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.

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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 methotrextate, 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). PGP’s are highly conserved
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. 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 (LmA1) 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 V/m, 2.25 kV/cm) using 20–40 μg of cosmid DNA. Typically, the PEN IC₅₀ for inhibition of LmA1 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, IC₅₀ calculations, and statistical tests for PEN were carried out as described [11].

Pentamidine and verapamil (VER) were purchased from Sigma, glucantime from Rhodia, SB61 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 IC₅₀ values for VER against wild-type cells (22.5 ± 1.7 μM).

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

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 HindIII and EcoRV, followed by self-ligation, as described [11]. Constructs were also generated by total digestion of cosPEN1-A ΔHindIII 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 TRI-ZOL 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 Prl 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-³²P]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 5kb SalI fragment in pSNBR. Primer-island sequencing was performed using primers MoskF: 5'-CCGAGAGAGAGGAGAAAATG-3' and MoskR: 5'-GGTTGACACTCTACAAGGTC-3'. Other specific primers were used as necessary, including one from an internal region of the PRP1 coding region PRP1 3'A: 5'-AGCGACATCGTGGTTTGTT-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™ Software. The nucleotide sequence data for pSNBR/5 kb SalI were deposited in GenBank®/EBI (accession number AV251609). Sequence data for the remaining regions were obtained from the Leishmania Genome Project Website (provided by the Sanger Institute as part of the Leishmania Database).
3. Results

3.1. Selection of the Leishmania major locus related to PEN

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 PEN (cosPEN1-A and cosPEN1-B) (Fig. 1B).

3.2. Mapping Leishmania major locus related to PEN

LmFA1 transfectants bearing cosPEN1-A showed modest increases of PEN (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 HindIII and self-ligation. Deletion cosPEN1-A ΔHindIII (with a ~15 kb insert) retained PEN (2.4-fold) (Table 1; Fig. 1C). A second round of deletions with EcoRV were generated from the cosPEN1-A ΔHindIII insert. None of these (cosPEN1-A ΔHindIIIΔEcoRV-I, cosPEN1-A ΔHindIIIΔEcoRV-II, cosPEN1-A ΔHindIIIΔEcoRV-III) showed resistance to PEN.

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

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Fig. 1. Functional mapping of the PRP1 locus. (A) Restriction map of genomic region related to PEN. The PRP1 gene is represented by the black box and the arrow indicates ORF orientation. Shaded boxes show the vector cLHYG. (B) Localization of PRP1 gene. cosPRP1-A was subjected to deletional analysis to localize the region encoding the gene related to PEN (cosPRP1-A ΔHindIII and cosPRP1-A ΔHindIIIΔEcoRV deletions). Restriction fragments from cosPRP1-A ΔHindIII were subcloned in the shuttle vector pSNBR [9] and tested for their ability to confer PEN. DNA inserts conferring PEN in LmFA1 cells after transfection are represented by plus (+) sign. (C) Organization of PRP1 locus containing in the 8 kb Smal fragment. The 0.8 kb PstI probe corresponding to the first NBD is represented (R:R). Restriction enzymes: B, BamHI; E, EcoRV; H, HindIII; K, KpnI; M, SmaI; N, NheI; No, NotI; P, HpaI; S, SacII; Sa, SalI; T, PstI; X, XhoI.
Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pentamidine</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IC50 (μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LmFA1</td>
<td>0.65 ± 0.15</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>cLHYG</td>
<td>0.66 ± 0.2</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>cosPEN1-A</td>
<td>1.04 ± 0.19</td>
<td>13</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>cosPEN1-A ΔHindIII</td>
<td>1.5 ± 0.31</td>
<td>15</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>cosPEN1-A ΔHindIII ΔEcoRV-I</td>
<td>0.5 ± 0.18</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>cosPEN1-A ΔHindIII ΔEcoRV-II</td>
<td>0.56 ± 0.11</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>cosPEN1-A ΔHindIII ΔEcoRV-III</td>
<td>0.58 ± 0.09</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>pSNBR</td>
<td>0.71 ± 0.06</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>pSNBR/5 kb SalI</td>
<td>0.64 ± 0.14</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>pSNBR/3 kb SmaI-A</td>
<td>2.25 ± 0.12</td>
<td>6</td>
<td>&lt; 0.001</td>
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<tr>
<td>pSNBR/8 kb SmaI-B</td>
<td>1.57 ± 0.18</td>
<td>12</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>pSNBR/8 kb SmaI-B ΔNheI</td>
<td>0.66 ± 0.09</td>
<td>8</td>
<td>ns</td>
</tr>
<tr>
<td>pSNBR/8 kb SmaI-B pΔNheI</td>
<td>0.87 ± 0.06</td>
<td>6</td>
<td>ns</td>
</tr>
</tbody>
</table>

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 IC50 for transfected parasites and LmFA1 cells. ns—not significant.

In grey are represented the result of PEN reversion after the indicated VER treatment.

3.3. Molecular characterization and analysis of the PRP1 gene

The sequence of the 5 kb Leishmania DNA of pSNBR/5 kb SalI 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 EcoRV and pSNBR/8 kb SmaI-A (Table 1, 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 access number AY251609). We showed that PRP1 was the active PEN gene, as a 1 kb NheI fragment deletion within the PRP1 coding region found in construct pSNBR/8 kb SmaI-B ΔNheI ablated the ability of this fragment to confer PEN (Table 1, Fig. 1C). This confirmed that PRP1 overexpression mediated PEN 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 NH2-terminal (873 LSGGQ877) but not in the COOH-terminal (1652 FSVGQ1656; Fig. 2A).
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™ 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 PPGs 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 Smal-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.

3.4. Role of PRP1 in PEN 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 Smal-B transfectants cells for PEN resistant at nontoxic concentrations of VER, and verified that PEN 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, even at toxic concentrations (data not shown).

The cross-resistance profile of LmFA1 transfected with pSNBR/8 kb Smal-A to structurally and functionally unrelated drugs is summarized in Table 2. Significant cross-resistance was observed only towards SbCl₃, while...
porter and/or at least two other transporters [35–37]. Significant differences between LmFA1 and pSNBR/8 kb in terms of drug resistance were observed in pSNBR/8 kb transfected parasites and LmFA1 cells. ns—not significant.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>LmFA1</th>
<th>pSNBR/8kb Sma-A</th>
<th>Fold resistance</th>
<th>n</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine[a]</td>
<td>2.625 ± 0.53</td>
<td>2.475 ± 0.16</td>
<td>0.95 ± 0.07</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>Glucantime[b]</td>
<td>33 ± 3.46</td>
<td>34 ± 3.65</td>
<td>1.05 ± 0.20</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>Miltefosine[a]</td>
<td>0.64 ± 0.03</td>
<td>0.65 ± 0.03</td>
<td>0.91 ± 0.08</td>
<td>4</td>
<td>ns</td>
</tr>
<tr>
<td>SbCl₃[b]</td>
<td>0.40 ± 0.11</td>
<td>1.52 ± 0.2</td>
<td>0.37 ± 0.41</td>
<td>6</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Fold resistance is the ratio of IC₅₀ for pSNBR/8 kb Sma-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. Identification of this drug resistance gene, genomic libraries were transfected in *L. major* and parasite 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 itraconazole, *DHFR-TS* and *PTR1* for methotrexate, and *TOR* for tuberculosis [11]. *PEN1* instead encodes the PRP1 (Figs. 1 and 2), a new member of the ABC transporter superfamily which maps to a 1500 kb chromosome, and thus identifies a gene clearly mediated PEN r in *L. major* and parasite bearing cosmids mediating PEN r [11]. 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).

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 transfers, and (3) as irrelevant DNA regions were eliminated from the starting cosmid coPEN1-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-A transfecteds (Fig. 3B).

Diamidines enter *T. brucei* cells via P2 nucleoside transporters and 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 SbCl₃ (Table 2), as seen previously for PGPA of *L. major* to ShIII tartrate, SbCl₃, and SbO₃ [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


