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ABC Proteins of *Leishmania*

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ABC proteins were first characterized in the protozoan parasite *Leishmania* while studying mechanisms of drug resistance. PGPA is involved in resistance to arsenite and antimonite and it most likely confers resistance by sequestering metal–thiol conjugates into an intracellular vesicle. PGPA is part of gene family with at least four more members which are in search of a function. *Leishmania* also contains a P-glycoprotein, homologous to the mammalian MDR1, that is involved in multidrug resistance. The ongoing genome project of *Leishmania* has pinpointed several novel ABC transporters and experiments are carried out to study the function of the ABC proteins in drug resistance and in host–pathogen interactions.

KEY WORDS: *Leishmania*; ABC proteins; efflux; drug resistance; glutathione; trypanothione; sequestration.

INTRODUCTION

ABC Proteins

The ATP-binding cassette (ABC) proteins are ubiquitous and most of these proteins mediate transport across biological membranes (Higgins, 1992). The ATP-binding domains of the ABC proteins include the Walker A and B motifs and the "signature" or "C" motif just upstream of the Walker B site which distinguish members of the ABC superfamily from other ATP-binding proteins. The sequence conservation of the ABC domains has allowed the isolation of new ABC genes by hybridization, degenerated PCR, and by inspection of DNA sequence databases. The latter strategy is now the most efficient one, and inventories and classification of ABC proteins have been made for several genomes (Decottignies and Goffeau, 1997; Klein *et al.*, 1999; Linton and Higgins, 1998; Quentin *et al.*, 1999; Saurin *et al.*, 1999; Taglicht and Michaelis, 1998; Tomii and Kanehisa, 1998).

In this report we will describe the ABC transporters known in the protozoan parasite *Leishmania*. In particular, we will describe ABC transporters found to be involved in drug resistance and the ongoing *Leishmania* genome effort undertaken by an international consortium (see www.ebi.ac.uk/parasites/leish.html) has highlighted several novel ABC proteins.

Leishmania

Leishmania are intracellular protozoan parasites and important human pathogens that cause a wide spectrum of diseases ranging from self-healing cutaneous lesions to visceral infections that can be fatal. It is estimated that there is over 2 million new cases of leishmaniasis each year in 88 countries, while 367 million people are at risk (Herwaldt, 1999). The treatment of choice for all forms of leishmaniasis relies on chemotherapy, with the first therapeutic choice being the administration of the pentavalent antimony-containing compounds (SbV) sodium stibogluconate (pentostam) or *N*-methylglucamine (Glucantime) (Berman, 1997; Herwaldt, 1999). The mechanism of action of antimonials is unknown. Cases refractory to treatment have been described more than 40 years ago, but

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more recently the incidence of antimony-resistant parasites has increased greatly (Faraut-Gambarelli *et al.*, 1997; Grogl *et al.*, 1992; Ibrahim *et al.*, 1994; Jackson *et al.*, 1990; Lira *et al.*, 1999; Sundar *et al.*, 1997). The underlying mechanisms that contribute to drug resistance in field isolates are poorly understood but *in vitro* work incriminates ABC proteins.

IN VITRO METAL-RESISTANT *LEISHMANIA* AND THE ABC TRANSPORTER PGPA

In order to understand the mechanisms of antimony resistance in *Leishmania* we, and others, have selected in a step-by-step fashion *Leishmania* cells for resistance to SbV. There is considerable data in the literature, suggesting that the active form of the metal is trivalent antimony (SbIII), and consequently several mutants were selected for SbIII resistance. Arsenite and antimonite are related metals that share several properties, and as a result *Leishmania* cells were also selected for arsenite resistance (AsIII) and these mutants are often used as a paradigm to study *in vitro* induced metal resistance in *Leishmania* (Ouellette *et al.*, 1998b). Parasites selected for resistance to one metal are usually cross-resistant to the other metal, and resistance mechanisms observed in SbIII- and AsIII-resistant cells share several similarities (Haimeur *et al.*, 2000).

Analysis of drug-resistant mutants indicated that resistance to metals is multifactorial and consistent with the step-by-step mode of selection for mutants. The model is illustrated in Fig. 1. We found that trypanothione (TSH) is increased in metal-resistant *Leishmania* (Haimeur *et al.*, 2000; Légaré *et al.*, 1997; Mukhopadhyay *et al.*, 1996). TSH is the major reduced thiol in *Leishmania* and is made of a bisglutathione–spermidine conjugate (Fairlamb and Cerami, 1992). The basis for increased TSH in AsIII- and SbIII-resistant cell lines is well understood. The gene *GSH1*, coding for γ-glutamylcysteine synthase (γ-GCS), the rate-limiting step in glutathione (GSH) biosynthesis

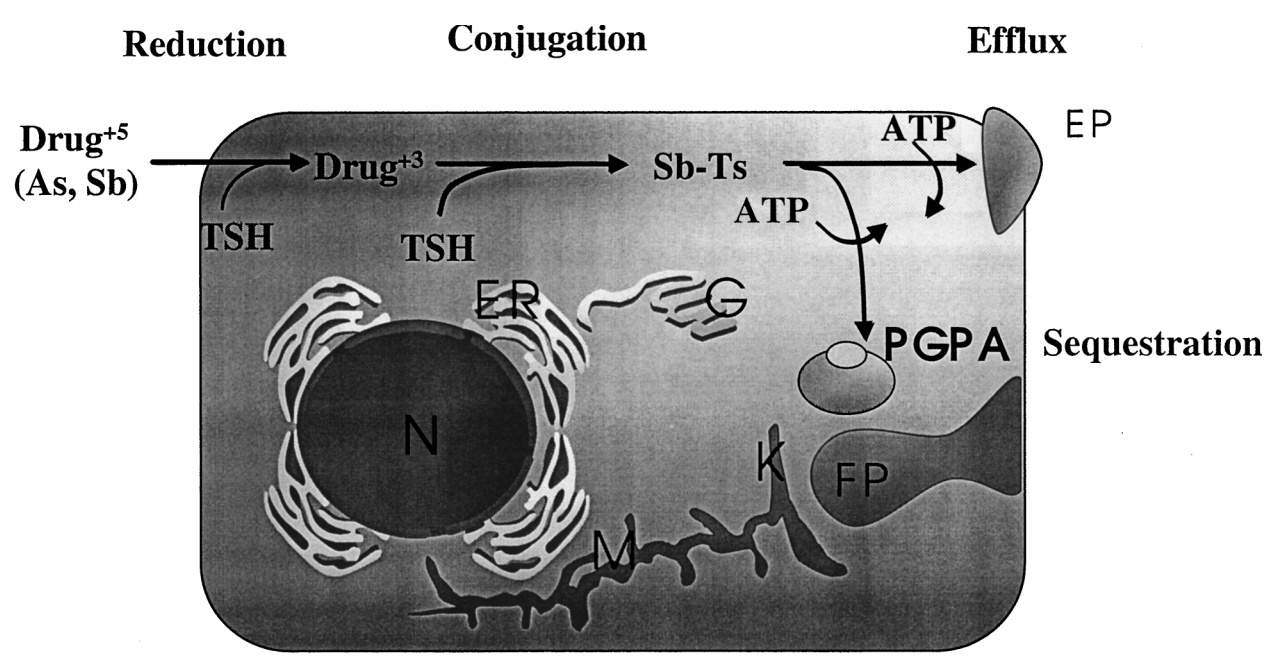

Fig. 1. Model for metal resistance in *Leishmania*. Pentavalent metals are likely reduced to the trivalent form which are thought to be the active form of the metals. The site of reduction is uncertain and could be either in the macrophage or in the parasite. An ORF on chromosome 1 of *L. major* shows weak similarities to an arsenate reductase (Myler *et al.*, 1999). This reduction may require trypanothione (TSH). Elevated levels of TSH are essential for resistance. This is achieved by amplification of *GSH1* (Grondin *et al.*, 1997) coding for γ-glutamylcysteine synthase and by overexpression of the *ODC* gene (Haimeur *et al.*, 1999) coding for the enzyme ornithine decarboxylase; these are the rate-limiting steps in glutathione and spermidine biosynthesis, respectively. A reduction in TSH levels by using specific inhibitors of glutathione and spermidine biosyntheses will revert resistance (Haimeur *et al.*, 1999). Although arsenite–TSH conjugates can form spontaneously in the test tube (Mukhopadhyay *et al.*, 1996), a putative TSH conjugate might be necessary inside the cell to increase the rate of generation of the substrate for the various X-thiol transporters. The metal–TSH conjugate can then be sequestered by being transported into an intracellular compartment by PGPA. These conjugates may then go outside the cell by exocytosis which occurs exclusively through the flagellar pocket (FP). Alternatively, the metal–TSH conjugate might be extruded outside the cell by a plasma membrane thiol-X-efflux pump (EP). N, nucleus; ER, endoplasmic reticulum; G, Golgi; M, mitochondria; K, kinetoplast.

is amplified (Grondin *et al.*, 1997; Haimeur *et al.*, 2000). In addition, the gene coding for ornithine decarboxylase (ODC), the rate-limiting step in spermidine biosynthesis is overexpressed in AsIII-resistant mutants (Haimeur *et al.*, 1999). A dual increase in GSH and spermidine levels, the two building blocks of TSH, leads to an increase in TSH levels in drug-resistant mutants. We found that TSH is essential for resistance but elevating levels of TSH alone is not sufficient for resistance. Indeed, transfection of either *GSH1* or *ODC* leads to an increase in TSH levels in wild-type cells that is even higher than TSH levels encountered in resistant cells; however, no increase in resistance is observed in the wild-type transfectants (Grondin *et al.*, 1997; Haimeur *et al.*, 1999). The γ-GCS and ODC specific inhibitors buthionine sulfoximine (BSO) and difluoromethyl ornithine (DFMO) can reduce the level of TSH into the resistant cells and do reverse the resistance phenotype in the mutants (Haimeur *et al.*, 1999, 2000). A strong correlative link therefore exists between TSH levels and resistance, but other gene products are implicated in the resistance phenotype.

The gene coding for the ABC transporter PGPA is frequently amplified in metal-resistant *Leishmania* (Ouellette *et al.*, 1998a). When discovered, PGPA was found to be the most divergent of eukaryotic ABC transporters (Ouellette *et al.*, 1990). When the MRP sequence became available, PGPA was found to be its closest homologue (Cole *et al.*, 1992). PGPA is now included in the MRP subfamily of ABC transporters. The results of *PGPA* gene transfection indicated clearly that this gene can contribute to AsIII and SbIII resistance (Callahan and Beverley, 1991; Légaré *et al.*, 1997; Papadopoulou *et al.*, 1994). The level of resistance conferred by PGPA differed, depending in which *Leishmania* species the gene was transfected. In *L. tarentolae,* only low level of resistance was observed and it was not possible to reach resistance levels observed in drug-resistant mutants (Légaré *et al.*, 1997; Papadopoulou *et al.*, 1994). This led to the suggestion that PGPA requires other factors for conferring high levels of resistance and that the availability of these factors may differ in various *Leishmania* species. The GS-X-mediated resistance pathway of mammalian cells was suggested to implicate sustained elevated GSH levels, increased activity of the GS-X-transporter, and increased conjugate activity (Ishikawa, 1992). By analogy to the GS-X pathway, we proposed that PGPA is recognizing metals conjugated to TSH. In order to test this hypothesis we first have done co-transfection experiments with *PGPA* and *GSH1* or *ODC*. When these genes were transfected into wild-type cells, we found only the low resistance levels mediated by PGPA; however, when the combination of genes was transfected in revertant cells (mutants grown in absence of the drug for prolonged period) we observed a strong synergy leading to high levels of resistance (Grondin *et al.*, 1997; Haimeur *et al.*, 1999) suggesting indeed that PGPA recognizes metals conjugated to TSH (Fig. 1). Since this synergy only occurs in revertant cells, it is clear that at least one other mutation is present in the mutant, and by analogy to the GS-X system, we are proposing that the missing mutation is a trypanothione-S-transferase. Work is in progress to unravel this activity.

ABC transporters often mediate resistance by increased extrusion of the drug outside the cell and our initial hypothesis was that PGPA corresponds to an efflux pump. Transport experiments indeed indicated that there was an active efflux of the metal outside the cell; however, this efflux system seems unrelated to PGPA (Dey *et al.*, 1994). Everted vesicles of fractions enriched for plasma membranes suggested that this efflux system recognizes metal–thiol conjugates (Dey *et al.*, 1996). The activity of this transporter is not increased in membranes derived from mutants or in cells overexpressing PGPA, suggesting that it corresponds to another gene product and that this transporter itself is not rate limiting. PGPA may therefore corresponds to an intracellular ABC transporter. This was tested by making a PGPA-green fluorescent protein (GFP) fusion. The PGPA-GFP fusion was totally active and conferred metal resistance in a TSH-dependent manner. The active fusion was indeed shown to be located into an intracellular membrane close to the flagellar pocket (Légaré *et al.*, 2001). This localization was also confirmed by electron microscopy. By ultracentrifugation in density gradient, we were able to isolate sealed cellular membrane vesicles enriched for PGPA and showed that they exhibit a basal ATPase activity. Preliminary transport experiments using these PGPA-enriched vesicles suggested indeed that PGPA transports metal–thiol conjugates in an ATP dependent fashion. PGPA therefore appears to confer resistance by sequestering thiol–metal conjugates in vesicles close to the flagellar pocket.

The ABC transporter HMT1 confers cadmium tolerance by sequestring phytochelatine (a glutathione-like molecule)–cadmium complexes in the fission yeast vacuole (Ortiz *et al.*, 1995). The yeast ABC transporter YCF1 confers cadmium and arsenite resistance by mediating the vacuolar accumulation of metal–glutathione complexes (Ghosh *et al.*, 1999; Li *et al.*, 1996; Tommasini *et al.*, 1996). Sequestration therefore seems to be a frequent mechanism used by cells for resisting metals (reviewed in Ishikawa *et al.*, 1997). The PGPA-mediated resistance phenotype was studied mainly in the promastigote form of the parasite. However, it is the intracellular form of the parasite that is treated with the drug. Recently, we have

transfected the *PGPA* gene in *Leishmania* cells that infect and survive well within macrophages and we found that these transfectants, once inside macrophages, are also resisting the activity of metals (unpublished observations) suggesting that PGPA can also confer resistance to metals in the intracellular stage of the parasite which is in contact with the drug.

MRP-Like Gene Family in *Leishmania*

Since the discovery of MRP1, several other MRP isoforms were found with now at least six members (Borst *et al.*, 1999). PGPA, which is part of the MRP subfamily of ABC proteins, is also part of a large gene family in *Leishmania* with at least four other members termed *PGPB*, *PGPC*, *PGPD*, and *PGPE* genes (Légaré *et al.*, 1994). The nucleotide sequence of *PGPB* and *PGPE* is known and the gene products are highly similar to PGPA. Partial sequence analysis of PGPC and PGPD also suggest that these genes are highly homologous to PGPA. Transfection experiments failed to show a role in resistance for any of the four novel genes (Légaré *et al.*, 1994) although co-transfection with *GSH1* has never been done and a limited number of drugs was tested. In a methotrexate (MTX)-resistant *L. tropica* cell line, a *PGPE* homologue was shown to be overexpressed (Gamarro *et al.*, 1994). With the recent demonstration that some members of the MRP family have the ability to produce MTX resistance (Hooijberg *et al.*, 1999), the role of PGPE in MTX resistance merits to be reinvestigated, although transfection of *PGPE* in *L. tarentolae* is not associated with resistance to MTX (Légaré *et al.*, 1994). Because of their sequence similarities to PGPA and MRP, it is likely that PGPB, PGPC, PGPD, and PGPE are thiol-X transporters and one of these may correspond to the thiol-X pump that is not PGPA and was seen in intact cells or everted vesicles and is responsible for metal efflux (Dey *et al.*, 1994, 1996). This possibility is now under investigation.

P-Glycoprotein

Leishmania contains in its genome at least one P-glycoprotein homologue. The *Leishmania* gene product is highly homologous to the mammalian MDR1 and it was characterized from several *Leishmania* species. The *Leishmania MDR1* gene was amplified in *Leishmania* mutants selected for vinblastine or daunomycin resistance and transfection experiments indeed indicated that this *MDR1* gene can cause multidrug resistance (Chiquero *et al.*, 1998; Chow *et al.*, 1993; Gueiros-Filho *et al.*, 1995; Henderson *et al.*, 1992; Katakura *et al.*, 1999). The interactions between flavenoids and the ABC domain of the *Leishmania* MDR1 were characterized and some derivatives with high affinity to the nucleotide-binding domain were reverting the multidrug resistance phenotype of resistant cells (Perez-Victoria *et al.*, 1999).

The high degree of homology between *Leishmania* and human MDR1 suggests that the former could confer resistance by active extrusion of the drug. The efflux of rhodamine 123 in *L. amazonensis* resistant cells (Gueiros-Filho *et al.*, 1995) and the absence of accumulation of puromycin in vinblastine resistant *L. donovani* (Henderson *et al.*, 1992) are consistent with this hypothesis. Efflux of either vinblastine or puromycin has not yet been shown, however. Moreover, subcellular localization studies in the laboratory of D. Wirth at Harvard suggest that the majority of *Leishmania* MDR1 protein is not located in the plasma membrane but in the mitochondria of *L. enriettii* (Chow and Volkman, 1998). Further work is required to understand how MDR1 confers drug resistance in *Leishmania*. Drugs that are part of the mammalian multidrug resistance spectrum are not used to treat leishmaniasis. However, *Leishmania* may encounter toxic natural products at one stage of its life cycle where MDR1 would be required for detoxification.

Other ABC Transporters

The sequence of the *L. major* genome is well underway and several of its 36 chromosomes are now being sequenced by an international consortium of laboratories and institutes (http://www.ebi.ac.uk/parasites/LGN). Chromosome 1 is completed (Myler *et al.*, 1999) and chromosomes 3, 4, 19, and 23 are nearly completed. The consortium estimates a total of about 9000 genes in the entire parasite genome. The number of unique sequences should be lower since significant proportion of the *Leishmania* genes are present in more than one copy. The ongoing sequencing project suggests that the majority of ORFs will be in search of a function. A recent survey of the available sequences, either as part of sequenced chromosomes, cosmids or genome survey sequences (GSS), revealed that *Leishmania* is likely to contain several ABC proteins (Table I).

Out of the 79 ORFs of chromosome 1 there is no ABC transporter, but on chromosome 3, a gene is present coding for a protein (ABCTP1) with two ABC domains and no apparent transmembrane segments. ABCTP1 shares extensive similarities with several other putative ABC transporters found in diverse organisms. The yeast YEF3 and GCN20 ABC proteins also contained duplicated ABC

Table I. ABC Transporters in *Leishmania*

Transporters	Comments	Chromosome	Accession number
PGPA	Involved in metal resistance	23	X171154
PGPB	Linked to PGPA	23	L29484
PGPC	Linked to PGPA and PGPB	23	AL135898[a]
PGPD	Linked to PGPE	33[b]	N.D.
PGPE	Linked to PGPD	33[b]	L29485
MDR1	Involved in multidrug resistance	33 or 34[b]	L08091
ABCTP1	No transmembrane domains	3	AAF31030[c] AAF28378
L4468.01	Possible ATP-dependent permease precursor	23	AL121864[a]

[a] Zimmermann, W., Wambutt, R., Ivens, A. C., Murphy, L., Quail, M., Rajandream, M. A., and Barrell, B. G., European *Leishmania major* Friedlin genome sequencing project, Sanger Center, The Wellcome Trust Genome Campus.

[b] Papadopoulou, B., unpublished observation.

[c] Myler, P. J., Sisk, E., Hixson, G., Kiser, P., Rickel, E., Hassebrock, M., Cawthra, J., Marsolini, F., Sunkin, S., and Stuart, K. D., Seattle Biomedical Research Institution, 4 Nickerson Street, Seattle, WA 98109-1651, USA.

domains without transmembrane segments and are involved in translation (Bauer *et al.*, 1999; Decottignies and Goffeau, 1997; Taglicht and Michaelis, 1998). ABCTP1 may serve a similar function. As part of an ongoing project to determine the function of ABC transporters in *Leishmania*, we are attempting to disrupt several ABC genes by homologous recombination. One of the two alleles of the *ABCTP1* gene was inactivated and no effect on growth properties of the mutants was observed (unpublished observation). Work is in progress to generate a null mutant.

It was already known that *PGPA*, *PGPB*, and *PGPC* were linked on the same chromosome while *PGPD* and *PGPE* and possibly *MDR1* are also linked on another chromosome (Légaré *et al.*, 1994). The sequencing effort has indicated that *PGPA*, *PGPB*, and *PGPC* are part of chromosome 23. A fourth ABC transporter was also found on chromosome 23. It contains one ABC domain and sequence similarities suggest a possible role as an ATP-dependent permease precursor. ABC transporter genes seem absent from the annotated sequences presently available for chromosomes 4 and 19. However, GSS revealed a plethora of novel ABC transporters in *Leishmania*.

Indeed, BLAST analysis of GSS done at Washington University in St. Louis (N. S. Akopyants and S. M. Beverley "A survey of the *L. major* Friedlin strain V1 genome by shotgun sequencing" and the Washington University Genome Sequencing Center) and at the Sanger Center (*L. major* Friedlin genome sequencing project, Sanger Center, The Wellcome Trust Genome Campus) clearly indicated the presence of several novel ABC transporters. Once translated, at least eight GSS have clearly recognizable and significant portions of ABC domains which are different than the ABC transporters of Table I. As sequences are partial it is difficult at this point to determine to which subfamily of ABC transporters these proteins belong. In addition, a number of GSS are clearly homologous to non-ABC domains of ABC transporters. Two other GSS were highly similar to a number of ABC proteins of bacteria. Two more GSS were highly similar to portions of two members (ABCA1 and ABCA3) of the ABCA subfamily of ABC transporters (Broccardo *et al.*, 1999) in the "switch region" downstream of the Walker B sequence of the second nucleotide-binding site. The sequence of the ABCA1 gene homologue of *Leishmania* is now completed (F. Gamarro, Grenada, personal communication). The ABCA subfamily is absent in the yeast genome and was thought to be restricted to multicellular organisms (Broccardo *et al.*, 1999). The presence of these transporters in the unicellular parasite *Leishmania* is interesting. ABCA1 appears to be involved in the control of membrane lipid composition and in a recessive disorder (called Tangier disease) of lipid metabolism in humans. The same protein has been implicated in the engulfment of cells (dying by apoptosis) by macrophages (Luciani and Chimini, 1996). The presence of ABC1-like protein in a parasite, engulfed by and living within macrophages, is noteworthy and may suggest a role for this ABC protein in host–pathogen interactions, possibly in the scavenging of host lipids.

CONCLUSION

Studies on the mechanisms of drug resistance have pinpointed the first ABC transporters of *Leishmania*. The sequence of the genome is progressing rapidly and its analysis indicated a number of novel ABC transporters. Inventories of ABC transporters are made for several of the organisms for which the genome is completed. ABC proteins are ubiquitous and, for example, constitute the largest family of paralogous proteins in *Escherichia coli* (Blattner *et al.*, 1997). Phylogenetic analysis with ABC protein orthologues from different organisms will help in functional assignment. Nonetheless, considerable work will be required to assess the function of several ABC proteins. Several recent tools developed in *Leishmania*, including episomal transfection, gene disruption mediated by homologous recombination, protein localization

using GFP fusions, and membrane and vesicles purification, should be useful to determine the function of ABC proteins in *Leishmania* and study their putative role in host–pathogen interactions and in drug resistance.

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