Heavy Metal Resistance: A New Role for P-glycoproteins in Leishmania*

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P-glycoproteins are responsible for multidrug resistance in tumor cell lines and are thought to have a physiologic role in exporting cellular metabolites. We now report that a P-glycoprotein gene in the H region of the trypanosomatid protozoan Leishmania confers resistance to heavy metals when present in multiple copies. The Leishmania H region is frequently amplified in drug-resistant lines and is associated with metal resistance. Leishmania expression vectors were used to introduce multiple copies of segments of the Leishmania major H region into wild-type L. major promastigotes. Only constructs bearing a segment of L. major DNA containing the P-glycoprotein ImpgpA conferred arsenite resistance. Deletional analysis of the arsenite-resistant construct mapped resistance to arsenite to the ImpgpA protein coding region. Lines expressing ImpgpA showed resistance to arsenite and trivalent antimonials, but not to pentavalent antimonials, zinc, cadmium, or the typical multidrug-resistant P-glycoprotein substrates vinblastine and puromycin. Transfection of the Leishmania tarentolae P-glycoprotein homologue ImpgpA resulted in a similar resistance profile. Thus, these pgpAs represent a functionally distinct group of P-glycoproteins which exhibit a substrate specificity similar to prokaryotic heavy metal pumps. Additionally, several arguments suggest that pgpAs may play a role in the susceptibility of Leishmania to clinically utilized antimonials.

P-glycoproteins are responsible for multidrug resistance in tumor cell lines and are thought to have a physiologic role in exporting cellular metabolites (Chen et al., 1986b; Endicott and Ling, 1988; Gottesman and Pastan, 1988; Gros et al., 1986). Recently, several P-glycoprotein homologues were identified in the trypanosomatid parasite Leishmania tarentolae (Ouellette et al., 1990). One of these (ImpgpA) maps within the Leishmania H region, a locus which is frequently amplified as extrachromosomal circular DNAs (Beverley et al., 1984) both in both unselected laboratory stocks (Hightower et al., 1988; Petrillio-Peixoto and Beverley, 1988; White et al., 1988) and in lines selected with methotrexate (MTX),1

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The abbreviations used are: MTX, methotrexate; MDR, multidrug resistance; kb, kilobase pairs.

primarine, terbinafine, and arsenite (Beverley et al., 1984; Detke et al., 1988; Ellenberger and Beverley, 1989; Ouellette et al., 1991).

Although the association of the H region and MTX resistance is well documented (Beverley et al., 1984; Detke et al., 1988; Ellenberger and Beverley, 1989; Hightower et al., 1988; Petrillio-Peixoto and Beverley, 1988; White et al., 1988), the role of the H region in metal resistance has remained largely unexplored. Detke et al. (1989) found that Leishmania amazonensis promastigotes selected for arsenite resistance contained an H region amplification not present in unselected cells. Subsequently, Ellenberger and Beverley (1989) found that Leishmania major promastigotes, selected for primarine resistance and expressing an H region amplification, were also resistant to arsenite. Additional studies with H-amplified lines support the association between H amplification and metal resistance.2 We chose to identify the H region gene responsible for heavy metal resistance using DNA transfection.

Since the H region is extensively transcribed into at least 20 polyadenylated RNAs (Ellenberger, 1989) whose coding potential and transcriptional signals have not been determined, we employed a strategy to allow expression of these RNAs following transfection into wild-type L. major promastigotes utilizing either endogenous signals or those provided by the transfection vector. In trypanosomatids, RNAs are transcribed in a polycistronic fashion; the 5' end of mRNAs is determined by addition of a small leader sequence by splicing (reviewed by Borst, 1986). This allows genes inserted downstream of a transcription initiation site to be correctly expressed using their own splice-acceptor sites (LeBowitz et al., 1990). We used Leishmania expression vectors to introduce, amplify, and express DNA fragments spanning the entire H region and thus localize the Leishmania metal resistance gene.

EXPERIMENTAL PROCEDURES

Transfections and Cell Culture—Wild-type L. major CC-1, a cloned derivative of LT252 which lacks the H region amplification, was transfected by electroperoration and plated on semisolid M199 medium containing 16 μg/ml G418 as described (Kaiper et al., 1990). Colonies were transferred to liquid medium and selected for growth in 512 μg/ml G418 before testing for metal resistance.

Molecular Constructs—Fragments spanning the H region were obtained from a genomic library of LT252 L. major DNA (Beverley et al., 1984); this unselected line contains low levels of an H region amplification of uncertain origin (Ellenberger and Beverley, 1989; Hightower et al., 1988). The large EcoRI fragments from XLTS-113, ALTM-HC3, ALTS-106, and ALTS-108 (Beverley et al., 1984) (designated fragments H1–H4, respectively) were inserted into the polylinker EcoRI site of pSNAR and pSNBR, in both orientations. The vectors pSNAR and pSNBR are modifications of pS45NEOA and B (LeBowitz et al., 1990). The EcoRI site present in the Leishmania-derived sequences was removed by a partial digestion with EcoRI, a Klencow fill-in reaction, and self-ligation. The designations A and B indicate that Leishmania DNA was either inserted in the same or opposite orientation, respectively, as the lac promoter contained in the bacterial vector pBluescript (the vectors (LeBowitz et al., 1990); arbitrarily assigning left to right as drawn in Fig. 1c).

Copy Number—Plasmid copy number was determined by slot blot analysis using cell dilutions of 2, 0.4, and 0.1 × 106 cells/well (Ellenberger and Beverley, 1989) followed by Southern blot hybridization.

2 H. L. Callahan and S. M. Beverley, unpublished data.
Identical blots were probed with a 0.9-kb SpeI fragment containing Neo, a 0.6-kb SstI-XmaI fragment containing DNA from the non-translated region downstream from *dhfr-ts* present in both chromosomal and transfected DNA, and a 3.9-kb SalI fragment from H2 present only in chromosomal DNA and H2-containing constructs.

**Pulse Field Gel Electrophoresis**—The chromosomal location of the transfected DNA was determined by pulse field gel electrophoresis on a CHEF apparatus. Short pulse times (20 s) were used to separate circular molecules from chromosomal DNA; longer pulse times (80 s) were used to separate chromosomes surrounding the H region. Blots were then probed with the Neo and H2 probes described above.

**PgpA Localization**—The location of *lmpgpA* was deduced by comparisons of the restriction maps of *ltpgpA* (Ouellette et al., 1990) and the *L. tarentolae* and *L. major* H regions (Petrillo-Peixoto and Beverley, 1988; White et al., 1988) and confirmed by Southern blot hybridization. Probes identifying the ends of *ltpgpA* (800-base pair 5’ and 700-base pair 3’ SauI-KpnI DNA fragments (Ouellette et al., 1990) were made from cloned *L. tarentolae* MG-C2 DNAs (Petrillo-Peixoto and Beverley, 1988).

**Drug Sensitivity Determinations**—Drug sensitivity was determined as described previously for terrinafine (Ellenberger and Beverley, 1989). The drug EC₅₀ is the concentration (in μM for MTX, μg/ml for all other drugs except Pentostam, which is mg/ml) which decreases the experimental and control lines. All experiments were done in triplicate. All antimodins and arsenicals were obtained from Aldrich except Pentostam, which was donated by P. Rainey (Yale Medical School). All other drugs were obtained from Sigma.

**RESULTS AND DISCUSSION**

Initially, four large DNA fragments spanning the H region (Fig. 1a) were introduced in both orientations into two *Leishmania* expression vectors, pSNAR and pSNBR, and transfected into *L. major* promastigotes. Transfectants were selected by plating on G418, since the vector expresses the bacterial neomycin resistance gene. Following amplification of the transfected DNA by growth in increased G418 concentrations, transfected lines were tested for arsenite resistance by growth inhibition assays. As expected, the vector-transfected controls showed susceptibilities comparable with those of the transfected DNA by growth in increased G418 concentrations, transfected lines were tested for arsenite resistance. All experiments were done in triplicate. All antimodins and arsenicals were obtained from Aldrich except Pentostam, which was donated by P. Rainey (Yale Medical School). All other drugs were obtained from Sigma.

**Fig. 1.** H region fragments and arsenite resistance. a, map of H region. Relevant restriction sites (*R, EcoRl; B, BglII; H, HindIII; S, SalI; T, SstI*). Arrows indicate the localization of the protein coding region. b, the percent growth of transfected lines (± S.D.) in arsenite relative to drug-free control at 72 h is shown. MTX was 10-fold more resistant to arsenite than vector alone (Fig. 1b). However, pSNBR-H3A transfected lines showed only 1.4-fold more resistance than lines transfected with pSNBR alone (Fig. 1, b and c). Although we were surprised that the pSNBR-H3A line showed significantly less resistance than the PSNAR-H3 lines, we did not pursue the discrepancy further since we were interested primarily in functional constructs. We presume that the orientation or some other incompatibility prevented expression in this construct. As shown below, further deletions of the resistant construct pSNAR-H3B increased the resistance to 10-fold.

Since there is also a close association between H amplification and MTX resistance we were surprised to find that none of these fragments conferred MTX resistance (Fig. 1, a and c). However, preliminary studies have localized the MTX resistance determinant in other H region constructs.

Recently, several P-glycoprotein homologues were identified in *L. tarentolae*, one of these (ltpgpA) maps within the H region (Ouellette et al., 1990). We mapped the *L. major* homologue of *ltpgpA* (*lmpgpA*) to a 7-kb HindIII fragment within the metal-resistant fragment H3 (Fig. 2a). Cell lines transfected with a deletion construct containing the intact *lmpgpA* were 10-fold resistant to arsenite (Fig. 2, b and c). However, lines transfected with deletions of pSNAR-H3B which contained either the left or right halves of the *lmpgpA* coding region did not show arsenite resistance (Fig. 2b and c). Since all transfected lines contained approximately 20 copies of extrachromosomal, circular, transfected DNA and no chromosomal alterations were observed by pulse field gel analysis and Southern blotting (data not shown), we attribute resistance to expression of the transfected pgpA. In addition, cell lines transfected with a larger 10-kb fragment containing *ltpgpA* (Fig. 2a) were more than 3-fold resistant to arsenite (Fig. 2b and c).

Subsequently, *lmpgpA* transfectants were tested for sensi-
activity or resistance to the related arsenical, arsenate, antimonials, and other metals (Table 1). *lmpgpA* transfectants were resistant to arsenate and all trivalent antimonials tested, including Sb(III) tartrate, but not to cadmium or zinc or the clinically utilized pentavalent antimonial Pentostam (Table 1). *ltpgpA* transfectants showed a similar resistance profile (generally 2-4-fold less resistant than the *lmpgpA* construct) to all drugs except to arsenate to which it was slightly more resistant (2.8 compared with 1.9-fold resistant; Table 1). Since it is known that point mutations in the mammalian *mdr1* gene can modify its resistance profile (Azzaria et al., 1989; Choi et al., 1988), amino acid sequence divergence between *lmpgpA* and *ltpgpA* may be responsible for the difference in resistance to arsenate relative to the other metals.

Members of the P-glycoprotein family are thought to mediate active efflux of toxic drugs and cellular metabolites (Chen et al., 1986b; Endicott and Ling, 1989; Gottesman and Pastan, 1988; Gros et al., 1986). Preliminary data show that *lmpgpA* transfectants accumulate 2-fold less antimony than wild-type and pSNAR-transfected cells when cultured in the presence of Sb(III) tartrate. Additional studies will be required to explain how a 2-fold decrease in drug accumulation mediates a 12-fold increase in drug resistance; however, similar disparities have been observed in cadmium-resistant *Staphylococcus aureus* (Tynecka et al., 1975, 1981) and *Bacillus subtilis* (Surowits et al., 1984; Laddaga et al., 1985). Resistance conferred by both *Leishmania* pgpA differs from previously studied mammalian and *Plasmodium* P-glycoproteins in substrate specificity. *Leishmania* pgpA transfectants show no resistance to puromycin or vinblastine (Table 1). Similarly, H-amplified lines of *L. tarentolae* show no resistance to puromycin, vincristine, doxorubicin, or chloroquine (Ouellette et al., 1990), drugs commonly associated with mammalian or *Plasmodium* multidrug resistance. In addition, drugs which are known to reverse multidrug resistance (verapamil, imipramine, desipramine, and quinidine; used at the highest nontoxic concentration) did not reverse resistance to Sb(III) tartrate or arsenite conferred by either *Leishmania* pgpA (data not shown). Recently, amplification of a P-glycoprotein gene highly divergent from *ltpgpA* has been reported in vinblastine-resistant *Leishmania donovani*; the resistance profile of these cells resembles that of multidrug-resistant mammalian cells.4

The unusual metal-resistant phenotype of pgpA transfectants points to the existence of a highly divergent class of P-glycoprotein genes. It is unknown whether *Leishmania* pgpA gene products interact directly with metals or in association with cellular factors such as trypanothione (Fairlamb et al., 1989; West, 1990). However, the pgpA drug resistance profile is similar to that of a bacterial heavy metal resistance system which exports arsenite, arsenate, and antimony(III) (Mobley and Rosen, 1982). This bacterial protein complex includes a membrane-embedded protein and associated ATPase and is thought to be analogous (not homologous) in function to P-glycoproteins (Chen et al., 1986a; Silver et al., 1989). However, sequence and structural comparisons show that *ltpgpA* belongs to the P-glycoprotein family (Ouellette et al., 1990) and is not closely related to the bacterial ATPase (data not shown).

These studies demonstrate one way that the newly developed *Leishmania* transfection methodology can be used to elucidate the function of specific genes, and they raise the possibility that P-glycoprotein genes play a role in the sensitivity and resistance of *Leishmania* to clinical drug treatment. Although pentavalent, not trivalent, antimonials are utilized to treat human Leishmaniasis, and *lmpgpA* does not confer Pentostam resistance in vitro, several workers have proposed that pentavalent derivatives are metabolized in vivo into the active species, possibly trivalent antimony (Croft et al., 1981; Goodwin and Page, 1943). Thus, it is possible that resistance to trivalent antimonials in vitro may signify resistance to pentavalent antimonials during infection in vivo. Correspondingly, Pentostam-resistant clinical isolates are cross-resistant to trivalent antimony when cultured in macrophages (Berman, 1982). Although the molecular basis for clinical resistance is unknown, some *Leishmania* selected for Pentostam resistance in vitro show reductions in antimony accumulation and increased binding of a P-glycoprotein monoclonal antibody (Grogl et al., 1991). If further studies show that pgpA is involved in clinical antimony sensitivity and resistance, then it may prove possible to develop effective agents to reverse P-glycoprotein-mediated metal resistance analogous to verapamil which reverses drug resistance in malaria and cultured mammalian cells.

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