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Biochimica et Biophysica Acta 1612 (2003) 195–207
BIOCHIMICA ET BIOPHYSICA ACTA
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The overexpression of a new ABC transporter in *Leishmania* is related to phospholipid trafficking and reduced infectivity

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Received 6 February 2003; accepted 24 April 2003

Abstract

This paper reports the characterization of a new ABC transporter (LtrABC1.1), related to the human ABCA subfamily, in the protozoan parasite *Leishmania tropica*. *LtrABC1.1* is a tandem duplicated gene flanked by inverted repeats. LtrABC1.1 is expressed mainly in the flagellar pocket of the parasite. Drug resistance studies in *Leishmania* overexpressing LtrABC1.1 showed the transporter not to confer resistance to a range of unrelated drugs. LtrABC1.1 appears to be involved in lipid movements across the plasma membrane of the parasite since overexpression reduces the accumulation of fluorescent phospholipid analogues. The activity of this protein may also affect membrane movement processes since secreted acid phosphatase (SAP) activity was significantly lower in promastigotes overexpressing LtrABC1.1. In vitro infection experiments with macrophages indicated *LtrABC1.1*-transfected parasites to be significantly less infective. Together, these results suggest that this new ABC transporter could play a role in lipid movements across the plasma membrane, and that its activity might influence vesicle trafficking. This is the first ABCA-like transporter described in unicellular eukaryotes.

Keywords: ABC transporter; *Leishmania*; Phospholipid trafficking

1. Introduction

Leishmania is a pathogenic, kinetoplastid, protozoan parasite, and is responsible for leishmaniasis. Its life cycle involves a flagellated promastigote form that lives in the insect vector, and intracellular amastigote forms that inhabit the vertebrate host. According to the World Health Organization, around 350 million people are exposed to the risk of infection by different species of *Leishmania*; the disease currently affects 12 million people and has an annual incidence of 2 million. Unfortunately, chemotherapy is confronted with ever more frequent cases of resistance. Usually, the resistant parasites amplify portions of the genome containing resistance genes, some of which encode members of the ATP binding cassette (ABC) family of transporters. For example, *Leishmania tarentolae* selected in vitro for resistance to arsenicals and antimonials amplifies the H circle containing the *PGPA* gene [1]. It has been suggested that this transporter is expressed in the membranes of an intracellular compartment where these drugs are accumulated [2]. When selected stepwise for resistance to daunomycin, *Leishmania tropica* amplifies and overexpress the *MDR1*-like gene as part of an extrachromosomal element [3]. The latter confers multidrug resistance (MDR) similar to that observed in tumour cells, which includes resistance to the alkyl-lysophospholipids miltefosine (hexadecylphosphocholine) and edelfosine (1-*O*-octadecyl-2-*O*-methyl-glycerophosphocholine), often considered promising anti-*Leishmania* agents [3,4].

ABC transporters are thought to be one of the largest of protein families, and they may have been present throughout evolution (see Ref. [5] for a review). They transport a range of structurally unrelated compounds using ATP hydrolysis as their energy source. These transporters are composed of two transmembrane domains (TMD1 and TMD2) and two nucleotide-binding domains (NBD1 and NBD2) that contain the conserved Walker A and Walker B motifs as well as the

Abbreviations: ABC, ATP binding cassette; NBD1, nucleotide-binding domain 1; NBD2, nucleotide-binding domain 2; PS, phosphatidylserine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; C_6-NBD-, [*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino]

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doi:10.1016/S0005-2736(03)00131-7

ABC signature. In humans, there are 48 different ABC proteins that have been organized into seven subfamilies http://www.med.rug.nl/mdl/humanabc.htm.). In recent years, it has been observed that several ABC transporters are involved in lipid movements across cell membranes [6]. This activity therefore influences several biological processes such as drug transport or the production of bile [6]. The members of the ABCA subfamily are an example of ABC transporters involved in lipid trafficking. One of the most characterized member of this subfamily, ABCA1, has a major role in cholesterol and phospholipid efflux across the plasma membrane [7]. Mutations in *ABCA1* are responsible for Tangier disease and familial HDL deficiency, disorders characterized by the almost complete absence of plasma HDL, cholesteryl ester accumulation in tissue macrophages, and low levels of plasma apolipoproteins [8–11]. In addition, ABCA1 is proposed to be involved in the engulfment and clearance of apoptotic cells [12] through the ability to expose phosphatidylserine (PS) on the external face of the plasma membrane [13].

Until now, only ABC transporters related to the human ABCB and ABCC subfamilies have been studied in *Leishmania* [14]. The present work reports sequences coding for ABCA-like transporters and describes the molecular and initial functional characterization of the *LtrABC1.1* gene of *L. tropica*. LtrABC1.1 appears to be involved in phospholipid trafficking across the plasma membrane of the parasite, an activity that seems to influence other cellular processes such as infectivity.

2. Materials and methods

2.1. Parasite cell culture

The parasite cell lines used in this study were *Leishmania infantum* strain 21578 (LEM 2592, Montpellier, France) and *L. tropica* strain LRC-L39 (LEM 2563, Montpellier, France). Promastigotes were grown in vitro at 28 °C in modified RPMI-1640 medium (Gibco BRL) supplemented with 20% heat-inactivated foetal bovine serum (FBS) (Gibco BRL) as previously described [15]. Promastigote cultures were initiated at 4×10^6 cells ml^{-1} and collected during the logarithmic phase of growth. For infection studies, parasites were collected during the stationary phase of growth. The transfected cell line (LABC1N) was cloned in solid medium containing RPMI plus 20% FBS/1% agar with 500 μg ml^{-1} of G418 at 28 °C. Isolated colonies were obtained after 10 days and transferred into liquid medium in the presence of G418.

2.2. Drug susceptibility assay

Drug susceptibilities were determined using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide (MTT)-based assay as previously described [16]. Briefly, log phase promastigotes in fresh medium were deposited (3×10^5 cells/well) in 96-well plastic plates and maintained for 72 h at 28 °C in the presence of different concentrations of drug compounds. After incubation, 0.5 mg ml^{-1} medium of MTT was added to each well, and the plates further incubated for 4 h. Finally, water-insoluble formazan crystals were dissolved by adding SDS, and absorbance was measured at 540 nm in a microplate reader (Beckman Biomek 2000). Cell viability was determined by dividing the absorbance at a given drug concentration by the absorbance of control cells grown in the absence of the drug.

2.3. Library screening and LtrABC1.1 cloning

A λEMBL3 genomic library of *L. tropica* was used for ABCA-like gene screening. Approximately 160,000 pfu of this library were transferred to nitrocellulose membranes (Schleicher & Schuell) and probed with a partial cDNA sequence for a putative ABCA-like transporter of *Trypanosoma cruzi* (23A18 clone, accession number AI057758) kindly provided by Dr. L. Aslund (Uppsala University, Sweden). A recombinant phage containing a 13,271-bp insert was then selected. Different fragments of this insert (see Fig. 1) were subcloned in the pBluescript II KS^+ vector

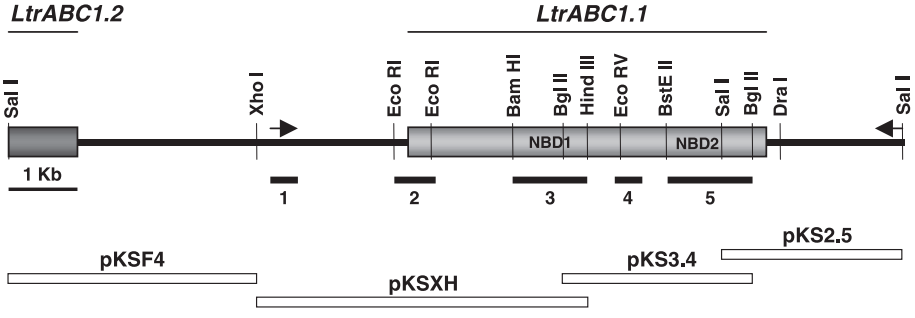

Fig. 1. Restriction map of the recombinant clone phage containing *LtrABC1.1*. The major restriction sites employed for cloning are indicated. Below: Distinct fragments subcloned into the pBluescript-KS vector. Fragments used as probes in Southern blot analysis are shown below as solid lines. (1) Probe for inverted repeat sequence. (2) Probe for 5′ end of *LtrABC1.1*. (3) Probe for NBD1 of *LtrABC1.1*. (4) Probe for extracytoplasmic loop, defined as the specific probe for *LtrABC1.1*. (5) Probe for NBD2 of *LtrABC1.1*. Arrows indicate the position of the inverted repeat sequence. Left—sequence corresponding to the 3′ end of *LtrABC1.2*.

(Stratagene) to provide different plasmids. The pKSF4 plasmid contained a 4kb *Sal*I/*Xho*I fragment corresponding to the 5′ end of the insert, pKSXH contained the adjacent 5 kb *Xho*I/*Hin*dIII fragment, pKS3.4 contained an overlapping 3 kb *Bgl*II fragment, and pKS2.5 contained an overlapping 2.5 kb *Sal*I fragment corresponding to the 3′ end of the phage insert. Nucleotide sequences were determined automatically as described by Lario et al. [17] using the ABI PRISM Big Dye™ Terminator Cycle Sequencing Ready Reaction (Applied Biosystems).

2.4. DNA constructs and transfection procedures

Plasmid constructs used for transfections were derived from the pX vector [18] with a modified polylinker (pKSNEOA). This plasmid contained the neomycin phosphotransferase gene (*NEO*) flanked by the 5′ and 3′ intergenic regions from the dihydrofolate reductase/thymidilate synthetase (DHFR-TS) gene. To construct plasmid bearing *LtrABC1.1* flanked with its natural 5′ and 3′ untranslated regions (UTRs), the following steps were performed. The pKS2.5 plasmid was digested with *Sal*I and *Dra*I and the 1500 bp resulting fragment containing the 3′ end of *LtrABC1.1* and 218 bp of 3′ UTR was subcloned into the *Sal*I/*Eco*RV site of the pBluescript II KS$^+$ vector. Part of the subcloned fragment was substituted by a 2600-bp *Bst*EII/*Xba*I fragment of pKS2.5 containing the 3′ end of the gene and a more extensive 3′ noncoding sequence. A 1800-bp *Sal*I fragment from pKS3.4 plasmid was inserted into *Sal*I site of the previous plasmid. Finally, a 5000-bp *Xho*I/*Hin*dIII fragment of pKSXH containing the 5′ portion of *LtrABC1.1* and its 5′ UTR was added. The resulting construct, named LABC1, bore the *LtrABC1.1* ORF flanked by 2171 bp of the 5′ and 1360 bp of the 3′ noncoding sequences. For expression in *Leishmania*, the *NEO* cassette of the pKSNEOA vector was inserted into the *Xho*I site of the LABC1 plasmid, resulting in the LABC1N plasmid. For transformation, 5×10^7 promastigotes of *L. infantum* were resuspended in 400 μl HBS buffer (21 mM HEPES, 137 mM NaCl, 5 mM KCl, 7 mM Na_2HPO_4, 6 mM glucose, pH 7.4) and transfected with 100 μg of LABC1N and pKSNEOA plasmids by electroporation at 450 V, 1300 μF, 13 Ωs (BTX ElectroCell Manipulator 600). Transfected parasites were grown in the usual growth medium in the presence of continually increasing concentrations of G418 (Gibco BRL) until a final concentration of 2 mg ml^{-1}.

2.5. Southern and Northern analysis

Genomic DNA was extracted from *Leishmania* with the "Puregene DNA isolation" kit (Gentra). Following the separation of digested total DNA on 0.8% agarose gel, the DNA was transferred to Hybond-N nylon membranes (Amersham) by standard methods [19]. Chromosome-sized DNA was separated by CHEF gel electrophoresis in a 1% agarose gel with 1 × TBE (100 mM Tris, 90 mM boric acid, 1 mM EDTA) as previously described [3]. Gels were transferred as described above. RNA was obtained using Trizol reagent (Gibco BRL). The poly (A+) fraction was obtained using the Quick Prep Micro mRNA Purification kit (Pharmacia) and electrophoresis was performed on denaturing gels containing formaldehyde. Filters were hybridized with a α^{32}P-dCTP random-primed labelled probes prepared from gel-isolated DNA fragments using the Gene Clean kit of Bio 101 Inc. Quantification was performed with an Instant Imager (Packard). The probes (see Fig. 1) employed in Southern, Northern and CHEF blot analyses were obtained as follows. Probe no. 3, corresponding to the sequence for NBD1 of *LtrABC1.1*, was obtained after digestion of the pKSXH plasmid with *Bam*HI and *Hin*dIII enzymes. Probe no. 5, corresponding to the sequence for NBD2, was obtained after digestion with *Bst*EII/*Bgl*II of the pKS2.5 plasmid. Specific probe no. 4 for *LtrABC1.1* was obtained by PCR from pKS3.4 by using LABC1-23 (5′-AGCACGCCGACGATCGTG) and LABC1-24 (5′-CGTACAGAGATGACTCCAC) primers. Probe no. 1 (which searches for inverted repeats) was obtained from PCR amplification of pKSXH with LABC1-26 (5′-GATCGTCGGCACGATTCG) and LABC1-27 (5′-GATGACGCCCACCACCTC) oligonucleotides. Probe no. 2, corresponding to the 5′ end of *LtrABC1.1*, was obtained after *Eco*RI digestion of the pKSXH plasmid. The *NEO* probe was obtained from the pKSNEOA plasmid after digestion with *Spe*I.

2.6. RT-PCR

The splice acceptor and polyadenylation sites of the *ABC1.1* transcript were determined by RT-PCR using poly(A+) RNA as a template. cDNAs were generated using a specific antisense 5′ primer (LABC1-11) corresponding to 655–673 nucleotides of the *LtrABC1.1* sequence (5′-GACTCGAAAGGGAATCTC) or an ANCH + oligo(dT) primer (5′-CCTCTGAAGGTTCCAGAATCGATAGGAATTC(T)$_{18}$VN) and M-MLV reverse transcriptase (Gibco BRL). The cDNAs were further amplified with the specific 5′ antisense LABC1-20 (5′-TCATCGGAGAGTACTCGC) or the 3′ LABC1-3 (5′-TGTCGAGCGTGTTCACTG) primers in combination with either a spliced leader (LSL) primer (5′-AACGCTATATAAGTATCAG) or the ANCH primer (5′-CCTCTGAAGGTTCCAGAATCGATA). The 3′ amplification products were reamplified with a 3′ primer corresponding to the noncoding sequence (5′-GGTCGCGTGAGTGAACTTG). PCR products were cloned into pGEM-T (Promega) and sequenced.

2.7. Antibodies to LtrABC1.1 and Western blot analysis

The recombinant protein corresponding to the region between the 7th and 8th transmembrane segments of LtrABC1.1, fused to a sequence for six histidines, was expressed in bacteria. It was then purified in a Ni^{2+}-NTA column. Polyclonal antiserum was obtained by several

subcutaneous immunizations of New Zealand White rabbits with 150 μg of purified recombinant peptide. The IgG fraction was obtained by passing the antiserum through a protein A-Sepharose CL-4B column (Pharmacia). For immunoblots, crude *Leishmania* extracts were prepared by suspending the washed parasites in urea cracking buffer (10 mM sodium phosphate pH 7, 1% β-mercaptoethanol, 1% SDS, 5M urea) at a concentration of 10^9 parasites ml^{-1}. Total cell proteins were separated on 10% polyacrylamide-SDS gel and electrotransferred onto immobilon-P membranes (Millipore) using a semi-dry blot apparatus (Hoefer Sci. Inst.). For immunodetection, membranes were incubated for 1 h at room temperature with a 1/5000 dilution of the anti-LtrABC1.1 immune serum in buffer A [PBS/1% bovine serum albumin (BSA)]containing 0.05% Tween-20. After washing twice with buffer A, the membranes were incubated for 30 min with phosphatase-conjugated goat anti-rabbit IgG antibodies (Sigma), washed twice, and then revealed with 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium substrates (Boehringer Mannheim).

2.8. Indirect immunofluorescence microscopy

Parasites (2×10^6 ml^{-1}) were harvested, washed five times in cold PBS, and allowed to settle onto slides with ten 6-mm-diameter circles. 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 antibody against LtrABC1.1 (dilution 1/200) or preimmune serum for 1 h at 37 °C. After three washes in PBS/0.5% BSA, 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 with Vectashield (Vecta Laboratories Inc.), the slides were observed using an epifluorescent microscopy Zeiss Axiophot (Germany). Images were captured with a SPOT camera (Diagnostic Instruments, Inc.) and analyzed using Adobe Photoshop 5.5 software.

2.9. Phospholipid analogue accumulation and flow cytometry analysis

Parasites (8×10^6 ml^{-1}) were preincubated in HPMI buffer (20 mM HEPES, 132 mM NaCl, 3.5 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose, 1 mM $CaCl_2$, pH 7.25) plus 0.3% BSA and 500 μM phenylmethylsulfonylfluoride for 30 min at 28 °C. In some cases, the parasites were preincubated in the absence of glucose and in the presence of 10 mM sodium azide (NaN_3) or 0.5 mM *N*-ethylmaleimide. The parasites were then incubated with 2 μM of [*N*-(7-nitrobenzo-2-oxa-1,3-diazol-4-yl)amino] (C_6-NBD)-PS, phosphatidylcholine (PC), or phosphatidylethanolamine (PE) (Avanti Polar Lipids) for 30 min at 28 °C or at 0 °C in HPMI plus 0.3% BSA. After washing twice with cold PBS, samples were maintained on ice and the cellular fluorescence measured. Flow cytometric analysis of C_6-NBD-PL accumulation in parasites was performed with a FACScan flow cytometer (Becton-Dickinson, San José, CA) equipped with an argon laser operating at 488 nm and using Cell Quest software. The experimental population was mapped using a two-parameter histogram of forward-angle light scatter versus side scatter. The mapped population was then analyzed for log green fluorescence (FL1) using a single-parameter histogram.

2.10. In vitro infection of macrophages

Before infection with parasites, macrophages from the J774G8 line were seeded into 24-well microtiter plates (20,000 cell/well) containing 12-mm coverslips and RPMI 1640 medium plus 10% FBS for 72 h. The macrophages were infected at 35 °C with stationary-phase promastigotes (metacyclic) of control and *LtrABC1.1*-transfected *L. infantum* at a ratio of 1:20 (cells/parasites). After 6 h, 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. After the incubation period, the cultures were fixed in methanol, stained with Giemsa, and the percentage of internalized parasites determined by light microscopy. Three independent experiments were performed with duplicates. The Student's *t*-test was used for the statistical analysis of data.

2.11. Secreted acid phosphatase (SAP) assay

To follow exocytosis, SAP activity was assayed daily in 200 μl of supernatants from *Leishmania* cultures starting with 2×10^6 parasites ml^{-1}, using *p*-nitrophenyl phosphate (Sigma) as a substrate, as previously described [20]. The assay was performed for 30 min at 37 °C. After the incubation period, the reaction was stopped with 800 μl of 0.25M NaOH and measured in a spectrophotometer at 410 nm. The results were expressed in nanomoles of substrate hydrolyzed per minute per milliliter (extinction coefficient: 17.8 mM^{-1}).

3. Results

3.1. Identification and characterization of LtrABC1.1

In order to identify genes related to the ABCA subfamily of transporters described in humans, a λEMBL3 *L. tropica* library was screened. This was performed using the 23A18 probe, a partial coding sequence for a putative ABCA-like transporter of the related protozoan parasite *T. cruzi*. Some 160,000 clones of the genomic library were plated and nearly 100 hybridized with the 23A18 probe. Twenty-four were plaque-purified for further characterization. Based on the restriction pattern shown by these

clones, together with the data obtained after hybridizing the restriction fragments with the 23A18 probe, a λ clone named 2B1 with an approximately 14-kb-long insert containing a full-length ABC gene was selected. After the subcloning of four overlapping fragments of this insert in the pBluescript II KS$^+$ vector (see experimental procedures and Fig. 1), the complete sequence was determined. The open reading frame (ORF) included within this insert comprises 5529 bp and codes for a 1843 amino acid protein with a predicted molecular weight of approximately 200 kDa. The sequence of this gene, named *LtrABC1.1*, has been submitted to GenBank (accession no. AF200948). A search of sequence databases using the FASTA algorithm revealed the deduced amino acid sequence of *LtrABC1.1* to best match with the ABC transporters of the human ABCA subfamily. LtrABC1.1 shared around 32% identity with human ABCA4 and ABCA1 and 28% with CED7 of *Caenorhabditis elegans*. When the homology of NBDs—the most conserved regions of these proteins—was compared, NBD1 of LtrABC1.1 was found to have 50%

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                MPGAGPLVPSPNSTTTTQNPLLLFASPGALPGLSFSRNNSEAFGEGQLFTPAVEGEYSPM   60
                SGGGSAFLRSDDRMIRGTALRTGEMRATTLEAGNHASPSAAENDVNDGSCVEMTAEQIPL  120
                AQADDQYDVLHRRSASANSPARCRPRAGCCQQLGHTILRVLILMKREWCSTLCEFLIPIL  180
                FVFGSVMLWAVFGTAQNSAGVYYSSTGPEKVSAMTDKISSSLCYNASLASGIAGLTPCLE  240
                GQKYYCPHGRGIPTGFCYDPPLKNVVAQLLGALPLTTSPMLPLDTLIACQWVGMDQSIRA  300
                STPFSYGGLLYFAPRTPEVESLVSYMRAQSKLFDNAYGGTFATAADATSHILSLTHDDPP  360
TMD1            TWGVVELDSYTADTFNVKIMVNSSALPATSDSILSGYLGGLDMSTNSPYIFSGYTTLQTL  420
                VYNHYTTSVLGKPATKPLTYTAMPTKAYNTSSFLLTGASLAPLILVLGFLYPVSQLTKRI  480
                VVEKELRIREAMLIMGLSEWTMYLAWLVVYGVWYTVVSIIITVLLRLTYLPESSPGYVFF  540
                MFLLFSWSTIALSGAIAAVFSKARLAAIIAPLIYFAMAIPLFAMERASGGAKMGIMILGP  600
                SAFAVGFALLFEHEVNGGAGVGALAYFRDEPKLIVVFVLLFVDIFVYLLLMMYFDRVVPK  660
                EWGTTKNPLFFVIDPVRWCFCRRRAGDADNDGDVPGDGRAEDGVFEAVDPAVEEAAAVRI  720
                RGLRKTFRRGGKAFAAVDDLCWSLNEGEISVLLGHNGAGKSTTMNLMTGMLEADGGDCYV  780
NBD1            YGHSVRHELSAVRQEIGLCPQHNILWPQLTVREHLDYYAAIKGLRGSEKEDAIRRLLAAV  840
                DLEDKEHYMSKALSGGQKRKLSVAVAFVGGSRLVILDEPTAGMDVGARRHTWSLLKEMAK  900
                WHTILLTTHFMDEADLLGDTVAIMSKGRLQCAGSNMFLKSKLGVGFVLTMSVVSHARRGP  960
                IEQMVQTLVPAAEAIGSGAGEVAYRLPMASKPMFPDLLCAVEEGIPGLGINAYSLSATTL 1020
                EEVFIKIAEGPDAERDADALAAKEKAEATAAVWNVEMEKGRWARRRLQFRAMMVKRLWNA 1080
                LRDRRTQFFQIVCPVACVLLAMLLTLVRLFSTPTIVLSSDVYGTAVDIPLANCEGVLDVT 1140
                TPFSTKAHMDIWTDVPDASAFSTKLNRTYQTHAKERYGGVSCAAAGSGELYHSVFYNTSA 1200
                LHEVAIETANVFAAHLRVATGRDNVSVTTAVASLPKTSQQRAVESSLYAMMIAVIIMIPF 1260
TMD2            TFIPSTFVGWIVRERECKARHLQNVSGLSFYIYWLSNFLFDLCSYIVTMCLVIVVFLAFG 1320
                RDEYVAVNNIGATFVVFLLYGVSGILMAYALSFAFDNHSTAQNVVMLVNFIVGFLLVLAV 1380
                SALSLKESTRNLAKVLRWIFRIVPSYCVGEAINNLASLKVTRAFGIDTSTWDMDVVGWVC 1440
                VYMAIEIPVFLFITLFIDHPGRRQRSQRLFHNPDGAAEVIEDEDEDVAAERRAVLEGGER 1500
                EGDLVRVLNLRKEYPNGKVAVRNIALGVRPGEVFGFLGTNGAGKTTTISILCQEFYPTSG 1560
NBD2            RAYVCGNDIVTESSEALRCIGYCPQFDACLDLLTVEEHLYLYAGVRGISSRACDRVVRGL 1620
                MKLCGLTEYRRTKSHELSGGNRRKLSVAVSLIGGPRVVFFDEPSAGMDPVARRGLWNAIE 1680
                TVADNCSVVLTTHHLEEVEALAHRVAIMVDGTLRCIGDKTHLKQKYGTGFEVAVRVADES 1740
                PEVMAGVELFFEEEFPSSKLTEVRAGRFTYQLPSTVRLSSVFTALEQQKEKLQICDYSVS 1800
                QTSIEQVFMRISEKAELEHEEEHRQRMESKKSCCVCCCGGLPR                   1843
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Fig. 2. Predicted sequence of LtrABC1.1. Putative transmembrane segments (TM), predicted by the Kyte and Doolittle algorithm [21], are underlined and numbered. Walker A (WA) and Walker B (WB) motifs are boxed and ABC family signature motifs double-underlined. Left—vertical lines represent the positions of TMD1 and TMD2 as well as NBD1 and NBD2. Right—amino acid positions are indicated.

identity with ABCA1 and 47% with ABCA4. NBD2 identity values reached 48% and 49%, respectively. In contrast, the alignment of LtrABC1.1 NBDs with other ABC transporters, such as human ABCB and ABCC subfamilies, revealed less than 25% identity. Analysis of the deduced amino acid sequence and prediction of the secondary structure [21] showed it to be a complete ABC protein with two TMDs and two NBDs (Fig. 2). Each TMD was composed of six hydrophobic segments. Within each, there was a stretch of 200–250 amino acids between the first two hydrophobic segments, predicting a large extracytoplasmic loop region (see Fig. 2). Similar membrane topology has been proposed for ABCA4 and other ABCA proteins [22]. It seems to be a characteristic feature of the ABCA subfamily that is absent in other ABC transporter subclasses.

3.2. LtrABC1.1 is duplicated in tandem

Restriction mapping of the insert of the 2B1 clone suggested the presence of a 3′ coding region of another ABC gene. This was further confirmed by sequencing. The sequence is located upstream of *LtrABC1.1* and is truncated by one phage arm of the recombinant clone (see Fig. 1 for a schematic representation). At the nucleotide level, the 1100-bp partial sequence of this gene is very similar to the 3′ region of *LtrABC1.1*, except at the 3′ end where it lacks the last six codons. In addition, there are five point changes that give rise to three changes in the predicted amino acid sequence (not shown). Given their proximity and their high sequence similarity, these two genes probably represent a tandem duplication. Consequently, this gene was named *LtrABC1.2*. The tandem duplication of *LtrABC1.1* was confirmed by Southern blot analysis using a specific probe for *LtrABC1.1* (the loop region located between the 7th and 8th transmembrane segments of the protein; probe number no. 4 in Fig. 1) (Fig. 3A). Further, analysis of sequences contained in the GenBank database revealed a cosmid sequence of *Leishmania major* (accession number AC098845) harbouring an ORF corresponding to *LtrABC1.2*. Sequence comparison revealed nearly 90% similarity at the nucleotide level between this and *LtrABC1.1*. The differences mainly lie at the 3′ (as described between *LtrABC1.1* and *LtrABC1.2*) and 5′ ends. Since genes from different species of *Leishmania* were compared, it can be predicted that *LtrABC1.1* and *LtrABC1.2* are very similar.

3.3. Identification of an inverted repeat sequence flanking LtrABC1.1

Analysis of the nucleotide sequence of the recombinant 2B1 clone revealed the presence of a perfect inverted repeat (IR) on both sides of the coding region of *LtrABC1.1*. The extension of this IR could not be determined because the sequence located at the 3′ end was truncated by the phage arm. However, the IR sequence is at least 219 nucleotides long. Southern blot analysis (with probe no. 1 of Fig. 1), performed to determine whether this belonged to a family of *Leishmania* repeat sequences, revealed it to be only infrequently repeated in the *Leishmania* genome (data not shown). Accordingly, after searching the *Leishmania*

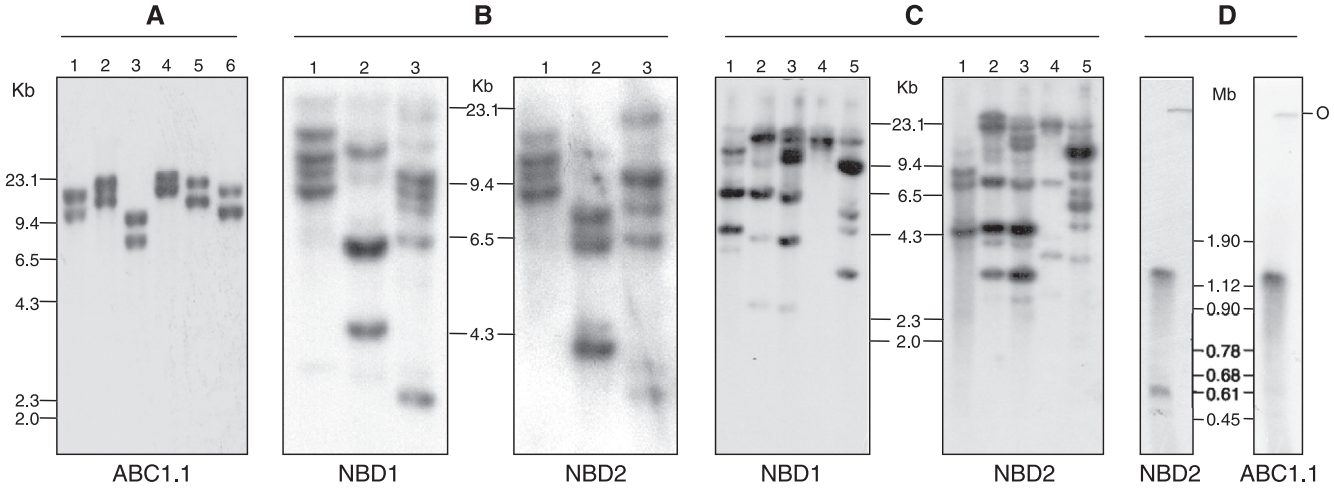

Fig. 3. Genomic organization and chromosomal localization of *LtrABC1.1* and ABCA-like genes in *Leishmania* sp. (A) Southern blot analysis of genomic DNA of *L. tropica* digested with restriction enzymes that do not cut the probe and hybridized with the specific probe for *LtrABC1.1*. Lanes: (1) *Apa*I; (2) *Eco*RI; (3) *Eco*RI/*Hin*dIII; (4) *Hin*dIII; (5) *Bgl*II; (6) *Hin*dIII/*Bgl*II. The molecular weight marker was lambda phage DNA digested with *Hin*dIII. (B) Southern blot analysis of genomic DNA of *L. tropica* digested with *Bsp*EI/*Eco*RI (1), *Bsp*EI/*Eco*RV (2), *Bsp*EI/*Bam*HI (3), enzymes that do not cut the probes, and hybridized with the NBD1 (left) and NBD2 (right) probes of *LtrABC1.1*. (C) Southern blot analysis of genomic DNA from different species of *Leishmania* digested with *Bsp*EI/*Eco*RV hybridized with the NBD1 (left) and NBD2 (right) probes of *LtrABC1.1*. Lanes: (1) *L. tropica*; (2) *L. infantum*; (3) *L. donovani*; (4) *L. braziliensis*; (5) *L. tarentolae*. (D) CHEF analysis of *L. tropica* hybridized with the NBD2 probe, which recognizes all ABCA-like genes (left), and the specific probe for *LtrABC1.1* (right). O represents origin of electrophoresis. The molecular weight marker was *S. cerevisiae* chromosomes from BioLabs.

genome database, no significant homology with any described sequences was seen for this IR.

3.4. Several sequences related to ABCA-like transporters are present in the genome of Leishmania

Since *LtrABC1.1* was found to be duplicated in tandem, experiments were performed to see whether there were any other sequences related to *LtrABC1.1* in the genome of *L. tropica*. Southern blot analysis using as probes conserved ABC transporter sequences such as those of the NBD1 and NBD2 of *LtrABC1.1* (probes no. 3 and no. 5, respectively, in Fig. 1), revealed several fragments. This supports the existence of a number of genes related to *LtrABC1.1* (Fig. 3B). Further, to determine whether this subfamily was represented by several members in other *Leishmania* species, we compared the hybridization patterns of the *L. tropica*, *L. infantum*, *L. donovani*, *L. braziliensis* and *L. tarentolae* genomes (after digestion with restriction enzymes that did not cut the probes and following hybridization with the NBD1 and NBD2 probes of *LtrABC1.1*). As observed in Fig. 3C, several fragments were recognized in all the species analyzed. These probably correspond to different genes. These results support the presence in *Leishmania* of a subfamily of transporters related to the human ABCA subfamily.

The chromosomal localization of this subfamily of transporters was then studied. The hybridization pattern of chromosomal bands of *L. tropica* was examined by clamped homogeneous electric field (CHEF) analysis using either the NBD2 probe or the specific *LtrABC1.1* probe (probes no. 5 and no. 4, respectively, in Fig. 1). The NBD2 probe hybridized with two chromosomal bands of 1.3 and 0.6 Mb, whereas the *LtrABC1.1* probe hybridized with the largest band (Fig. 3D). These results indicate that *LtrABC1.1* lies on a 1.3 Mb chromosome while the other ABCA-like genes must lie on the smallest chromosome.

3.5. Expression and processing of ABC1.1 mRNA

The mRNA expression of *ABC1.1* was analyzed by Northern blotting. No transcript was detected in wild-type promastigotes of *L. tropica* or *L. infantum* when 40 μg of total RNA or 5 μg of the poly(A)+ RNA fraction were blotted and hybridized with the *LtrABC1.1*-specific probe. This indicates that it is a low-expression gene. Indeed, RT-PCR experiments performed with RNA from *L. infantum* showed the presence of *ABC1.1* mRNA. The 5′ and 3′ processing sites in the mRNA of ABC1.1 were then characterized. To locate the spliced leader addition sites of *ABC1.1*, cDNA was synthesized from promastigotes by reverse transcription. This was amplified with spliced leader primer (LSL) and antisense primer (LABC1-20) (which hybridizes to a region 163 nucleotide downstream of the ATG triplet of *ABC1.1*). Two different *trans*-splicing sites were identified as shown in Fig. 4A, located 443 and 463

A

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TCTTTCATCGCATCGTTCGTGTGTCTCCCCTATCCATTGCCCGCTCTCGT  -736
CTTACCCCCGTTTCAAACGCGTGGTCGCGCGCGCTCTGTGCTTGCTTTGGG  -686
CCTGCACACACACACACACACACACACACTTCCCTTCCCTCCCTGGGC  -636
GTCCACATCCAGTTTTGTTCGTTTTCTCTCTTGTTCTTCCCTTTCGGCGT  -586
GACTGTGTGGGACAGGCCTCGGCACGCCAAGTCGCACGCACCGCCGTTTC  -536
GCCCTGTTTTGGCTTCTGCTTTGCTCGCTTCACCTCTCTAACCTTTTTTA  -486
TTTCTGTCGCCTTCCTTTTACAGCTCTGCGGACTGATTCACAGTGTGCTT  -436
CCGCGCCTCTTTCACCCCTTCCTTGACCACCCGCATTCGCCACCCTTTCC  -386
TCTCCTCAGAACGGAGACTCCTGGTAGCTCTGGCGGCTGTCCTCTTTCCC  -336
TCTCCCTCTGACACACTCTCTCTTGTGATTTCGCCAAGAACGTCTCTCTT  -286
GCTTGGACAGATCCGCCACGAAAAAAATCCCGAAACGGACGTCAAGCGCA  -236
TATACGGCTGAAGCCGCTGCTTTACACCAACGTTGGGCTTCACAGACTCA  -186
GCTGGCCGGTGCAGGCGCATTCGTTTTCTCGTTGCCGCTTCACACGAGCC  -136
TCCTCCCTCAGAATTCGAGAAAAGTGGGAGTTGCCGCTCACGAGCTCTCT  -86
GCACAGCGAGGAGGAAAGCGCTGACGGCGTGTGACGTGGTATCCGCGAAG  -36
AGCGGACTACTCCCTTCAACCAGAGCGAGGCGCCTATGCCTGGGGCTGGC  +15
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B

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AGCAAGAAGTCGTGCTGCGTTTGCTGCTGCGGAGGCTTACCGCGCTGATGA 5.535
GCATAAAGTAAGTGTTTGTAAGAGGTCGCGTGAGTGAACTTGTATATAGTT 5.586
TTTTTGTGCTTGTTTTTTGTCCTCGATCGTTGTCTATTGTGTAGAAGACTG 5.637
CTTTGAGGAGCGTTGGCATCAGTCGATTGGTGCTGAGTGAAGAACCGCGCA 5.688
CCTGTGCCTCTCTGTACAAGCTCAGTTGTATATATGTCGGCTCAGCTGTTT 5.739
TCGTGGTTTTTAAATTGCGCTGCTGGACAGCATAACATCAGAAGAGTTGTG 5.790
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Fig. 4. Processing sites of mRNA of LtrABC1.1. (A) The acceptor splicing AG dinucleotides at positions −443 and −463 from the translation initiation site are in bold and underlined. The polypyrimidine tract preceding the spliced leader acceptors are underlined. Underlined ATG indicates the start of *LtrABC1.1*. (B) The sites for polyadenylation at positions +246 and +248 downstream of stop codon (underlined) are in bold and are underlined.

nucleotide upstream from the putative translation initiation site of *ABC1.1*. For the identification of the polyadenylation sites, a similar strategy was used. cDNA was synthesized with an antisense primer consisting of a stretch of poly T fused to an anch sequence (ANCH + T), and was amplified with an ANCH primer and an antisense primer corresponding to the 3′ end of *ABC1.1* (LABC1-3). To increase specificity, amplification products were further amplified using a primer of the 3′ noncoding region of *ABC1.1*. Two polyadenylation sites of *ABC1.1* were identified 246 and 248 nucleotide downstream of the stop codon (see Fig. 4B). From these results, it can be deduced that the mRNA of *LtrABC1.1* is 6240 nucleotide long, with a 5′ UTR at least 443 nucleotide long and a 3′ UTR at least 246 nucleotide long.

3.6. Obtaining parasites overexpressing LtrABC1.1

To study functional aspects of the LtrABC1.1 transporter, *Leishmania* parasites were obtained that overexpress LtrABC1.1. Since the *L. tropica* strain usually maintained in our laboratory had lost its infectivity, *LtrABC1.1* was transfected into the more infective *L. infantum*. In *Leishmania*, it is known that UTRs often determine the expression efficiency or stability of mRNA. Accordingly, in the construction of the expression plasmid, the natural 5′ and 3′ noncoding regions of *LtrABC1.1* were included. After transfection, the presence of the episomal gene was revealed by cleaving of total DNA of transfected parasites with *Bam*HI

and hybridizing with the 5′ end probe of *LtrABC1.1* (probe 2 in Fig. 1). Since *Bam*HI cleaves *LtrABC1.1* and also the 3′ end of the *NEO* cassette, two hybridization bands were expected corresponding to the episomal and genomic genes in the lanes containing the DNA of transfected cell lines (Fig. 5A). Using quantitative Southern blot analysis, it was found that LABC1N-transfected parasites have around 80 gene copies. Northern blot analysis showed overexpression of a transcript longer than 6 kb corresponding to *LtrABC1.1* in parasites transfected with the LABC1N plasmid (Fig. 5B).

3.7. Expression and localization of LtrABC1.1

To obtain specific antibodies against LtrABC1.1, the region spanning the loop between the 7th and 8th transmembrane segments was selected. Amino acid sequence alignment of the ABCA subfamily identified this region as the most divergent (data not shown). Consequently, it was expected that antibodies against this polypeptide would recognize only ABC1.1 in *Leishmania*. Polyclonal antiserum against this region of LtrABC1.1 was obtained and

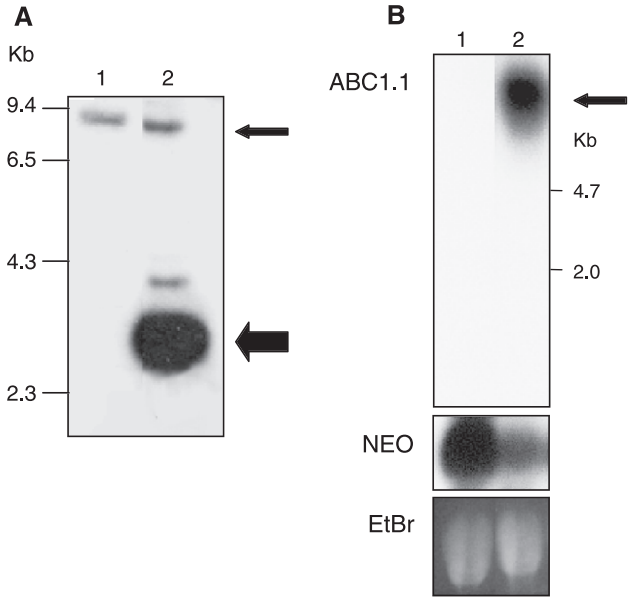

Fig. 5. Molecular characterization of transfected parasites. Lanes: (1) control parasites transfected with the pKSNEOA plasmid; (2) parasites transfected with the LABC1N plasmid. (A) Southern blot analysis of genomic DNA digested with *Bam*HI from control and *LtrABC1.1*-transfected parasites hybridized with the 5′ probe of *LtrABC1.1*. Thin arrows indicate hybridization bands of genomic copies of *ABC1.1*; thick arrows indicate episomal hybridization bands. The molecular weight marker was lambda DNA digested with *Hin*dIII. (B) Northern blot analysis of total RNA from control and *LtrABC1.1*-transfected parasites, hybridized with the specific probe for *LtrABC1.1*. Arrow indicates position of *LtrABC1.1* transcript. Lower panel corresponds to the same blot reprobed with the *NEO* probe to monitor its transcript levels. The final panel corresponds to ethidium bromide staining for monitoring the quantities of RNA.

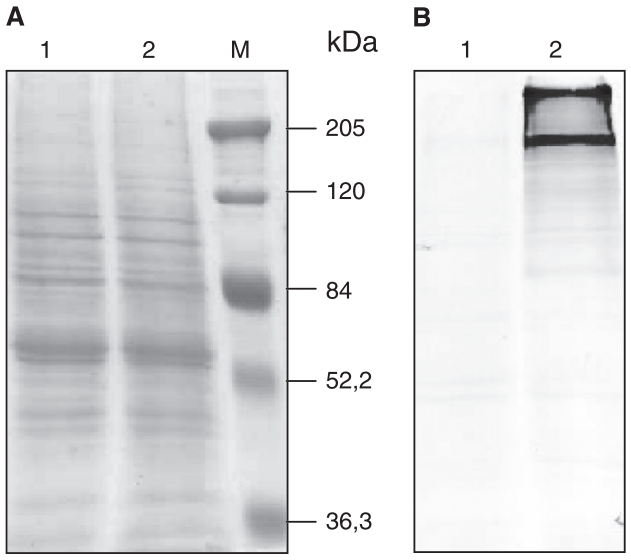

Fig. 6. LtrABC1.1 expression in *Leishmania*. Lanes: (1) parasites transfected with the pKSNEOA plasmid; (2) parasites transfected with the LABC1N plasmid. (A) Proteins stained with Coomassie Blue as loading control. (B) Western blot analysis of total proteins from transfected parasites with antiserum raised with LtrABC1.1. Molecular mass standards (kDa) are from Bio-Rad.

its specificity confirmed by Western blot of cell lysates from control and LtrABC1.1-transfected logarithmic promastigotes. As observed in Fig. 6, the antiserum detected a band of around 200 kDa in transfected parasites overexpressing LtrABC1.1. This band was weakly perceptible in control transfected parasites. To determine whether this protein was differentially expressed during promastigote growth, LtrABC1.1 expression levels were examined throughout the logarithmic and stationary phases. No changes were observed in expression levels (data not shown). To determine the subcellular localization of LtrABC1.1, indirect immunofluorescence was performed with *LtrABC1.1*-transfected promastigotes. Antiserum against LtrABC1.1 predominantly stained the flagellar pocket but also the plasma membrane and flagellum (Fig. 7B,D) in parasites transfected with the LABC1N plasmid. Control transfected parasites showed a faint stain, also mainly located in the flagellar pocket (Fig. 7A,C). No fluorescence was observed with a rabbit preimmune serum (not shown).

3.8. Drug resistance profiles of Leishmania overexpressing LtrABC1.1

Since some ABC transporters are involved in drug resistance in *Leishmania*, the question arose as to whether LtrABC1.1 overexpression could confer resistance to different compounds. Different unrelated drugs were tested including some antileishmanial agents such as the alkyllysophospholipids edelfosine and miltefosine, ketoconazole and amphotericin B, and other known ABC substrates or

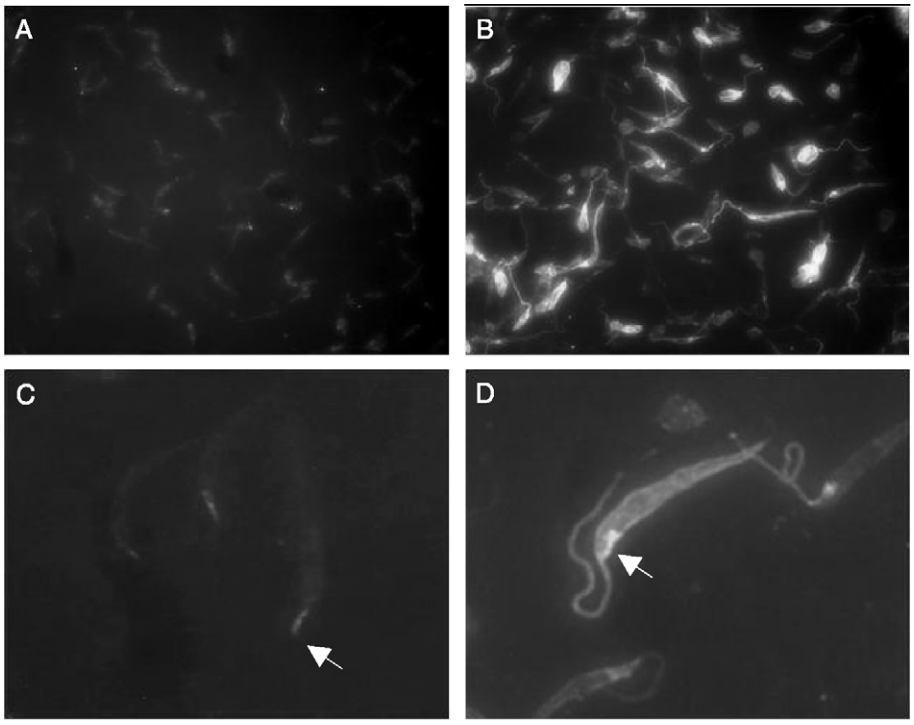

Fig. 7. Immunofluorescence localization of LtrABC1.1 in *Leishmania* promastigotes. Parasites overexpressing LtrABC1.1 were stained with anti-LtrABC1.1 polyclonal rabbit antiserum followed by fluorescein-conjugated secondary antibody. (A and C) Parasites transfected with control plasmid. (B and D) Parasites transfected with the LABC1N plasmid. Conditions of exposure were forced to visualize label in control cells. Arrowheads indicate region of flagellar pocket.

inhibitors such as doxorubicin, retinoic acid, all-*trans* retinol and glyburide. LtrABC1.1 was found to confer resistance to none of the drugs tested (data not shown).

3.9. Accumulation of fluorescent phospholipid analogues

To study the possible involvement of LtrABC1.1 in lipid transport, phospholipid accumulation was examined using short-chain fluorescent labelled phospholipids. Parasites were previously incubated in the presence of phenylmethylsulfonylfluoride to maintain labelled phospholipid integrity, and then incubated with C_6-NBD-PS, C_6-NBD-PE or C_6-NBD-PC for 30 min at 28 °C and the cell-associated fluorescence analyzed by flow cytometry. Under these conditions, the accumulation of the three assayed analogues was significantly lower in LtrABC1.1-overexpressing cells (Fig. 8). Thus, the accumulation differences observed may be a consequence of the activity of LtrABC1.1. To assess this, cells were loaded with C_6-NBD-phospholipids (C_6-NBD-PL) in an accumulation experiment performed under different conditions. While accumulation of C_6-NBD-PL at 28 °C with glucose was lower in transfected than in control parasites (ratios values of 2.5 ± 0.3 for PC, 2.5 ± 0.3 for PE, and 1.9 ± 0.1 for PS, $n = 3$), C_6-NBD-PL accumulation at 0 °C or in the presence of NaN_3 were similar for both lines (see Fig. 8). Similarly, no differences were observed when accumulation of C_6-NBD-PC occurred in the presence of the sulfhydryl modifying reagent *N*-ethylmaleimide (data not shown). These results strongly suggest that the activity of LtrABC1.1 is responsible for the observed differences in phospholipid accumulation.

3.10. LtrABC1.1 overexpression leads to a decrease in the infectivity of Leishmania

Based on the results concerning the involvement of LtrABC1.1 in phospholipid trafficking across plasma mem-

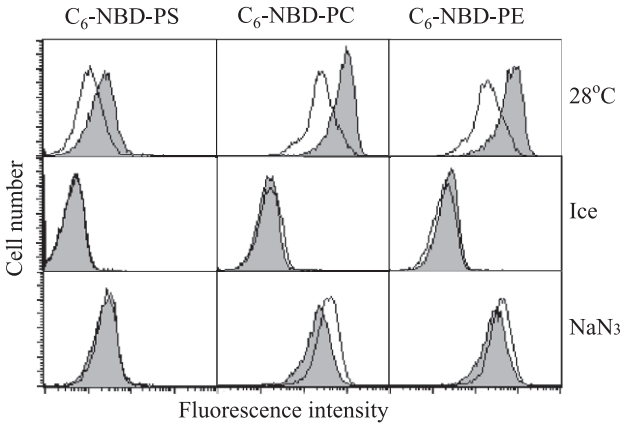

Fig. 8. C_6-NBD-PL accumulation. Parasites were incubated with short-chain fluorescent analogues of phospholipids at 28 °C or on ice (with glucose), or at 28 °C in the presence of 10 mM NaN_3 (without glucose). Cell-associated fluorescence was measured by flow cytometry analysis. A representative histogram is shown for each analogue. The grey histogram represents control transfected cells, the uncoloured histogram that of *LtrABC1.1*-transfected parasites.

brane, the question arose as to whether this activity might influence the infective capacity of *Leishmania*. In vitro infection experiments using a J774G8 macrophage cell line were therefore performed. Parasites overexpressing LtrABC1.1 were significantly ($P<0.005$) less infective ($11.8 \pm 4.2\%$) compared to transfected controls ($29.1 \pm 2.7\%$) while the ratios of number of parasites/cells were similar in both lines. The low infectivity showed by these transfected parasites is not due to an intrinsic characteristic of the clone employed in these studies since the noncloned parental line also showed low infectivity (not shown).

3.11. Vesicle trafficking may be modified in LtrABC1.1 transfected parasites

The significantly lower infectivity of parasites overexpressing LtrABC1.1 may be due to altered expression, at the parasite surface, of molecules involved in the interaction with and/or invasion of parasites into host cells. To investigate this, the exocytosis process of these parasites was studied. Secretion of acid phosphatases of *Leishmania* has been studied extensively and it has been employed as a marker for the secretory pathway [20,23]. The analysis of SAP activity, as a measure of exocytosis, indicated that parasites transfected with *LtrABC1.1* showed a significantly lower SAP activity with respect to transfected control parasites (Fig. 9). Transfected noncloned parental line also showed a similar lower SAP activity (not shown). These results indicate that the exocytosis pathway is modified in *LtrABC1.1*-transfected organisms.

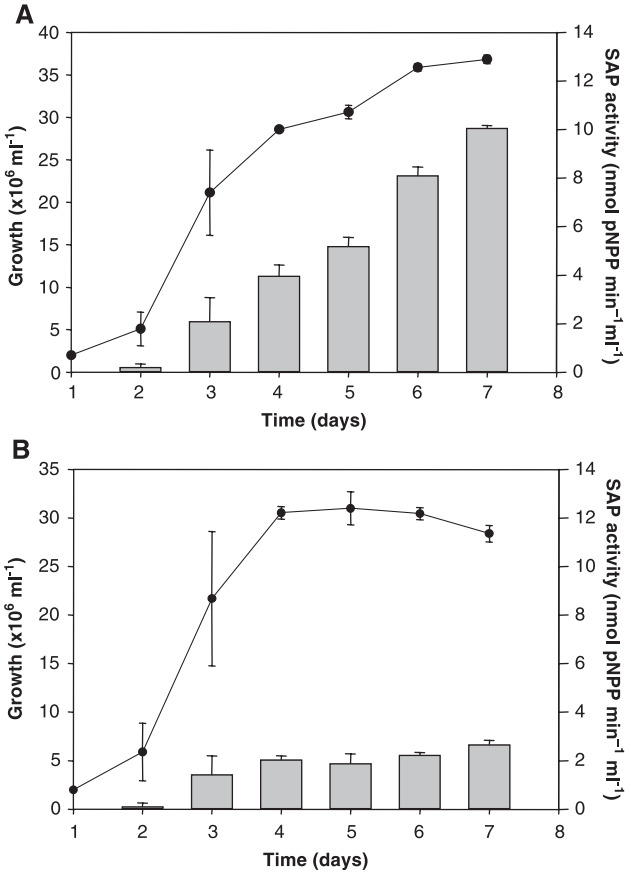

Fig. 9. SAP activity in *Leishmania* overexpressing LtrABC1.1. Bars represent the SAP activity in *Leishmania* promastigotes as nanomoles pNPP hydrolyzed per minute per milliliter (right scale). The line plot represents the growth rate of parasites over culture time (left scale). (A) Control-transfected parasites. (B) LABC1N-transfected parasites. Data are means of two independent experiments performed in duplicate ± S.D.

4. Discussion

This work identifies a new ABC transporter in the protozoan parasite *Leishmania*. Sequence homology comparison of LtrABC1.1 suggests that this transporter is related to the ABCA subfamily described in humans. The hydropathy profile of LtrABC1.1 is similar to that of other ABCA transporters, which predicts a particular membrane topology for this group, characterized by these proteins having a very large exocytoplasmic domain in both their N- and C-terminal halves [22]. This is the first report of an ABCA-like transporter in unicellular eukaryotes. It is noteworthy that, until now, it was thought that this subfamily was confined to multicellular eukaryotic organisms. No sequence related to this subfamily of transporters has been found in the complete sequenced genome of *Saccharomyces cerevisiae*. Since the order Kinetoplastidae, to which *Leishmania* belongs, is thought to have diverged very early during eukaryotic evolution, the presence of this subfamily in *Leishmania* raises the question of whether yeasts have lost genes encoding transporters related to the ABCA subfamily during their evolution. It is possible that functions normally carried out by these transporters have been acquired by others proteins of the ABC family.

4.1. Genomic and chromosomal organization of the ABCA-like genes of Leishmania

This study reveals that in the *Leishmania* genome, there are several sequences related to the ABCA-like transporters. Southern blot analysis and the examination of sequences deposited in the database of the *Leishmania* genome project http://www.ebi.ac.uk/parasites/leish.html) support the presence of at least four genes related to the ABCA subfamily. Checking for the presence of these genes in several species of *Leishmania* revealed this subfamily to be conserved and to be represented by several genes in all the species studied. The presence of the *LtrABC1.1* and *LtrABC1.2* genes in tandem and the high degree of sequence similarity between them are consistent with gene duplication and sequence divergence. Similarly, a high degree of similarity suggests that both gene products may have similar functions in the parasite.

LtrABC1.1 ORF is flanked on both sides by an IR sequence that is conserved at the nucleotide level. It is

known that *Leishmania* has repeat sequences, some of which are involved in recombination processes that give rise to genomic rearrangements finally resulting in amplification of particular chromosomal regions [24]. Therefore, the locus containing *LtrABC1.1* might be considered a "hot site" that suffers rearrangements under particular circumstances, for example when under drug pressure. Indeed, *Leishmania* is very efficient at using directed and inverted repeat sequences to induce amplicons, as in the H circle [1]. It has been demonstrated that, in vitro, the H circle is amplified using repeat sequences as a response to drugs [25]. However, *LtrABC1.1* does not seem to be a resistant gene since no correlation between resistance and LtrABC1.1 expression was observed with the unrelated compounds tested in this study.

LtrABC1.1 appears to be a low-expression gene since its mRNA is not revealed in Northern blot analysis. However, RT-PCR studies for identifying the processing sites at the 5′ and 3′ ends revealed that this gene is indeed expressed in *Leishmania* promastigotes. Therefore, to know the function of this ABC transporter in *Leishmania*, transfected parasites that overexpress LtrABC1.1 were obtained. Since the 5′ and 3′ UTRs of *Leishmania* genes are important for suitable expression, the natural 5′ and 3′ noncoding regions of *LtrABC1.1* gene were included in the plasmid construct. This might account for the good expression level obtained with this construct.

4.2. Role of LtrABC1.1 in lipid and vesicle trafficking

The results presented here provide evidence that LtrABC1.1 is involved in lipid transport across the plasma membrane of this parasite. The *Leishmania* line overexpressing LtrABC1.1 showed significantly less accumulation of C_6-NBD analogues of phospholipids. In fact, accumulation levels reverted to values similar to those of control cells when accumulation was performed on ice or in the presence of sodium azide (conditions that inhibit energy-dependent protein activities). Similar results were obtained when C_6-NBD-PC accumulation was performed in the presence of the sulfydryl modifying agent *N*-ethylmaleimide, further supporting the idea that the accumulation differences observed are the consequence of protein activities. It has been reported that the mammalian ABC transporters Mdr1 and Mdr2 function as lipid floppases. However, while Mdr2 has been described as a PC translocator, Mdr1 appears to be a more general phospholipid transporter [26]. Similarly, the activity of LtrABC1.1 appears to be independent of the polar heads of phospholipids since parasites overexpressing this protein accumulated less of all three analogues tested (PS, PC and PE). It is striking that *LtrABC1.1*-transfected parasites do not show resistance to the ether lipid edelfosine, which is structurally similar to the ester lipid C_6-NBD-PC. However, structural differences between these two compounds might explain the absence of resistance. LtrABC1.1 may be responsible for the decreased accumulation of phospholipid analogues as a consequence of efflux activity across the plasma membrane or through facilitating the sorting and packaging of lipids into their transport vesicles for exocytosis from cells, as proposed for mammalian ABCA1 [27]. ABCA1 is involved in an apolipoprotein-mediated lipid efflux pathway (reviewed in Ref. [28]) and its activity also influences vesicle trafficking. For example, it has been described that ABCA1 induces the rearrangement of actin cytoskeletons through the possible interaction with the Cdc42/N-WASP pathway [29]. Besides, it has been demonstrated that the expression of members of the Rho GTPase family is altered in cells from Tangier disease patients [30,31]. All these observations suggest that ABCA1 is involved in vesicular transport through interaction with the components of this pathway. In fact, endocytosis is enhanced in Tangier cells [32]. It is noteworthy that LtrABC1.1 protein appears to be predominantly expressed at the flagellar pocket and that this region is thought to be the sole site for exocytosis and endocytosis in *Leishmania* [33]. It is therefore possible that at this location, LtrABC1.1 may regulate membrane movements such as endocytosis, exocytosis and vesicle trafficking. Indeed, in this study, it was observed that exocytosis (measured as SAP activity) in parasites overexpressing LtrABC1.1 is altered. These findings could explain why parasites overexpressing LtrABC1.1 are less infective than controls. It is suggested that altered vesicle traffic would result in surface alterations where specific molecules, some involved in the infection process, may be affected. It has been proposed that ABCA1 functions as a PS floppase [13]. However, the lipid substrates for this transporter have not yet been clearly defined, and it has been proposed that ABCA1 may be a protein with regulatory functions that directs membrane trafficking [34,35]. In *Leishmania*, it remains to be clarified whether LtrABC1.1 is a primary transporter that directly translocates phospholipids across the plasma membrane or whether it is a secondary transporter with mainly regulatory functions.

The majority of eukaryotic cell membranes are asymmetric in phospholipid distribution across the bilayer, and loss of asymmetry in multicellular organisms (which induces the surface exposure of PS) triggers important biological events such as the coagulation cascade in platelets, the recognition and clearance of apoptotic cells by phagocytic cells, and lymphocyte differentiation [36]. However, little is know about the physiological significance of phospholipid distribution and movement in unicellular organisms such as *Leishmania*. It is possible that the membrane lipids and protein machinery have specific functions in parasite differentiation and/or infectivity.

Acknowledgements

The authors would like to thank Pilar Navarro for help with parasite cultures. A.P.T. was the recipient of a

fellowship from the Agencia Española de Cooperación Internacional (MUTIS grant). This work was supported by the Spanish Grants SAF2001-1039 (S.C.) and PPQ2000-1655-C02-02 (F. G.), and the Plan Andaluz de Investigación (Research Group CVI130).

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