequencing in M-1M parasites revealed a single allele with a point mutation abolishing the start codon (a G → T nucleotide substitution). The next in-frame ATG codon is placed after the first TM domain, which would yield a truncated protein that must be inactive. The wild-type parental line showed two different alleles, as assessed by polymorphisms in the 5′-untranslated region of the gene. Because *Leishmania* is a diploid organism, either gene replacement or an allele deletion had to occur in the *LdRos3* locus in M-1M parasites. Taking advantage of the mutation in the start codon, which abolishes an NarI restriction site, we performed Southern blots to discriminate between these two options. Indeed, all *LdRos3* putative loci in M-1M have lost the NarI site

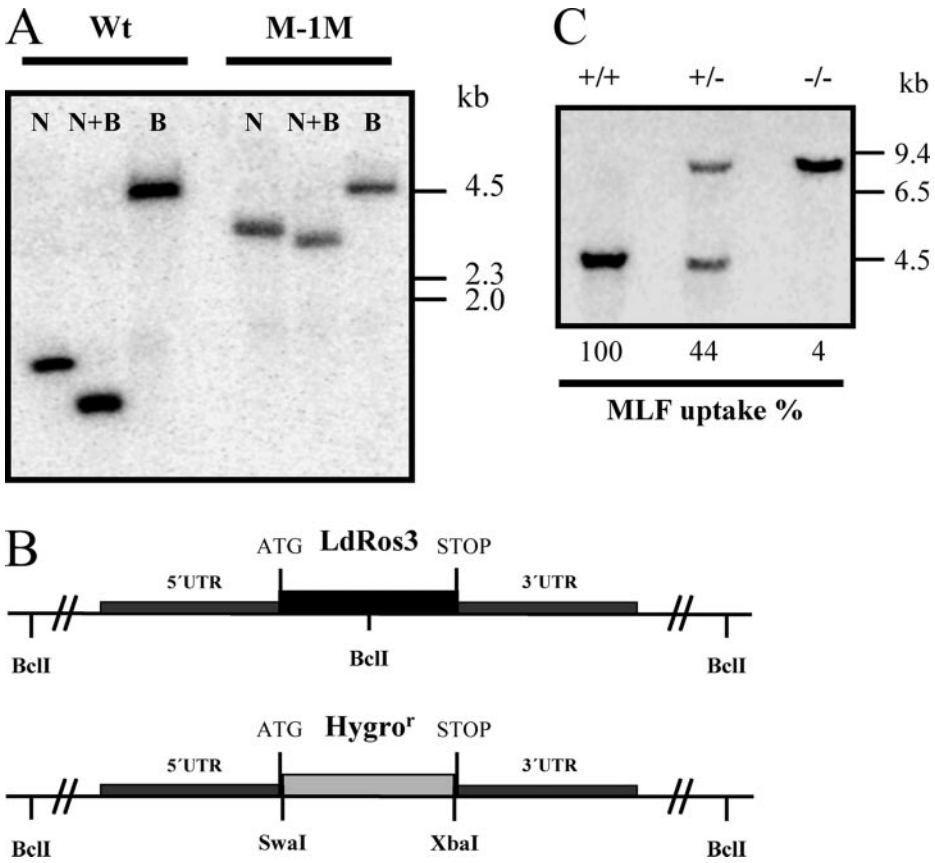

FIGURE 4. **Genetic characterization of *LdRos3* loci in M-1M parasites and deletion mutants.** *A*, Southern blot of genomic DNA from wild-type (*Wt*) and M-1M parasites digested with either NarI (*N*), BclI (*B*), or a combination of the two restriction enzymes (*N*+*B*) and labeled with a *LdRos3* ORF probe. Molecular markers are shown in the *right*. All *LdRos3* loci in M-1M parasites have lost the NarI restriction site present near the initiation codon of the gene. *B*, design of the disruption cassette for *LdRos3*. *C*, Southern blot of genomic DNA from wild-type parasites (+/+), heterozygous *LdRos3*+/− (+/−), and null mutants *LdRos3*−/− (−/−), which were digested with BglI and hybridized with a labeled probe of the 5′-untranslated region (*5′-UTR*) of *LdRos3*. The intragenic BglI site is lost in disrupted alleles. [^{14}C]MLF uptake values (in % with regard to wild-type parasites) are given for the three parasite lines *below* the blot. *kb*, kilobases.

present at the start codon (Fig. 4*A*). Densitometry analysis of the bands revealed that signals in M-1M parasites represent around 50% those of wild-type parasites even though genomic DNA loading was similar in all lanes, suggesting a deletion of the *LdRos3* locus in one of the two homologous chromosomes.

Because LdRos3 were able to only partially rescue the MLF uptake phenotype, we decided to sequence both *LdMT* alleles in these parasites. One of them contained a nonsense mutation at residue 630, which creates a truncated protein after the fifth TM segment that must be inactive.

To rule out any dominant negative effect of the mutated LdRos3 or the presence of other deleterious mutations in M-1M parasites, we generated gene knock-out mutants for *LdRos3* as performed for *LdMT* (Fig. 4*B*). Southern blots determined the proper genotype of the deletion mutants (Fig. 4*C*). Interestingly, *LdRos3*+/− parasites showed a 2-fold resistance to MLF (Table 1) and around 50% the uptake of the parental line (Fig. 4*C*). As expected, the homozygous mutant (*LdRos3*−/−) was completely defective in flippase activity (Fig. 4*C*) and, thus, highly resistant to MLF (Table 1).

Functional Characterization of LdRos3—To study the functional relationship between the two proteins uncovered to be essential for the inward-directed translocation activity of phospholipids and MLF across the PM, LdRos3, and LdMT, we cloned both genes with or without the GFP chimera in episomal multicopy vectors that allow to modulate the levels of each protein independently.

As stated above, transfection with LdRos3 was able to increase MLF uptake levels 15–20-fold in M-1M parasites (Fig. 5). Transfection with the LdRos3-GFP chimera yielded similar levels of MLF uptake (Fig. 5), implying that the chimera is fully functional when expressed from an episomal vector. With both constructions, selection of transfected parasites with the minimal amount of drug-selective agent produced a maximal recovery in MLF uptake levels, similar to those observed at the highest G418 or hygromycin B concentrations. These data suggest that M-1M-transfected parasites were saturated of LdRos3 molecules and that levels in another protein involved in lipid translocation from the PM, likely LdMT, were limiting. Indeed, M-1M parasites were found heterozygous for *LdMT*, with one allele disrupted with a nonsense mutation that produces an inactive protein, and could be considered as *LdMT*+/− parasites.

M-1M parasites transfected with *LdMT* were still deficient in the inward translocation of MLF and NBD-PC, -PE, and -PS from the PM but were supposed to produce higher levels of LdMT. When these parasites were cotransfected with LdRos3-GFP, the recovery in the MLF uptake phenotype was complete and even higher than that of wild-type parasites (Fig. 5). Selection with different G418 concentrations permitted the regulation of LdRos3-GFP expression levels, as assessed by Western blot (Fig. 5). MLF uptake levels increased concomitantly with LdRos3-GFP overexpression until a saturation point was again reached at around 100 μg/ml G418 (Fig. 5).

Finally, when LdRos3 was overexpressed in wild-type parasites, no further increase in the uptake of MLF was observed (Fig. 5), as opposed to the overexpression of LdMT, which further increased MLF uptake levels in wild-type parasites around 2-fold (Fig. 5). These data together with the previous observation of a direct correlation between LdMT expression levels and flippase activity in MLF resistant (M-40 R) and wild-type parasites (13) clearly indicate that the expression of both proteins LdRos3 and LdMT is essential for the flippase activity at the parasite PM and that either one can behave as the limiting molar factor in such activity.

MLF is not the only possible substrate of the translocation machinery, and thus, other compounds are required to meas-

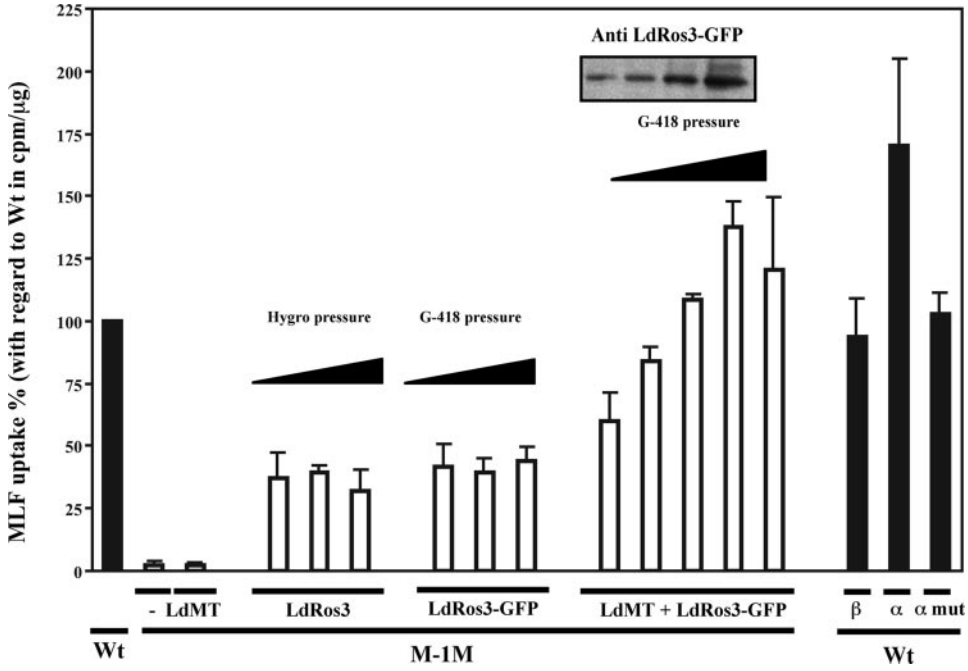

FIGURE 5. **Dependence of both LdMT and LdRos3 for the uptake of [^{14}C]MLF.** The uptake of [^{14}C]MLF was measured after 60-min incubations for wild-type parasites (*Wt*), M-1M parasites (*M-1M*), M-1M parasites transfected with LdMT (*M-1M LdMT*), M-1M parasites transfected with the pXGHYG-LdRos3 construction (*M-1M LdRos3*) and selected against increasing concentrations of hygromycin B (20, 50, and 200 μg/ml), M-1M parasites transfected with the pXG′-LdRos3-GFP construction (*M-1M LdRos3-GFP*) selected against increasing concentrations of G418 (10, 20, and 50 μg/ml), M-1M parasites double-transfected first with LdMT and selected with 100 μg/ml hygromycin B and later with LdRos3-GFP (*M-1M + LdMT + LdRos3-GFP*) selected with increasing concentrations of G418 (10, 20, 50, 100, and 500 μg/ml; a Western blot of the latter lines showing the correlation between G418 pressure selection and LdRos3-GFP expression levels is shown *above* the *bars*), and wild-type parasites transfected with either LdRos3 (*Wt β*), LdMT (*Wt α*), or the mutant T420N-LdMT-GFP (*Wt α mut*). *Bars* shown are the mean ± S.D. of three independent experiments and are expressed as the percentage uptake relative to control wild-type cells.

ure the substrate specificities of the complex. NBD-fluorescent glycerophospholipids are well established probes representative of more endogenous glycerophospholipids. Moreover, yeast cells defective for Lem3 were shown to be deficient in the uptake of NBD-PC and -PE, but surprisingly not -PS (12, 20, 21), whereas the inactivation of the ATPases Dnf1 and Dnf2 produced a defect in NBD-PS uptake as well. In M-1M parasites, the uptake of NBD-PC, -PE, and -PS but not -sphingomyelin was also defective independently of the overexpression of LdMT (Table 2). When LdRos3/LdRos3-GFP were expressed in M-1M or M-1M parasites transfected with *LdMT*, a rescue in the uptake of NBD-lipids was also observed, only partial for the former case and complete when both LdMT and LdRos3 were overexpressed (Table 2). These results clearly indicate that the absence of LdRos3 produces exactly the same phenotype as that observed in *LdMT*−/− parasites, including a defective translocation of NBD-PS, further suggesting that both proteins may work in the same machinery.

LdRos3-GFP Localizes to the PM Only in the Presence of LdMT—To study the localization of LdRos3, its GFP chimera was expressed in M-1M parasites cotransfected with *LdMT* and in *LdMT*−/−. In the former case an important fraction of the LdRos3-GFP pool was able to reach the PM, as happens normally with LdMT-GFP (Fig. 6*A*). However, in the absence of LdMT, LdRos3-GFP cannot reach the PM and is retained in intracellular organelles (Fig. 6*B*), which colocalized with the ER protein BiP (Fig. 3*B*). Preparation of PM fragments after incubation in hypotonic buffer further discarded any PM localization of LdRos3-GFP in *LdMT*−/− parasites (Fig. 6*B*). Thus, both LdMT and LdRos3 are mutually dependent on each other to reach the PM.

Inactivating Point Mutations in LdMT Prevents Both LdMT and LdRos3 Proper Location at the PM—If LdMT and LdRos3 depend on each other to reach the PM, one may wonder which situation occurs when single inactivating mutations are introduced in the system. We have taken advantage of our previous characterization of two single point mutations at positions T420N and L856P in LdMT that are sufficient to inactivate the transporter and are, thus, responsible of the drug-resistant phenotype in the M-40 R *Leishmania* line (13). Cloning of these mutated alleles in-frame with GFP allowed tracking of each mutated protein either in the absence of any other LdMT allele (transfecting *LdMT*−/− parasites), in the presence of only inactivated LdMT (transfecting M-40 R parasites), or even in the presence of other active LdMT polypeptides (transfecting wild-type parasites).

Whatever the situation, L856P-LdMT-GFP and T420N-LdMT-GFP were always retained in intracellular organelles, being unable to reach the PM (Fig. 7, *A* and *B*). They also colocalized with the ER protein BiP (data not shown). Similarly, wild-type LdRos3-GFP did not reach the PM in the M-40 R line, indicating again that both proteins are mutually dependent to reach the PM (Fig. 7*C*). The L856P point mutation is placed just before TM segment 5, inside the large TM4-TM5 cytoplasmic loop, whereas the T420N point mutation is found inside the conserved phosphorylation motif present in all P-type ATPases. It seems likely that both mutations affect the proper folding and/or recognition of LdMT by ER resident chaperones.

There seems to be no productive LdMT-LdMT interactions during its trafficking to the PM. Mutant GFP-tagged proteins did not reach the PM in wild-type parasites, and moreover, their overexpression did not modify MLF uptake (Fig. 5), therefore, not behaving as dominant negative mutations. Thus, LdMT-LdMT interactions in the ER, if present, are not important for sorting of the translocation machinery to the PM, as opposed to the Pma1 case in yeast, in which the presence of mutated Pma1 triggers the degradation of wild-type proteins (28, 29).

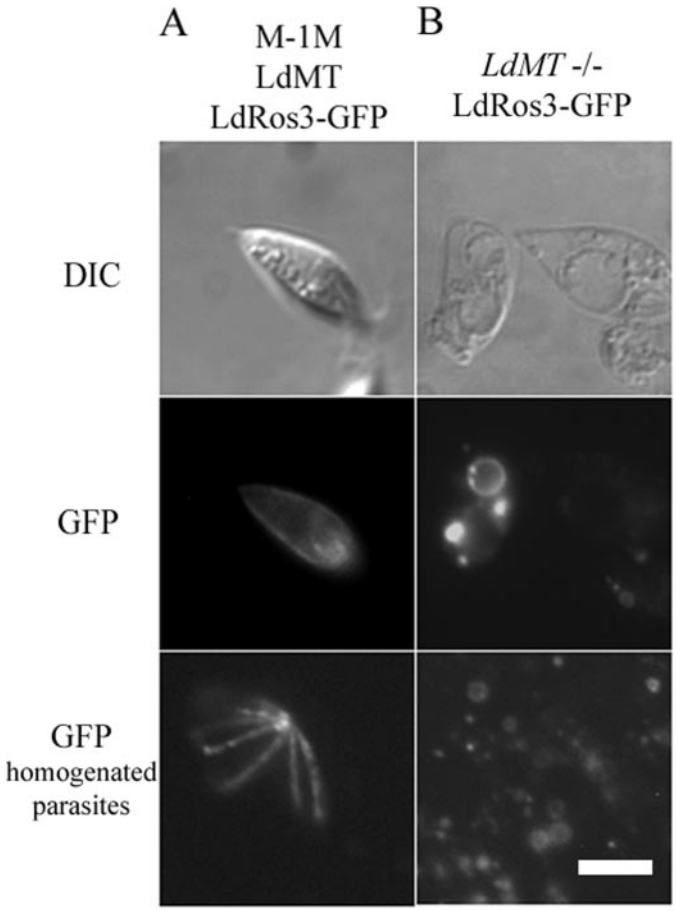

FIGURE 6. **LdRos3-GFP localizes at the parasite PM in the presence of LdMT.** *A*, M-1M parasites transfected with LdMT and LdRos3-GFP. *B*, *LdMT−/−* parasites transfected with LdRos3-GFP. *Upper panels*, Nomarski differential interference contrast images (*DIC*) of hypotonic buffer-treated parasites. *Intermediate panels*, fluorescence images showing localization of LdMT-GFP in the corresponding parasites. *Lower panels*, fluorescence images of Dounce-homogenized parasites. *Bar*, 5 μm.

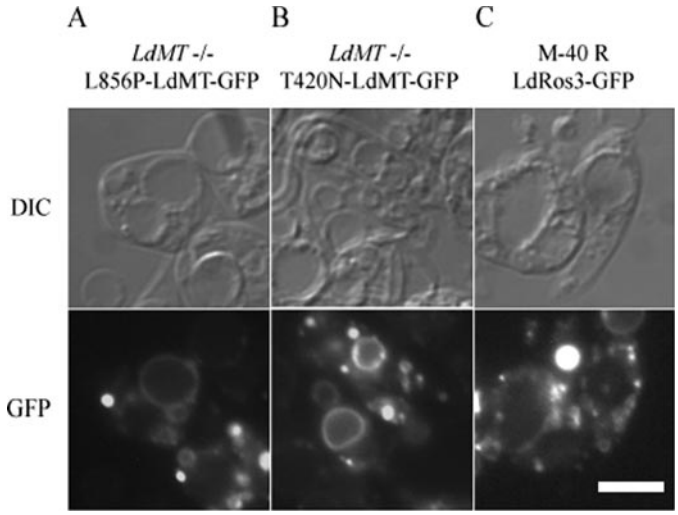

FIGURE 7. **Inactivating point mutations inside LdMT prevents both LdMT-GFP and LdRos3-GFP trafficking to the parasite PM.** *A*, *LdMT−/−* parasites transfected with L856P-LdMT-GFP. *B*, *LdMT−/−* parasites transfected with T420N-LdMT-GFP. *C*, M-40 R parasites transfected with LdRos3-GFP. The fluorescence of the GFP chimeras were monitored as described in Figs. 2 and 6. Nomarski differential interference contrast images (*DIC*) images are also shown. *Bar*, 5 μm.

DISCUSSION

In this report we show the identification and functional characterization of LdRos3, a new member of the Lem3/CDC50 protein family, which together with the P_4-type ATPase LdMT forms part of the phospholipid inward-directed translocation machinery at the cell surface of *Leishmania* parasites.

We propose that LdRos3 functions as a specific β subunit for the ATPase LdMT based on the following evidence. (i) LdRos3 is essential for the proper function of LdMT. Its inactivation through either point mutations or gene replacement produces a phenotype undistinguishable from that of *LdMT−/−* or LdMT-mutated parasites, including a defect in the internalization of NBD-PS. (ii) LdRos3 and LdMT are mutually dependent on each other for their proper localization at the parasite PM, their place of action. In the absence of any of the two proteins, the other is retained in the ER, not reaching the parasite PM. Furthermore, both LdMT and LdRos3 are retained in intracellular organelles when inactivating point mutations are present in LdMT. Thus, both proteins seem to travel together to the PM, provided a functional LdMT is synthesized at the ER. (iii) The expression levels of either LdRos3 or LdMT can behave as the limiting molar factor for the phospholipid translocation activity, which implies some stoichiometric relationship between both proteins in the putative translocation machinery.

This evidence is further supported by the recent results found in yeast with other P_4-ATPases and CDC50/Lem3 protein pairs. (iv) Yeast Dnf1p and Lem3p, the functional homologs of leishmanial LdMT and LdRos3, respectively, appear to be mutually dependent for their function (12, 14, 20, 21). Furthermore, both proteins can co-immunoprecipitate with each other (20). Finally, in Lem3 knock-out yeast, Dnf1p is retained in the ER, not reaching the PM (20). (v) Yeast Drs2p, another P_4-ATPase, and the Lem3/CDC50 protein CDC50p localize to the trans-Golgi network, and endosomes and seem to depend on each other. The phenotype of Drs2 knock-out yeast is strikingly similar to that of CDC50 knock-out. Both proteins co-immunoprecipitate with each other, and when any one of them is missing, the partner is retained at the ER (20).

Thus, taking all these data together, we propose that the CDC50/Lem3 protein family should be considered as the specific β subunits for P_4-ATPases. This situation remarkably resembles the case of the Na,K-ATPase and the H,K-ATPase of mammalian origins. Unfortunately, we have so far been unable to demonstrate a direct interaction between LdMT and LdRos3 by co-immunoprecipitation using different epitope tags. This failure should not be seen as an absence of a possible interaction with LdRos3.

Do all P_4-ATPases require a β subunit of the Lem3/CDC50 family to be functional? There are three members of the Lem3/CDC50 protein family in yeast and *Leishmania* parasites but five members of the P_4-ATPase subfamily in both organisms. Furthermore, in mammalian organisms we also find three Lem3/CDC50 proteins (30) but 14–15 P_4-ATPases (31). Therefore, either some of the P_4-ATPases do not require a β subunit or some of the β subunits behave promiscuously. In the case of P_2-ATPases, only Na,K-ATPases and H,K-ATPases pos-

ses specific β subunits, whereas the sarcoplasmic reticulum Ca-ATPase gets by without any β subunit (19). Interestingly, it has been proven that Ca-ATPase can transiently associate to the Na,K-ATPase and H,K-ATPase β subunits in *Xenopus* oocytes during its biogenesis, helping for the proper folding of the ATPase (32). Whether this transient association occurs in natural systems remains to be demonstrated. Therefore, in the case of P_4-ATPases, it would be feasible that either some enzymes get by completely without a β subunit or that some can transiently share the β subunit normally bound to a relative ATPase. In *Leishmania*, the PM LdMT seems to specifically require LdRos3 to exert its function. Indeed, in M-1M parasites (lacking LdRos3), the overexpression of the other two members of the Lem3/CDC50 protein family does not rescue the translocation-deficient phenotype. Nevertheless, in yeast, the Golgi-localized Drs2p, which normally associates to CDC50p, can co-immunoprecipitate Lem3p, and Lem3 is a multicopy suppressor of the cold-sensitive phenotype of CDC50 knock-out yeast (20), suggesting productive interactions between Drs2p and Lem3p. It will be interesting to study the three mammalian Lem3/CDC50 proteins (called CDC50/TMEM30A, -B, and -C) that also share the main features of the protein family but without any evident similarity between putative functional homologs (32).

The definite identity of the P_4-ATPases as the real phospholipid translocases responsible of the direct flip of phospholipids remains to be unequivocally demonstrated by reconstitution of the purified candidates into chemically defined liposomes, a task still elusive. The results presented here together with those of Tanaka and co-workers (20) strongly suggest that apart from the P_4-ATPase, its corresponding β subunit from the Lem3/CDC50 family will be needed to reconstitute the translocation machinery. Thus, previous results reporting the biochemical characterization of purified P_4-ATPases after heterologous expression should be revised with care (33).

Finally, we must consider the clinical relevance of these findings. *Leishmania* parasites are highly sensitive to MLF and other ether-lipid analogs because of the high inward-directed translocation activity across the parasite PM (10). We have been able to characterize the main determinants for this translocation activity and, thus, for the exquisite *Leishmania* sensitivity to MLF, namely LdMT and LdRos3. Consequently, these determinants could be used as drug sensitivity/resistance markers in natural *Leishmania* populations from geographical areas in which MLF is used to treat kala azar or cutaneous leishmaniasis. MLF sensitivity absolutely correlates with the level of drug uptake, and this one depends on the expression level of the translocation machinery at the parasite PM, its place of action (Refs. 10 and 13 and this work). Any factor decreasing the steady-state levels of the LdMT-LdRos3-dependent machinery at the PM (*i.e.* protein expression levels, fate of these proteins, vesicular trafficking and endocytosis of the complex, efficiency in the folding, etc.) will generate parasites less sensitive to MLF. Gaining of inactivating point mutations in LdMT or LdRos3 will have more dramatic consequences. Because *Leishmania* is a diploid organism, the inactivation of one allele for *LdMT* or *LdRos3* produces around a 2-fold decrease in drug sensitivity (Table 1). The loss of the second allele yields parasites that are absolutely defective in the translocation of the drug (Fig. 1) and, thus, are highly resistant to MLF (Table 1).

In summary, we have identified and characterized LdRos3 as a new protein factor required for the phospholipid inward-directed translocation activity across the PM of *Leishmania* parasites, which together with the recent observations in yeast claims consideration of the Lem3/CDC50 protein family members as the specific β subunits for the P-type ATPases phospholipid translocases.

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