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The overexpression of an intracellular ABCA-like transporter alters phospholipid trafficking in *Leishmania*

José María Araújo-Santos [a], Adriana Parodi-Talice [b], Santiago Castanys [a], Francisco Gamarro [a,*]

[a] *Instituto de Parasitología y Biomedicina "López-Neyra", C.S.I.C, Parque Tecnológico de Ciencias de la Salud, Avda del Conocimiento s/n 18100 Armilla, Granada, Spain*
[b] *Sección de Genética, Facultad de Ciencias, Universidad de la República, Montevideo, Uruguay*

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Abstract

In parasites, ATP-binding cassette (ABC) transporters represent an important family of proteins related to drug resistance and other biological activities. Here we report the characterization of LtrABCA2, a new ABC transporter of the ABCA subfamily in the protozoan parasite *Leishmania tropica*, localized at the flagellar pocket region and in internal vesicles. The overexpression of this transporter reduced the accumulation of fluorescent glycerophospholipid analogs, increased the exocytic activity, and decreased infectivity of macrophage, but did not confer resistance to drugs. Together, these results suggest that this new ABC transporter plays a role in phospholipid trafficking, which may be modifying the vesicular trafficking and the infectivity of the parasite.

Keywords: ABC transporter; ABCA subfamily; Phospholipid trafficking; Exocytic activity; Infectivity; Parasites; *Leishmania*

Protozoan parasites of the genus *Leishmania* are the causative agent of leishmaniasis, a disease that affects more than 6 million people worldwide, with 400,000 new cases each year. Leishmaniasis is endemic in 88 countries, and the overall prevalence is estimated at 12 million cases [1]. The standard treatment against leishmaniasis is based on pentavalent antimonials but resistance to these compounds has been a persistent problem that limits their use. Alkyl-phospholipids such as miltefosine and edelfosine have shown a significant antiproliferative activity against *Leishmania* spp. [2–7] being considered the most promising antileishmanial agents; however, we recently described in vitro resistances to alkyl-phospholipids related to overexpression of membrane proteins such as ATP-binding cassette (ABC) proteins [8] or by mutation of a P-type ATPase from the aminophospholipid translocase subfamily [9].

The ABC superfamily of transporters is one of the largest families of proteins found in both eukaryotic and prokaryotic cells [10–14]. Mammalians P-glycoprotein MDR1 (ABCB1) and multidrug resistance-associated protein 1 MRP1 (ABCC1) are the most extensively studied in relation with drug resistance. ABCA1 has a major role in cholesterol and phospholipid efflux across the plasma membrane [15] and is proposed to be involved in the phagocytosis of apoptotic cells [16,17].

In *Leishmania*, MDR (ABCB) and MRP (ABCC) subfamily transporters have been characterized [18,19]. Recently, we have identified the ABCA-like subfamily in *Leishmania*, and one of its members, localized at the plasma membrane, could be involved in phospholipid trafficking [20]. In the present study, we report the characterization of *LtrABCA2* gene in *Leishmania tropica*, which encodes for a new member of the ABCA subfam-

* Corresponding author. Fax: +34 95 8181 632.
E-mail address: gamarro@ipb.csic.es (F. Gamarro).

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ily, localized at the flagellar pocket region and in internal vesicles. The overexpression of LtrABCA2 in promastigotes reduced the accumulation of fluorescent glycerophospholipid analogs and decreased macrophage infectivity but it did not seem to be involved in drug resistance. Also, an increased secreted acid phosphatase activity was observed in parasites that overexpressed LtrABCA2, an activity that could influence other cellular processes of this protozoan parasite.

Materials and methods

Leishmania culture and cytotoxicity assays. Promastigotes of *Leishmania infantum* (strain 21578, LEM 2592, Montpellier, France) were grown at 28 °C in modified RPMI-1640 medium (Gibco-BRL) supplemented with 20% heat-inactivated fetal bovine serum (FBS) (Gibco-BRL). The L9ABCA2/NeoA-transfected parasite line was cloned in solid medium containing RPMI plus 20% FBS/1% agar with 500 μg/ml G418 (Gibco-BRL) at 28 °C. A growth inhibition test was performed using the anticancer drug doxorubicin, different leishmanicidal drugs (amphotericin B, miltefosine, edelfosine, and ketoconazol) and the antimalarial drug chloroquine. Drug susceptibilities were determined in 96-well plates using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT)-based assay as previously described [21].

Library screening and LtrABCA2 cloning. A λEMBL3 genomic library of *L. tropica* was used for ABCA-like gene screening. This library was transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with a partial cDNA sequence for a putative ABCA-like transporter of the protozoan parasite *Trypanosoma cruzi* (23A18 clone, Accession No. AI057758) kindly provided by Dra. L. Aslund (Uppsala University, Sweden). Two different recombinant phages were selected. Different fragments of these inserts were subcloned in the pBluescript II KS^+ vector (Stratagene). Nucleotide sequences were determined automatically as described previously [22] using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction (Applied Biosystems).

RNA manipulations. RNA was extracted by a Trizol reagent (Gibco-BRL). Messenger RNA was purified using the Quick Prep Micro mRNA Purification Kit (Pharmacia). Electrophoresis was performed on denaturing gels containing formaldehyde. RNA was transferred to a nylon membrane in 20× SSC for Northern blot hybridization. The splice acceptor sites of *LtrABCA2* transcript were determined by RT-PCR using poly(A^+)RNA as a template. cDNAs were generated using a specific antisense 5′ primer RT1 (5′-CACA ACAGCGTCGGCAG) and MLV reverse transcriptase (Gibco-BRL). The cDNAs were further amplified with the specific 5′ antisense primer RT12 (5′-CAGGATGAGCATGCGC) in combination with a spliced leader (LSL) primer (5′-AACGCTATATAAGTATCAG). PCR products were cloned into pGEM-T (Promega) and sequenced.

DNA constructs and transfection. To generate L7ABCA2 plasmid, an approximately 7.2 kb *Cla*I/*Bam*HI fragment of one of the phages containing 5591 bp of the open reading frame and untranslated 5′ region was subcloned into a pBluescript II KS^+ vector. The rest of the translated region (100 bp) and untranslated 3′ region (1598 pb) were amplified by PCR after adding a *Xba*I restriction site and subcloned into *Bam*HI/*Xba*I sites of L7ABCA2 obtaining the plasmid L9ABCA2. The neomycin phosphotransferase gene (NEO) flanked by the 5′ and 3′ intergenic regions from the dihydrofolate reductase/thymidilate synthetase gene of *Leishmania* was inserted into the *Kpn*I/*Cla*I site of the L9ABCA2 plasmid resulting in the expression vector L9ABCA2/NeoA. This vector was transfected into wild-type promastigotes by electroporation [23]. Cells were then cultured in the presence of increasing concentrations of G418 until a final concentration of 1 mg/ml to get parasites that overexpress LtrABCA2. To obtain parasites overexpressing unfunctional LtrABCA2, a described mutation in ABC transporters was induced in the first nucleotide binding domain at the 2317 nucleotide (K772M) using Quik Change XL Site-Directed (Roche). The resulting plasmid was sequenced and called L9ABCA2/NeoA/Mut7.

Antibodies against LtrABCA2 and Western blot analysis. Rabbit anti-LtrABCA2 antibodies were produced against a recombinant protein corresponding to positions 2–130 of the NH_2 terminus of LtrABCA2 fused to a sequence for six histidines. This recombinant protein was expressed in bacteria and purified in a Ni^{2+}–NTA column. The polyclonal antiserum was obtained by several subcutaneous immunizations of a New Zealand White rabbits with 150 μg of purified peptide. Wild-type and transfectant promastigotes were harvested in different growth phases and lysed by suspending the washed parasites in an urea-cracking buffer (10 mM Na_2HPO_4, 1% β-mercaptoethanol, 1% SDS, and 5 M urea, pH 7.0) at 37 °C for 30 min. Each sample (equivalent to 5×10^6 cells) was electrophoresed on 10% SDS–polyacrylamide gels. Proteins were transferred onto Immobilon-P membranes (Millipore) and reacted with rabbit anti-LtrABCA2 antibodies at a 1:5000 dilution.

Indirect immunofluorescence microscopy. Promastigotes were harvested, washed three times in cold phosphate-buffered saline (PBS; 130 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, and 8.1 mM Na_2HPO_4, pH 7.4), and settled onto slides. Fixation was allowed to proceed sequentially at −20 °C in ethanol for 5 min and in acetone for 8 min. The slides were then incubated with anti-LtrABCA2 antibodies (dilution 1:200) or preimmune serum for 1 h at 37 °C. After three washes in PBS/0.5% bovine serum albumin, the slides were further incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) for 1 h at 37 °C and washed as above. After mounting, images were acquired with a TCS-SP confocal microscope (Leica) and processed with Adobe Photoshop software.

Labelling with [N-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino] (NBD)-phospholipids and flow cytometry analysis. Promastigotes in the logarithmic phase of growth (day 4) were harvested by centrifugation, washed twice with PBS, and resuspended at 2×10^5 parasites/ml in HPMI buffer (132 mM NaCl, 3.5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM glucose, and 20 mM Hepes, pH 7.2). 1-Palmitoyl-(NBD-hexanoyl)-glycerophospholipids and NBD-sphingomyelin (NBD-SM) were from Avanti Polar Lipids (Birmingham, AL). Label suspension was prepared as previously described [24]. Labelling was initiated by adding 80 μl label solution to 1170 μl cell suspension. Then, cells were incubated at 28 °C for 30 min, diluted 1:1 with ice-cold PBS containing 5% fatty-acid free bovine serum albumin (w/v), and analyzed after 5 min by flow cytometry on a Becton–Dickinson FACS (San Jose, CA) equipped with an argon laser operating at 488 nm using Cell Quest software. HPMI was routinely supplemented with 5 mM di-isopropyl fluorophosphate to block the catabolism of NBD-phospholipids as previously described [24]. Even at 28 °C, less than 10% of the probes were converted to other NBD-phospholipids. The amount of NBD-phospholipid added to the cells corresponded to about 2.5 mol% of the total cell phospholipid concentration. Total phospholipid was quantified after lipid extraction by phosphate determination [25].

Secreted acid phosphatase assay and endocytosis measurements. To follow exocytosis, secreted acid phosphatase (SAP) activity was daily assayed in 200 μl of supernatants from *Leishmania* cultures starting at 2×10^6 parasites/ml, using *p*-nitrophenyl phosphate (Sigma Chemicals, St. Louis, MO, USA) as a substrate, as described [26]. Total SAP activity was measured using lysed parasites with 1% Triton X-100. The results were expressed in nanomoles of substrate hydrolyzed per 10^6 promastigotes for 30 min (extinction coefficient: 17.8 mM^{-1}). To determine if the overexpression of LtrABCA2 affected the endocytic activity of the parasite, we studied by flow cytometry the accumulation of the fluorescent compound FM4-64, previously used as an endocytosis marker in *Leishmania* spp. [27].

In vitro infection of macrophages. Macrophages from the J774G8 line were infected at 35 °C with control, *LtrABCA2* and *LtrABCA2*-

mutant transfected stationary-phase *L. infantum* promastigotes at a ratio of 1:20 (cells:parasites) as previously described [20]. After 6 h of infection, excess parasites were removed by washing with serum-free medium. The infected macrophages were further incubated in RPMI 1640 medium plus 10% FBS for 72 h at 37 °C in a 5% CO_2 atmosphere to allow intracellular parasite proliferation. Then, the cultures were fixed in methanol, stained with Giemsa, and the percentage of internalized parasites determined by light microscopy.

Statistical analysis. The results given are means ± SD of three independent experiments performed in duplicate and the Student *t* test was used for the statistical analysis of data.

Results and discussion

Identification and characterization of LtrABCA2

We have characterized a new ABC protein LtrABCA2 in the protozoan parasite *Leishmania*. The open reading frame of *LtrABCA2* gene (GenBank Accession No. AI057758) encoded a protein of 1896 amino acid, with a predicted molecular weight of 210 kDa consisting of two similar halves, each containing six putative transmembrane segments, Walker A and B motifs, and the ABC signature sequence (Fig. 1). The analysis of deduced amino acid sequence of LtrABCA2 demonstrated that it has a long region between the first two hydrophobic segments, predicting a large extracytoplasmic loop region. LtrABCA2 showed a comparable predictive membrane topology to that proposed for other eukaryotic ABCA proteins [28]. A search on the sequence databases using the FASTA algorithm revealed the best match with previously described ABC transporters from the ABCA subfamily. LtrABCA2 showed 52% amino acid identity to *Leishmania* LtrABCA1.1, 41% to TcABCA1 from *T. cruzi*, 26% to human ABCA3 and mouse ABCA1, and less than 20% to ABC proteins belonging to other subfamilies. Recently, a gene se-

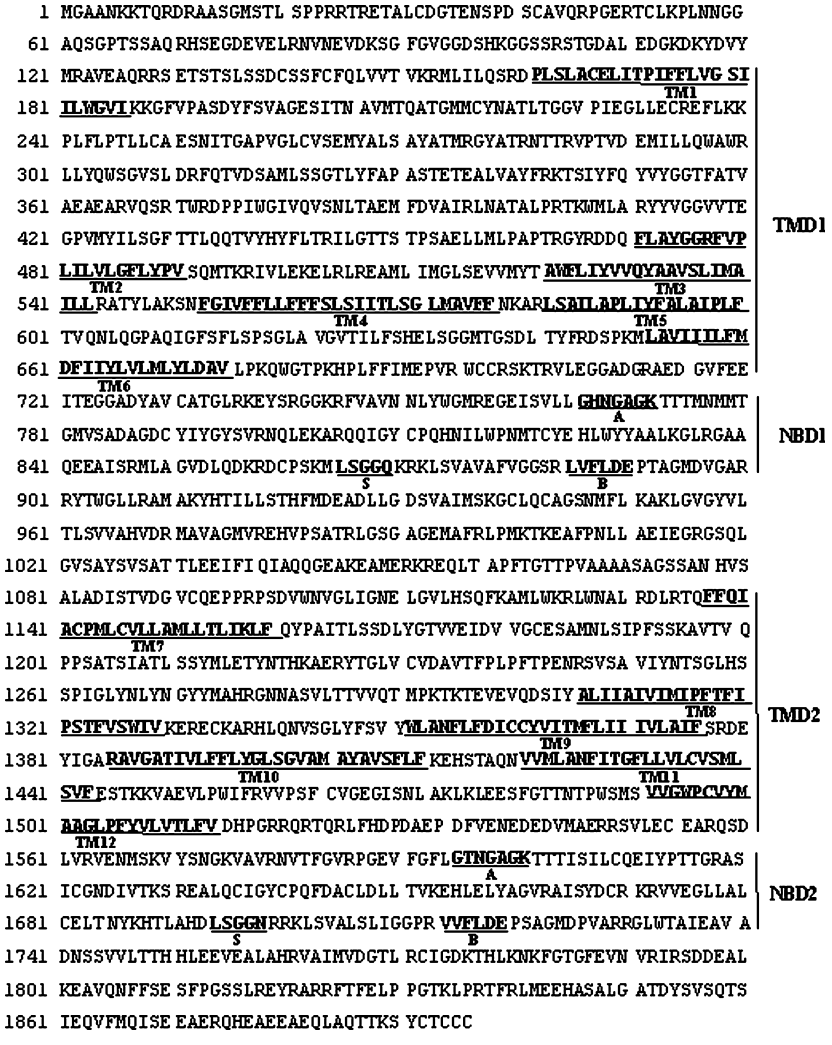

Fig. 1. Predicted sequence of LtrABCA2. Putative transmembrane segments (TM), predicted by the Kyte and Doolittle algorithm, and Walker A (A), Walker B (B), and ABC family signature motifs (S) are underlined. Vertical lines represent the positions of transmembrane domains (TMD1 and TMD2) as well as nucleotide binding domains (NBD1 and NBD2). Amino acid positions are indicated.

quence with a 99% identity to LtrABCA2 has been described in the Genome Project of *L. infantum*.

Northern blot analysis does not detect any transcript in wild-type promastigotes of *L. tropica* or *L. infantum*, indicating that *LtrABCA2* is a low expression gene. Indeed, RT-PCR experiments showed the presence of *LtrABCA2*-mRNA. Two different trans-splicing sites were identified, located 335 and 390 nucleotides upstream from the putative translation initiation site of *LtrABCA2* (data not shown).

Overexpression of LtrABCA2 in transfectant parasites

To study the functionality of LtrABCA2, we obtained infective *L. infantum* parasites that overexpress this transporter. Anti-LtrABCA2 peptide antibodies recognized a single protein of approximately 215 kDa in parasites transfected with L9ABCA2/NeoA and L9ABCA2/NeoA/Mut7, absent in parasites transfected with the control plasmid (Fig. 2A). Considering that overexpression of ABC transporters has been involved in drug resistance in *Leishmania*, we determine if the LtrABCA2 overexpression could confer resistance to different compounds among others antileishmanial agents amphotericin B and alkyl-lysophospholipids miltefosine and edelfosine. We observed that overexpression of LtrABCA2 does not confer resistance to the compounds tested (data not shown) showing a different behavior to other ABC proteins [8].

Localization of LtrABCA2

Indirect immunofluorescence was performed with both *LtrABCA2*-transfected promastigotes and amastigotes infecting macrophages. A significant portion of the fluorescent signal was localized to the flagellar pocket region and to internal vesicles which configure an asymmetrical tubular structure of the *LtrABCA2*-transfectants (Fig. 2B, b and e). The flagellar pocket is the sole site for exocytosis and endocytosis in *Leishmania* and the tubular structure may correspond to the multivesicular tubule lysosome of the parasite [27,29,30]. Control transfected parasites showed a faint stain, also mainly located in internal structures (Fig. 2B, a and d). The same location was observed in parasites transfected with the mutant-LTRABCA2 (Fig. 2B and C). No fluorescence was observed with a rabbit preimmune serum (Fig. 2B and F). This subcellular localization is similar for TcABCA1 in *T. cruzi* [31]. However, *Leishmania* LtrABCA1.1 is mainly located in the flagellar pocket and plasma membrane [20]. The different localization between both *Leishmania* ABCA-like proteins could be due to the high divergence in the sequence at the NH_2-terminus of both proteins as described for other proteins in parasites [32].

Accumulation of fluorescent phospholipid analogs

Fluorescent phospholipid analogs of PC, PE, and PS are internalized in *Leishmania* and require an energy-

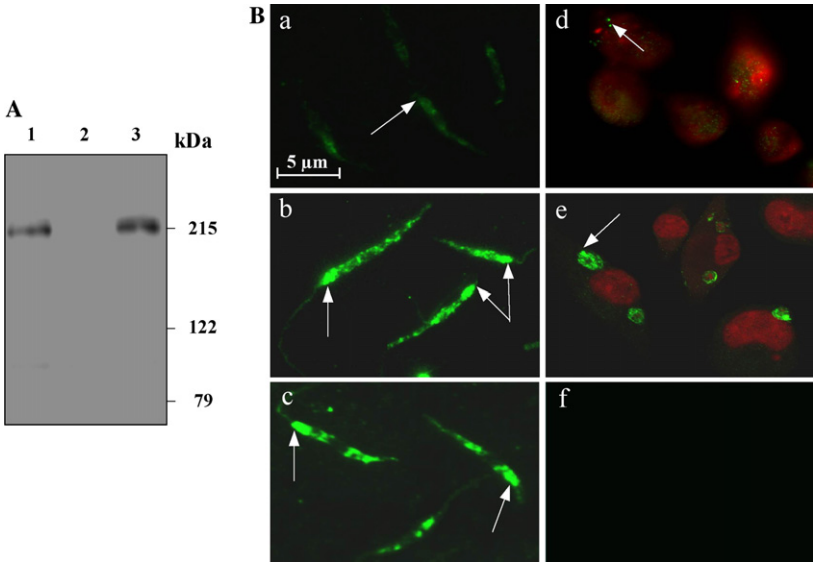

Fig. 2. Expression and immunolocalization of LtrABCA2. (A) Western blot analysis of total proteins from: parasites transfected with *LtrABCA2* (1), the control plasmid (2), and mutant-*LtrABCA2* (3), using an anti-LtrABCA2 antibody. Molecular mass standards (kDa) are from Bio-Rad. (B) Immunofluorescence localization of LtrABCA2 in promastigotes and intracellular amastigotes. Promastigotes (a) and amastigotes (d) transfected with the control plasmid, promastigotes (b) and amastigotes overexpressing LtrABCA2 (e), and promastigotes overexpressing the mutant-LtrABCA2 (c) were stained with polyclonal rabbit antiserum followed by fluorescein-conjugated secondary antibody (green). Nuclei of macrophages are stained with propidium iodide (red). Promastigotes overexpressing the transporter and stained with pre-immune serum were used as control (f). Arrows indicate the region of flagellar pocket. (For interpretation of the references to colors in this figure legend, the reader is referred to the web version of this paper.)

coupled transport and accumulated in intracellular compartments [24]. Moreover, we have identified a P-type ATPase from the aminophospholipid translocase subfamily to be involved in the transbilayer movement of PC, PE, and PS in *Leishmania* [9]. LtrABCA2-overexpressing parasites accumulated significantly lower levels ($P < 0.005$) of glycerophospholipid analogs compared to control parasites (Fig. 3) (ratio values of 2.8 ± 0.2 for C_6-NBD-PC, 2.7 ± 0.3 for C_6-NBD-PE, and 2.2 ± 0.3 for C_6-NBD-PS). On the contrary, no differences within accumulation were observed when the parasites were labelled with the sphingolipid analog (Fig. 3). Promastigotes overexpressing mutant-LtrABCA2 showed similar behavior as control parasites (not shown). This role in phospholipid trafficking appears to be independent of their polar heads, but not of the total phospholipid structure, which might explain the absence of resistance to alkyl-phospholipids or the normal internalization of the sphingolipid analog. LtrABCA2 is located in intracellular vesicles and we proposed that phospholipid analogs are pumped into secretory vesicles and exported from the parasite by exocytosis. Similarly, *Leishmania* MDR1 P-glycoprotein-like transporter is expressed in a number of secretory and endocytic compartments, and confers resistance to some hydrophobic drugs that are extruded out of the cell [33]. Also, this transport confers resistance to miltefosine (hexadecylphosphocholine) in *Leishmania* and reduced the accumulation of a fluorescent lipid analog of PC [8].

Exocytosis may be modified in LtrABCA2-transfected parasites

Secretion of SAP of *Leishmania* has been studied extensively and it has been employed as an exocytosis marker [34]. Parasites overexpressing LtrABCA2 showed a significantly ($P < 0.005$) higher secreted SAP activity with respect to control parasites (Fig. 4). Considering that total SAP activity was similar in both parasite lines, these results suggest that overexpression of the transporter induced an increase in the exocytic activity. Transfected non-cloned parental line also showed a comparably higher secreted SAP activity (not shown). Also, *Leishmania* promastigotes overexpressing the mutant-LtrABCA2 showed similar SAP activity as control parasites. These results differ from that obtained with the overexpression of *Leishmania* LtrABCA1.1 that showed a significantly lower SAP activity [20]. No differences were observed in the accumulation of the endocytosis marker FM4-64 between these two lines of *Leishmania*, indicating that LtrABCA2 does not affect the endocytosis activity of the promastigotes. The involvement of ABCA transporters in regulation of lipid transport and vesicular trafficking has been described for each one of the members of human ABCA subfamily. For instance, ABCA3 is critical for the proper for-

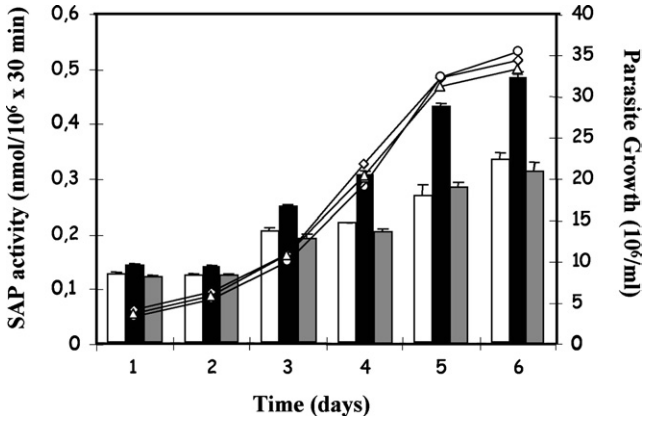

Fig. 4. SAP activity in *Leishmania*. Bars represent the SAP activity in supernatants from *Leishmania* cultures as nanomoles p-nitrophenyl phosphate hydrolyzed in 30 min per 10^6 promastigotes (left scale). The line plot represents the growth rate of parasites over culture time (right scale). Control-transfected parasites (white bars, triangles), *LtrABCA2*-transfected parasites (black bars, circles), and promastigotes overexpressing mutant-LTRABCA2 (grey bars, rhombus).

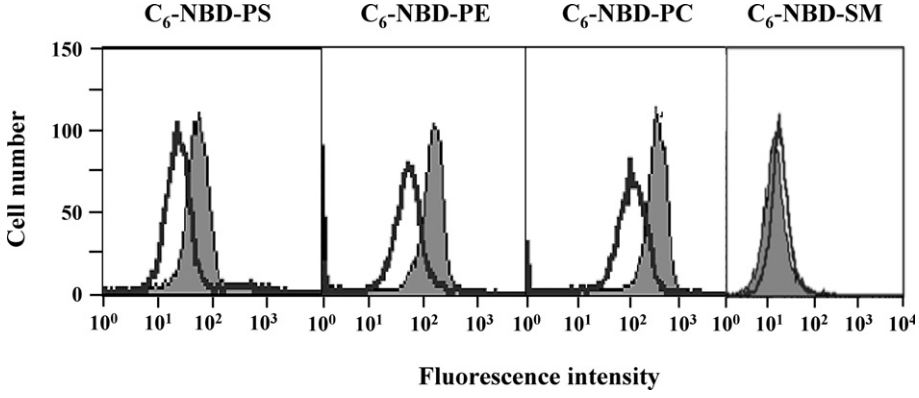

Fig. 3. C_6-NBD-phospholipid analog accumulation in *Leishmania*. Promastigotes were incubated with 6.4 μM short-chain fluorescent analogs of phospholipids for 30 min at 28 °C. Cell-associated fluorescence was measured by flow cytometry analysis. A representative histogram is shown for each analog. The grey histogram represents control transfected cells, the uncolored histogram that of *LtrABCA2*-transfected parasites.

mation of lamellar bodies and surfactant secretion by exocytosis in alveolar type II cells [35]. Human ABCA1 expression may influence cholesterol efflux, in part by enhancing vesicular trafficking from the Golgi to the plasma membrane [36].

Decreased infectivity of transfectant parasites overexpressing LtrABCA2

Parasites overexpressing LtrABCA2 were significantly ($P < 0.005$) less infective ($22.8 \pm 3.1\%$) compared to control parasites ($43.5 \pm 4.8\%$) while the ratio of the number of parasites/macrophage was similar in both lines. The lower infectivity showed by these transfected parasites is not due to an intrinsic characteristic of the clone used on these studies since the non-cloned parental line also showed similar low infectivity (not shown). Moreover, transfectant parasites overexpressing the mutant-LtrABCA2 showed the same infectivity as control parasites. We suggest that the expression levels of LtrABCA2 may alter phospholipid and vesicle trafficking that would result in surface modifications where specific molecules, some involved in the parasite infectivity, may be affected.

In summary, LtrABCA2 seems to play a role in phospholipid trafficking, which may be modifying the vesicular trafficking and the infectivity of the parasite. Further experiments including functional reconstitution into proteoliposomes are an obligatory step for providing information that this protein is directly responsible for phospholipid transport.

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