The heat shock protein HSP70 and heat shock cognate protein HSC70 contribute to antimony tolerance in the protozoan parasite *Leishmania*

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Abstract Antimony-containing drugs are still the drugs of choice in the treatment of infections caused by the parasite *Leishmania*. Resistance to antimony is now common in some parts of the world, and several mechanisms of resistance have been described. By transfecting cosmid banks and selecting with potassium antimonyl tartrate (SbIII), we have isolated a cosmid associated with resistance. This cosmid contains 2 copies of the heat shock protein 70 (HSP70) and 1 copy of the heat shock cognate protein 70 (HSC70). Several data linked HSP70 to antimony response and resistance. First, several *Leishmania* species, both as promastigotes and amastigotes, increased the expression of their HSP70 proteins when grown in the presence of 1 or 2 times the Effect Concentration 50% of SbIII. In several mutants selected for resistance to either SbIII or to the related metal arsenite, the HSP70 proteins were found to be overexpressed. This increase was also observed in revertant cells grown for several passages in the absence of SbIII, suggesting that this increased production of HSP70 is stable. Transfection of HSP70 or HSC70 in *Leishmania* cells does not confer resistance directly, though these transfectants were better able to tolerate a shock with SbIII. Our results are consistent with HSP70 and HSC70 being a first line of defense against SbIII until more specific and efficient resistance mechanisms take over.

INTRODUCTION

The protozoan parasite *Leishmania* is responsible for several pathologies ranging from cutaneous lesions to visceral infections that can be fatal if untreated (Herwaldt 1999). The incidence of leishmaniasis increases, and its control is further exacerbated by the high incidence of resistance to pentavalent antimony (SbV)-containing drugs, which are still the mainstay in the treatment of *Leishmania* infections in most parts of the world (Murray 2001; Guerin et al 2002; Berman 2003). Miltefosine is now used in treating leishmaniasis in parts of India, but resistance to miltefosine, at least in vitro, can readily occur (Perez-Victoria et al 2003), suggesting that SbV is likely

to remain an important drug in the control of *Leishmania* infections. The mode of action of SbV is poorly understood, but it is believed that SbV is a prodrug that needs to be reduced to trivalent antimony (SbIII), which would be the toxic species against *Leishmania*. It is not known yet whether most of the reduction occurs in the parasite or in the host cell (Serenio et al 1998; Shaked-Mishan et al 2001). Analysis of mutants selected for resistance to SbIII and to its related metal arsenite (AsIII) led to the development of a model for metal resistance. In this model, SbV and SbIII appear to enter *Leishmania* cells by means of different transporters (Brochu et al 2003), and mutations in one of these could lead to resistance. As part of the detoxification process, the metal would be conjugated to trypanothione, a spermidine-glutathione conjugate specific to kinetoplastidae (Fairlamb and Cerami 1992), which is increased in resistant cells by virtue of the amplification or overexpression (or both) of genes coding for enzymes involved in glutathione or spermidine bio-

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synthesis (Grondin et al 1997; Haimeur et al 1999; Guimond et al 2003). The Sb-trypanothione conjugate is then either sequestered inside a vacuole by the ATP-binding cassette transporter PGPA (Légaré et al 2001) or extruded from the cell by a thiol-X pump (Dey et al 1996).

Although the above model has received considerable support from in vitro work, it is possible that other resistance mechanisms operate in field isolates. In fact, it was suggested in a recent report that amplified deoxyribonucleic acid (DNA) derived from chromosome 9 might be involved in clinical SbV resistance (Singh et al 2003). Other mechanisms of resistance have also been described in antimony-resistant mutants (Haimeur and Ouellette 1998; Shaked-Mishan et al 2001). One powerful strategy to isolate novel resistance genes in *Leishmania* is functional cloning. This technique was useful to isolate transporters involved in resistance to nucleoside (Vasudevan et al 1998), folate (Kündig et al 1999), and phospholipid analogs (Perez-Victoria et al 2003) or soluble proteins mediating resistance to inhibitors of nucleoside and ergosterol metabolism (Cotrim et al 1999). This strategy was used here in an attempt to isolate novel genes mediating resistance to SbIII. Characterization of 1 cosmid, named X6, revealed the presence of genes coding for the homolog of the heat shock protein HSP70 and heat shock cognate protein HSC70.

Leishmania have a dimorphic life cycle and are found as promastigotes in the sandfly vector (25°C) and amastigotes in the mammalian macrophage (37°C). The *Leishmania* life cycle can be mimicked in vitro, and temperature is indeed an important trigger of differentiation (Zilberstein and Shapira 1994), and some HSP homologs were suggested to be important for *Leishmania* differentiation (Wiesgigl and Clos 2001). The HSP70s of *Leishmania* are part of a multigene family, with varying numbers in different species (Quijada et al 1997; Zurita et al 2003)(www.genedb.org). In contrast to most eukaryotes, the regulation of HSPs in *Leishmania* was suggested to occur exclusively at the post-transcriptional level (Hunter et al 1984). This was first demonstrated for the HSP83 gene (Argaman et al 1994), for several *Leishmania* HSP genes (Brandau et al 1995), and in details for HSP70 (Quijada et al 1997). Recently, gene transfection experiments have shown that HSP70 can bestow a heat-inducible increase in resistance to peroxide (Miller et al 2000). The role of HSPs in *Leishmania* in response to antimony is unexplored, with only 1 report showing that HSPs of varying molecular weight were increased in the presence of AsIII (Lawrence and Robert-Gero 1985). The isolation of a cosmid coding for HSP70 has allowed us to analyze the association between antimony resistance and HSP70 in *Leishmania* in more detail.

MATERIALS AND METHODS

Reagents

Potassium antimonyl tartrate (SbIII) was obtained from Aldrich. Sodium m-arsenite (AsIII) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) were obtained from Sigma.

Cell lines and cultures

The *Leishmania tarentolae* cell lines TarII wild-type, As50.1, As20.3rev (selected for resistance to AsIII), and Sb400.4 (selected for resistance to SbIII) have been described previously (Ouellette et al 1991; Dey et al 1994; Haimeur et al 2000), as have *L. infantum* strains MHOM/MA/67/IT-MAP-263 and Sb4000.3 (selected for resistance to SbIII) (Brochu et al 2003). *L. tarentolae* and *L. infantum* strains were grown in SDM-79 (Brun and Schonenberger 1979) at 25°C as promastigotes and in MAA/20 for *L. infantum* (Serenio et al 1998) at 37°C as axenic amastigotes.

Viability test

The MTT micromethod was used to estimate the viability of cells after heat or metal treatments (Serenio et al 1998). Briefly, cells in their exponential growth phase were grown in SDM-79 for 12 hours with 2 times the EC₅₀ of SbIII (0.1 µM for *L. tarentolae* and 225 µM for *L. infantum*) or for 2 hours at 42°C. A 100-µL aliquot was transferred to a 96-well flat-bottom microtray, where 10 µL of MTT (7.5 mg/mL) was added to each well for 4 hours. The reaction was stopped by the addition of 100 µL of 50% isopropanol–10% sodium dodecyl sulfate. The plates were incubated for 30 minutes under agitation before observing the optical density at 570 nm. Statistical significance was calculated by the Fisher's protected least significant difference test using StatView SE + Graphic software v1.03.

DNA manipulations

The pSP72α-NEO-α vector (Papadopoulou et al 1994) transfected in the wild-type strains of *L. tarentolae* or *L. infantum* was used as a control in viability tests. Polymerase chain reaction fragments, using the cosmid X6 as template for the *L. tarentolae* HSC70 (5'-GACGACCACTG CCGCAGAGATG-3' and 5'-GTCCTTGCTCAGCCGGC CCTTG-3'), and the *L. tarentolae* HSP70 (5'-CATCGATGAGCGTCACAGAG-3' and 5'-ATGCACCGAGTTGCAC AGTC-3') were cloned in the expression vector pSP72α-NEO-α and were transfected in *L. tarentolae* or *L. infantum*. Pulse field gel electrophoresis of *Leishmania* chromosomes was done as described previously (Tamar and Papadopoulou 2001) using a Bio-Rad CHEF DR III apparatus.

Functional cloning

The genomic cosmid library, made from a partial *Sau3AI* digest of the DNA of the mutant As20.4, was described previously (Kündig et al 1999). *L. tarentolae* promastigotes were transfected by electroporation (Papadopolou et al 1992) using 20 µg of the cosmid library, and transfectants were grown in 5 mL of SDM-79 medium. After 24 hours of growth, an equal volume of fresh medium was added with hygromycin B (final concentration 300 µg/mL) and incubated for an additional 24 hours. The cells were transferred to a 15-mL conical tube, centrifuged at 2500 rpm at 4°C for 10 minutes, and resuspended in fresh SDM-79 medium at a density of 2.5×10^7 cells/mL. Aliquots (200 µL) were plated carefully on hygromycin B-containing plates, which were then sealed with parafilm and incubated at 29°C. Colonies usually appeared between 5 to 10 days of incubation. Colonies were scraped off the plates with a glass spreader in 1 mL of medium and resuspended in SDM-79 medium at a concentration of 5×10^6 cells/mL. Cells were grown for 24 hours in the presence of 1 µM of hygromycin B, and 5×10^6 cells were spread on plates containing SbIII (0.5 to 2.5 µM) and hygromycin (300 µM) and incubated at 29°C. Colonies appearing after 2 to 3 weeks of incubation were then picked and grown individually in 5 mL of SDM-79 medium with hygromycin at 300 µM. Cosmids were isolated as previously described (Kündig et al 1999).

DNA sequencing

DNA sequencing was carried out on an Applied Biosystem 377 automated sequencer. Analysis of the sequences was performed using the GCG software package (Genetics Computer Group, 1994) and PAUP* 4.0 (Swofford 2003). The nucleotide sequences reported here will appear in the GenBank sequence database under the accession numbers AY423867 for *L. tarentolae* HSP70 and AY423868 for *L. tarentolae* HSC70. The following sequences, with their accession number in parentheses, were used for the phylogenetic analysis: *L. donovani* HSP70 (X52314), *L. infantum* HSP70 (X85798), *L. braziliensis* HSP70 (AF291716), *Trypanosoma brucei* HSP70 (P11145), *T. brucei* HSC70 (P20030), Human HSP70 (P08107) and *Escherichia coli* DNAK (P04475). The *L. major* HSP70 (LmjF28.2780) and *L. major* HSC70 (LmjF28.2820) sequences are available at www.genedb.org.

Immunoblotting

Cells were grown to log-phase then harvested and lysed by sonication on ice 3 times, (30 seconds of sonication, followed by a 30-second recovery) and diluted in sodium dodecyl sulfate (SDS) containing Laemmli buffer. Equal

Table 1 Susceptibility to metals in *Leishmania*

Strains	AsIII		SbIII	
	EC ₅₀ ^a (µM)	Fold	EC ₅₀ ^a (µM)	Fold
TarIIWT	0.4	1	0.1	1
TarIIWT + cosmid X6	10	25	60	600

^a EC₅₀ values are an average of at least 3 independent determinations.

amounts of total proteins were loaded on a 10% SDS-polyacrylamide gel electrophoresis gel followed by immunoblotting using anti-HSP70 and anti-α-tubulin antibodies (Harlow and Lane 1988). The reaction was detected by using the enhanced chemiluminescence method (Amersham). The mouse monoclonal antibody anti-heat shock protein 70 Mab (5A5) (Alexis Biochemical) reacts with HSP70 members across a broad range of species, including those from *Leishmania*. It recognizes an epitope located between amino acids 122–264 of human HSP70, a very conserved region shown to be involved in adenosine triphosphate (ATP) binding. The mouse monoclonal antibody anti-α-tubulin (bovine) 236–10 501 (Molecular Probes) was also used as a control. The intensity of the reactions was determined by densitometry analysis, using an alphaImager 2000 (Alpha Innotech) with the software AlphaEase version 3.3b and compared with the signal intensity of α-tubulin.

RESULTS

Isolation of an HSP70-containing cosmid by functional cloning in *Leishmania*

To isolate SbIII resistance genes, we transfected in wild-type *L. tarentolae* cells a cosmid bank of genomic DNA derived from As20.4, an AsIII-selected mutant highly cross-resistant to SbIII (Dey et al 1994). Transfectants were selected on SbIII-containing plates, and a few colonies were grown on plates with 2.5 µM SbIII. Five different cosmids were isolated from several transfectants, whereas the same cosmids were isolated from different colonies. Only 2 of these cosmids, termed "XS" and "X6", were still associated with resistance on retransfection (A. Haimeur and M. Ouellette, personal communication). The gene on cosmid XS is currently under investigation, and the retransfection of cosmid X6, isolated from 1 of these colonies, in wild-type *L. tarentolae* conferred a 25-fold increase in resistance to AsIII (10 µM) and more than a 600-fold resistance to SbIII (60 µM) (Table 1). The link between X6 and resistance to SbIII was not straightforward, however, because all further attempts to isolate the episomal cosmid from secondary transfectants failed. Instead, it was found that the cosmid had integrated in the genome of these highly resistant selected cells. We found

that in the initial transfectant, the cosmid X6 was both existing as an extrachromosomal circle and integrated in the genome (not shown). The transfection of X6 repeatedly gave SbIII-resistant colonies on selective plates, and despite its integration in the genome, we reasoned that a gene present on this cosmid might contribute to the metal resistance phenotype. Cosmid X6 was partially mapped and sequenced and found to contain 2 copies of the *HSP70* gene. The evidences for integration of the cosmid (or part of it) in the chromosome encoding *HSP70* are provided in Figure 1. One large molecular weight *L. tarentolae* chromosome hybridized to the *HYG* probe in transfectant X6, whereas no bands hybridized in the wild-type cells (Fig 1A, lanes 1 and 2). Extrachromosomal circles, as shown in cells transfected with the control cosmid cLHyg, do not migrate similarly and usually give rise to smears (Fig 1A, lane 3) representing different forms of circular DNA (White et al 1988; Grondin et al 1993). A similar CHEF blot run at the same time was hybridized to an *HSP70* probe. One high-molecular weight chromosome hybridized to the *HSP70* probe in the wild-type cell (Fig 1B, lane 2), whereas the same band and 1 slightly higher hybridized in transfectant X6 (Fig 1B, lane 1). It is this higher band that also hybridized to the *HYG* probe. To further prove that the transfected cosmid (or part of it) integrated in the *L. tarentolae* chromosome harboring the *HSP70* locus, a similar blot was hybridized to the *Leishmania*-activated protein kinase C receptor gene (*LACK*) that is, according to the *L. major* genome, ~8 kb upstream of the *HSP70* genes on *L. major* chromosome 28 (www.genedb.org). The same hybridization profile was seen with the *LACK* probe as with the *HSP70* probe (Fig 1 B,C), showing that the cosmid had indeed integrated in the *HSP70*-coding chromosome. The integration of cLHyg increases the size of the chromosome 28 version of *L. tarentolae*. This could be the result of the integration of the whole cosmid, hence increasing the copy number of *HSP70*. This does not seem to be the case, however, because the copy number of *HSP70*, as determined by Southern blot analysis, is not drastically changed in the X6 transfectant compared with wild-type cells, although some minor rearrangements did occur, as suggested by the presence of additional hybridizing bands (Fig 1D). The *HYG* gene and plasmid sequences were clearly integrated in the *Leishmania* genome (Fig 1 E,F). Multiple bands hybridized with these markers (Fig 1 E,F, lanes 4 and 6), suggesting that the rearrangement probably selected during drug selection is complex, but contained mostly plasmid-derived sequences.

The cosmid X6 was further characterized, and 8.5 kb downstream of *HSP70*, a copy of the more distantly related *HSC70* was found (Fig 1G). The *HSP70* gene is 1971 base pairs and encodes a putative protein of 657 amino acids for a predicted molecular weight of 71.6 kDa. The

protein shares more than 96% identity with the *HSP70* of *L. major*, *L. donovani*, and *L. infantum* and is also very close to *HSP70* of *T. brucei* (87.8%) and humans (73.2%) (Fig 2). The other gene, *HSC70*, is 1992 base pairs and encodes a putative protein of 664 amino acids for a predicted molecular weight of 72.4 kDa. According to the phylogenetic tree (Fig 2A), *L. tarentolae* *HSC70* is distinct from the *HSP70* proteins. It is highly related (89.4% identity) to a new copy of a *HSP70*-related gene on chromosome 28 of *L. major* and to the *T. brucei* *HSC70* (Fig 2). The *L. tarentolae* *HSC70* shares 58% identity with the *T. brucei* *HSC70* but less than 50% identity with the different *HSP70*s of *Leishmania* sp. The N-terminal domain, including the ATP-binding domain, is very conserved between the *L. tarentolae* *HSC70* and the other *HSP70* proteins, with most differences located in the C-terminal 10 kDa of the protein.

Induction of HSP70 by SbIII in *Leishmania*

To look further into the role of *HSP70* and antimony resistance, we tested whether antimony could induce the expression of the *HSP70* proteins. To monitor *HSP70* expression, we used a commercial monoclonal antibody (Mab 5A5) that reacts with *HSP70* members of a broad range of species because it recognizes a highly conserved epitope located in the ATP-binding domain of *HSP70*. This antibody can react equally well to *HSP70* and *HSC70* of *L. tarentolae* (Fig 3A), both having the conserved ATP-binding domains. We also observed that the *HSP70*-*HSC70* proteins were overproduced in the X6 transfectant with its integrated cosmid (Fig 3B). We showed that the *L. tarentolae* *HSP70* (and or *HSC70*) was induced by several fold (>4) when cells were incubated for 12 or 24 hours with either 1 or 2 times the EC_{50} of SbIII (Fig 3C). Similarly, the *HSP70*s of *L. infantum* promastigotes were also induced dramatically (>4-fold) on shock with the EC_{50} of SbIII (Fig 3D). The induction of *HSP70*s was more rapid in *L. infantum* promastigotes compared with *L. tarentolae* because we could not observe induction of *HSP70*s in the latter species after a 2-hour shock (results not shown). *L. infantum* can be grown as axenic amastigotes, and although the basal level of *HSP70*s is higher in these cells that are grown at 37°C, a shock with SbIII can also increase (~2-fold) the level of *HSP70*s (Fig 3E). Results presented in Figure 3C–E clearly demonstrate that on SbIII exposure, *Leishmania* cells respond by increasing their *HSP70*s.

HSP70 and SbIII resistance

We next tested the status of *HSP70*s in metal-resistant mutants of *Leishmania*. We first studied *L. tarentolae* selected for either AsIII or SbIII resistance. These analyses were done with cultures that were passaged at least twice

without drugs to discriminate between an induction of HSP70 due to the presence of the drug and a stable HSP70 overexpression correlated with resistance. The HSP70s of *L. tarentolae* cells selected for arsenite or antimonite resistance were increased compared with wild-type cells (Fig 4, lanes 1, 3, and 4). In the mutants As50.1 and Sb400.4, HSP70s were increased more than 4-fold compared with wild-type cells. Remarkably, HSP70s were still higher in the *L. tarentolae* mutant As20.3rev (Fig 4, lane 2) (~4-fold compared with wild-type cells), a mutant grown in the absence of arsenite for several hundreds of passages. This revertant has an EC_{50} of 5 μ M for arsenite, which is considerably higher than that for wild-type cells (0.4 μ M) but nonetheless lower than that for the parent mutant (50 μ M). The overexpression of HSP70s thus appears to be a stable phenotype in these revertant cells. Similarly, HSP70s were slightly increased in the *L. infantum* mutant selected for SbIII resistance (Fig 4, lanes 5 and 6).

HSP70s are increased on SbIII shock and in SbIII-resistant mutants. To test further the link between HSP70s and resistance and in an attempt to discriminate whether the observed phenotype is due to either HSP70 or HSC70, we cloned individually the 2 genes in *Leishmania* expression vectors. These constructs were transfected independently, and both HSP versions were overexpressed as determined by Western blot analysis (Fig 3A). We then performed growth curves in the presence of varying concentrations of SbIII but found no increase in resistance compared with control cells transfected with the vector alone (result not shown). Similar negative results were obtained when AsIII was tested. We also cotransfected both genes (as part of NEO and HYG-containing vectors) in a wild-type cell and also found no increase in SbIII resistance. We thus looked for more subtle ways by which HSP70s could contribute to metal tolerance. To do so, we compared the viability of transfected cells overexpressing or not overexpressing HSP70s after a brief shock with SbIII. In this assay, we found that the *L. tarentolae* HSP70 and HSC70 protein conferred a low but significant protection to an SbIII shock compared with control cells (Fig 5). When transfected in *L. tarentolae*, HSC70 and HSP70 conferred 21–29% increased viability after 12 hours of SbIII exposure to either 1 or 2 times the EC_{50} of SbIII (0.1 and 0.2 μ M) compared with the control cells. Despite the higher basal level of HSP70 expression in *L. infantum*,

overexpression of both HSP70 and HSC70 conferred a low (10–25%) but significant protection against a SbIII shock (Fig 5). HSP70s are expected to provide heat tolerance (reviewed in Kiang and Tsokos 1998), although this has never been formally reported for *Leishmania*. We found that transfectants overexpressing HSP70 and HSC70 had increased viability after a heat shock at 42°C, with a 60–100% increase in survival compared with control cells (Fig 5).

DISCUSSION

By functional cloning we have isolated a cosmid that harbors 2 copies of *HSP70* and 1 copy of *HSC70* (Fig 1G). The copy number of *HSP70* genes varies considerably among the various *Leishmania* species. In *L. infantum*, a cluster of 6 *HSP70* genes has been described, with the most distal gene expressed constitutively at high levels (Quijada et al 1997). The *L. major* genome sequence (www.genedb.org) has revealed a multitude of HSP70-like proteins and other homologs. It is possible that the *HSP70* cluster of *L. tarentolae* is larger, but this is unlikely because the *L. major* sequence on chromosome 28 has also revealed 2 copies of *HSP70* and 1 copy of an *HSC70*. Interestingly, however, the gene order between *L. major* and *L. tarentolae* seems to differ (Fig 1G). The reason why the copy number of *HSP70* differs greatly in different *Leishmania* species is unknown, from 1 in *L. Viannia braziliensis* (Zurita et al 2003) to 6 in *L. infantum* (Quijada et al 1997), but does not seem to relate to the temperature that *Leishmania* species are likely to encounter. Despite some differences in copy number and organization, the *Leishmania* HSP70 members, including the new *L. tarentolae* sequences presented here, are highly conserved with more than 96% identity (Fig 2). The HSC70 proteins form a separate phylogenetic branch and are highly homologous among one other but show only 40–45% identity with the various *Leishmania* HSP70 members (Fig 2). In systems in which it was studied, the HSC70 gene and protein are expressed constitutively, although this was difficult to assess in *L. tarentolae* using the antibody available. Most sequence differences between HSP70 and HSC70 were localized in the last 10 kDa of the protein, a region known in HSC70 to be involved in the stability of the bound unfolded polypeptides and possibly in self-association (Chou et al 2003).

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Fig 1. Chromosomal integration of the cosmid X6 and its characterization in antimony-resistant transfectant. *Leishmania* cells were isolated and their chromosomes resolved by pulse field gels. Deoxyribonucleic acid (DNA) was transferred and hybridized to an *HYG* (probe a) (A), *HSP70* (probe b) (B), and *LACK* (C) probes. *LACK* and *HSP70* are linked on chromosome 28 of *Leishmania major* (www.genedb.org). 1, *L. tarentolae* cells retransfected with X6 and selected for resistance to SbIII; 2, *L. tarentolae* wild-type cells; and 3, *L. tarentolae* cells transfected with the cosmid cLHYG (Ryan et al 1993). The smear in lane 3 is indicative of the episomal nature of the transfected DNA. The integration event was further studied by Southern blot analysis of total genomic DNA hybridized to *HSP70* (D), *HYG* (E), and vector sequences (probe

b) (F). The DNA was derived from wild-type cells (odd numbers) or the X6 retransfectant (even numbers) and digested with *Xba*I, 1, 2; *Not*I, 3, 4; *Bgl*II, 5, 6. (G) A partial map of cosmid X6 was determined using a selection of restriction enzymes. The equivalent region in the *L. major* genome (taken from www.genedb.org) is found below the map of the cosmid. Arrows without gene names indicate hypothetical proteins. The location of the probes (a,b,c) is indicated below the map. Only the HSPs were sequenced in cosmid X6. B, *Bgl*II; H, *Hind*III; N, *Not*I, X, *Xba*I.

Fig 2. Phylogenetic analysis and sequence conservation of the *Leishmania* HSP70 protein. (A) Phylogenetic analysis was performed using the GCG software package and PAUP*4.0 and the amino acid sequences of various members of the HSP70 family. (B) Pairwise identities (% identity) among different proteins. *L. tarentolae* HSP70 (LTHSP70), *L. tarentolae* HSC70 (LTHSC70), *L. major* HSP70 (LMHSP70), *L. major* HSC70 (LMHSC70), *L. donovani* HSP70 (LDHSP70), *L. infantum* HSP70 (LIHSP70), *L. braziliensis* HSP70 (LBHSP70), *Trypanosoma brucei* HSP70 (TBHSP70), *T. brucei* HSC70 (TBHSC70), human HSP70 (HHSP70), and *Escherichia coli* DNAK (DNAK).

The HSP70 and HSC70 proteins are ATP-dependent molecular chaperones assuming diverse essential cellular functions, including the assembly, folding, and translocation of oligomeric proteins (reviewed in Hartl and Hayer-Hartl 2002). Under a variety of stresses, including heat, radiation, infection, chemical or biochemical stress, induction of HSP70s can provide cytoprotection, possibly by binding to misfolded proteins (Morimoto and Santoro 1998; Hartl and Hayer-Hartl 2002). HSP70s were also found to be immunodominant molecules of many microbial pathogens and are a major target of the humoral immune response during *Leishmania* infections (Quijada et al 1998). Although the inducible expression of HSP70s can lead to cross-resistance to a selected number of toxic substances, this has been less studied in *Leishmania*. *Leishmania* cycles from an insect vector (25°C) to its mammalian host (37°C), and this temperature shift is a likely triggering factor of their developmental program (Zilberstein and Shapira 1994). Several of the *Leishmania* HSP70 transcripts are increased on heat exposure (Quijada et al 1997), and in contrast to most other organisms, this control is at the posttranscriptional level and is controlled by the 3'-untranslated region of the gene (Hunter et al 1984; Brandau et al 1995; Quijada et al 2000). Similar regulation has also been described in the related parasite *T. brucei* (Lee 1998). It is likely that an increase in HSP70 amounts leads to heat protection, although this appears to be shown formally for the first time using transfected genes in *Leishmania* in Figure 5. Interestingly, both HSP70 and HSC70 can provide this increased heat tolerance. It has been shown previously that transfection of *HSP70* leads

to a heat-inducible increase in resistance to hydrogen peroxide, suggesting that HSP70 can also be implicated in oxidant stress in *Leishmania* (Miller et al 2000). Although arsenite is a known potent inducer of HSPs in general (reviewed in Del Razo et al 2001) and has been shown to induce some HSPs in *Leishmania* (Lawrence and Robert-Gero 1985), much less is known about the role of SbIII as an inducer of HSPs (Snawder et al 1999).

In this study we isolated a cosmid by functional cloning. In all subsequent retransfection, this cosmid was found to be integrated in the genome (Fig 1A), but its presence was associated with a very high level of resistance. Only part of the cosmid seems to integrate as the copy number of HSP70s does not change notably (Fig 1D), although some weakly hybridizing extra bands (Fig 1D, lanes 4 and 6) are visible. Instead, on integration and subsequent complex rearrangements, the vector portion of the cosmid is mostly retained, and this is consistent with the multiple *HYG* and plasmid hybridization bands (Fig 1 E,F). This complex rearrangement was likely selected using hygromycin B, and the multimers of vector sequences did increase the size of the *L. tarentolae* chromosome 28 equivalent (Fig 1 B,C, lane 1). More importantly, this complex integration event led to an increase in the expression of the HSP70 and or HSC70 proteins (Fig 3B). Additional work is required to understand how the cosmid has exactly integrated and rearranged and to understand how these events led to increase in HSP70 expression. In the initial transfectant, the cosmid was found both integrated and as part of an episome. Characterization of this cosmid revealed that it contains *HSP70*

Fig 3. Analysis of HSP70 expression on shock with potassium antimonyl tartrate (SbIII). (A) Western blots showing expression of HSP70 in *Leishmania tarentolae* transfected with pSP72 α -NEO- α (ctrl), pSP72 α -NEO- α -HSC70 (HSC70), or pSP72 α -NEO- α -HSP70 (HSP70). (B) Western blots showing the increase in expression of HSP70 in the X6 transfectant with the integrated cosmid. (C–E) HSP70 and α -tubulin immunoblots of *Leishmania* after an SbIII exposure. Wild-type *L. tarentolae* (C), *L. infantum* in the promastigote stage (D), or in the amastigote stage (E) were incubated in the presence of 1 or 2 times the EC_{50} of SbIII for 0, 2, 12, or 24 hours. The EC_{50} for *L. tarentolae* is 0.1 μ M, 225 μ M for *L. infantum* promastigotes, and 60 μ M for *L. infantum* amastigotes. Total proteins were extracted and then reacted with monoclonal antibodies directed against HSP70 or to α -tubulin. The approximate fold increase in HSP70 expression was adjusted with the α -tubulin signal. One experiment representation of at least 3 replicates is shown.

and *HSC70* genes. Our inability to directly link HSP70 to metal resistance suggests that HSP70s play a secondary role, which could serve as a first non-specific stress response that could allow the cells to develop more specific and efficient resistance mechanisms. Indeed, HSP70 proteins were induced in cells subjected to a short pulse of low SbIII concentration. Moreover, we were able to show that in some *Leishmania* mutants resistant to either AsIII or SbIII, the HSP70s were increased constitutively. This increase seems stable because cells grown in the absence

of drugs for several hundreds of passages still had an increase in HSP70 expression compared with susceptible cells (Fig 4, lane 2). Transfection of either *HSP70* or *HSC70* showed that these proteins can directly confer both heat tolerance and protection against an SbIII shock (Fig 5). The effects are small but statistically significant and under stressful conditions may confer a sufficient advantage to allow the cell to develop more specific resistance mechanisms. These data show a novel role for HSP70s in *Leishmania* and for the first time experimen-

Fig 4. HSP70 overexpression in *Leishmania* metal-resistant mutants. All mutants were grown without drugs for at least 2 passages, and then total proteins were extracted, resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel (SDS-PAGE) gels, transferred and then reacted with monoclonal antibodies directed against HSP70 or to α -tubulin. 1, *Leishmania tarentolae* wild-type; 2, *L. tarentolae* As20.3rev; 3, *L. tarentolae* As50.1; 4, *L. tarentolae* Sb400.4; 5, *L. infantum* wild-type; 6, *L. infantum* Sb4000.3. The approximate fold increase in HSP70 expression was adjusted with the α -tubulin signal. One representative experiment of 3 independent experiments is shown.

Fig 5. The role of the *Leishmania* HSC70 and HSP70 proteins in heat and metal protection. Viability of *Leishmania* cells transfected with the control plasmid pSP72 α -NEO- α (in black), by pSP72 α -NEO- α -HSC70 (in gray), and by pSP72 α -NEO- α -HSP70 (in white) after an incubation of 2 hours at 42°C, or a 12-hour incubation to SbIII at a concentration corresponding to 1 or 2 times the EC₅₀ for SbIII (0.1 and 0.2 μ M for *Leishmania tarentolae* and 450 μ M for *L. infantum*). The standard error is represented for each experimental condition; 100% corresponds to the viability of cells without treatment. The viability was estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) method as described in Materials and Methods. Each experiment was done at least 4 times. Results that are significantly different ($P < 0.05$ as calculated by the Fisher's protected least significant difference test) are marked with asterisks. Bars with different number of asterisks are statistically different among each other.

tally link HSC70 with a function. Overall, our data show that HSP70s are not directly involved in resistance to antimony but clearly give some protection to the cell against this metal. This may explain how we were able to select an HSP70-encoding cosmid in the first place using a functional screen with SbIII. The stable overexpression of HSP70s in mutant cells (Fig 4) suggests that HSP70s are indeed required during the genesis of the metal resistance phenotype in the protozoan parasite *Leishmania*.

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REFERENCES

- Argaman M, Aly R, Shapira M. 1994. Expression of heat shock protein 83 in *Leishmania* is regulated post-transcriptionally. *Mol Biochem Parasitol* 64: 95–110.
- Berman J. 2003. Current treatment approaches to leishmaniasis. *Curr Opin Infect Dis* 16: 397–401.
- Brandau S, Dresel A, Clos J. 1995. High constitutive levels of heat-shock proteins in human-pathogenic parasites of the genus *Leishmania*. *Biochem J* 310: 225–232.
- Brochu C, Wang J, Roy G, Messier N, Wang XY, Saravia NG, Ouellette M. 2003. Antimony uptake systems in the protozoan parasite *Leishmania* and accumulation differences in antimony-resistant parasites. *Antimicrob Agents Chemother* 47: 3073–3079.
- Brun R, Schonenberger M. 1979. Cultivation and in vitro cloning or procyclic culture forms of *Trypanosoma brucei* in a semi-defined medium. Short communication. *Acta Trop* 36: 289–292.
- Chou CC, Forouhar F, Yeh YH, Shr HL, Wang C, Hsiao CD. 2003. Crystal structure of the C-terminal 10-kDa subdomain of Hsc70. *J Biol Chem* 278: 30311–30316.
- Cotrim PC, Garrity LK, Beverley SM. 1999. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. *J Biol Chem* 274: 37723–37730.
- Del Razo LM, Quintanilla-Vega B, Brambila-Colombres E, Calderon-Aranda ES, Manno M, Albores A. 2001. Stress proteins induced by arsenic. *Toxicol Appl Pharmacol* 177: 132–148.
- Dey S, Ouellette M, Lightbody J, Papadopolou B, Rosen BP. 1996. An ATP-dependent As(III)-glutathione transport system in membrane vesicles of *Leishmania tarentolae*. *Proc Natl Acad Sci U S A* 93: 2192–2197.
- Dey S, Papadopolou B, Haimeur A, Roy G, Grondin K, Dou D, Rosen BP, Ouellette M. 1994. High level arsenite resistance in *Leishmania tarentolae* is mediated by an active extrusion system. *Mol Biochem Parasitol* 67: 49–57.
- Fairlamb AH, Cerami A. 1992. Metabolism and functions of trypanothione in the Kinetoplastida. *Annu Rev Microbiol* 46: 695–729.
- Grondin K, Haimeur A, Mukhopadhyay R, Rosen BP, Ouellette M. 1997. Co-amplification of the gamma-glutamylcysteine synthetase gene gsh1 and of the ABC transporter gene pgpA in arsenite-resistant *Leishmania tarentolae*. *Embo J* 16: 3057–3065.
- Grondin K, Papadopolou B, Ouellette M. 1993. Homologous recombination between direct repeat sequences yields P-glycoprotein containing amplicons in arsenite resistant *Leishmania*. *Nucleic Acids Res* 21: 1895–1901.
- Guerin PJ, Olhario P, Sundar S, Boelaert M, Croft SL, Desjeux P,

- Wasunna MK, Bryceson AD. 2002. Visceral leishmaniasis: current status of control, diagnosis, and treatment, and a proposed research and development agenda. *Lancet Infect Dis* 2: 494–501.
- Guimond C, Trudel N, Brochu C, et al. 2003. Modulation of gene expression in *Leishmania* drug resistant mutants as determined by targeted DNA microarrays. *Nucleic Acids Res* 31: 5886–5896.
- Haimeur A, Brochu C, Genest P, Papadopoulos B, Ouellette M. 2000. Amplification of the ABC transporter gene PGPA and increased trypanothione levels in potassium antimonyl tartrate (SbIII) resistant *Leishmania tarentolae*. *Mol Biochem Parasitol* 108: 131–135.
- Haimeur A, Guimond C, Pilote S, Mukhopadhyay R, Rosen BP, Poulin R, Ouellette M. 1999. Elevated levels of polyamines and trypanothione resulting from overexpression of the ornithine decarboxylase gene in arsenite-resistant *Leishmania*. *Mol Microbiol* 34: 726–735.
- Haimeur A, Ouellette M. 1998. Gene amplification in *Leishmania tarentolae* selected for resistance to sodium stibogluconate. *Antimicrob Agents Chemother* 42: 1689–1694.
- Harlow E, Lane D. 1988. *Antibodies, a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Hartl FU, Hayer-Hartl M. 2002. Molecular chaperones in the cytosol: from nascent chain to folded protein. *Science* 295: 1852–1858.
- Herwaldt BL. 1999. Leishmaniasis. *Lancet* 354: 1191–1199.
- Hunter KW, Cook CL, Hayunga EG. 1984. Leishmanial differentiation in vitro: induction of heat shock proteins. *Biochem Biophys Res Commun* 125: 755–760.
- Kiang JG, Tsokos GC. 1998. Heat shock protein 70 kDa: molecular biology, biochemistry, and physiology. *Pharmacol Ther* 80: 183–201.
- Kündig C, Haimeur A, Légaré D, Papadopoulos B, Ouellette M. 1999. Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*. *Embo J* 18: 2342–2351.
- Lawrence F, Robert-Gero M. 1985. Induction of heat shock and stress proteins in promastigotes of three *Leishmania* species. *Proc Natl Acad Sci USA* 82: 4414–4417.
- Lee MG. 1998. The 3' untranslated region of the hsp 70 genes maintains the level of steady state mRNA in *Trypanosoma brucei* upon heat shock. *Nucleic Acids Res* 26: 4025–4033.
- Légaré D, Richard D, Mukhopadhyay R, Stierhof YD, Rosen BP, Haimeur A, Papadopoulos B, Ouellette M. 2001. The *Leishmania* ABC protein PGPA is an intracellular metal-thiol transporter ATPase. *J Biol Chem* 276: 26301–26307.
- Miller MA, McGowan SE, Gantt KR, et al. 2000. Inducible resistance to oxidant stress in the protozoan *Leishmania chagasi*. *J Biol Chem* 275: 33883–33889.
- Morimoto RI, Santoro MG. 1998. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. *Nat Biotechnol* 16: 833–838.
- Murray HW. 2001. Clinical and experimental advances in treatment of visceral leishmaniasis. *Antimicrob Agents Chemother* 45: 2185–2197.
- Ouellette M, Hettima E, Wust D, Fase-Fowler F, Borst P. 1991. Direct and inverted DNA repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania*. *Embo J* 10: 1009–1016.
- Papadopoulos B, Roy G, Dey S, Rosen BP, Ouellette M. 1994. Contribution of the *Leishmania* P-glycoprotein-related gene *ltpgpA* to oxyanion resistance. *J Biol Chem* 269: 11980–11986.
- Papadopoulos B, Roy G, Ouellette M. 1992. A novel antifolate resistance gene on the amplified H circle of *Leishmania*. *Embo J* 11: 3601–3608.
- Perez-Victoria FJ, Gamarro F, Ouellette M, Castanys S. 2003. Functional cloning of the miltefosine transporter: a novel P-type phospholipid translocase from *Leishmania* involved in drug resistance. *J Biol Chem* 278: 49965–49971.
- Quijada L, Requena JM, Soto M, Alonso C. 1998. Analysis of the antigenic properties of the *L. infantum* Hsp70: design of synthetic peptides for specific serodiagnosis of human leishmaniasis. *Immunol Lett* 63: 169–174.
- Quijada L, Soto M, Alonso C, Requena JM. 1997. Analysis of post-transcriptional regulation operating on transcription products of the tandemly linked *Leishmania infantum* hsp70 genes. *J Biol Chem* 272: 4493–4499.
- Quijada L, Soto M, Alonso C, Requena JM. 2000. Identification of a putative regulatory element in the 3'-untranslated region that controls expression of HSP70 in *Leishmania infantum*. *Mol Biochem Parasitol* 110: 79–91.
- Ryan KA, Dasgupta S, Beverley SM. 1993. Shuttle cosmid vectors for the trypanosomatid parasite *Leishmania*. *Gene* 131: 145–150.
- Sereno D, Cavaleyra M, Zemzoumi K, Maquaire S, Ouassii A, Lemesre JL. 1998. Axenically grown amastigotes of *Leishmania infantum* used as an in vitro model to investigate the pentavalent antimony mode of action. *Antimicrob Agents Chemother* 42: 3097–3102.
- Shaked-Mishan P, Ulrich N, Ephros M, Zilberstein D. 2001. Novel intracellular SbV reducing activity correlates with antimony susceptibility in *Leishmania donovani*. *J Biol Chem* 276: 3971–3976.
- Singh N, Singh RT, Sundar S. 2003. Novel mechanism of drug resistance in kala azar field isolates. *J Infect Dis* 188: 600–607.
- Snawder JE, Tirmenstein MA, Mathias PI, Toraason M. 1999. Induction of stress proteins in rat cardiac myocytes by antimony. *Toxicol Appl Pharmacol* 159: 91–97.
- Swofford DL. 2003. *PAUP* Phylogenetic Analysis Using Parsimony* and Other Methods, Version 4*. Sinauer Associates, Sunderland, MA.
- Tamar S, Papadopoulos B. 2001. A telomere-mediated chromosome fragmentation approach to assess mitotic stability and ploidy alterations of *Leishmania* chromosomes. *J Biol Chem* 276: 11662–11673.
- Vasudevan G, Carter NS, Drew ME, Beverley SM, Sanchez MA, Seyfang A, Ullman B, Landfear SM. 1998. Cloning of *Leishmania* nucleoside transporter genes by rescue of a transport-deficient mutant. *Proc Natl Acad Sci U S A* 95: 9873–9878.
- White TC, Fase-Fowler F, van Luenen H, Calafat J, Borst P. 1988. The H circles of *Leishmania tarentolae* are a unique amplifiable system of oligomeric DNAs associated with drug resistance. *J Biol Chem* 263: 16977–16983.
- Wiesgigl M, Clos J. 2001. Heat shock protein 90 homeostasis controls stage differentiation in *Leishmania donovani*. *Mol Biol Cell* 12: 3307–3316.
- Zilberstein D, Shapira M. 1994. The role of pH and temperature in the development of *Leishmania* parasites. *Annu Rev Microbiol* 48: 449–470.
- Zurita AI, Rodriguez J, Pinero JE, Pacheco R, Carmelo E, del Castillo A, Valladares B. 2003. Cloning and characterization of the *Leishmania* (*Viannia*) *braziliensis* Hsp70 gene. Diagnostic use of the C-terminal fragment rLb70(513–663). *J Parasitol* 89: 372–378.