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Functional genetic identification of *PRP1*, an ABC transporter superfamily member conferring pentamidine resistance in *Leishmania major*☆

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Abstract

Pentamidine (PEN) is a second-line agent in the treatment of leishmaniasis whose mode of action and resistance is not well understood. Here, we used a genetic strategy to search for loci able to mediate PEN resistance (PEN^r) when overexpressed in *Leishmania major*. A shuttle cosmid library containing genomic DNA inserts was transfected into wild-type promastigotes and screened for PEN-resistant transfectants. Two different cosmids identifying the same locus were found, which differed from other known *Leishmania* drug resistance genes. The PEN^r gene was mapped by deletion and transposon mutagenesis to an open reading frame (ORF) belonging to the P-glycoprotein (PGP)/MRP ATP-binding cassette (ABC) transporter superfamily that we named pentamidine resistance protein 1 (*PRP1*). The predicted PRP1 protein encodes 1807 amino acids with the typical dimeric structure involving 10 transmembrane domains and two nucleotide-binding domains (NBDs). *PRP1*-mediated PEN^r could be reversed by verapamil and *PRP1* overexpressors showed cross-resistance to trivalent antimony but not to pentavalent antimony (glucantime). Although the degree of PEN^r was modest (1.7- to 3.7-fold), this may be significant in clinical drug resistance given the marginal efficacy of PEN against *Leishmania*.

Keywords: *Leishmania major*; Pentamidine; Drug resistance; Gene transfection; Overexpression; P-glycoprotein

1. Introduction

Chemotherapy based on pentavalent antimonials is the primary means for treatment of leishmaniasis, and has been used for more than 50 years. Pentamidine (PEN) is a second-line drug used for the treatment of leishmaniasis, and although it may inhibit many different cellular processes, its cellular target remains unclear [1,2]. PEN competes with polyamines for nucleic acid binding and may also preferentially bind to kinetoplast DNA interfering with replication and transcription at mitochondrial level [2,3]. Recently, it was shown that the mitochondrion is an important target of PEN action in kinetoplastids, and that PEN resistance (PEN^r) in some resistant lines involves decreased drug accumulation in this organelle [4].

Gene amplification has been frequently observed in *Leishmania* following stepwise selection [5–7]. Amplification of *Leishmania major* H-circle genomic region, obtained by this procedure, confers resistance to diverse compounds, including methotrexate, primaquine, terbinafine, arsenite, and antimonials [8–10]. In contrast, a stepwise-selected PEN-resistant *L. major* line (PT-R20) did not show H region or other amplifications [8]. Recently, we described a method exploiting libraries of *Leishmania* transfected with episomal vectors bearing random DNA fragments for the identification of genes whose overexpression leads to drug resistance in *Leishmania* [11]. In this work it has been applied to the identification of loci implicated in PEN^r.

We have applied the overexpression/selection method here to the identification of loci implicated in PEN^r. We found a new PEN^r gene active in *L. major* promastigotes, encoding a protein termed pentamidine resistance protein 1 (PRP1). PRP1 is a member of the ATP-binding cassette (ABC) transporters superfamily which includes the P-glycoprotein (PGP). PGPs are highly conserved

Abbreviations: ABC, ATP-binding cassette; kb, kilobase; LmFA1, *Leishmania major* Friedlin A1; MDR, multidrug resistance; NBD, nucleotide-binding domain; *NEO*, neomycin phosphotransferase gene; ORF, open reading frame; PEN, pentamidine; PEN^r, PEN resistance; PGP, P-glycoprotein; PRP, pentamidine resistance protein

☆ *Note:* Nucleotide sequence obtained in this work has been deposited in the GenBank™/EBI Database under accession number AY251609.

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transmembrane proteins in eukaryotic cells, consisting of duplication of two segments which each contain six transmembrane domains and one nucleotide-binding domain (NBD) [12,13]. In *Leishmania*, the MRP-like family includes at least five genes. *PGPA* was identified in amplified circular DNA (H-circle) from methotrexate-resistant *L. tarentolae* and *L. major* promastigotes, and mediates arsenite and antimony resistance [9,10,14]. *PGPB* and *PGPC* are closely related and genetically linked to *PGPA* but have not been shown to mediate drug resistance. Finally, *PGPD* and *PGPE* are more divergent and located on another chromosome [15]. Although their physiological functions are unknown, it has been demonstrated that overexpression of PGPA leads to resistance to antimonials and arsenite, while disruption of *PGPA* by gene replacement showed an increased sensitivity to arsenite and antimonite [9,16]. The PGP-like family includes *MDR1*, a gene identified in the amplified circular DNA (V-circle) that mediates resistance to hydrophobic drugs, like vinblastine and puromycin, in *Leishmania* spp. [17–19]. Data emerging from the *Leishmania* Genome Project suggest the presence of additional members of this superfamily [20].

2. Materials and methods

2.1. Parasites, cultures, transfections, and drugs

Leishmania (*Leishmania*) *major* Friedlin A1 strain (LmFA1) is an avirulent clonal line derived from the Friedlin V1 strain (MHOM/IL/1980/Friedlin), after multiple passages in vitro [21]. Promastigotes were grown in M199 medium supplemented as described [11,22]. Parasites from late log phase cultures were transfected by electroporation (500 μF, 2.25 kV/cm) using 20–40 μg of cosmid DNA. Typically, the PEN IC_{50} for inhibition of LmFA1 cells was 0.65 μg/ml in liquid media, however, higher concentrations were used in PEN selections as described below and previously [11,23]. Parasite cell determinations, IC_{50} calculations, and statistical tests for PEN^r were carried out as described [11].

Pentamidine and verapamil (VER) were purchased from Sigma, glucantime from Rhodia, $SbCl_3$ from Merck, and Cyclosporin-A from Calbiochem. Miltefosine was provided by Zentaris/ASTA Medica AG (Frankfurt am Main, Germany). For PEN reversal resistance studies, same culture experiments were done by just adding VER in a constant and nontoxic concentration, previously determined by the IC_{50} values for VER against wild-type cells (22.5 ± 1.7 μM).

2.2. Identifying and mapping the Leishmania major locus related to PEN^r

In this work we began with two transfected *L. major* cell populations, each bearing a separate cosmid from an *L. major* library constructed in the shuttle vector cLHYG [11,24]. Library or control cultures were plated on M199 semisolid medium containing increasing concentrations of PEN, 4.8–15.6 μg/ml of PEN, as described [11]. Cosmid DNA from the primary PEN-resistant transfectants was recovered by transformation of *Escherichia coli* DH5α strain and analyzed by restriction enzyme digestion [24]. Deletions of cosPEN1-A insert were obtained by partial digestion with *Hin*dIII and *Eco*RV, followed by self-ligation, as described [11]. Constructs were also generated by total digestion of cosPEN1-A Δ*Hin*dIII DNA insert with the indicated restriction enzyme and subcloned into pSNBR [9] as described [11].

2.3. Southern and Northern blot analyses

For Southern blots, genomic DNA was purified as described [25], digested and separated by electrophoresis in 0.9% agarose gels. Total RNA was prepared with TRIZOL reagent (Gibco-BRL) according to the manufacturer's instructions and separated by 1% formaldehyde–agarose gel electrophoresis [26]. Nucleic acids were transferred to nylon membranes (Gibco-BRL), immobilized by a UV-cross-linking (BioRad), and hybridized using high stringency conditions [26]. A 0.8 kb *Pst*I fragment corresponding to the first NBD of *PRP1* was excised from an agarose gel following electrophoresis, purified by glass milk (GeneClean II; Bio 101, Inc.) and labeled with [α-^{32}P]dCTP using the Random Primers DNA Labeling System (Gibco-BRL).

2.4. Nucleotide sequencing of PRP1 gene

DNA sequencing was done on an ALF Express System (Amersham Pharmacia) automated sequencer. A PCR-based sequencing reaction was done using a Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia). The nucleotide sequence of *PRP1* gene was determined by primer-island sequencing, using arbitrarily selected clones from a random in vitro transposon insertion library using the *Mosk* mariner transposable element [27]. We created several different insertions in a partial *PRP1* 5 kb *Sal*I fragment in pSNBR. Primer-island sequencing was performed using primers *Mosk*F: 5′-CCGAGAGAGATGGGAAAAATG-3′ and *Mosk*R: 5′-GGTTGACACTTCACAAGGTC-3′. Other specific primers were used as necessary, including one from an internal region of the *PRP1* coding region *PRP1* 3′A: 5′-AGCGACATCGTTGTGGTT-3′. All listed primers were labeled with 5′Cy and synthesized by Gibco-BRL.

Analysis of the sequence was performed using Lasergene Software (DNASTAR, Inc.) and Clone Manager 5^{TM} Software. The nucleotide sequence data for pSNBR/5 kb *Sal*I were deposited in GenBank^{TM}/EBI (accession number AY251609). Sequence data for the remaining regions were obtained from the *Leishmania* Genome Project Website (provided by the Sanger Institute as part of the *Leishmania*

Genome Network with support by The Wellcome Trust; www.sanger.ac.uk/Projects/L_major).

3. Results

3.1. Selection of the Leishmania major locus related to PEN^r

Following the overexpression/selection strategy previously described [11], a library of 17,900 independent genomic cosmid transfectants in the Friedlin derivative LmFA1 were plated on semisolid media in the presence of increasing concentrations of PEN. Colonies from plates exhibiting higher numbers relative to controls were recovered and grown briefly. DNAs were prepared and the cosmids recovered by transformation into *E. coli*, and analyzed by restriction enzyme digestion. Fingerprint analysis of seven colonies identified two cosmid populations from the same locus designated *PEN1* (cosPEN1-A and cosPEN1-B) (Fig. 1B).

3.2. Mapping Leishmania major locus related to PEN^r

LmFA1 transfectants bearing cosPEN1-A showed modest increases of PEN^r (1.68-fold resistance), when compared to wild-type LmFA1 (Table 1). To map the active gene, cosPEN1-A deletions were made by partial digestion with *Hin*dIII and self-ligation. Deletion cosPEN1-A Δ*Hin*dIII (with a ∼15 kb insert) retained PEN^r (2.4-fold) (Table 1; Fig. 1C). A second round of deletions with *Eco*RV were generated from the cosPEN1-A Δ*Hin*dIII insert. None of these (cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-I, cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-II,

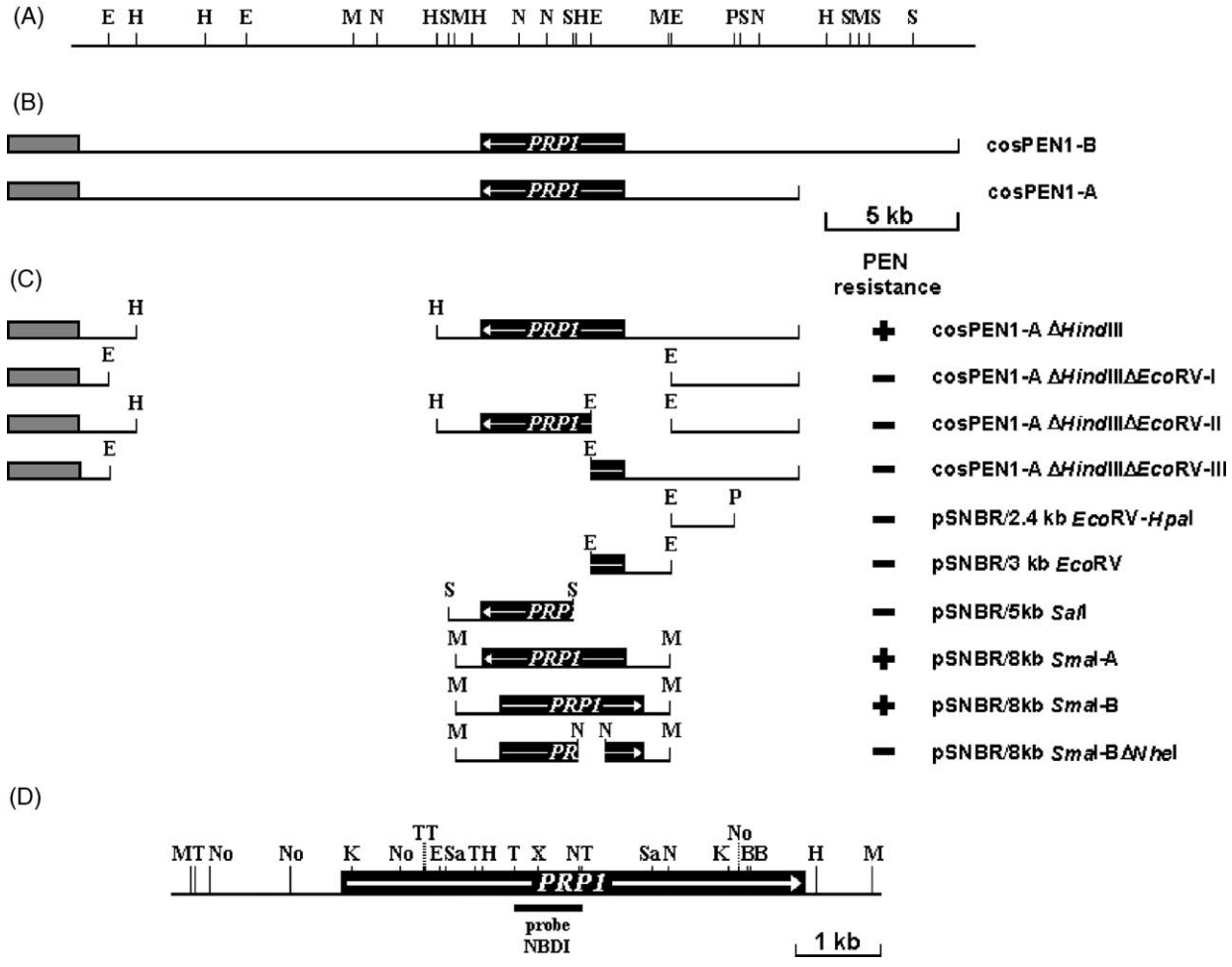


Fig. 1. Functional mapping of the *PRP1* locus. (A) Restriction map of genomic region related to PEN^r. (B) Map of cosmids isolated by overexpression/selection in the presence of PEN. The *PRP1* gene is represented by the black box and the arrow indicates ORF orientation. Shaded boxes show the vector cLHYG. (C) Localization of *PRP1* gene. cosPEN1-A was subjected to deletional analysis to localize the region encoding the gene related to PEN^r (cosPEN1-A Δ*Hin*dIII and cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV deletions). Restriction fragments from cosPEN1-A Δ*Hin*dIII were subcloned in the shuttle vector pSNBR [9] and tested for their ability to confer PEN^r. DNA inserts conferring PEN^r in LmFA1 cells after transfection are represented by plus (+) sign. (D) Organization of *PRP1* locus containing in the 8 kb *Sma*I fragment. The 0.8 kb *Pst*I probe corresponding to the first NBD is represented (NBDI). Restriction enzymes: B, *Bam*HI; E, *Eco*RV; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; N, *Nhe*I; No, *Not*I; P, *Hpa*I; S, *Sal*I; Sa, *Sac*I; T, *Pst*I; X, *Xho*I.

Table 1
Resistance level of the PEN-resistant constructs after transfection in LmFA1 wild-type cells

Cell line	Pentamidine		n	P
	IC_{50} (μg/ml)	Fold resistance		
LmFA1	0.65 ± 0.15	1	15	
cLHYG	0.66 ± 0.2	1.08 ± 0.37	7	ns
cosPEN1-A	1.04 ± 0.19	1.68 ± 0.5	13	< 0.001
cosPEN1-A Δ*Hin*dIII	1.5 ± 0.31	2.4 ± 0.83	15	< 0.001
cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-I	0.5 ± 0.18	0.8 ± 0.25	8	ns
cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-II	0.56 ± 0.11	0.95 ± 0.26	8	ns
cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-III	0.58 ± 0.09	0.93 ± 0.25	8	ns
pSNBR	0.71 ± 0.06	1.14 ± 0.24	8	ns
pSNBR/5kb *Sal*I	0.64 ± 0.14	1.05 ± 0.35	12	ns
pSNBR/8kb *Sma*I-A	2.25 ± 0.12	3.7 ± 1.11	6	< 0.001
pSNBR/8kb *Sma*I-B	1.57 ± 0.18	2.85 ± 0.84	12	< 0.001
pSNBR/8kb *Sma*I-B Δ*Nhe*I	0.66 ± 0.09	1.11 ± 0.36	8	ns
pSNBR/8kb *Sma*I-B plus (15 μM VER)	0.87 ± 0.06	0.52 ± 0.08	6	ns

The mean ± S.D. of *n* independent experiments are given. Values (*P*) significantly different from the wild-type value (1-fold resistance) by Student's *t*-test are designed. The fold resistance is the ratio of IC_{50} for transfected parasites and LmFA1 cells. ns—not significant.
In gray are represented the result of PEN^r reversion after the indicated VER treatment.

and cosPEN1-A Δ*Hin*dIIIΔ*Eco*RV-III) were able to confer PEN^r after transfection, suggesting that the PEN^r gene contained an *Eco*RV site (Table 1; Fig. 1C). Relevant portions of cosPEN1-A Δ*Hin*dIII were then subcloned into the shuttle vector pSNBR [9]. Two of these constructs contain an 8 kb *Sma*I fragment in opposite orientations (Fig. 1C). Interestingly, both constructs were able to confer PEN^r in LmFA1 after transfection (Table 1), confirming that the phenotype does not depend on the direction of transcript within insert in relation to the neomycin phosphotransferase resistance gene (*NEO*) [9,28,29]. A number of smaller deletion derivatives (pSNBR/5 kb *Sal*I, pSNBR/3 kb *Eco*RV, and pSNBR/2.4 kb *Eco*RV-*Hpa*I) did not confer PEN^r (Table 1; Fig. 1C; data not shown).

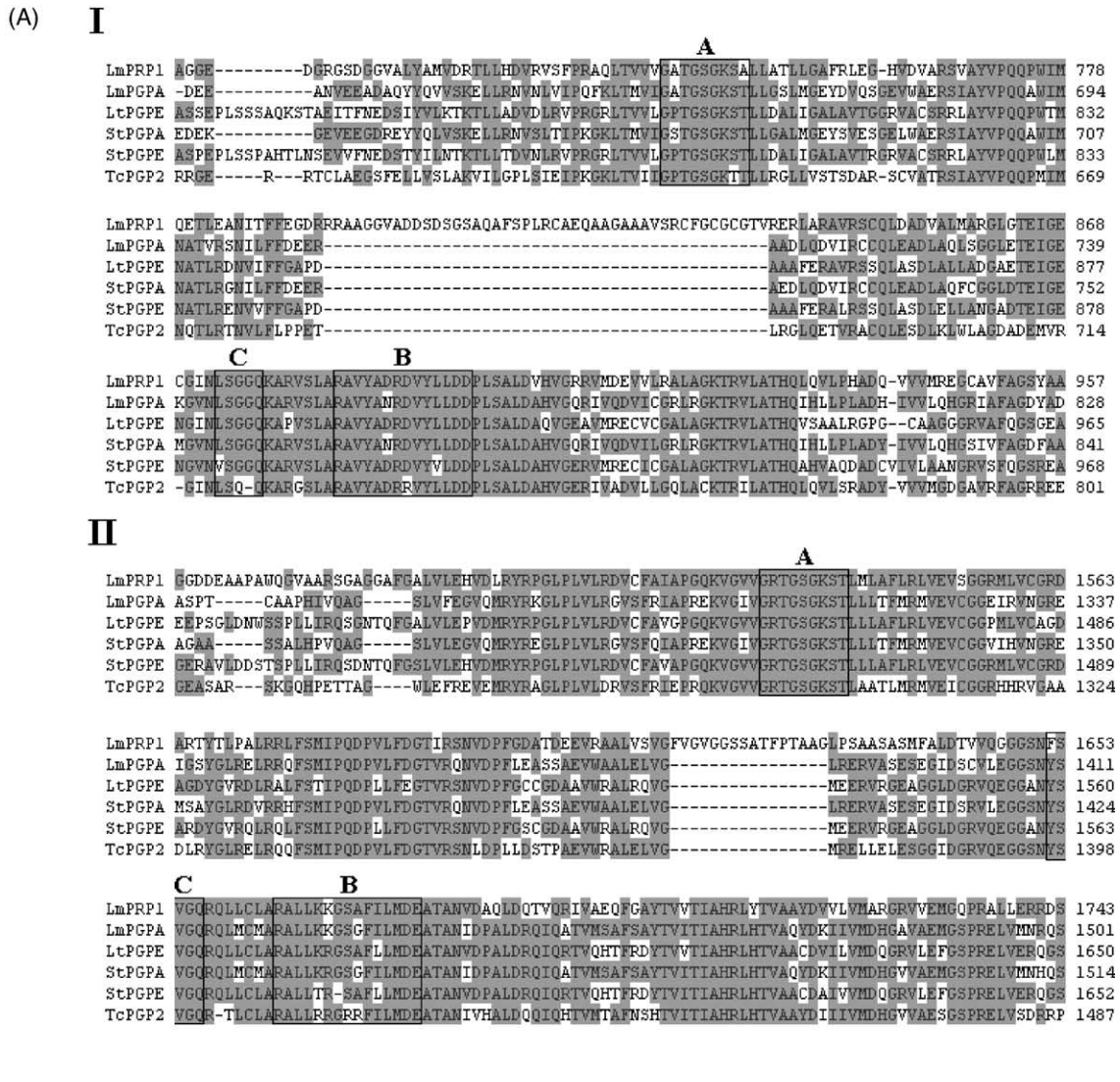
3.3. Molecular characterization and analysis of the *PRP1* gene

The sequence of the 5 kb *Leishmania* DNA of pSNBR/5 kb *Sal*I was determined by transposon-mediated primer-island sequencing [27]. An apparently truncated 1230 amino acid open reading frame (ORF) was found, which showed identity to a region found within a 1500 kb chromosome of *L. major* present in the *Leishmania* Genome Project Database at Sanger Center Web Server. The sequence was then completed by sequencing parasite insert ends in constructs pSNBR/3 kb *Eco*RV and pSNBR/8 kb *Sma*I-A (Fig. 1C), and ultimately filled in from data arising from the *L. major* Genome Project.

The entire *PRP1* gene contained 5424 nucleotides, encoding a predicted polypeptide of 1807 amino acids with an estimated molecular mass of 190 kDa (GenBank™ accession number AY251609). We showed that *PRP1* was the active PEN^r gene, as a 1 kb *Nhe*I fragment deletion within the *PRP1* coding region found in construct pSNBR/8 kb *Sma*I-B Δ*Nhe*I ablated the ability of this fragment to confer PEN^r (Table 1; Fig. 1C). This confirmed that *PRP1* overexpression mediated PEN^r in *L. major*.

The primary structure of polypeptide chain predicted three potential Asn-linked glycosylation sites, although it is likely that none of these are extracellular as they occur in conserved NBDs (^{785}NITF^{788}; ^{872}NLSG^{875}; ^{1651}NFSV^{1654}; Fig. 2A). Hydrophobicity plots of PRP1 showed a structure similar to other PGPs, with a set of putative transmembrane domains followed by regions of hydrophilicity corresponding to the two NBDs. Both predicted NBDs contain the consensus sequences involved in Mg-ATP binding (the Walker motifs A and B) and the ABC transporter signature LSGGQ that has been completely retained in NH_2-terminal (^{873}LSGGQ^{877}) but not in the COOH-terminal (^{1652}FSVGQ^{1656}) (Fig. 2A).

(A)



(B)

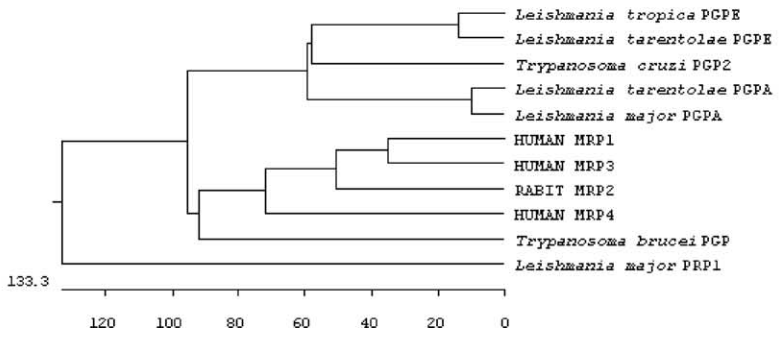


Fig. 2. Protein sequence comparison of *Leishmania major* PRP1 with other members of ABC transporters. (A) An amino acid sequence alignment from the two NBDs (I and II) of PRP1 from *L. major* (LmPRP1), PGPA from *L. major* (LmPGPA); PGPE from *L. tropica* (LtPGPE) (GenBank^{TM} accession number AAB51191); PGPA and PGPE from *L. tarentolae* (StPGPA and StPGPE) (P21441 and AAA65541), and PGP2 from *Trypanosoma cruzi* (TcPGP2) (CAA89197). Alignment was performed using the ClustalW algorithm implemented in the Lasergene software (DNASTAR, Inc.). Identical residues are shaded gray and the Walker A/B motifs and the ABC signature motif regions are boxed and labeled by A, B, and C, respectively. LmPRP1 is 38, 34, 38.6, 34.5, and 29% identical to LmPGPA, LtPGPE, StPGPA, StPGPE, TcPGP2, respectively. (B) An unrooted dendogram was prepared by comparing the full-length amino acid sequences of 11 members of the ABC transporter superfamily using ClustalW algorithm (DNASTAR, Inc.) in standard parameters. The scale at the bottom measures distance between sequences. The units indicate the number of nucleotide substitutions (×100).

The predicted protein has 10 transmembrane domains (data not shown) according to TMHMM Server v. 2.0 [30]. Sequence comparison shows the highest similarity in the two regions containing NBDs of PRP1 with others PGPs from the MRP-like family of *Leishmania* spp. and *Trypanosoma cruzi* (Fig. 2A). However, we noted that PRP1 contain specific sequences or features, such as the spacing between the conserved Walker motifs A and B that differ from others members of MRP-like family (Fig. 2A). A phylogenetic comparison of PRP1 sequence with six other ABC transporters of MRP-like family from trypanosomatids and four multidrug resistance (MDR) proteins from mammalian indicated that PRP1 of *L. major* was the most divergent member, and thus defines a new PGP family (Fig. 2B).

Southern blot analysis for *PRP1* gene organization showed that it was present as a single copy within *L. major* genome (Fig. 3A), in agreement with genome sequencing. We observed faint bands in some digestions even with high stringency washes, which may reflect hybridization to other ABC transporter genes within the *L. major* genome (Fig. 3A). Nucleotide sequence analysis of genomic region of *PRP1* gene indicated that no other gene of ABC transporter superfamily occurred nearby. Upstream of *PRP1* there was an ORF of 335 amino acids that showed 15% amino acid identity to GP46 of *L. chagasi* (GenBank™ accession number AAB62271), while a downstream ORF comprised 877 amino acids did not show any relationship to other proteins in database.

Northern blot analysis identified a major transcript of 6 kb, as well as a minor transcript of 4.4 kb, in total RNA of *L. major* transfectant pSNBR/8 kb *Sma*I-A (Fig. 3B). No hybridization was observed with logarithmic and stationary LmFA1 wild-type RNA (Fig. 3B). The 6 kb transcript would be large enough to encode PRP1 according to the predicted ORF and a better characterization will be necessary to determine the minor transcript. Attempts to map the 5′ trans-splicing site of the *PRP1* transcript were not successful.

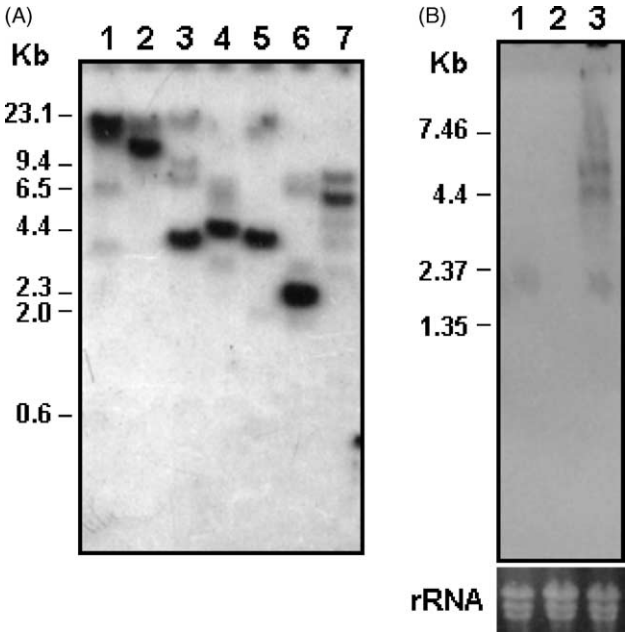


Fig. 3. (A) Southern blot analysis of *Leishmania major* genomic DNA. Genomic DNA (5 μg) was digested, fractionated on 0.9% agarose gel, and probed with a 0.8 kb *Pst*I fragment corresponding to NBDI (Fig. 1D). None of the enzymes used cut the probe except *Xho*I (Fig. 1D). Size markers (kb) are derived from λ phage DNA digested with *Hin*dIII. Restriction enzymes are: 1, *Bam*HI; 2, *Eco*RV; 3, *Hin*dIII; 4, *Kpn*I; 5, *Not*I; 6, *Sac*I; 7, *Xho*I. (B) Northern blot analysis of transfectants with the *PRP1* gene and wild-type LmFA1. Total RNA (5 μg) of logarithmic (1), stationary (2), and logarithmic transfectants pSNBR/8 kb *Sma*I-A LmFA1 promastigotes (3) were separated by electrophoresis, transferred to a nylon membrane, and hybridized with the 0.8 kb *Pst*I fragment corresponding to NBDI (Fig. 1D). Ribosomal RNA (rRNA) was used as a loading control.

3.4. Role of PRP1 in PEN^r and cross-resistance studies

Classical modulators of mammalian MDR phenotype, such as VER and Cyclosporin-A, can reverse drug resistance mediated by these transporters [31], although they are not effective with MDR in *Leishmania* [17,32]. We tested pSNBR/8 kb *Sma*I-B transfectants cells for PEN^r at nontoxic concentrations of VER, and verified that PEN^r was reverted when compared with transfected cells not treated with VER and/or LmFA1 wild-type cells (Table 1, gray line; Fig. 4). Similar tests were performed with Cyclosporin-A, however, this compound failed to reverse PEN^r, even at toxic concentrations (data not shown).

The cross-resistance profile of LmFA1 transfected with pSNBR/8 kb *Sma*I-A to structurally and functionally unrelated drugs is summarized in Table 2. Significant cross-resistance was observed only towards $SbCl_3$, while

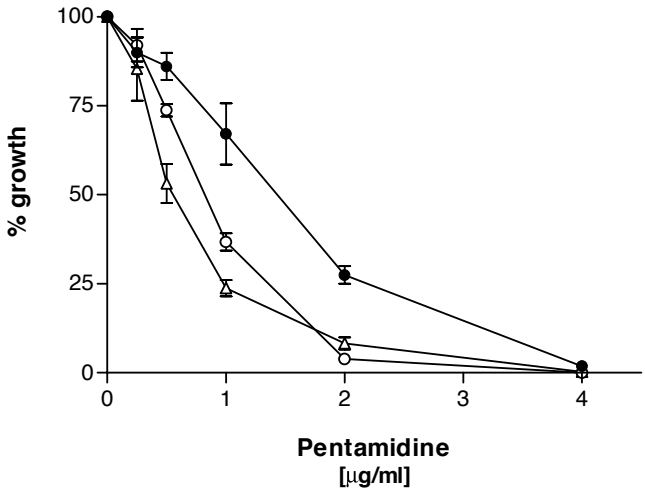


Fig. 4. Analysis of *PRP1* gene overexpression and reversal of PEN^r. Growth properties of PEN-resistant *Leishmania major* transfectant pSNBR/8 kb *Sma*I-B and reversal of resistance in constant and nontoxic concentration of VER. Symbols are: LmFA1 (△); transfectant pSNBR/8 kb *Sma*I-B (●); transfectant pSNBR/8 kb *Sma*I-B treated with nontoxic concentration of VER (15 μM) (○).

Table 2
Cross-resistance profile (IC_{50} value) verified for transfected LmFA1 cells resistant to PEN

Drugs	LmFA1	pSNBR/8 kb *Sma*I-A	Fold resistance	*n*	*P*
Cyclosporin-A[a]	2.625 ± 0.53	2.475 ± 0.16	0.95 ± 0.07	4	ns
Glucantime[b]	33 ± 3.46	34 ± 3.65	1.05 ± 0.20	4	ns
Miltefosine[a]	0.64 ± 0.03	0.65 ± 0.03	1.01 ± 0.08	4	ns
$SbCl_3$[b]	0.40 ± 0.11	1.52 ± 0.2	3.87 ± 0.81	6	<0.001

Fold resistance is the ratio of IC_{50} for pSNBR/8 kb *Sma*I-A transfected parasites and LmFA1 cells. ns—not significant.
[a] Concentration in μg/ml.
[b] Concentration in mg/ml.

cross-resistance was not observed to glucantime (meglumine antimoniate), miltefosine, or Cyclosporin-A (Table 2).

4. Discussion

ABC transporters have been identified from different species of *Leishmania* [33]. In this report, we described a new member of ABC transporter superfamily in *Leishmania* related to PEN^r. For identification of this drug resistance gene, genomic libraries were transfected in *L. major* and parasites bearing cosmids mediating PEN^r were isolated [11].

Hybridizations and sequence comparisons showed that the *PEN1* locus was not related to other loci implicated in drug resistance in *Leishmania* previously characterized by overexpression selection, including *SQS1* for terbinafine and itraconazol, *DHFR-TS* and *PTR1* for methotrexate, and *TOR* for tubercidin [11]. *PEN1* instead encodes the PRP1 (Figs. 1 and 2), a new member of the ABC transporter superfamily with high similarity with members of MRP-like family (around 30–40%) (Fig. 2A). *PRP1* is a single copy gene which maps to a 1500 kb chromosome, and thus identifies a new ABC transporter *locus* (Fig. 3A). Phylogenetic analysis showed that *Leishmania* PRP1 represents the most evolutionarily distant group of the PGPs, diverging even before the mammalian and trypanosomatids MRP-like members (PGPA, PGPE, TcPGP2) diverge from each other (Fig. 2B). By these criteria, PRP1 represents a new family of ABC transporters.

Although the modest levels of PEN^r observed in transfected cells, *PRP1* gene clearly mediated PEN^r in LmFA1 cells: (1) the resistance levels of diverse constructs containing *PRP1* were statistically significant (Table 1; Fig. 1); (2) the level of resistance seen has proven to be significant in other drug selection tests involving cosmid transfections selected with terbinafine and itraconazol [11], or from endogenous amplification, as vinblastine [17,19], or primaquine [8]; and (3) as irrelevant DNA regions were eliminated from the starting cosmid cosPEN1-A, an increase of the resistance level was found (Table 1) [9,11,34]. While we were unable to visualize the endogenous PRP1 transcript, a 6 kb transcript sufficient to encode PRP1 was observed in pSNBR/8 kb *Sma*I-A transfectants (Fig. 3B).

Diamidines enter *T. brucei* cells via P2 nucleoside transporter and/or at least two other transporters [35–37]. Significantly, Basselin et al. showed that PEN uptake in *L. mexicana* was not mediated by nucleoside transporters but by a carrier that recognizes diamidines with high affinity [4]. These authors showed that the mitochondrion may be the main target of PEN action in *Leishmania*, as resistance involved decreased mitochondrial PEN accumulation in resistant parasites [4]. Our data raise the possibility that PRP1 encodes a transporter involved in the decrease of mitochondrial PEN uptake observed by these authors. Consistent with this possibility, reversion of PEN resistant by VER was also observed in the PEN^r *L. mexicana* cells [4].

PRP1 also conferred cross-resistance to $SbCl_3$ (Table 2), as seen previously for PGPA of *L. major* to Sb(III) tartrate, $SbCl_3$, and SbO_3 [9]. Although trivalent antimonials are not used to treat human leishmaniasis, it has been proposed that pentavalent derivatives are metabolized in vivo into trivalent antimony [38].

In conclusion, we have described a new member of the ABC transporter superfamily which mediates PEN^r following overexpression in *Leishmania*. The properties of this transporter suggest that it may be related to ones implicated in *L. mexicana* in PEN^r mutants [4]. Future studies will address this possibility, and the role of PRP1 in *Leishmania* metabolism.

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References

[1] Herwaldt BL. Leishmaniasis. Lancet 1999;354:1191–9.
[2] Sands M, Kron MA, Brown RB. Pentamidine: a review. Rev Infect Dis 1985;7:625–34.
[3] Basselin M, Badet-Denisot MA, Robert-Gero M. Modification of kinetoplast DNA minicircle composition in pentamidine-resistant *Leishmania*. Acta Trop 1998;70:43–61.

[4] Basselin M, Denise H, Coombs GH, Barrett MP. Resistance to Pentamidine in *Leishmania mexicana* involves exclusion of the drug from the mitochondrion. Antimicrob Agents Chemother 2002;46:3731–8.
[5] Segovia M. *Leishmania* gene amplification: a mechanism of drug resistance. Ann Trop Med Parasitol 1994;88:123–30.
[6] Beverley SM. Gene amplification in *Leishmania*. Annu Rev Microbiol 1991;45:417–44.
[7] Borst P, Ouellette M. New mechanisms of drug resistance in parasitic protozoa. Annu Rev Microbiol 1995;49:427–60.
[8] Ellenberger TE, Beverley SM. Multiple drug resistance and conservative amplification of the H region in *Leishmania major*. J Biol Chem 1989;264:15094–103.
[9] Callahan HL, Beverley SM. Heavy metal resistance: a new role for P-glycoproteins in *Leishmania*. J Biol Chem 1991;266:18427–30.
[10] Papadopoulou B, Roy G, Dey S, Rosen BP, Ouellette M. Contribution of the *Leishmania* P-glycoprotein-related gene *ltpgpA* to oxyanion resistance. J Biol Chem 1994;269:11980–6.
[11] Cotrim PC, Garrity LK, Beverley SM. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. J Biol Chem 1999; 274:37723–30.
[12] Endicott JA, Ling V. The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 1989;58:137–71.
[13] Higgins CF. ABC transporters: from microorganisms to man. Annu Rev Cell Biol 1992;8:67–113.
[14] Ouellette M, Fase-Fowler F, Borst P. The amplified H circle of methotrexate-resistant *Leishmania tarentolae* contains a novel P-glycoprotein gene. EMBO J 1990;9:1027–33.
[15] Legare D, Hettema E, Ouellette M. The P-glycoprotein-related gene family in *Leishmania*. Mol Biochem Parasitol 1994;68:81–91.
[16] Papadopoulou B, Roy G, Dey S, Rosen BP, Olivier M, Ouellette M. Gene disruption of the P-glycoprotein related gene pgpa of *Leishmania tarentolae*. Biochem Biophys Res Commun 1996;224: 772–8.
[17] Henderson DM, Sifri CD, Rodgers M, Wirth DF, Hendrickson N, Ullman B. Multidrug resistance in *Leishmania donovani* is conferred by amplification of a gene homologous to the mammalian *mdr1* gene. Mol Cell Biol 1992;12:2855–65.
[18] Katakura K, Iwanami M, Ohtomo H, Fujise H, Hashiguchi Y. Structural and functional analysis of the *LaMDR1* multidrug resistance gene in *Leishmania amazonensis*. Biochem Biophys Res Commun 1999;255:289–94.
[19] Chow LM, Wong AK, Ullman B, Wirth DF. Cloning and functional analysis of an extrachromosomally amplified multidrug resistance-like gene in *Leishmania enriettii*. Mol Biochem Parasitol 1993;60:195–208.
[20] Akopyants NS, Clifton SW, Martin J, Pape D, Wylie T, Li L, et al. A survey of the *Leishmania major* Friedlin strain V1 genome by shotgun sequencing: a resource for DNA microarrays and expression profiling. Mol Biochem Parasitol 2001;113:337–40.
[21] da Silva RP, Sacks DL. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect Immun 1987;55:2802–7.
[22] Kapler GM, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol Cell Biol 1990;10:1084–94.
[23] Ellenberger TE, Beverley SM. Reductions in methotrexate and folate influx in methotrexate-resistant lines of *Leishmania major* are independent of R or H region amplification. J Biol Chem 1987;262:13501–6.
[24] Ryan KA, Dasgupta S, Beverley SM. Shuttle cosmid vectors for the trypanosomatid parasite *Leishmania*. Gene 1993;131:145–50.
[25] Medina-Acosta E, Cross GA. Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. Mol Biochem Parasitol 1993;59:327–9.
[26] Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. 2nd ed. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press; 1989.
[27] Tosi LR, Beverley SM. *cis* and *trans* factors affecting *Mos1* mariner evolution and transposition in vitro, and its potential for functional genomics. Nucleic Acids Res 2000;28:784–90.
[28] Pedrosa AL, Cruz AK. The effect of location and direction of an episomal gene on the restoration of a phenotype by functional complementation in *Leishmania*. Mol Biochem Parasitol 2002;122:141–8.
[29] Callahan HL, Beverley SM. A member of the aldoketo reductase family confers methotrexate resistance in *Leishmania*. J Biol Chem 1992;267:24165–8.
[30] Krogh A, Larsson B, von Heijne G, Sonnhammer EL. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 2001;305:567–80.
[31] Gottesman MM, Pastan I. Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 1993;62:385–427.
[32] Chiquero MJ, Perez-Victoria JM, O'Valle F, Gonzalez-Ros JM, del Moral RG, Ferragut JA, et al. Altered drug membrane permeability in a multidrug-resistant *Leishmania tropica* line. Biochem Pharmacol 1998;55:131–9.
[33] Perez-Victoria JM, Parodi-Talice A, Torres C, Gamarro F, Castanys S. ABC transporters in the protozoan parasite *Leishmania*. Int Microbiol 2001;4:159–66.
[34] Singh AK, Papadopoulou B, Ouellette M. Gene amplification in amphotericin B-resistant *Leishmania tarentolae*. Exp Parasitol 2001;99:141–7.
[35] Carter NS, Berger BJ, Fairlamb AH. Uptake of diamidine drugs by the P2 nucleoside transporter in melarsen-sensitive and -resistant *Trypanosoma brucei brucei*. J Biol Chem 1995;270:28153–7.
[36] de Koning HP, Jarvis SM. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by the P2 adenosine transporter and at least one novel, unrelated transporter. Acta Trop 2001;80:245–50.
[37] De Koning HP. Uptake of pentamidine in *Trypanosoma brucei brucei* is mediated by three distinct transporters: implications for cross-resistance with arsenicals. Mol Pharmacol 2001;59:586–92.
[38] Roberts WL, Berman JD, Rainey PM. In vitro antileishmanial properties of tri- and pentavalent antimonial preparations. Antimicrob Agents Chemother 1995;39:1234–9.