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Co-amplification of the γ-glutamylcysteine synthetase gene *gsh1* and of the ABC transporter gene *pgpA* in arsenite-resistant *Leishmania tarentolae*

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Resistance to the oxyanion arsenite in the parasite *Leishmania* is multifactorial. We have described previously the frequent amplification of the ABC transporter gene *pgpA*, the presence of a non-PgpA thiol–metal efflux pump and increased levels of glutathione and trypanothione in resistant cells. Other loci are also amplified, although their role in resistance is unknown. By gene transfection, we have characterized one of these novel genes. It corresponds to *gsh1*, which encodes γ-glutamylcysteine synthetase, an enzyme involved in the rate-limiting step of glutathione biosynthesis. Transfection of *gsh1* in wild-type cells increased the levels of glutathione and trypanothione to levels found in resistant mutants. These transfectants were not resistant to metals. However, when *gsh1* was transfected in partial revertants, it conferred resistance. As *pgpA* is frequently co-amplified with *gsh1*, we co-transfected the two genes into both wild-type and partial revertants. Arsenite resistance levels in wild-type cells could be accounted for by the contribution of PgpA alone. In the partial revertant, the *gsh1* and *pgpA* gene product acted synergistically. These results support our previous suggestion that PgpA recognizes metals conjugated to thiols. Furthermore, amplification of *gsh1* overcomes the rate-limiting step in the synthesis of trypanothione, contributing to resistance. In addition, the results suggest that at least one more factor acts synergistically with the *gsh1* gene product.

Keywords: ABC transporter/drug resistance/glutathione/*Leishmania*/trypanothione

Introduction

Leishmania are protozoan parasites that affect millions of people worldwide. The only effective way to control *Leishmania* is chemotherapy. The first line of drugs used against *Leishmania* are pentavalent antimonials, and clinical resistance to these drugs is now frequently encountered (Ouellette and Papadopoulou, 1993). To understand the mechanisms of drug resistance to antimony, we have selected, in a step by step protocol, several different *Leishmania* species for *in vitro* resistance to antimony but also for the related metal arsenic (Ouellette *et al.*, 1995). Gene amplification is observed frequently in arsenite-resistant *Leishmania* cells (Detke *et al.*, 1989; Ouellette *et al.*, 1991; Grondin *et al.*, 1993; Singh *et al.*, 1994). The first amplified gene characterized was the P-glycoprotein-related gene homolog *pgpA* (Ouellette *et al.*, 1990), a member of the ever growing family of ABC transporters which are involved in resistance to drugs in mammalian tumor cells (Gottesman and Pastan, 1988) and in parasites (Borst and Ouellette, 1995). PgpA is more similar to the human multidrug-associated protein MRP than to P-glycoproteins (Cole *et al.*, 1992; Légaré *et al.*, 1994). Transfection experiments implicated *pgpA* in both arsenite and antimonite resistance, but resistance levels differed depending on which *pgpA* allele was used and in which *Leishmania* species the genes were transfected (Callahan and Beverley, 1991; Papadopoulou *et al.*, 1994a).

We have also observed, in *L.tarentolae* mutants selected for resistance to arsenite or antimonite, the presence of a non-*pgpA* efflux system (Dey *et al.*, 1994; Papadopoulou *et al.*, 1996) that recognizes metals conjugated to various thiols including glutathione (GSH) (Dey *et al.*, 1996) and trypanothione (TSH) (Mukhopadhyay *et al.*, 1996). GSH is the tripeptide γ-Glu–Cys–Gly and plays an important role in the cellular defense against oxidative stress and in the detoxification of several drugs and xenobiotics (Meister and Anderson, 1983). TSH (Fairlamb *et al.*, 1985), which consists of a spermidine moiety linked to two GSH molecules, is the major reduced thiol in *Leishmania* (Fairlamb and Cerami, 1992). Recently, we found a large increase in TSH and smaller increases in cysteine and GSH in arsenite-resistant cell lines (Mukhopadhyay *et al.*, 1996).

Resistance to oxyanions in *Leishmania* is, therefore, clearly multifactorial, with contributions by several independent mechanisms. Several loci in addition to *pgpA* are also amplified (Ouellette *et al.*, 1995). One of these loci was first observed as part of a 50 kb linear amplicon derived from an 800 kb chromosome (Grondin *et al.*, 1993). We have now studied the role of this amplicon in resistance by gene transfection. We show that the amplicon contains the *gsh1* gene, which codes for the heavy subunit of γ-glutamylcysteine synthetase (γ-GCS), the enzyme that catalyzes the rate-limiting step of GSH biosynthesis (Meister and Anderson, 1983). The *gsh1* gene was transfected into wild-type cells and into revertants of arsenite-resistant strains. There was no increase in arsenite resistance in the wild-type transfectants. In contrast, transfection of *gsh1* into the revertant conferred arsenite resistance. These results indicate that the *gsh1* gene product is necessary but not sufficient for resistance. In addition, co-transfection of the *pgpA* gene produced synergistic resistance, suggesting that there are several independent pathways that result in resistance.

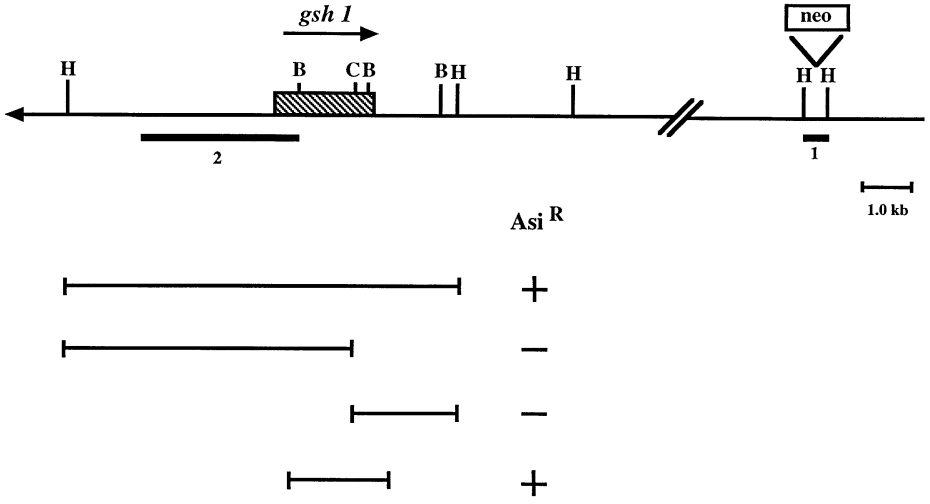

Fig. 1. Map of the *gsh1* locus of *L.tarentolae*. A portion of the linear amplicon containing the *gsh1* gene is shown. The site of integration of the *neo* gene into the linear amplicon is also shown. Probes (1 and 2) used in this study are located below the map. The location of probe 1 relative to probe 2 is unknown and could be upstream of the *gsh1* gene. Below the map the sizes of the fragments cloned into *Leishmania* expression vectors are indicated. These fragments were transfected into TarIIAs20.3rev, and the resistance to arsenite of the transfectants was compared with that of strains containing the *neo*-containing linear amplicon. B, *Bam*HI; C, *Cla*I; H, *Hin*dIII

Results

DNA amplification in arsenite-resistant mutants

We have already described 12 independent *L.tarentolae* mutants highly resistant to arsenite. The H locus *pgpA* gene was amplified as part of extrachromosomal circles in half the mutants (Ouellette *et al.*, 1991; Grondin *et al.*, 1993). Another unrelated locus was amplified as part of a 50 kb linear amplicon (Grondin *et al.*, 1993). Amplification of that locus was first observed when we used probe 1 (Figure 1) (Grondin *et al.*, 1993). When probe 2 (see Figure 1) derived from the linear amplicon was used, we observed that this region is amplified in all 12 mutants studied, either as part of a 50 kb linear amplicon (eight mutants) (Figure 2, lane 3) or as part of a circular amplicon (four mutants) (Figure 2, lane 1) containing ~13 kb of unique sequences. When mutants containing either the 50 kb linear amplicon (TarIIAs20.2) or the circular amplicon (TarIIAs20.3) were maintained in culture in the absence of arsenite selection for 2 months, the amplicons were lost and the partial revertants were 2- to 10-fold less resistant to arsenite compared with the parent mutants (Table I). The circumstantial link between the presence of the amplicons and oxyanion resistance was studied more directly by gene transfection.

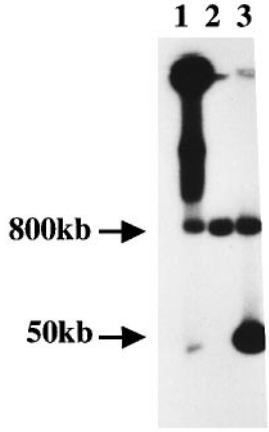

Fig. 2. Linear and circular gene amplification in arsenite-resistant *L.tarentolae* cells. Chromosomes were separated by TAFE, and Southern blots were hybridized with probe 2 (see Figure 1). Lane 1, TarIIAs20.3; lane 2, TarIIWT; lane 3, TarIIAs20.2. The 800 kb band present in the three strains corresponds to the chromosomal copy of the *gsh1* locus. In mutant TarIIAs20.3, the locus was amplified as a circle whereas in TarIIAs20.2 it was present as a 50 kb linear amplicon. Molecular sizes were estimated from the *S.cerevisiae* chromosomes.

Transfection of the linear amplicon

We first attempted to transfect the 50 kb linear amplicon into wild-type cells. We have shown previously the feasibility of transfecting linear chromosomes into *Leishmania* (Papadopoulou *et al.*, 1993). A fragment derived from the linear amplicon into which the neomycin phosphotransferase gene (*neo*) was cloned (Figure 1) was transfected in the mutant TarIIAs20.2 in order to integrate the *neo* gene into the linear amplicon by homologous recombination (Figure 3, lane 4). Whereas the amplicon in TarII As20.2 hybridized only to the probe 1 (Figure 3A and B, lane 2), the amplicon present in transfectant TarIIAs20.2*neo* hybridized with both the amplicon-specific (Figure 3A, lane 4) and *neo* (Figure 3B, lane 4) probes, showing that the *neo* gene has indeed integrated into the linear amplicon but apparently not at the chromosomal copy of this locus. Chromosomes of the transfectant TarIIAs20.2*neo* were resolved by transalternative field electrophoresis (TAFE). The *neo*-containing linear amplicon was isolated and transfected into the *L.tarentolae* TarIIWT strain. Transfectants were selected for G418 resistance and hybridized with probe 1 and with a *neo* probe to test for the presence of the linear amplicon. Linear fragments at 50, 200 and 800 kb hybridized with both probes in transfectant TarIIwt+20.2*neo* (Figure 3, lanes 5). At lower exposure times, the 50 kb band was shown in fact to be made of a 50 and a 100 kb band (data not shown). These results suggest that multimerization of the amplicon occurred during transfection, since the linear amplicon is present

Table I. Arsenite resistance and total thiol levels in *L.tarentolae* transfectants

Cell lines	Arsenite resistance		Total thiols (nmol/10^8 cells)	Fold increase[a]
	EC_{50} (μM)[b]	Fold increase[a]		
TarII WT	0.4	1	5.3 ± 0.2	1
TarIIWT + 20.2neo[c]	0.4	1	7.6 ± 0.2	1.5
TarIIWT + *gsh1*[d]	0.4	1	ND	
TarIIWT + *pgpA*	0.8	2	ND	
TarIIWT + 20.2neo + *pgpA*	0.8	2	ND	
TarIIAs20.2	50	2	ND	
TarIIAs20.2rev	25	1	ND	
TarIIAs20.2rev + 20.2neo	50	2	ND	
TarIIAs20.2rev + *pgpA*	50	2	ND	
TarIIAs20.2rev + 20.2neo + *pgpA*	160	8	ND	
TarIIAs20.3	50	10	7.6 ± 0.1	1.5
TarIIAs20.3rev	5	1	5.9 ± 0.2	1.1
TarIIAs20.3rev + *gsh1*	10	2	9.4 ± 1.1	1.8
TarIIAs20.3rev + *pgpA*	10	2	ND	
TarIIAs20.3rev + *gsh1* + *pgpA*	80	16	ND	

[a]Values refer to fold increase compared with either wild-type or the revertants of As20.2 or As20.3.
[b]Average of at least three independent measurements.
[c]The linear amplicon.
[d]The 8 kb *Hin*dIII–*Hin*dIII fragment derived from the linear amplicon (see Figure 1).
[e]Nd, not determined.

in the transfectant as a monomer (50 kb), a dimer (100 kb) and a tetramer (200 kb). We have observed a similar phenomenon while transfecting H locus linear amplicons (Papadopoulou *et al.*, 1993). After few passages, only the 50 kb linear amplicon was retained (not shown). Since the *neo* probe recognizes the 800 kb chromosome in TarIIwt+20.2*neo*, the amplicon, or at least part of it, also integrated at the chromosomal locus (Figure 3B, lane 5).

The linear amplicon produces arsenite resistance

Resistance in wild-type *L.tarentolae* cells with an increased copy number of the linear amplicon was examined in the presence of varying concentrations of arsenite. The transfectant TarIIWT+20.2*neo* was no more resistant to arsenite than was the TarII wild-type strain (Table I). Although gene amplification events unrelated to drug resistance (Beverley, 1991; Ouellette *et al.*, 1995) have been described in *Leishmania*, the above negative result was unexpected, especially when taking into account that loss of the amplicon in mutants was associated with decreased resistance (Table I). Since oxyanion resistance has several components (see Borst and Ouellette, 1995; Ouellette *et al.*, 1995), we reasoned that to give rise to resistance, the gene present on the linear amplicon may need other mutations absent in wild-type cells.

The neo-containing linear amplicon was transfected in TarIIAs20.2rev to yield the transfectant TarIIAs20.2 rev+20.2*neo*. Probe 1 hybridized to the 800 kb chromosomal locus but also to a 50 kb band (which at shorter exposure time was also shown to consist of 50 and 100 kb bands). Similarly to the wild-type transfectants, after few passages only the 50 kb amplicon was retained (not shown). The *neo* probe hybridized to the 50 kb band but not, as expected, to the chromosomal locus (Figure 2B, lane 6). The introduction of the linear amplicon in TarIIAs-20.2rev consistently restored the resistance to the level of the parental strain (Table I), clearly demonstrating that the linear amplicon is involved in oxyanion resistance.

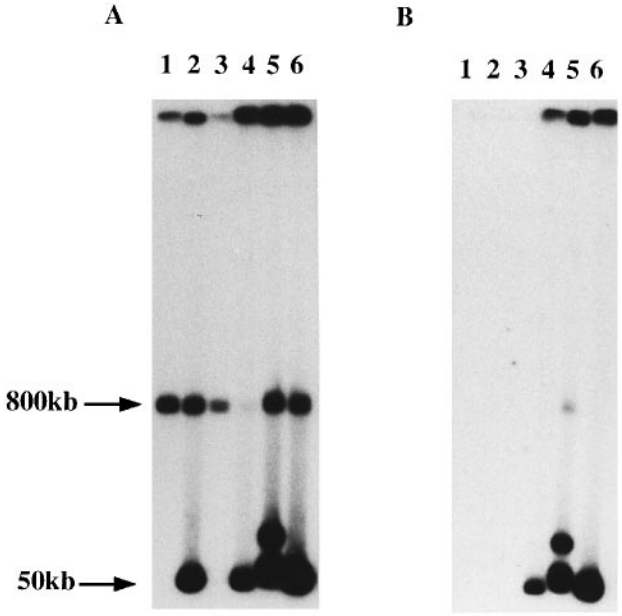

Fig. 3. Transfection of the linear amplicon isolated from TarIIAs20.2. The 50 kb linear amplicon is present in TarIIAs20.2 (lane 2). When this mutant was kept in culture without selection, the amplicon was lost, yielding a partial revertant (lane 3). The *neo* gene (cloned into the 0.5 kb *Hin*dIII–*Hin*dIII fragment of Figure 1) was transfected into TarIIAs20.2 to integrate *neo* into the linear amplicon (lane 4). The linear amplicon targeted with *neo* was isolated and transfected into the wild-type (lane 5) or the partial revertant (lane 6). (**A**) Hybridized with probe 1 and (**B**) with a *neo* probe. Lanes 1, TarIIWT; 2, TarIIAs20.2; 3, TarIIAs20.2rev; 4, TarIIAs20.2*neo*; 5, TarIIWT+20.2*neo*; 6, TarIIAs20.2rev+20.2*neo*.

The results further indicate that other factors found in the partial revertant but absent in the wild-type strain are required to produce resistance. The gene present on the linear amplicon and implicated in resistance was subcloned and characterized.

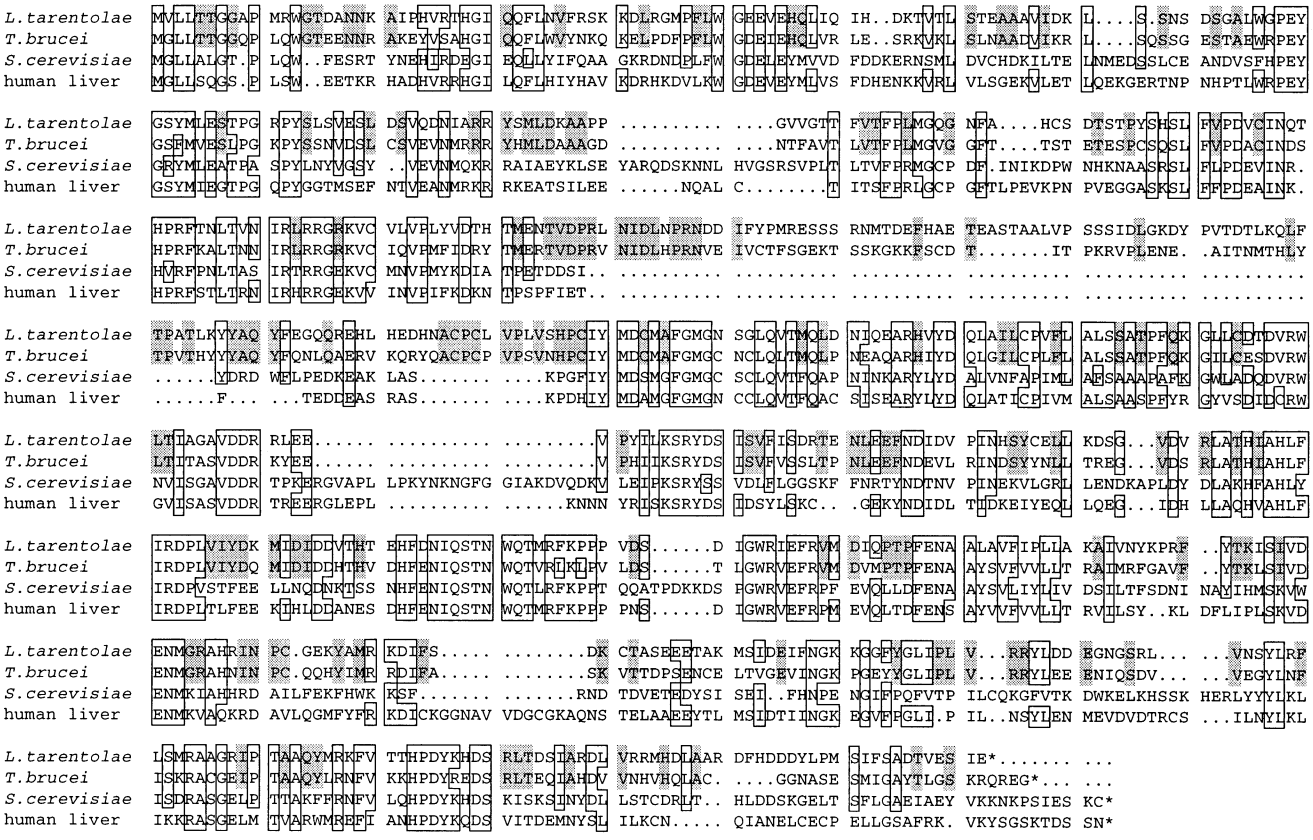

Fig. 4. Alignment of the deduced amino acid sequences of four γ-GCS, from *L.tarentolae*, *T.brucei* (Lueder and Phillips, 1996), human liver (Gipp *et al.* 1992) and *S.cerevisiae* (Ohtake and Yabuuchi, 1991). The *Schizosaccharomyces pombe* γ-GCS (Mutoh *et al.*, 1995) is not shown in the alignment but is highly homologous to the other proteins. The protein sequences were aligned for maximum identity by introduction of gaps using the UWGCG PileUp Program (Genetics Computer Group, 1994). Identical residues present in at least three sequences are shown within open boxes. Amino acids in common between *L.tarentoale* and *T.brucei* but absent in the two other sequences are shaded. Gaps are shown as dots (.). Nucleotide sequence accession numbers are for *L.tarentolae*, Y10049; human liver, M90656; *T.brucei*, U56818; *S.cerevisiae*, M87066.

The linear amplicon encodes the catalytic subunit of γ-glutamylcysteine synthetase

To clone the gene present on the linear amplicon that was responsible for arsenite resistance, we used probe 2 (Figure 1) to screen a cosmid library made from the mutant TarIIAs20.4. An 8 kb *Hin*dIII–*Hin*dIII fragment derived from one isolated cosmid was found to be associated with resistance when transfected into TarIIAs20.2rev (Figure 1). Transfection of this fragment in the partial revertant TarIIAs20.3rev was also associated with resistance (Table I). In contrast, transfection of this 8 kb fragment into the wild-type did not increase resistance. Subcloning, transfection and growth curves in the presence of arsenite suggested that the gene involved in resistance was within or close to a unique *Cla*I site (Figure 1), and DNA sequence was initiated at this site. An open reading frame of 2064 bp with a deduced amino acid sequence sharing significant similarities with the heavy subunit of γ-GCS was identified (Figure 4). The enzyme γ-GCS (EC 6.3.2.2), which catalyzes the ATP-dependent conjugation of glutamic acid and cysteine, has been shown to be the rate-limiting step of GSH biosynthesis (Meister and Anderson, 1983). The *L.tarentolae* γ-GCS is closely related to the recently characterized *Trypanosoma brucei* enzyme (Lueder and Phillips, 1996). Both enzymes are slightly larger than other homologs, the *Leishmania* enzyme being 688 amino acids in length, but are nevertheless highly similar to yeast and mammalian homologs, with 40–43% overall identities and several long stretches of high identity (Figure 4, open boxes). The *gsh1* gene encoding γ-GCS was amplified by PCR and cloned into pSPY-neo. Transfection of a fragment containing only the *gsh1* gene was sufficient to confer resistance to arsenite in partial revertants (Figure 1).

Increased level of thiols in gsh1 transfectants

The amount of *gsh1* RNA was correlated with the copy number of the *gsh1* gene. A 2.4 kb overexpressed RNA was observed in mutants and in transfectants (Figure 5) compared with wild-type cells or revertants grown in the absence of arsenite (Figure 5, lanes 1 and 4). We often observed that in *L.tarentolae*, genes present in transfection vectors give rise to more RNAs than genes with a similar copy number but present in natural amplicons (see also Papadopoulou *et al.*, 1992). The γ-GCS enzymatic activity of mutants and *gsh1* transfectants was increased compared with wild-type cells or with the revertant (Table II). This enzymatic activity was inhibited by the addition of the γ-GCS inhibitor L-buthionine-(*SR*)-sulfoximine (BSO) (Figure 6A), demonstrating the specificity of the assay. At 1 mM BSO, 70% of the enzymatic activity present in cell lysates was inhibited (Figure 6A), a value similar to the one reported for the purified *T.brucei* enzyme (Lueder and Phillips, 1996). To examine the effect of BSO on

in vivo reversal of resistance, we pre-treated the cells with 5 mM BSO, as indicated in Materials and methods. Pre-treatment of cells with BSO in defined medium was shown to sensitize arsenite-resistant cells to arsenite (Figure 6B).

Using 5,5′-dithio-bis 2-nitrobenzoic acid (DTNB), the level of total thiols was found to be increased in mutants and *gsh1* transfectants (either the 8 kb *Hin*dIII fragment or the *gsh1* coding region obtained by PCR) (Table I, and not shown). The thiols from mutants and *gsh1* transfectants were separated by HPLC (Table II). As described previously (Mukhopadhyay *et al.*, 1996), arsenite-resistant mutants had a large increase in TSH and smaller increases in cysteine and GSH compared with wild-type. Here we show that GSH and TSH levels are decreased in the partial revertant (Table II). More interestingly, transfection of *gsh1* into both wild-type cells and revertants increased the amounts of GSH to levels even higher than those found in the original mutants. Remarkably, the levels of TSH were increased 20-fold in the *gsh1* transfectants (Table II). These results indicate that in *Leishmania*, the *gsh1* gene product is the rate-limiting step of both GSH and TSH biosynthesis. Although high levels of thiols appear to be required for resistance, this is clearly not sufficient, since wild-type cells with high levels of thiols (*gsh1* transfectants) are not resistant to oxyanions (Table I).

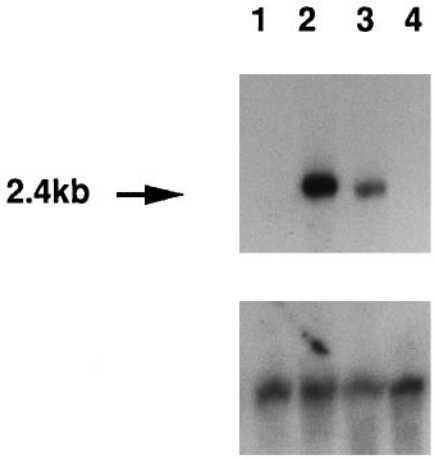

Fig. 5. Northern blot analysis of total RNA isolated from *L.tarentolae*. Five μg of total RNA was loaded per lane. Lane 1, TarIIWT; lane 2, TarIIWT transfected with *gsh1*; lane 3, TarIIAS20.2; 4, TarIIAs20.2rev. The blot was hybridized with probe 2 (Figure 1) and reprobed with a tubulin gene to monitor the amount of RNA layered in each lane.

gsh1 and pgpA co-transfection

In addition to *gsh1*, the ABC transporter gene *pgpA* frequently is amplified. We have proposed previously that PgpA may confer resistance by recognizing metals conjugated to thiols (Borst and Ouellette, 1995; Papadopoulou *et al.*, 1996), and indeed we have shown recently that metals conjugated to thiols can be pumped out from *Leishmania* cells by an ATP-coupled As–thiol pump different from PgpA (Dey *et al.*, 1996). As described previously (Papadopoulou *et al.*, 1994a), transfection of *pgpA* into wild-type *L.tarentolae* yields a 2-fold increase in resistance (Table I). The *pgpA* and *gsh1* genes were also co-transfected into wild-type cells. When both genes were in high copy number, the level of thiols was increased. However, the level of resistance was due solely to the contribution of PgpA (Table I). When *gsh1* and *pgpA* were co-transfected into the two arsenite-independent revertants, TarIIAs20.2rev and TarIIAs20.3rev, we observed resistance levels higher than expected from the individual contribution of both genes (Table I). These results suggest a synergistic interaction between the transporter PgpA and the synthesis of a putative transport precursor requiring high levels of GSH and/or TSH.

Discussion

Characterization of the gene present on a linear amplicon observed in *L.tarentolae* cells selected for arsenite resistance (Grondin *et al.*, 1993) revealed that it encodes the catalytic subunit of γ-GCS, the rate-limiting enzyme in GSH biosynthesis. This finding is consistent with the central role that thiols have been proposed to play in oxyanion resistance in *Leishmania* (Borst and Ouellette, 1995; Dey *et al.*, 1996; Mukhopadhyay *et al.*, 1996; Papadopoulou *et al.*, 1996). Increases in γ-GCS activity has been described in tumor cells resistant to cisplatin (Godwin *et al.*, 1992; Ishikawa *et al.*, 1994; Goto *et al.*, 1995; Kurokawa *et al.*, 1995; Yao *et al.*, 1995), to melphalan (Bailey *et al.*, 1992) to BSO (Yokomizo *et al.*, 1995) and to other xenobiotics. In all the cases studied, the increase in γ-GCS activity was due to transcriptional up-regulation. The role of γ-GCS in animal cells was established more directly by the recent transfection of human *gsh1* into COS cells, which led to resistance to the alkylating agent melphalan (Mulcahy *et al.*, 1995) or to cisplatin (Kurokawa *et al.*, 1995). As described in this study, *gsh1* can also be overexpressed in arsenite-resistant mutants. In *Leishmania* this is due to gene amplification, by far the preferred resistance mechanism in this organism

Table II. γ-GCS activity and thiol analysis in *Leishmania* mutants and transfectants

Cell lines	γ-GCS activity (U/mg)[a]	Cys (nmol/10^8 cells)[b]	GSH	TSH
TarII WT	<1	0.10	0.14	0.15
TarIIAs20.3	7.0 ± 0.4	0.19	0.46	1.5
TarIIAs20.3rev	2.0 ± 0.6	0.19	0.26	0.35
TarII WT + *gsh1*[c]	12.3 ± 1.2	0.13	1.04	3.05
TarIIAs20.3rev + *gsh1*	11.8 ± 0.8	0.14	0.99	2.97

[a]One unit corresponds to 1 μM of product made in 1 h.
[b]Average of two independent measurements.
[c]The 8 kb *Hin*dIII–*Hin*dIII fragment derived from the linear amplicon (see Figure 1).

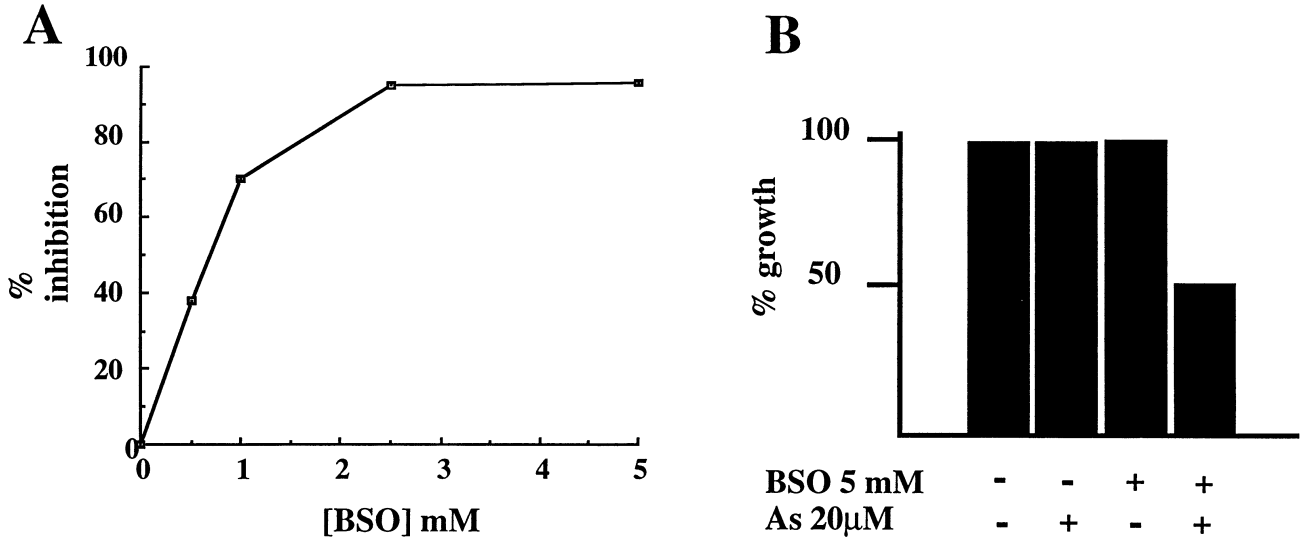

Fig. 6. The *in vitro* and *in vivo* effect of the specific γ-GCS inhibitor L-buthionine-(*SR*)-sulfoximine (BSO). (**A**) The percentage inhibition of γ-GCS activity was measured in cell lysates of the cell line TarIIAs20.3rev + *gsh1* in the presence of varying concentrations of the inhibitor BSO. (**B**) Reversal of arsenite resistance by BSO. Mutant TarIIAS20.3 was grown in defined medium treated or not with 5 mM BSO for 24 h. Sodium arsenite at 20 μM was added and cells were grown for 72 h and counted. The results shown are from one experiment. Two other experiments gave essentially identical results.

(Beverley, 1991; Ouellette and Papadopoulou, 1993). In several mammalian cell lines selected for arsenite resistance, GSH was found to be increased (Lee *et al.*, 1989; Li and Chou, 1992). Based on the results presented here, we would predict that the increase of GSH in animal cells may have been due to increased γ-GCS activity.

Recently, several GSH conjugate transporters have been described. One is the multidrug resistance-associated protein MRP (Cole *et al.*, 1992), a distant homolog of PgpA which was shown to transport GSH-linked substrates (Leier *et al.*, 1994; Müller *et al.*, 1994). Transfection of MRP is associated with oxyanion resistance (Cole *et al.*, 1994), and arsenite was shown to stimulate the extrusion of GSH by MRP (Zaman *et al.*, 1995). PgpA is a member of the same subfamily of ABC transporters as MRP or YCF1, a yeast GSH conjugate pump conferring resistance to cadmium (Li *et al.*, 1996). MRP was shown recently to complement a yeast *YCF1* deletion mutant (Tommasini *et al.*, 1996). A large family of *MRP* genes was characterized recently in *Caenorhabditis elegans*; some members of this family were found to contribute to arsenite and cadmium resistance (Broeks *et al.*, 1996). In *L.tarentolae pgpA* transfectants, the level of resistance observed is not proportional to the copy number of *pgpA* (Papadopoulou *et al.*, 1994a). This led us to suggest that resistance requires additional factors, possibly related to GSH (Borst and Ouellette, 1995).

The results of co-transfection of *pgpA* and *gsh1* into revertants are fully consistent with this hypothesis (Table I). The *gsh1* gene is amplified in all strains of *L.tarentolae* selected for arsenite. High levels of thiols are probably required for both PgpA and the non-PgpA plasma membrane As–thiol pump (Dey *et al.*, 1996; Mukhopadhyay *et al.*, 1996; this work). Independent genomic amplification of two genes, one involved in the synthesis of the substrates and the other in the transport of these substrates, is novel. Overexpression of γ-GCS often is correlated with augmentation of transport of GSH conjugates (Kondo *et al.*, 1993; Ishikawa *et al.*, 1994; Goto *et al.*, 1995; Kurokawa *et al.*, 1995). However, it is not known whether the transporter itself is increased, or whether an increase in the concentration of substrate is sufficient to increase transport and resistance. Overexpression of MRP by itself is sufficient to confer resistance (Cole *et al.*, 1994; Zaman *et al.*, 1995). Although GSH is required, wild-type levels are sufficient to produce resistance (Zaman *et al.*, 1995). Nevertheless, cisplatin was found recently to induce the human MRP and γ-GCS genes coordinately (Ishikawa *et al.*, 1996).

The role of PgpA in resistance is still unclear, and conflicting results have been reported on the transport properties of *Leishmania* cells overexpressing PgpA (Callahan *et al.*, 1994; Papadopoulou *et al.*, 1994a; Singh *et al.*, 1994). Our present working hypothesis is that PgpA is a pump that transports As–thiol conjugates into intracellular compartments (Papadopoulou *et al.*, 1996). It would confer resistance by sequestering the metal in intracellular organelle/vesicles, similar to what has been demonstrated for YCF1-catalyzed cadmium resistance in yeast (Li *et al.*, 1996; Tommasini *et al.*, 1996). Similarly, the putative GS-X pump may catalyze cisplatin resistance in animal cells by intracellular sequestration (Ishikawa *et al.*, 1994).

Transfection of *gsh1* alone is not enough to confer resistance to arsenite in wild-type strains of *L.tarentolae*. Similarly, transfected *pgpA* and *gsh1* act synergistically to confer resistance only in revertants, not in wild-type cells. These results indicate that another factor (or factors), absent in wild-type cells, is (are) required in addition to γ-GCS for resistance, and an increase in GSH or TSH alone is not sufficient to give rise to resistance mediated via either PgpA or the plasma membrane As–thiol pump. Since *gsh1* transfectants have high levels of TSH, it is unlikely that another enzyme in TSH synthesis is rate limiting. Although arsenite–thiol conjugates can form spontaneously (Delnomdedieu *et al.*, 1994; Mukhopadhyay

et al., 1996), there is circumstantial evidence that complex formation may be accelerated by a glutathione *S*-transferase (GST), as increased levels of GST were found in arsenite-resistant animal cells (Wang and Lee, 1993). Therefore, an unidentified step in the pathway of arsenite resistance in *Leishmania* may be a thiol transferase. Although GSH is increased in arsenite-resistant *Leishmania*, TSH is increased much more (Mukhopadhyay *et al.*, 1996), suggesting that the physiological substrate of both the plasma membrane As–thiol pump and PgpA may be a TSH conjugate.

This report provides a molecular explanation for our previous observation that GSH and TSH are increased in arsenite-resistant *L.tarentolae* (Mukhopadhyay *et al.*, 1996). Pre-treatment with BSO can partly revert the resistance phenotype (Figure 6), and BSO treatment similarly was shown to sensitize animal cells to arsenite (Lee *et al.*, 1989; Li and Chou, 1992). Mutants selected for resistance to antimonials are cross-resistant to arsenite and vice versa. The transport properties of antimony-resistant mutants are indistinguishable from those of arsenite-resistant cells (Dey *et al.*, 1994). Thus it may be of interest to examine the effect of BSO administration on the resistance of field strains of *Leishmania* to antimonials.

Materials and methods

Cell lines and cultures

The *L.tarentolae* cell line TarIIWT has been described previously (White *et al.*, 1988). Cell lines were grown in SDM-79 medium (Brun and Schönenberger, 1979). Arsenite-resistant mutants and revertants of *L.tarentolae* have been described previously (Ouellette *et al.*, 1991; Grondin *et al.*, 1993). Relative drug resistance values were obtained as reported previously (Ouellette *et al.*, 1990). Reversion of resistance with 5 mM BSO, a specific inhibitor of γ-GCS (Griffith and Meister, 1979), was done by first growing the cells in the defined medium α-MEM (Kar *et al.*, 1990). Cells were treated with 5 mM BSO for 24 h, washed with fresh medium, put in the presence of a sub-inhibitory concentration of arsenite and cells were counted after 72 h of growth.

DNA and RNA manipulations

Chromosomes in agarose blocks were resolved by TAFE (Beckman) as described previously (Grondin *et al.*, 1993). The 50 kb linear amplicon was isolated as described (Papadopoulou *et al.*, 1993). Total RNA was isolated using Trizol (Gibco-BRL). Southern and Northern blotting, hybridization and washing conditions followed standard protocols (Sambrook *et al.*, 1989). Probe 1 is a 0.5 kb *Hin*dIII–*Hin*dIII fragment isolated from the linear amplicon, probe 2 is a 6 kb *Bgl*II–*Bgl*II fragment recognizing both the 50 kb linear amplicon and the 13 kb circular amplicon. A probe containing the entire coding sequence of neomycin phosphotransferase was obtained by PCR.

DNA constructs

The 0.5 kb *Hin*dIII–*Hin*dIII fragment used as probe 1 was isolated from the linear amplicon and partially sequenced. The DNA sequence revealed no significant homology to sequences present in data banks (unpublished). However, a unique *Sma*I site approximately in the middle of the fragment was noted. The *neo* gene derived from pSPY-neo (Papadopoulou *et al.*, 1994b) was introduced into this unique *Sma*I site. This *neo*-containing fragment was used to introduce *neo* into the linear amplicon. A genomic cosmid library of TarIIAs20.4 (where the novel region is amplified as circles) in vector cL-Hyg (Ryan *et al.*, 1993) was made following the protocol detailed in Descoteaux *et al.* (1994). The library was screened with probe 2 (Figure 1). Several positives were obtained, one of which was studied further and found to contain a repeated 13 kb region, suggesting that the circles present in mutants are multimers. The isolated cosmid was digested with *Hin*dIII, and fragments were subcloned into *Leishmania* expression vectors (Papadopoulou *et al.*, 1994b). The *L.tarentolae pgpA* gene construct has been described previously (Papadopoulou *et al.*, 1994a).

Transfections

Promastigotes of *L.tarentolae* were transfected by electroporation as reported previously (Papadopoulou *et al.*, 1992). Selections were done with 40 μg/ml of G418 (Gibco-BRL) or with 100 μg/ml of hygromycin B (Calbiochem).

DNA sequence analysis

DNA sequencing was carried out on a Applied Biosystems 373 DNA automated sequencer. Analysis of the sequence was performed using the GCG software package (Genetics Computer Group, 1994). The nucleotide sequence reported here will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession No. Y10049.

Analysis of cellular thiols

Total thiols were measured in deproteinized cell extracts using DTNB as described previously (Mukhopadhyay *et al.*, 1996). Thiols were also derivatized with monobromobimane and separated by HPLC as described (Mukhopadhyay *et al.*, 1996).

γ-GCS enzymatic activity

Late-log phase *Leishmania* cells were pelleted and resuspended in 5 mM Tris–HCl pH 8.0. Cells were disrupted by homogeneization (Dyna-Mix, Fisher). The solution was freed of particulate material by centrifugation (12 000 r.p.m., 40 min) and ultracentrifugation (45 000 r.p.m., 60 min). The γ-GCS activity was determined by following the formation of ADP spectrophotometrically using a coupled assay with pyruvate kinase and lactate dehydrogenase (Seelig and Meister, 1985). The reaction mixture (final volume, 1.0 ml) contained Tris–HCl buffer (100 mM, pH 8.2), sodium L-glutamate (10 mM), L-cysteine (10 mM), magnesium chloride (20 mM), disodium ATP (5 mM), sodium phosphoenolpyruvate (2 mM), potassium chloride (150 mM), NADH (0.2 mM), pyruvate kinase (10 U) and lactate dehydrogenase (10 U). The reaction was initiated by adding the protein extract, and the rate of decrease in absorbance at 340 nm was followed at 25°C. One unit of enzyme activity is defined as the amount that catalyzes the formation of 1 μmol of product per hour. Specific activity is expressed as units/milligram of protein.

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