Overexpression of LaMDR2, a novel multidrug resistance ATP-binding cassette transporter, causes 5-fluorouracil resistance in *Leishmania amazonensis*[1]

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Abstract The ATP-binding cassette (ABC) proteins play an important role in drug resistance and detoxification in various organisms. Here we isolated LaMDR2, a new member of the multidrug resistance (MDR) subfamily of ABC proteins in *Leishmania amazonensis*. LaMDR2 exhibited 47% amino acid identity to its most closely related protein, LaMDR1, which was previously isolated from the same species. Promastigotes that overexpressed LaMDR2 showed significant resistance to 5-fluorouracil (5-FU), but not to LaMDR1 substrates. Expression of LaMDR2 in the transfectants was relatively higher in the log phase than the stationary phase, and a lower accumulation of [^{3}H]5-FU was observed in the log-phase cells. These results suggest that LaMDR2 is involved in extrusion of xenobiotics, but functionally different from LaMDR1.

Key words: ATP-binding cassette transporter; Multidrug resistance; 5-Fluorouracil; *Leishmania*

1. Introduction

Protozoan parasites of the genus *Leishmania* cause a wide spectrum of human diseases ranging from mild cutaneous leishmaniasis to fatal visceral leishmaniasis (kala-azar). Leishmaniasis is endemic in 88 countries, and the overall prevalence is estimated at 12 million [1]. The primary control measure against leishmaniasis is treatment of patients with pentavalent antimonial drugs, although the precise mechanism of action of antimonials is unknown. However, the emergence of antimony-resistant *Leishmania* parasites is evident in different endemic areas [2–4]. Studies on the mechanisms of drug resistance are thus crucial for a more rational use of drugs to minimize or overcome resistance. Resistance in clinical cases is not easily defined in biological terms, but studies on parasites selected for drug resistance in the laboratory have revealed that several mechanisms are involved in drug resistance, including a decrease in drug uptake, active drug extrusion, loss of drug activation, and alteration of drug targets [5–7].

The ATP-binding cassette (ABC) protein superfamily is one of the largest families found in both eukaryotic and prokaryotic cells [8–12]. In humans, the 48 known ABC proteins are classified into seven distinct subfamilies and implicated in a number of serious diseases [12]. Multidrug resistance protein (MDR1) in the ABCB (MDR/TAP) subfamily and multidrug resistance-associated protein 1 (MRP1) in the ABCC (CFTR/MRP) subfamily are the most extensively studied in relation to drug resistance. MDR1 transports a wide variety of structurally unrelated anticancer drugs in addition to physiological substrates, including ions, phospholipids, steroids, amino acids and peptides by coupling drug/lipid efflux with energy derived from the hydrolysis of ATP [13]. MRP1 transports organic anions and drugs conjugated to glutathione, glucuronate or sulfate [5].

In *Leishmania*, MDR (ABCB), MRP (ABCC) and ABCA subfamily genes have been identified and characterized [6,7,14], and 25 ABC transporter family-like proteins are listed in the recent *Leishmania major* Gene DB, (http://www.genedb.org/genedb/leish). Previously, we isolated the *Leishmania amazonensis MDR1* gene, which shared 78% and 91% amino acid identity with *LeMDR1* and *LdMDR1* from different *Leishmania* species, respectively [15–17]. Overexpression of these leishmanial MDR1 genes in their transfectants conferred resistance to certain anticancer drugs, including vinblastine, although their physiological functions remain unknown. Analysis of arsenite-resistant *Leishmania tarentolae* led to the discovery of the *LtPGPA* gene, the first eukaryotic MRP-like gene [18]. Overexpression of *LtPGPA* in the transfectants conferred resistance to oxyanions, such as arsenite and antimony [19]. The LtPGPA tagged with green fluorescent protein (GFP) was shown to be located in the intracellular vesicular membrane close to the flagellar pocket, suggesting that PGPA confers resistance by sequestering metal-thiol conjugates into intracellular vesicles [20]. Five other PGP subfamily proteins

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Abbreviations: ABC, ATP-binding cassette; MDR, multidrug resistance; 5-FU, 5-fluorouracil

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were identified in *Leishmania*, but MDR1 was the only MDR subfamily protein reported [7].

In the present study, we report the isolation and characterization of the *LaMDR2* gene in *L. amazonensis*, which encodes a new member of the MDR (ABCB) subfamily of *Leishmania* ABC proteins. We found that overexpression of the *LaMDR2* gene in wild-type promastigotes conferred resistance to 5-fluorouracil (5-FU), a fluoropyrimidine used in cancer chemotherapy. A decreased accumulation of 5-FU was observed in the transfectants that overexpressed LaMDR2 proteins. These results suggest that MDR2 plays a role in efflux of toxic compounds. Although 5-FU and vinblastine are not clinically used for treatment of leishmaniasis, an understanding of the molecular mechanisms of drug resistance mediated by ABC transporters in *Leishmania* will provide further insight into novel chemotherapeutic strategies against this worldwide tropical disease.

2. Materials and methods

2.1. Growth study

Promastigotes of *L. amazonensis* (MPRO/BR/72/M1845, LV78) were cultivated at 26°C in Medium 199 supplemented with 15% heat-inactivated fetal calf serum and 25 mM HEPES [21]. A growth inhibition test was performed against anticancer drugs (vinblastine, puromycin, doxorubicin and 5-FU), anti-leishmanial drugs (Pentostam® and amphotericin B), anti-malarial reagents (chloroquine, mefloquine and artesunate), heavy metals (sodium arsenite and $CdCl_2$), and nucleoside analogues (formycin B and tubercidin). These chemicals were obtained from Sigma Chemicals (St Louis, MO, USA), except for Pentostam (Wellcome Foundation, London, UK), mefloquine and artesunate (Mepha, Aesch-Basel, Switzerland). Promastigotes were incubated in each well of a 96-well, flat-bottomed microplate at a concentration of 2×10^5 cells/ml in a volume of 200 μl in the presence of two-fold serial dilutions of the drug. The plates were incubated at 26°C for 4–5 days until control cultures without the drug reached maximum growth [17]. The number of parasites in culture was counted using a hemocytometer. Growth curves of promastigotes were also determined by measuring the absorbance for the cultures at 600 nm [18]. The effective concentrations of the drug, defined as concentrations at which parasite growth was inhibited by 50% (ED_{50}), were calculated.

2.2. Cloning and sequencing

The sense (5′-GGCTCGTCGGGCTGCGGG/CAA-3′) and antisense (5′-GTCCAGCGCC/TGAC/TGTCGCCTCG/ATC-3′) primers were synthesized according to conserved amino acid residues (GSSGCGK and DEATSALD) within the ABC region [17]. A polymerase chain reaction (PCR) product of 408 bp amplified from *L. amazonensis* DNA was cloned into the *Sma*I site of pUC18 plasmid. Two clones showed the same nucleotide sequence (136 amino acids), which was 67% identical to the NH_2-terminal ABC region of the *LdMDR1* gene [15]. One clone (LA10) was used as a probe to isolate genomic counterparts from a λGEM-11 genomic library prepared from *L. amazonensis* DNA. Thirteen phages (λ-01 to λ-13) were obtained with the LA10 probe. Four *Sac*I fragments (0.34, 1, 2.5 and 6 kb) of λ-13 phage were subcloned into pUC19 or pBluescript plasmid. The 1-kb *Sac*I fragment containing the 5′ region corresponding to the NH_2-terminal ABC region of the *LdMDR2* gene was first sequenced by a primer walking method. Finally, a total of 4526 nucleotides, including 3801 nucleotides for an open reading frame from four *Sac*I fragments, were sequenced in both directions using a total of 27 primers that were designed according to the nucleotide sequences determined in each sequencing process.

2.3. DNA constructs and transfection

An approximately 7.0-kb *Hin*dIII fragment of the λ-13 phage was subcloned into the pcDNA3 plasmid. To generate p6.5-LaMDR2/7, the *Hin*dIII fragments were ligated to *Bam*HI linker and inserted into the *Bam*HI site of the *Leishmania*-specific expression vector p6.5, which contained the *N*-acetylglucosamine-1-phosphate transferase gene (*nagt*) as a selective marker for tunicamycin selection [22,23]. The entire *LaMDR2* was amplified by PCR using the sense primer (5′-GGG**GGATCC**ATGTCCGCCGATAAAAAG-3′) and the antisense primer (5′-AGCT**GGATCC**TCACGTCTGGGCTAGCTCCCA-3′). Both primers contained a *Bam*HI site (indicated by bold letters). The sense and antisense primers contained the start codon (underlined) and the stop codon (doubly underlined) of LaMDR2, respectively. The PCR products were digested with *Bam*HI and cloned into the *Bam*HI site of p6.5 to generate p6.5-LaMDR2/3.8. These expression vectors were transfected into wild-type promastigotes by electroporation [17]. Cells were then cultured in the presence of tunicamycin (Sigma) at 10 μg/ml. Drug concentration was increased to 20 and 40 μg/ml, and transfectant cell lines at each drug concentration were obtained.

2.4. DNA and RNA manipulations

DNA extract (500 ng) was digested with different endonucleases and blotted to a nylon membrane (Hybond-N^+, Amersham) in 20×SSC (1×SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.4) for Southern hybridization. RNA extract (10 μg) was also transferred to a nylon membrane in 20×SSC for Northern hybridization. The PCR-amplified entire 3.8-kb *LaMDR2* and 1.34-kb *L. amazonensis* β-tubulin genes were used as probes [17]. Probes were labeled with digoxigenin (DIG), hybridized to blots in a hybridization solution (5×SSC, 2% non-fat milk, 0.02% sodium dodecyl sulfate (SDS), 0.1% sarcosyl and 50% formamide) at 42°C, and detected using a DIG DNA labeling and detection kit (Boehringer-Mannheim, Mannheim, Germany). Messenger RNA was purified with Oligotex-dT30 column (Takara Bio, Tokyo, Japan) and subjected to reverse transcription-PCR (RT-PCR). The sense primer (5′-CGACCGTTCGCCAGCTG-3′), the antisense primer (5′-GCCGGCCATTGCCACAA-3′) and SuperScript One-Step RT-PCR system (Invitrogen) were used for amplification of 337-bp DNA fragments from the *LaMDR2* mRNA.

2.5. Western blot analysis

Rabbit anti-LaMDR2 peptide antibodies were produced against a synthetic peptide of 13 amino acids (DGEYKSRWELAQT), corresponding to positions 1255–1267 of the COOH-terminus of LaMDR2 protein. Mouse anti-LaMDR2 recombinant antibodies were produced by immunization of BALB/c mice with histidine-tagged recombinant proteins purified from *Escherichia coli*, which were transfected with pQE-32 (Qiagen) carrying a PCR product (LA10) corresponding to 136 amino acids of the NH_2-terminal ABC region of LaMDR2. Wild-type promastigotes and transfectants were harvested in different growth phases: the early-log (EL), mid-log (ML), late-log (LL), early-stationary (ES), and mid-stationary (MS) phase. Cells were lysed in a sample buffer (50 mM Tris–HCl, 1% SDS, 20% glycerol and 1% 2-mercaptoethanol, pH 6.8) at 85°C for 10 min. Each sample (equivalent to 5×10^5 cells) was electrophoresed on 5–20% gradient SDS–polyacrylamide gels. Proteins were transferred to a 0.22-μm polyvinylidene difluoride (PVDF) membrane and reacted with rabbit anti-LaMDR2 peptide antibodies, mouse anti-LaMDR2 recombinant antibodies, and rabbit anti-*Leishmania* surface membrane protein gp63 antibodies [23]. Mouse monoclonal antibody against chicken α-tubulin was obtained from ICN Biomedicals (Aurora, OH, USA). The sera were used at a 1:250–1000 dilution. Reactions were visualized with the ECL-Plus Western Blotting Detection System on Hyperfilms (Amersham).

2.6. Transport study

Wild-type cells and transfectants in the ML, LL, and ES phase were harvested. Cells were washed twice with a washing medium (15 mM KCl, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$ and 50 mM triethanolamine-HCl, pH 6.9), and suspended in an incubation medium (15 mM KCl, 10 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1 mM glucose and 50 mM triethanolamine-HCl, pH 6.9) at 25°C [19]. The cells (1×10^7 cells/400 μl) were incubated in the presence of 0.1 or 3 μM [^{3}H]5-FU (37 MBq/ml, Du Pont, France), and 1 or 3 μM [^{3}H]vinblastine (9.25 MBq/ml, Amersham) at 25°C for up to 120 min. At several time points, cells were fixed with 10% formaldehyde in ice-cold washing medium, and washed four times with the washing medium. Cells were then lysed with the lysis solution (Cell Culture Lysis Reagent, Promega) and added with the scintillator solution. The radioactivity was measured and accumulation of [^{3}H]5-FU or [^{3}H]vinblastine was expressed as pmol/10^9 cells.

3. Results

3.1. Homology of LaMDR2 to other ABC proteins

The open reading frame of the *LaMDR2* gene encoded a protein of 1267 amino acids, consisting of two similar halves, each containing six putative transmembrane domains, Walker A, motif C and Walker B (Fig. 1). LaMDR2 protein showed no potential Asn-linked *N*-glycosylation sites at the putative extracellular side. Alignment of deduced amino acid sequences of LaMDR1 and LaMDR2 indicates that LaMDR2 has a longer hydrophilic region between NH_2-terminal Walker B and the seventh transmembrane domain than LaMDR1 (Fig. 1). LaMDR2 showed 47% amino acid identity to LaMDR1 [17], 29% to LtPGPA [18] and less than 40% to ABC proteins in other species, including human MDR1 (ABCB1), human MDR3 (ABCB4), and *Plasmodium falciparum mdr1* gene products (PGH-1).

3.2. Southern and Northern blot analysis of transfectants

The p6.5 vector contained an ~7.5-kb *Leishmania* DNA, in which the upstream p36 gene was replaced with a *Bam*HI site for cloning and the downstream *nagt* gene was kept as the selective marker for tunicamycin [22]. The p6.5-LaMDR2/7 carried an insert ~7-kb DNA fragment consisting of the 5′-flanking (~1.3 kb), coding (3.8 kb) and 3′-flanking (~1.9 kb) regions of the *LaMDR2* gene. Southern blot analysis indicated that the gene is present as a single copy (data not shown). Based on the hybridization intensities against the 1.2-kb *Sal*I fragments (arrowhead in Fig. 2A), the copy numbers of p6.5-LaMDR2/7 were estimated at approximately 13, 24 and 42 in cell lines maintained in the presence of tunicamycin at 10, 20 and 40 μg/ml, respectively (Fig. 2A). By Northern blot analysis, a major 7.2-kb and a minor 5.9-kb *LaMDR2* gene transcript were detected in total RNA samples from these transfectants (Fig. 2B). Transfectants with p6.5-LaMDR2/3.8, which carried only the PCR-amplified coding region of the *LaMDR2* gene, produced a major 5.2- and a minor 7.4-kb *LaMDR2* RNA (Fig. 2B). Although the corresponding transcripts were hardly detected from wild-type cells and transfectants with p6.5 by Northern blots, the *LaMDR2* mRNAs were detected by RT-PCR (data not shown).

3.3. Expression of LaMDR2 proteins in transfectants

Total protein was extracted from promastigotes in different growth phases and reacted with anti-LaMDR2 peptide antibodies, which were raised against the COOH-terminal 13 amino acids of LaMDR2 protein. The antibodies clearly recognized a single protein of approximately 140 kDa in transfectants with p6.5-LaMDR2/7, but hardly detected the corresponding protein band in transfectants with p6.5 (Fig. 3A). The antibodies also detected LaMDR2 proteins in transfectants with p6.5-LaMDR2/3.8, although the LaMDR2 ex-

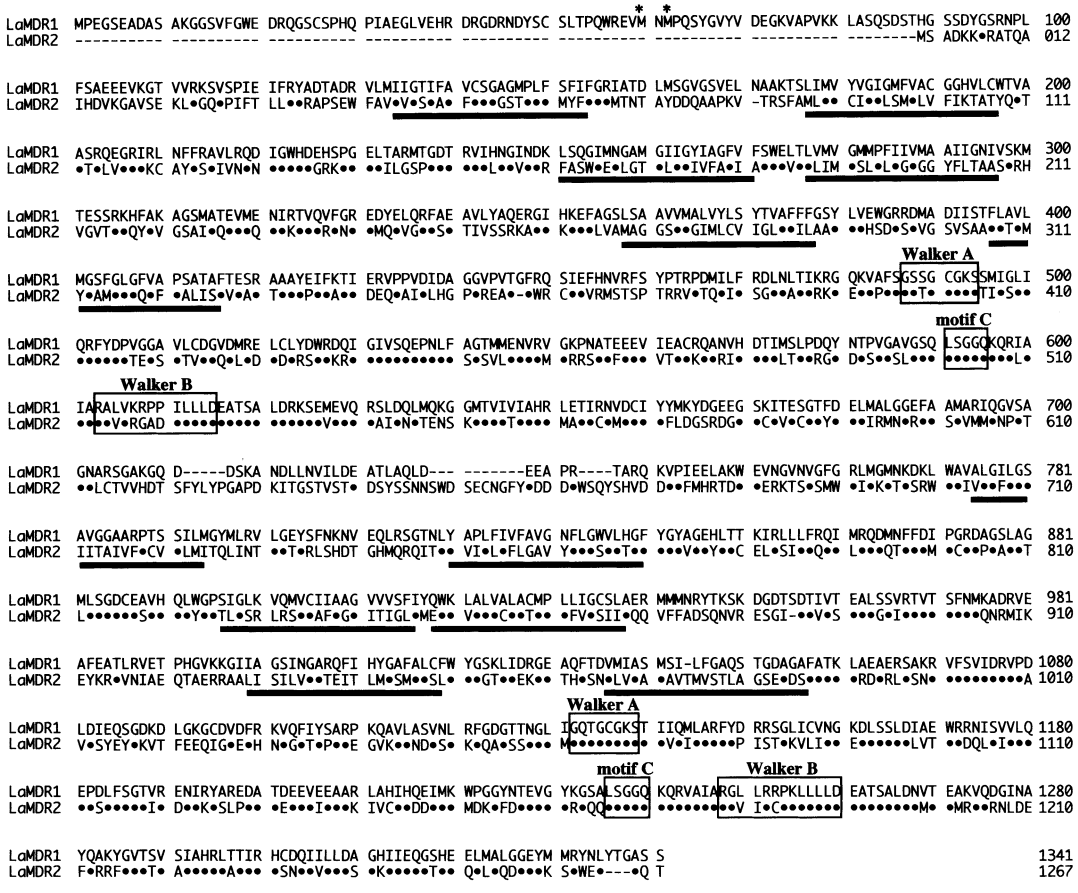

Fig. 1. Comparison of the deduced amino acid sequences of LaMDR2 (DDBJ accession number AB008469) to LaMDR1 (DDBJ AB003329) in *L. amazonensis*. Amino acids identical to LaMDR1 are indicated with dots. Twelve putative transmembrane domains, which are derived from a computer prediction, are thickly underlined. Two other in-frame methionine translation start sites of LaMDR1 are indicated with asterisks. Walker A, Walker B and motif C are indicated with boxes.

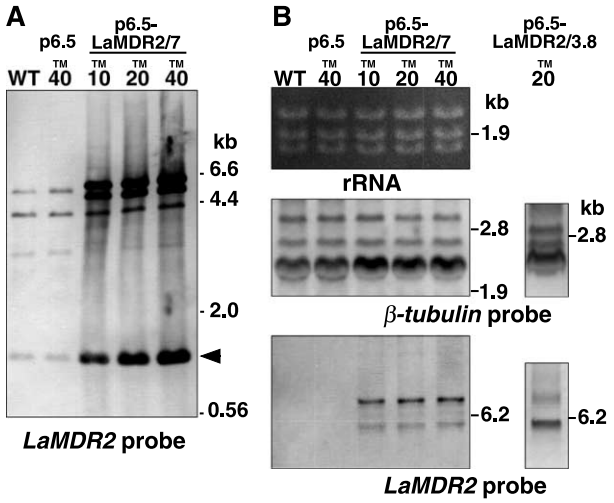

Fig. 2. Southern and Northern blot analyses of transfectants with the *LaMDR2* gene. Total DNA was isolated from wild-type cells, transfectants with p6.5 maintained in the presence of tunicamycin at 40 μg/ml, and transfectants with p6.5-LaMDR2/7 maintained in the presence of tunicamycin at 10, 20 or 40 μg/ml. The DNA samples were digested with *Sal*I, electrophoresed on 0.8% agarose gel, transferred to a nylon membrane and hybridized with the DIG-labeled *LaMDR2* gene probe (A). The arrowhead in A indicates the hybridization signals to the 1.2-kb *Sal*I DNA fragment. Total RNA was isolated from the same cell lines and in addition from transfectants with p6.5-LaMDR2/3.8 maintained with tunicamycin at 20 μg/ml. The RNA samples were electrophoresed on 0.8% RNA agarose gel, transferred to a nylon membrane and hybridized with DIG-labeled β-tubulin gene or *LaMDR2* gene probe (B).

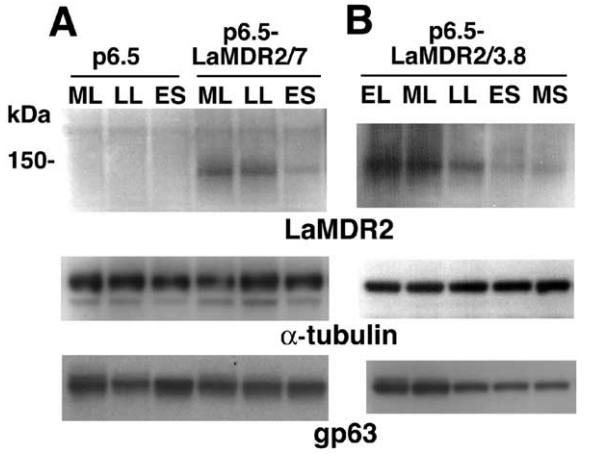

Fig. 3. Western blot analysis of LaMDR2 expression in transfectants with the *LaMDR2* gene. Promastigotes were harvested from transfectants with p6.5, p6.5-LaMDR2/7 and p6.5-LaMDR2/3.8 in the EL, ML, LL, ES and MS growth phases. Each cell lysate equivalent to 5×10^5 was electrophoresed on a 5–20% gradient polyacrylamide gel and transferred to a PVDF membrane. The membrane was immunostained with rabbit anti-LaMDR2 peptide antibodies, mouse anti-chicken α-tubulin monoclonal antibodies, or rabbit anti-*Leishmania* gp63 antibodies. Reactions were visualized with the ECL-Plus chemiluminescence system.

pression was gradually decreased in the following order: EL, ML, LL, ES and MS phase (Fig. 3B). However, we found no degradation of LaMDR2 proteins in these immunoblots. Mouse anti-LaMDR2 recombinant antibodies, which were raised against a recombinant protein of 136 amino acids in the NH_2-terminal ABC region of LaMDR2, also recognized a protein of similar size in the same transfectant cell lines (data not shown). Expression of gp63 surface membrane proteins was slightly decreased in the stationary phase, whereas α-tubulin was constitutively expressed regardless of the cell growth phase (Fig. 3A,B).

3.4. Involvement of LaMDR2 in 5-FU resistance

The ED_{50} value against 5-FU was 2.5-fold higher (11.2 ± 2.3 vs. 4.5 ± 0.6 μM, $P<0.01$) in transfectants with p6.5-LaMDR2/7 than in transfectants with p6.5 (Table 1). Although the degree of resistance was two- to three-fold, the difference was always observed and reproducible. No cross-resistance was observed against the following compounds: puromycin and doxorubicin as anticancer drugs, Pentostam and amphotericin B as anti-leishmanial drugs, chloroquine, mefloquine and artesunate as anti-malarial reagents, formycin B and tubercidin as nucleoside analogues, and $CdCl_2$. Unexpected hypersensitivities were observed with respect to sodium arsenite and vinblastine (Table 1). We verified that the *LaMDR2* gene alone was sufficient to confer 5-FU resistance, since the ED_{50} value of 5-FU was 9.5–11.5 μM in transfectants with p6.5-LaMDR2/3.8, which

Table 1
Drug susceptibility of *L. amazonensis* overexpressing LaMDR2

Drug	ED_{50}[a]		Relative drug Resistance[b]
	p6.5 (*n*)	p6.5-LaMDR2/7 (*n*)	
Sodium arsenite (μM)	6.0 ± 1.6 (4)	4.2 ± 0.8 (4)	0.70*
Puromycin (μM)	1.9 ± 0.3 (6)	2.0 ± 0.5 (3)	1.05
Vinblastine (μM)	7.8 ± 0.6 (3)	5.0 ± 0.9 (3)	0.64**
Doxorubicin (μM)	1.40 ± 0.05 (3)	1.71 ± 1.1 (3)	1.24
Amphotericin B (μg/ml)	3.4 ± 0.7 (3)	3.2 ± 0.2 (3)	0.94
Pentostam (mg/ml)	220 ± 59 (5)	222 ± 20 (3)	1.01
5-FU(μM)	4.5 ± 0.6 (8)	11.2 ± 2.3 (6)	2.50**
Chloroquine (μM)	13.3 ± 3.9 (3)	13.8 ± 1.3 (3)	1.04
Mefloquine (μg/ml)	1.3 ± 0.5 (5)	1.3 ± 0.4 (3)	1.00
Artesunate (μM)	19.7 ± 13.3 (3)	23.5 ± 8.5 (3)	1.18
$CdCl_2$ (nM)	113 ± 30 (3)	99 ± 34 (3)	0.88
Formycin B (nM)	104 ± 17 (4)	93 ± 11 (3)	0.89
Tubercidin (nM)	155 ± 51 (4)	146 ± 31 (4)	0.94

Significance: *$P<0.05$; **$P<0.01$.
[a]The ED_{50} value is the mean ± S.D. of three to eight independent experiments.
[b]Relative drug resistance is the ratio of the p6.5-MDR2/7 ED_{50} divided by the p6.5 ED_{50}.

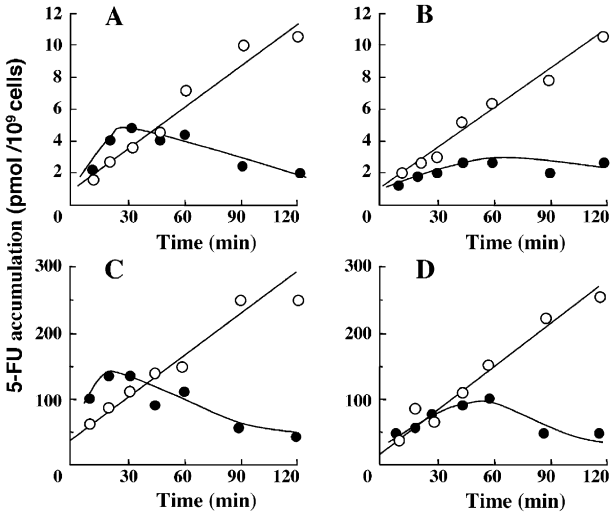

Fig. 4. Accumulation of [^{3}H]5-FU in transfectants with the *LaMDR2* gene in different growth phases. Transfectants with p6.5 (open circles) and p6.5-LaMDR2/7 (filled circles) in the ML (A,C) and LL (B,D) phase were incubated in the presence of [^{3}H]5-FU at 0.1 μM (A,B) and 3 μM (C,D). The radioactivity in these cells was then measured. A typical result in three independent experiments is shown.

contained only the PCR-amplified coding region of the *LaMDR2* gene.

3.5. Decreased accumulation of 5-FU in transfectants overexpressing LaMDR2

We examined the uptake and accumulation of [^{3}H]5-FU in transfectants in different growth phases. We used two different 5-FU doses, 3 μM and 0.1 μM. The former dose was just below the ED_{50} value of wild-type cells and transfectants with p6.5, and the latter concentrations showed no effects on cell growth. In transfectants with p6.5, 5-FU accumulation was continually increased up to 120 min, regardless of the cell growth phases and the drug concentrations (Fig. 4A–D). In contrast, in transfectants with p6.5-LaMDR2/7 in the ML phase, 5-FU uptake was 1.5-fold greater than that in control transfectants in the first 30 min, and the drug accumulation was gradually decreased to 20–30% of the control level at 90 min at both drug concentrations (Fig. 4A,C). Although this initial increase of drug accumulation was not observed in the LL phase cells, the drug accumulation was also markedly decreased at both drug concentrations (Fig. 4B,D). However, when cells from the ES phase were used, 5-FU accumulation was continually increased in both transfectants with p6.5 and p6.5-LaMDR2/7 up to 120 min, and no difference was found in the accumulation kinetics between these transfectant cell lines (data not shown). In addition, accumulation of [^{3}H]vinblastine was similarly increased up to 120 min in both transfectant cell lines regardless of the growth phase, suggesting that overexpression of LaMDR2 was specific to a decreased accumulation of 5-FU (data not shown).

4. Discussion

In the present study, we isolated and characterized a novel ABC protein, LaMDR2, in the protozoan parasite *Leishmania*. Putative LaMDR2 had a typical MDR structure, but exhibited only 47% amino acid identity to its most closely related protein, LaMDR1, which was recently isolated from the same species [17]. Promastigotes that overexpressed LaMDR2 showed significant resistance to 5-FU, but not to *Leishmania* MDR1 substrates, such as vinblastine, puromycin or doxorubicin [15–17]. In addition, transfectants with *LaMDR2* exhibited a reduced accumulation or active extrusion of 5-FU. These results clearly suggest that LaMDR2 is structurally and functionally different from LaMDR1, and represents a new member of the MDR (ABCB) subfamily of leishmanial ABC proteins.

Overexpression of LaMDR2 conferred 5-FU resistance in *Leishmania* promastigotes. This is the first report of a MDR (ABCB) subfamily protein of ABC transporters causing 5-FU resistance in eukaryotic cells. Human MRP1 of the ABCC subfamily conferred 5-FU resistance in NIH 3T3 cells [24]. A variety of substrate specificity or functional diversity exists for each ABC subfamily protein among different organisms [8,9,11]. In *Leishmania*, two MDR (ABCB) subfamily genes and five PGP (ABCC) subfamily genes have been characterized ([20], present study). Recently, the first leishmanial ABCA subfamily protein was reported to be involved in phospholipid trafficking [14].

The subcellular localization of LaMDR2 proteins is unknown, although our preliminary indirect immunofluorescence studies showed that anti-LaMDR2 antibodies predominantly stained the flagellar pocket and asymmetrical tubular structure in the transfectants. The tubular structure may correspond to the multivesicular tubule lysosome in *Leishmania* [25–27]. The *Plasmodium falciparum* MDR protein (PGH-1) is located in the food vacuole membrane and associated with chloroquine resistance [28]. Studies on further immunofluorescence and immunoelectron microscopy are under way to clarify whether LaMDR2 is associated with the lysosomal membrane. Alternatively, LaMDR2 may stimulate or regulate other molecule(s), which enhance the uptake or export of 5-FU. A highly selective and high-affinity H^+-dependent transporter for uracil was reported in procyclic forms of *Trypanosoma brucei brucei* [29]. This uracil uptake was inhibited by 5-FU, but not by a broad range of purine and pyrimidine nucleosides and nucleobases, suggesting a nucleobase-H^+-symporter model for this carrier. It is unknown whether leishmanial carrier-dependent nucleobase transporters are involved in 5-FU uptake or export.

Finally, the physiological function of LaMDR2 remains to be elucidated. Expression of LaMDR2 in wild-type promastigotes was undetectable by Western blot analysis, although the corresponding transcripts were detected by RT-PCR. Mammalian MDR2, the other member of the MDR subfamily, is involved in the transport of long-chain phospholipids such as phosphatidylcholine [12]. A daunomycin-resistant *L. tropica* cell line, which overexpressed a P-glycoprotein-like transporter (probably LtMDR1), displayed significant cross-resistance to miltefosine (alkyl-lysophospholipid or hexadecylphosphocholine) [30], a new chemotherapeutic agent against visceral leishmaniasis. However, we found no cross-resistance to miltefosine in transfectants with p6.5-LaMDR2/7 (unpublished data). Further studies are required to determine if LaMDR2 transports lipid molecules.

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