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Leishmania major Hsp100 Is Required Chiefly in the Mammalian Stage of the Parasite

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In *Leishmania major* a 100-kDa heat shock protein, Hsp100, is abundant in the intracellular amastigote stage which persists in the mammalian host. A replacement of both *clpB* alleles which encode Hsp100 does not affect promastigote viability under standard culture conditions but impairs thermotolerance in vitro. In experimental infections of BALB/c inbred mice, the lack of Hsp100 in the gene replacement mutants results in a markedly delayed lesion development compared with that in infections with wild-type *L. major*. Overexpression of exogenous *clpB* gene copies can partly restore virulence to the gene replacement mutants. Genetic-selection experiments also reveal a strong pressure for Hsp100 expression in the mammalian stage. This requirement for Hsp100 was also observed in in vitro infection experiments with mouse peritoneal macrophages. These experiments indicated a role for Hsp100 during the development from the promastigote to the amastigote stage. Our results suggest an important role for this parasite heat shock protein during the initial stages of a mammalian infection.

Under environmental stress all organisms examined to date respond with the synthesis of a subset of chaperone molecules, the so-called heat shock proteins. The synthesis of heat shock proteins confers protection against the adverse effects of cellular stress. This is best exemplified by the phenomenon of inducible stress tolerance: cells can survive otherwise-lethal conditions, e.g., heat, when they are pretreated under sublethal stress conditions. The proteins which mediate this inducible protection can vary from organism to organism. In *Drosophila melanogaster* constitutive overexpression of the 70-kDa heat shock protein (Hsp70) leads to added stress protection, while expression of Hsp70 antisense RNA blocks the induction of stress tolerance (38). Hsp72 and Hsp27 have both been implicated in inducible stress tolerance in human cells (19, 20, 22). The best-investigated system is the budding yeast *Saccharomyces cerevisiae*. In this system the stress-induced expression of Hsp104, a member of the ClpB family of heat shock proteins (7, 26), leads to increased tolerance to heat stress and various chemical stresses (25, 33, 34). Hsp104 forms a homohexamer (28) and has an ATP-dependent chaperone function. It can dissolve aggregates of denatured proteins and plays a crucial role in the reconstitution of heat-damaged splicing complexes in the nucleus, in accordance with its predominantly nuclear localization (27, 41).

Obligate endoparasites of mammals, such as the leishmaniae, encounter heat stress as an integral part of their life cycle after transmission from insect vectors into mammalian hosts. This fact has led several researchers to investigate the stress response in *Leishmania*. Early work using metabolic labelling techniques showed that temperatures which correspond to those in the mammalian tissue can indeed elicit a markedly increased synthesis of several protein species, namely, of the Hsp70 and Hsp90 families (15, 21, 40). It was inferred from this that heat shock protein levels are increased during heat stress and thus may confer thermotolerance to the parasite during its mammalian stage (29, 40, 42). However, the major heat shock proteins of *Leishmania*, Hsp70 and Hsp83, are already extremely abundant under standard culture conditions (3), and no direct evidence pertaining to the role of specific stress proteins during the parasitic life cycle has been produced to date.

In contrast to that of Hsp70 and Hsp83, the expression of the Hsp104 homolog in *Leishmania*, Hsp100, is chiefly restricted to conditions of heat stress (13). This protein is barely detectable in unstressed insect stages of the parasite, but its intracellular level is increased by 1 order of magnitude by temperatures equivalent to those in the mammalian target tissues (17b).

Smejkal et al. (37) had shown that heat treatment increased the virulence of *Leishmania braziliensis panamensis* in the hamster model. A minimum of several hours of 34°C heat stress prior to inoculation led to a shortened latency period in the experimental infections. The expression pattern of Hsp100 (13) suggests that this protein was the effector molecule which mediated the heat inducibility of virulence observed by Smejkal et al. We therefore investigated whether *Leishmania* Hsp100 is required for efficient infection of a mammalian host.

(The data contributed by S. Krobitsch will be part of her doctoral thesis.)

MATERIALS AND METHODS

Construction of replacement vectors. The 3.4-kb *Bam*HI and 3.5-kb *Xho*I fragments of the *Leishmania major clpB* gene, which contain the 5′ half and the 3′ half of the gene, respectively, were subcloned (13), and the plasmids were designated p*L.m.ClpB*/B3.4 and p*L.m.ClpB*/X3.5, respectively. Two kilobases of the 5′-flanking region of *L. major clpB* was amplified enzymatically from p*L.m. ClpB*/B3.4 with the two primers *Hsp100-4* (GGAATTCTAGCCGCGTTCCT TCTC) and *Hsp100-5* (GCTCTAGAGGTACCAGAAAGAGAGAAGGATG). The primers provided an *Eco*RI site at the 5′ end of the amplification product and *Kpn*I and *Xba*I sites at the 3′ end. The 3′-flanking DNA of *clpB* was amplified with primer *Hsp100-6* (GCTCTAGAGGATCCAGCGGCGGTTGC GGACTC), which introduces synthetic *Xba*I and *Bam*HI sites, and primer *Hsp100-7* (GCTCTAGACGGCTCAACACACGCGAG), which introduces an *Xba*I site at the 3′ end. The amplified 5′ flank was ligated into pUC19 opened with *Eco*RI and *Xba*I to create plasmid pUC19-5′Clp. The amplified 3′ flank was ligated into the *Xba*I site of pUC19-5′Clp. Then the 900-bp *Eco*RI-*Sca*I fragment of the resulting plasmid was replaced by the corresponding fragment from

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pUC18 to provide a multiple-cloning site at the 5′ end of the insert. Neo^r and Hyg^r genes were isolated from pBSneo or pBShyg (9) by a *Kpn*I-*Bam*HI double digestion and placed between the *clpB* 5′ and 3′ flanks to yield either p*L.m.ClpB*-NEO or p*L.m.ClpB*-HYG.

Construction of *clpB* expression vectors. The complete *clpB* gene was reconstructed from the plasmids p*L.m.ClpB*/B3.4 and p*L.m.ClpB*/X3.5 to yield the plasmid p*L.m.ClpB*. The correct relative orientation of the gene fragments was confirmed by analytical restriction analysis. A 2.2-kb *Xho*I fragment from pHMPAC (6) which contains the puromycin resistance gene was integrated into the single *Xba*I site of p*L.m.ClpB* by using S1 nuclease digestion and blunt-end ligation. This led to a plasmid which contains the protein-coding regions of *clpB* and the Pac^r gene in head-to-tail orientation. The plasmid was named pSAR+.

Parasite culture. Promastigote stages of *L. major* (MHOM/SU/73/5ASKH) were grown in M199 medium supplemented with 25% fetal bovine serum, 100 μM adenine, 10 μg of heme per ml, 40 μM HEPES (pH 7.4), 1.2 μg of 6-biopterin per ml, and 20 μg of gentamicin per ml. Clonal derivatives of transfected parasites were obtained by plating on M199 agar plates as previously described (17) except that the agar plates contained 50 μg of G418 per ml and/or 50 g of hygromycin B per ml to select for transfectants. Selection and maintenance of pSAR+-transfected Δ*clpB* strains were carried out in modified M199 medium supplemented with 50 μg each of puromycin, hygromycin B, and G418 per ml.

Electrotransfection. (i) Preparation of DNA. If linear DNA was required for homologous recombination, fragments were excised from p*L.m.ClpB*-NEO or p*L.m.ClpB*-HYG by a single *Xba*I digestion. Single-stranded overhangs were removed by S1 nuclease treatment. For episomal stable transfection, supercoiled plasmid DNA purified over two consecutive density gradients was used.

(ii) Electroporation. Electrotransfection of *Leishmania* parasites was carried out as previously described (18). Briefly, cells were harvested during the late log phase of growth, washed twice in ice-cold phosphate-buffered saline and once in prechilled electroporation buffer, and suspended at a density of 10^8 cells/ml in electroporation buffer. Chilled DNA was mixed with 0.8 ml of the cell suspension, which was immediately used for electroporation with a Bio-Rad Gene Pulser apparatus. Electrotransfection of linear DNA was carried out at 3,000 V/cm and 25 μF in a 4-mm electroporation cuvette, whereas circular DNA was transfected at 3,500 V/cm and 25 μF. Mock transfection of the *L. major* wild type was performed in identical fashion, but without plasmid DNA, to obtain positive control strains for phenotypical analysis. After electroporation, cells were kept on ice for 10 min before being transferred to 10 ml of drug-free medium. The appropriate drugs for selection of transfectants were added after 24 h.

Western blot analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western transfer were performed as previously described (3). Anti-Hsp70 and anti-Hsp83 immunoglobulin from yolk (IgY) were previously described (13). Anti-Hsp100 IgY was isolated from eggs from laying hens immunized with the recombinantly expressed complete Hsp100 protein (3a) as previously described (30, 31). Anti-chicken immunoglobulin G–alkaline phosphatase conjugate (diluted 1:3,000) was obtained from Jackson Immunolabs.

Southern blot analysis of transfected cells. *Sca*I-digested genomic DNA (10 μg) of each *L. major* clone was separated by electrophoresis through a 1% agarose gel in Tris-acetate-EDTA buffer (32) and transferred onto a nylon membrane. Blots were hybridized to various DNA probes: a 1,300-bp *clpB*-specific amplification product (13) which represented the *clpB* open reading frame (ORF) and probe 5′-0.6, which was isolated from a *Bam*HI-*Sca*I digest of the originally obtained phage DNA (13). This DNA fragment is located immediately upstream of the 3.4-kb *Bam*HI fragment and is not part of the DNA constructs used to replace the *clpB* alleles. Probes specific for the Neo^r and Hyg^r genes were isolated from pBSneo and pBShyg. Radiolabelling and hybridization were carried out according to standard procedures (32).

Experimental infections. Stationary-phase promastigotes were collected by centrifugation (1,000 × *g*, 10 min, 4°C), washed twice in ice-cold phosphate-buffered saline, and resuspended at a density of 4×10^8 or 4×10^7/ml. Parasites were inoculated in a volume of 50 μl into the right hind footpads of 5- to 6-week-old BALB/c mice. Footpad size was measured weekly with an ODITEST caliper (Kroeplin, Schlüchtern, Federal Republic of Germany). Parasite loads were determined by limiting dilution of homogenized host tissue in supplemented M199 medium (see above).

Limiting-dilution analysis. Mice infected with wild-type *L. major*, *L. major* Δ*clpB* strain 1, and *L. major* Δ*clpB*(pSAR+) were sacrificed after 11 weeks of infection, during which significant lesions had developed. The proximal lymph nodes were removed and homogenized, and the homogenates were subjected to a limiting-dilution assay in supplemented M199 medium by using 96-well microtiter plates. After 2 weeks the plates were evaluated for *Leishmania* proliferation. In previous tests we had established that one *Leishmania* cell per well proliferates in supplemented M199 medium.

In vitro infections. Peritoneal macrophages from BALB/c mice were seeded into sterile chamber slides (Nunc) at 10^6 cells per ml in supplemented M199 medium (see above). After allowing 1 h at 37°C for adhesion of the cells, a 10-fold excess of *L. major* stationary-phase promastigotes was added. After 4 h at 37°C, the supernatant was removed and the cells were washed once with fresh medium. After addition of fresh medium, the infected macrophages were incubated for up to 3 days. Cells were then fixed with ice-cold methanol, stained with Giemsa, and analyzed microscopically.

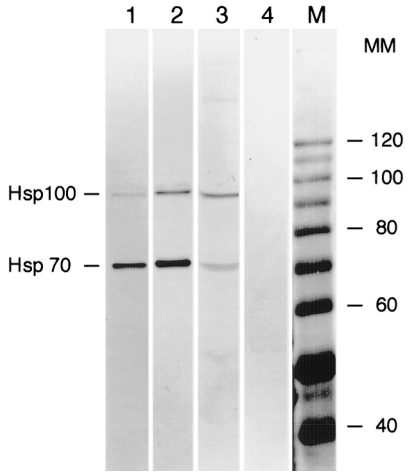

FIG. 1. Western blot analysis. A total of 5×10^6 *L. major* promastigotes incubated for 24 h at 25°C (lane 1) or 35°C (lane 2) were analyzed alongside 5×10^6 *L. major* amastigotes from infected lymph node material (lane 3) and an equivalent amount of uninfected lymph node material (lane 4) by SDS-PAGE and immunoblotting. The blot was probed with antibodies specific for *L. major* Hsp100 and Hsp70. The molecular masses (MM) of selected marker proteins (lane M) are indicated in kilodaltons.

Thermotolerance experiments. Growth of the different *Leishmania* strains was synchronized by daily dilution to 10^6 cells/ml. Synchronized log-phase promastigotes were seeded at 10^6/ml and incubated at either 25 or 35°C. Cell density was measured daily with a microscope and a counting chamber. Live cells were defined as showing flagellum motility.

Imaging. Autoradiographs and Western blots were digitalized on a flatbed scanner. Photographic images on 35-mm slides were digitalized in a Kodak Photo CD format. Digital images were cropped and juxtaposed by using Adobe Photoshop software, version 3.0.5, on an Apple Power PC computer. Line drawings were generated and combined with halftone images by using Claris Draw software.

RESULTS

Hsp100 is expressed in *L. major* amastigotes. The heat inducibility of Hsp100 synthesis suggests that this protein should be expressed in amastigotes of *Leishmania*. We therefore investigated the abundance of Hsp100 in tissue amastigotes of *L. major*. Promastigotes from a stationary-phase culture were inoculated into the footpads of BALB/c mice. After 11 weeks, during which substantial footpad lesions developed, the mice were sacrificed, and amastigotes were recovered from lymph node tissue and frozen. The number of parasites per milliliter was determined by limiting-dilution assay (data not shown). An equivalent of 5×10^6 frozen amastigotes was analyzed by SDS-PAGE and immunoblot analysis (Fig. 1, lane 3). A control sample with lymph node material prepared from uninfected mice did not react with the antibodies against *Leishmania* heat shock proteins (Fig. 1, lane 4). Control lanes with 5×10^6 *L. major* promastigotes cultivated at 25 and 36°C, respectively, showed signals for both Hsp70 and Hsp100, with an increase of Hsp100 after heat shock (Fig. 1, lanes 1 and 2). The amastigote material showed a dominant signal for Hsp100, even more prominent than for Hsp70. The high Hsp100 concentration in amastigotes suggests an important role for this protein during the mammalian stage of *L. major*.

Replacement of *clpB* alleles in *L. major*. To obtain direct evidence for Hsp100's role during the mammalian stage of *L. major*, we performed a gene disruption analysis. The cloning and sequence analysis of the *clpB* gene from *L. major* (13), which encodes Hsp100, allowed us to perform a gene knockout and subsequent phenotype analyses of resulting mutant *L.*

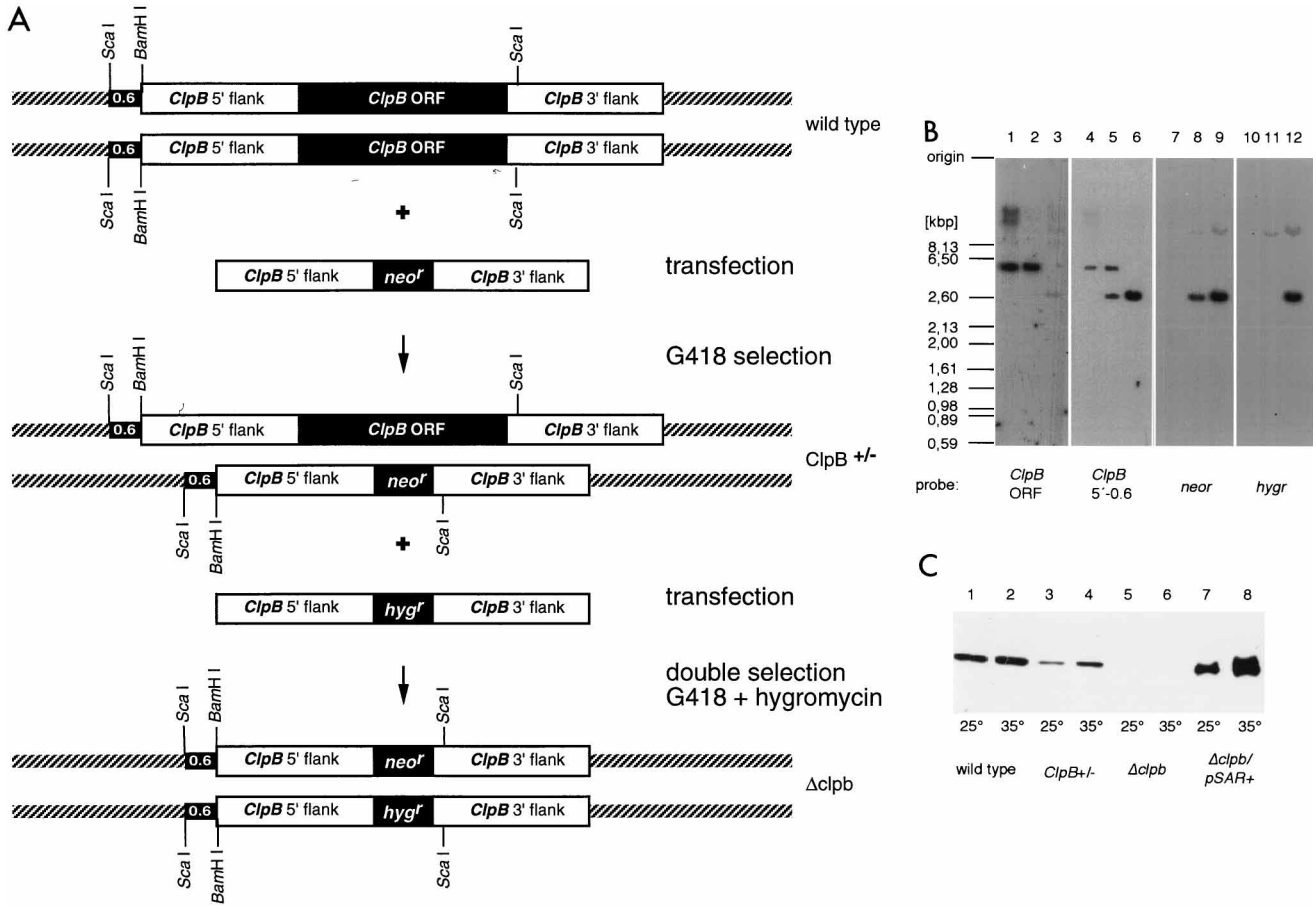

FIG. 2. Deletion of *clpB* alleles in *L. major*. (A) Strategy for homologous recombination as detailed in Materials and Methods. The boxes marked "0.6" represent hybridization probe 5′-0.6. (B) Southern analysis of *Sca*I digests of genomic DNA from wild-type (lanes 1, 4, 7, and 10), *clpB*$^{+}$/$^{-}$ (lanes 2, 5, 8, and 11), and Δ*clpB* (lanes 3, 6, 9, and 12) *L. major*. Filters were probed with either *clpB* ORF DNA, the *clpB* restriction fragment 5′-0.6, or Neo^{r} or Hyg^{r} gene DNA. The positions of marker DNA fragments are indicated on the left. (C) Immunoblot analysis of wild-type and mutant *L. major* strains. Equal amounts of protein equivalent to 10^{6} promastigotes of wild-type *L. major* or two mutant strains, *clpB*$^{+}$/$^{-}$ and Δ*clpB* strains, cultivated at either 25°C or 35°C for 24 h, were subjected to SDS-PAGE and Western transfer. A total of 2×10^{5} promastigote equivalents of *L. major* Δ*clpB*(pSAR+) was loaded in lanes 7 and 8 to achieve comparable staining. Membranes were probed with anti-Hsp100 antibodies.

major strains. The technique of homologous recombination we employed was developed for *Leishmania* by Cruz and Beverley (4). The strategy used is shown schematically in Fig. 2A. The coding sequences of the two *clpB* alleles were replaced sequentially with the ORFs encoding neomycin phosphotransferase and hygromycin phosphotransferase. We confirmed the correct insertion of the selection marker genes by Southern blot analysis (Fig. 2B). Probing the filters with a *clpB* ORF probe (Fig. 2B) showed the incremental loss of the 5-kb *Sca*I fragment which includes the Hsp100-coding DNA in the mutant strains. The Neo^{r} gene probe (Fig. 2B) hybridizes in both mutant strains to a 2.6-kb *Sca*I fragment as predicted for a correct insertion (Fig. 2A). The Hyg^{r} gene probe (Fig. 2B) hybridizes only in the double knockout mutant Δ*clpB* strain 1 and to the predicted 2.6-kb *Sca*I fragment. The correct insertion was further ascertained by hybridization with a 0.6-kb *Sca*I-*Bam*HI fragment (probe 5′-0.6 [Fig. 2B]) which is located immediately upstream of the transfected *clpB*-flanking DNA. This fragment hybridizes to 5-kb *Sca*I fragments in the wild type, to one 5-kb and one 2.6-kb fragment in the single-replacement *clpB*$^{+}$/$^{-}$ strain, and to 2.6-kb fragments in the double-replacement strain Δ*clpB* strain 1. Analysis by pulse-field gel electrophoresis of intact chromatin and Southern blotting (data not shown) confirmed that the marker genes were inserted exclusively in the correct chromosome.

The replacement of one *clpB* allele (*clpB*$^{+}$/$^{-}$) results in an approximately 50% reduction of Hsp100 levels in heat-stressed promastigotes (Fig. 2C, lanes 3 and 4) compared with those in the wild type (Fig. 2C, lanes 1 and 2). The second round of gene replacement results in a complete elimination of Hsp100 expression in the knockout mutant (Δ*clpB* strain 1) (Fig. 2C, lanes 5 and 6). The elimination of one *clpB* allele is not compensated for by an activation of the remaining gene copy, which indicates that expression of the *clpB* locus is maximally induced under the conditions of the experiment. To rescue the Hsp100 expression in the knockout mutant, we constructed plasmid pSAR+, which contains the complete *clpB* gene locus and the puromycin resistance gene under the control of the dihydrofolate reductase gene-flanking sequences. The knockout mutant was transfected with the pSAR+ plasmid by electroporation, and puromycin-resistant parasites were selected. The rescue mutant *L. major* Δ*clpB*(pSAR+) expresses 5- to 10-fold-higher levels of Hsp100 than the wild type (Fig. 2C, lanes 7 and 8). Note that only one-fifth as much protein was loaded in lanes 7 and 8 of Fig. 2C as in the other lanes. Nevertheless, expression of Hsp100 from the episomal gene

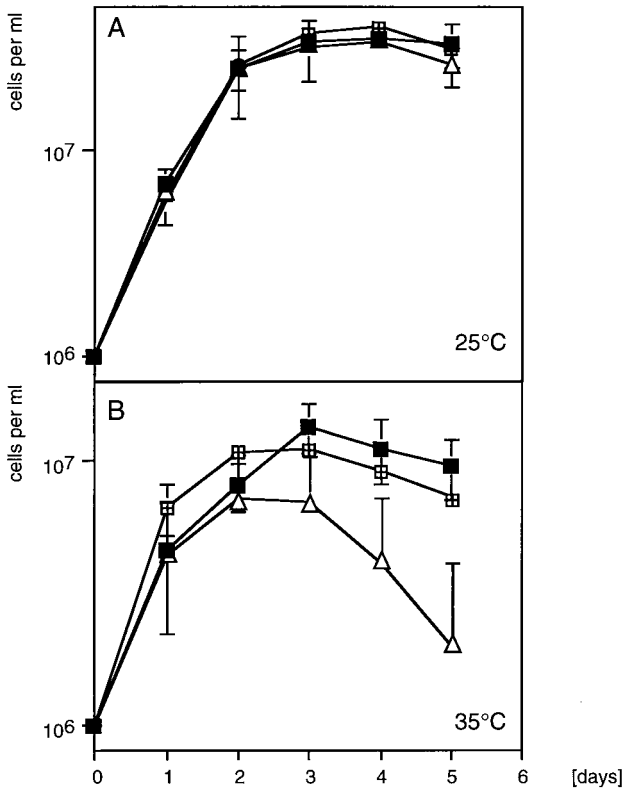

FIG. 3. Proliferation in vitro of promastigotes of mock-transfected wild-type (solid squares) or Δ*clpB* (triangles) *L. major* or *L. major* Δ*clpB*(pSAR+) (crossed squares). Cells were seeded at 10^6/ml in supplemented M199 medium and incubated at either 25 or 35°C. Live cells, i.e., cells showing flagellar motility, were counted daily with a hemocytometer. The data are mean values obtained from a series of eight independently conducted experiments, and the error bars represent the standard deviations. Another series of seven experiments with different seed concentrations showed the same tendency, albeit with variant values (data not shown).

copies appears to be upregulated under heat stress. This indicates that Hsp100 expression is not feedback regulated. However, downregulatory elements may be lacking from the 2,000 bp each of the 5′- and 3′-flanking regions which control Hsp100 synthesis in the plasmid pSAR+.

Hsp100 contributes to thermotolerance. The *clpB* gene knockout does not affect the general viability. Proliferation rates of wild-type *L. major*, Δ*clpB* strain 1, and *L. major* Δ*clpB*(pSAR+) were indistinguishable under normal (25°C) culture conditions (Fig. 3A). At elevated culture temperatures, however, Hsp100 appeared to contribute to survival and/or proliferation of promastigotes. At 35°C both the mock-transfected wild-type *L. major* and the rescue strain *L. major* Δ*clpB*(pSAR+) grew steadily for several days and entered a stationary growth phase (Fig. 3B). Cell counts for Δ*clpB* strain 1, however, plateaued after 2 days of incubation at 35°C and then dropped sharply. Since at least 24 h is required for Hsp100 to appear in significant concentrations (13), it was expected that differences between wild-type and mutant strains would not become manifest before 1 or more days. We conclude that expression of Hsp100 extends the upper limit of the permissive temperature range of the promastigote, i.e., it confers additional thermotolerance in *L. major*. Note that the prolonged incubation at elevated temperatures did not lead to the development of amastigote-like forms as in other leishmaniae.

We also tested whether Hsp100 is required for survival or proliferation under conditions of heat stress. We performed limiting-dilution analysis of wild-type and mutant strains after subjecting promastigote cultures to defined heat stress (24 h at 36, 37, or 38°C). No differences in survival could be detected (data not shown). We conclude that Hsp100 contributes to proliferation rather than survival at elevated temperatures.

Experimental infection with wild-type and mutant *L. major*. BALB/c inbred mice are a well-established animal host for *L. major* infection (2, 10–12). In order to assess the role of Hsp100 during the intracellular stage in a mammalian host, we tested pathogenicity and virulence of mock-transfected wild-type and mutant *L. major* by inoculating stationary-phase promastigotes into the footpads of BALB/c mice.

Leishmaniae cultured in liquid growth media for prolonged periods of time may lose their infectivity spontaneously (35). To eliminate any such artifacts due to the in vitro culture necessary for mutagenesis, we first passaged the mock-transfected wild type and the gene replacement strains once in the mouse infection model to select for the most virulent parasites of each strain. We inoculated promastigotes (2×10^7) from each strain into the footpads of BALB/c mice, monitored the progression of the infection as indicated by swelling of the infected footpad, and harvested the parasites from the spleen tissue of the sacrificed animals. In this initial infection we already observed a delayed development of footpad lesions in animals infected with Δ*clpB* strain 1 (data not shown). In contrast, the mock-transfected wild-type strain, which prior to transfection procedures had been in culture for more than 2 years, nevertheless induced rapidly developing lesions (data not shown). We conclude that strain MHOM/SU/73/5ASKH is not prone to random losses of virulence due to electrotransfection or prolonged in vitro culture.

The reisolated strains were then inoculated into the footpads of BALB/c mice at infection doses of 2×10^6 promastigotes per animal. Figure 4A displays the time course of the infection experiment. We observed a lowered virulence of Δ*clpB* strain 1 indicated by the delayed appearance of lesions compared with those of the wild type. The difference between the wild type and Δ*clpB* strain 1 is significant ($\alpha = 0.005$) in a U test performed according to the method of Mann and Whitney (24). The reduced virulence is due to the lack of Hsp100: overexpression of Hsp100 from extrachromosomal exogenous gene copies in *L. major* Δ*clpB*(pSAR+) significantly ($\alpha = 0.05$) speeds up lesion formation by the knockout mutant.

We also performed limiting-dilution analyses to determine the parasite load in the proximal draining lymph nodes of infected animals. The graphic representation (Fig. 4B) shows that total parasite numbers in the draining lymph nodes of animals infected with wild-type *L. major* are 75-fold higher than the numbers in animals infected with Δ*clpB* strain 1. Overexpression of Hsp100 from extrachromosomal *clpB* copies in *L. major* Δ*clpB*(pSAR+) quadruples parasite loads compared with the Δ*clpB* replacement mutant. These data are in agreement with the lesion sizes observed (Fig. 4A) and confirm earlier reports (11) that skin lesion size correlates with parasite load in *L. major*-infected BALB/c mice.

Our inability to fully restore the gene replacement mutant to the wild-type phenotype by overexpression of Hsp100 (Fig. 4A) raised the possibility of a spontaneous loss of virulence in Δ*clpB* strain 1 mixing with the effect of the gene replacement. To exclude this possibility, we produced new single-allele-replacement mutants of an *L. major* wild-type strain freshly derived from animal passage. We isolated two strains, strains 2 and 12. These strains were separately subjected to replacement of the second *clpB* allele. After clonal selection on agar plates, three clones were derived from each single-replacement

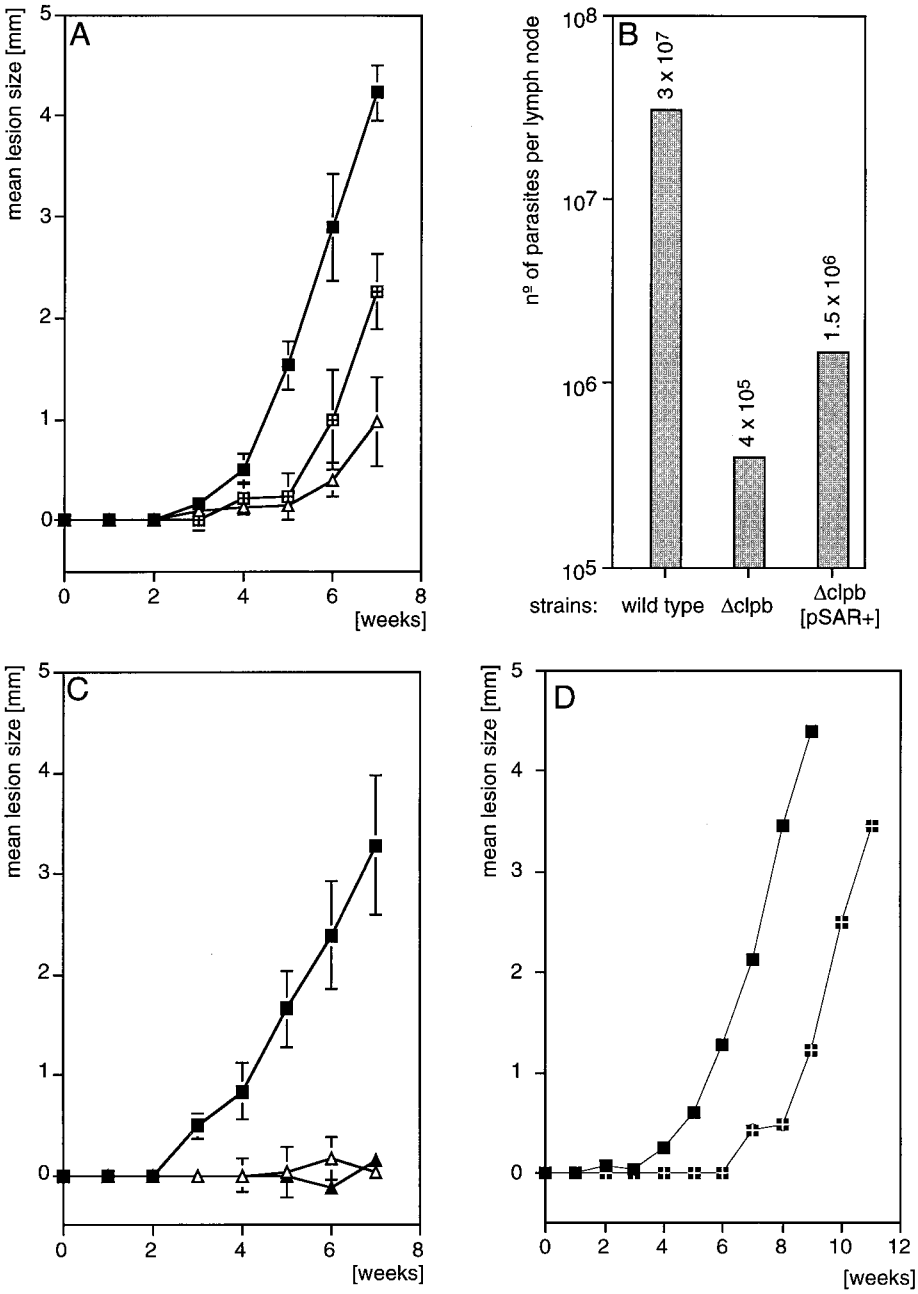

FIG. 4. Time course of *L. major* infections. Mock-transfected wild-type and mutant *L. major* promastigotes from stationary-phase cultures were inoculated into the right hind footpads of BALB/c mice. (A) A total of 2×10^6 wild-type (solid squares; $n = 5$), Δ*clpB* (triangles; $n = 5$), or *L. major* Δ*clpB*(pSAR+) (crossed squares; $n = 5$) promastigotes were inoculated. Lesion size, defined as difference of foot thickness between the right and left hind feet in millimeters, was measured weekly. The error bars represent the standard deviations. (B) Mean numbers of parasites in draining lymph nodes of BALB/c mice 11 weeks after infection with wild-type *L. major* ($n = 3$), *L. major* Δ*clpB* strain 1 ($n = 3$), or *L. major* Δ*clpB*(pSAR+) ($n = 3$). (C) A total of 2×10^7 promastigotes of wild-type *L. major* (solid squares; $n = 4$) or 2×10^7 promastigotes of six individual Δ*clpB* strains were inoculated and analyzed as for panel A. The mean lesion sizes caused by replacement mutant groups 2.× (open triangles; $n = 1$) and 12.× (solid triangles; $n = 1$) are plotted individually. Each group contains three individual mutant strains. The error bars represent the standard deviations. (D) Mean lesion size versus time in BALB/c mice infected with 10^7 promastigotes of either wild-type *L. major* (solid squares; $n = 2$) or *L. major* (pSAR+) (crossed squares; $n = 2$).

mother strain, yielding a total of six independent Δ*clpB* double-replacement strains, 2.1 to 2.3 and 12.1 to 12.3.

All six new strains were used in an infection experiment (Fig. 4C). The average lesion sizes of four mice infected with wild-type *L. major* increased rapidly after 3 weeks. The footpads of mice infected with Δ*clpB* strains 2.× and 12.× showed no significant swelling over the course of the experiment. Thus, seven of seven Δ*clpB* strains displayed a delayed or impaired lesion formation in mice. This result confirms the specificity of the gene replacement and makes a random loss of virulence highly unlikely as an explanation for the observed phenotypes.

Overexpression of Hsp100 in *Leishmania* is not beneficial. Another explanation for the incomplete restoration of the wild-type phenotype by the introduction of exogenous *clpB*

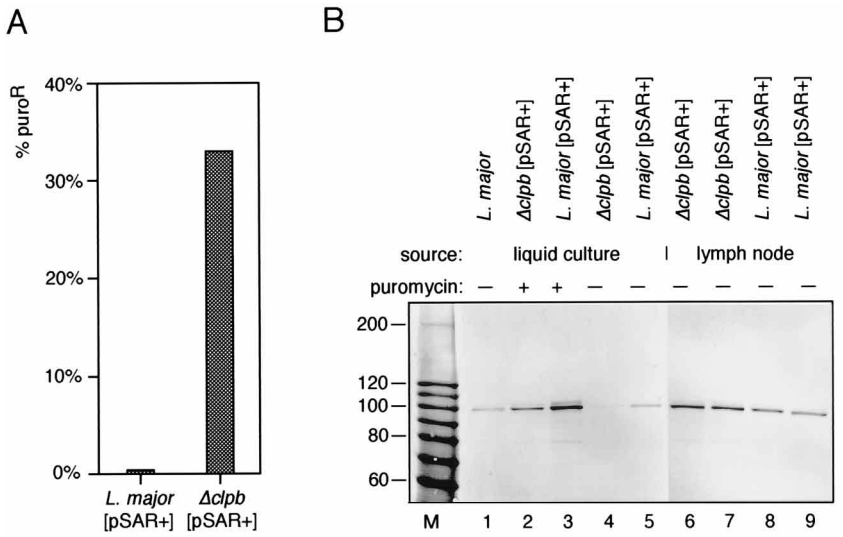

FIG. 5. Stability of the pSAR+ episome in wild-type and mutant *L. major*. Wild-type and Δ*clpB L. major* strains were transfected with the pSAR+ plasmid, and recombinant cells were selected in the presence of puromycin. *L. major*(pSAR+) and *L. major* Δ*clpB*(pSAR+) were inoculated into two BALB/c mice each and reisolated from the draining lymph nodes 11 weeks postinfection. (A) The number of live parasites of either recombinant strain and from either of two mice was determined by limiting dilution. In parallel, the numbers of puromycin-resistant (puro^R) parasites were determined by limiting dilution in puromycin-containing growth medium. From this set of data the mean percentage of puromycin-resistant leishmaniae was calculated for each strain. (B) Parasites of either recombinant strain and from either animal were grown as promastigotes for 1 week without antibiotic selection. A total of 10^6 promastigotes derived from either of two mice infected with *L. major*(pSAR+) (lanes 8 and 9) and *L. major* Δ*clpB*(pSAR+) (lanes 6 and 7) were compared in an immunoblot by using anti-Hsp100 antibodies with equal numbers of promastigotes from the wild type (lane 1) and from both recombinant strains, which had been cultivated for 12 weeks in liquid culture with and without puromycin. The marker lane was stained with Coomassie blue, and the molecular masses of marker proteins are indicated in kilodaltons. Note that lanes 1 to 5 and lanes 6 to 9 are derived from separate areas of the same immunoblot and thus can be compared directly.

gene copies (Fig. 4A) is the considerable overexpression of Hsp100 in the *L. major* Δ*clpB*(pSAR+) strain (Fig. 2C). To test whether such overexpression may have interfered with lesion formation, we transfected the pSAR+ plasmid into wild-type *L. major*. The lesions caused by this overexpressing strain, *L. major*(pSAR+), indeed appeared later than those caused by wild-type *L. major* (Fig. 4D). In addition, the parasites reisolated from mouse lymph node tissue 12 weeks postinoculation expressed normal levels of Hsp100 (data not shown) and, as shown below, had lost the pSAR+ episome.

A selective pressure for expression of Hsp100. Another way to evaluate the importance of a protein is to test whether it is a selectable marker under any given condition. The stability without antibiotic selection of the pSAR+ construct in the genetic backgrounds of wild-type *L. major* and the Δ*clpB* double-replacement strain served as a measure for selective pressure.

Parasite strains *L. major*(pSAR+) and *L. major* Δ*clpB* (pSAR+) were inoculated into BALB/c mice. In parallel, the strains were cultivated in liquid medium with and without antibiotic selection. Eleven weeks postinoculation, parasites were reisolated from the lymph nodes of infected mice. Cell suspensions were then subjected to limiting dilution both in the presence and in the absence of puromycin to determine the percentage of parasites which had retained the puromycin resistance marker gene on the pSAR+ plasmid. The result presented in Fig. 5A shows a dramatic difference between the wild type and the Δ*clpB* mutant: 33% of the Δ*clpB* mutants had retained puromycin resistance through 11 weeks of mouse passage in the absence of any antibiotic pressure. In contrast, the same plasmid was all but lost from wild-type *L. major* during the same period of time.

To test whether Hsp100 expression was affected in the same way, we performed an immunoblot analysis with anti-Hsp100 antibody (Fig. 5B). Wild-type *L. major* (Fig. 5B, lane 1) was compared with *L. major*(pSAR+) and *L. major* Δ*clpB*(pSAR+) passaged in liquid culture with antibiotic selection or in the absence of puromycin for 12 weeks. In the presence of puromycin the episomes are predictably stable. Without selection, however, both the wild type and the Δ*clpB* mutant lose the episome: the Δ*clpB* mutant contains no Hsp100 and the wild type shows normal levels of expression. In contrast, *L. major* Δ*clpB*(pSAR+) parasites reisolated from infected mice and cultivated for 1 week in the absence of puromycin still expressed high levels of Hsp100 (Fig. 5B, lanes 6 and 7). Reisolated *L. major*(pSAR+) (Fig. 5B, lanes 8 and 9), however, showed Hsp100 expression only barely above normal, which is in agreement with its loss of the episome (Fig. 5A).

We conclude that during the mouse infection the pSAR+ episome is stabilized in the Δ*clpB* mutant by a strong selection for Hsp100 expression. This selective pressure to maintain the episome is obviously absent from wild-type *L. major* because the two chromosomal *clpB* alleles are sufficient and overexpression appears to be a disadvantage.

Loss of *Leishmania* Hsp100 affects in vitro infection of macrophages. We have also tested the ability of wild-type *L. major* and the Δ*clpB* knockout mutants to survive and differentiate in isolated mouse peritoneal macrophages in vitro. Mouse peritoneal macrophages were presented with a 10-fold excess of wild-type *L. major* and with each of the seven Δ*clpB* strains. After 2 days the infection was evaluated. Figure 6A to D show representative views of infected macrophage populations. While over 90% of the macrophages presented with wild-type *L. major* harbored amastigotes (Fig. 6B), only 29% (±16% [standard deviation]) of those challenged with any Δ*clpB* strains showed intracellular parasites (Fig. 6C and D). More importantly, those macrophages infected with Δ*clpB* strains 1 and 2.3 (Fig. 6G and H) and the other five strains (data not shown) contained only small numbers of parasites per cell which were still mostly in an elongated shape and resembling

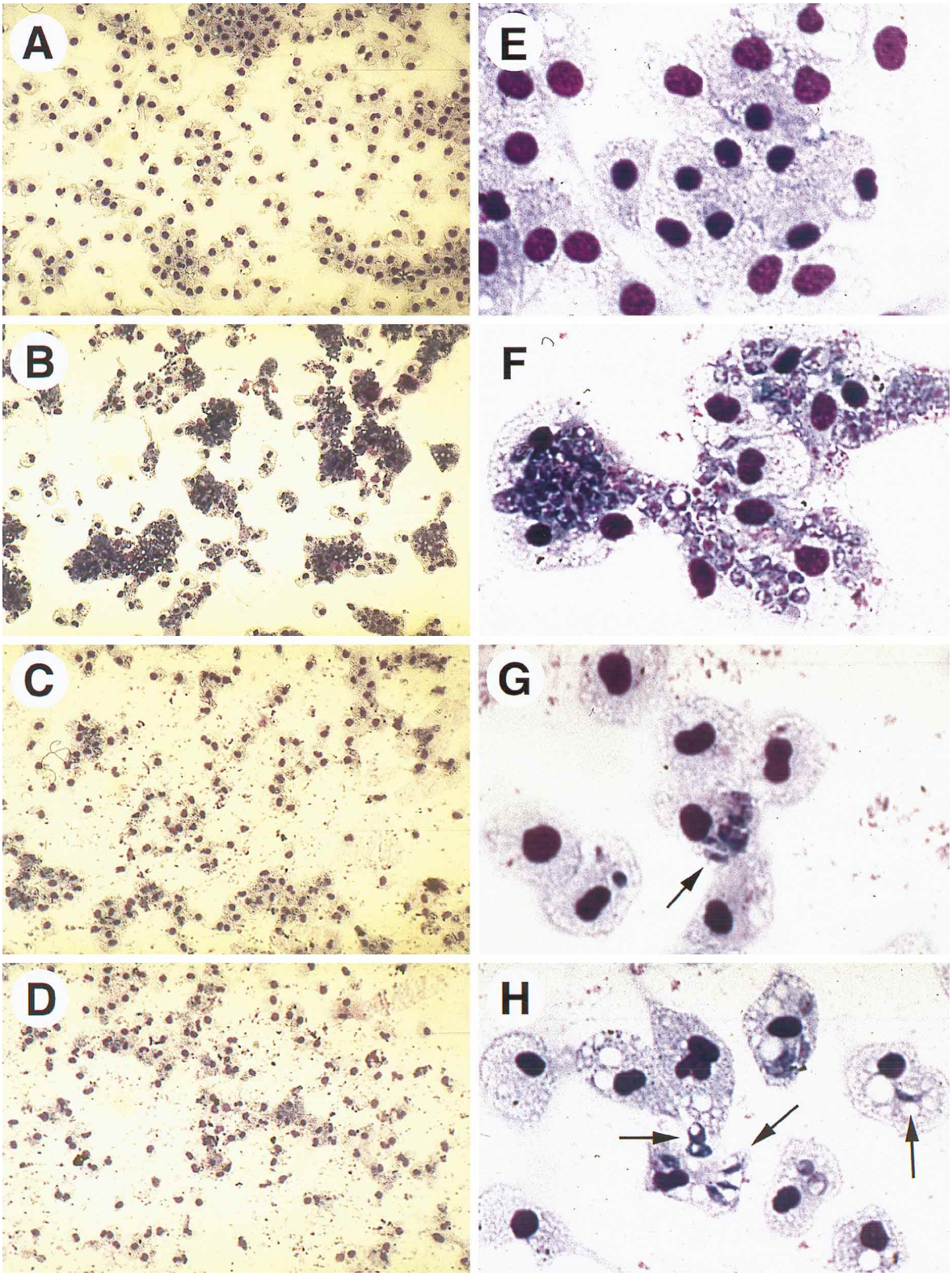

FIG. 6. In vitro infection of mouse macrophages with mock-transfected wild-type and mutant *L. major*. Isolated mouse peritoneal macrophages were infected with a 10-fold excess of *Leishmania* promastigotes. After 2 days, the macrophages were fixed and stained with Giemsa. Microphotographs were taken in overview at a magnification of 180 (A through D) and in detail at a magnification of 567 (E through H). Macrophages were infected with wild-type *L. major* (B and F), Δ*clpB* strain 1 (C and G), or Δ*clpB* strain 2.3 (D and H). Noninfected control macrophages are shown in panels A and E. Arrows in panels G and H point at intracellular parasites.

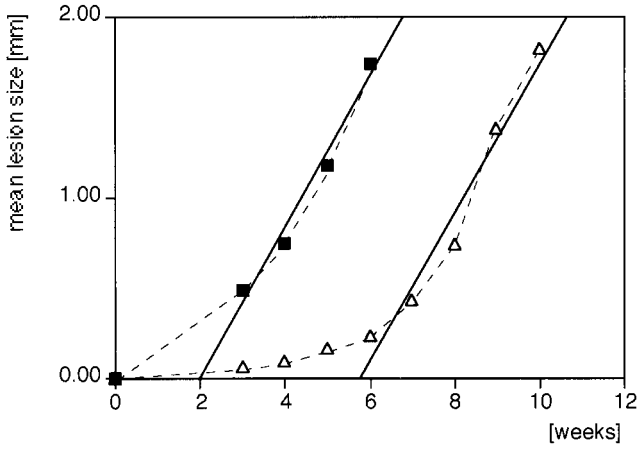

FIG. 7. Prolonged observation of lesion growth in infected mice. A total of 2×10^7 promastigotes both of *L. major* (squares) and of Δ*clpB* strain 1 mutant (triangles) were inoculated into footpads of BALB/c mice, and lesion development was monitored for 10 weeks. The linear functions of lesion size versus time were calculated from all data pairs with mean lesion sizes of >0.2 mm and are shown as solid lines.

promastigotes. In contrast, wild-type *L. major* (Fig. 6F) showed considerable intracellular proliferation of amastigote stages after 2 days.

These in vitro results confirm the in vivo infection data and suggest that Hsp100 is important early in infection during the differentiation from promastigotes to amastigotes in the host cell.

DISCUSSION

Hsp100 contributes to thermotolerance. Members of the ClpB family of heat shock proteins are expressed in cells to cope with heat or chemical stresses (23, 25, 33, 34, 39). *Leishmania* Hsp100 appears to play a similar role: at 35°C, which is comparable to the temperature of mammalian skin tissue, the loss of Hsp100 impairs viability (Fig. 3B). At temperatures beyond the limits of viability of the promastigote stage (e.g., 37°C) the Δ*clpB* mutant succumbs faster than the wild type (data not shown). At lower temperatures which already induce Hsp100 synthesis in wild-type cells, the proliferation rates of the wild type and Δ*clpB* mutants do not vary to a significant extent (data not shown). This and the fact that growth and/or viability under normal culture conditions is unaffected by the gene replacement (Fig. 3A) are evidence that expression of Hsp100 in the promastigote stage is required only under conditions of severe heat stress.

Loss of Hsp100 retards lesion development in infected mice. The parasite loads caused in mice by the Δ*clpB* mutants are reduced by 2 orders of magnitude compared with the loads caused by mock-transfected wild-type *L. major* (Fig. 4B). This correlates with a delayed onset of lesion progression observed for the gene replacement mutants (Fig. 4A). Once initiated, however, lesions caused by mutant strain 1 progress with rates comparable to those caused by mock-transfected wild-type *L. major* and eventually reach a similar size (Fig. 7). This argues against a requirement of Hsp100 for the intracellular proliferation of fully differentiated amastigotes and suggests that the loss of Hsp100 chiefly affects processes in the initial phase of an infection, i.e., development from the promastigote to the amastigote stage. This view is supported by the in vitro infection experiments (Fig. 6), in which we observed that the gene replacement strains showed a low incidence of development towards amastigote-like morphology. Such an impaired development could explain the delayed appearance of lesions.

The phenotypic effects are specific. The observed phenotypes are not the result of random gene disruption or random loss of virulence due to prolonged in vitro culture or the stress of electroporation. The correct insertion of selective marker genes was carefully verified (Fig. 2B). This argues against replacements in other gene loci. Also, the transfected constructs are void of other protein-coding sequences as evidenced by sequence analysis of the complete *clpB* gene locus (3b).

The wild type was always subjected to the same electroporation regimen, and random losses of virulence were never observed. In contrast, seven of seven gene replacement strains show identical phenotypes, with a loss of virulence and impaired amastigote development.

More is not better. One point of concern was our inability to fully restore virulence to the gene replacement strain by overexpression of Hsp100 from multiple episomal gene copies (Fig. 4A). This concern was mitigated by our finding that transfection of wild-type *L. major* with the same overexpression construct also leads to a delayed lesion development (Fig. 4D). We assume that temperature-induced overexpression of Hsp100 inside a mammalian host from multiple extrachromosomal gene copies does not constitute an advantage for *Leishmania.*

This assumption was corroborated by our finding that wild-type *L. major* rapidly loses episomal *clpB* gene copies in infected mice (Fig. 5). In contrast, the Δ*clpB* mutant which lacks chromosomal *clpB* alleles maintains the episome stably through animal passage. This result is clear proof that overexpression of Hsp100 does not give *Leishmania* a selective advantage. Therefore, more Hsp100 is not better, but a lack of Hsp100 is worse.

This preference for a limited expression is also reflected in the fact that Hsp100 is encoded by single-copy genes only in *L. major* and *Leishmania donovani* (13, 17b). In contrast, other known heat shock protein genes, e.g., *Hsp70* and *Hsp83*, are present in multiple copies in *Leishmania* and are organized in gene clusters (14, 21a, 36). Hsp70 and Hsp83 are consequently far more abundant than Hsp100 (3).

A stage-specific role for Hsp100. The loss of any *Leishmania* gene may reduce general viability and thus virulence. Both the impact and the expression of Hsp100, however, are largely restricted to conditions encountered during host invasion. In the promastigote stage of *L. major*, Hsp100 is obviously dispensable under normal culture conditions. This was evidenced by the successful gene replacement and the fact that the replacement mutants are indistinguishable from the wild type under standard culture conditions (Fig. 3A). The stage-specific impact of Hsp100 is best demonstrated by the selection experiment whose results are shown in Fig. 5: in the absence of chromosomal *clpB* copies there is a strong selective pressure to maintain episomal *clpB* genes as long as the parasite resides in the mammalian host. Therefore, Hsp100 has a protective role which it plays during the establishment of the parasite within a mammalian host but not during the insect stage.

The function of Hsp100 in other leishmaniae. We have also observed high levels of Hsp100 in axenic amastigote-like forms of *L. donovani* (17b). Such axenically derived amastigotes of *L. donovani* have been in use for several years, and in every respect analyzed so far they are indistinguishable from true lesion-derived amastigotes (1, 5, 8, 16, 43). Replacement of the *L. donovani clpB* alleles indeed affects differentiation to the axenic amastigote stage (17a). We therefore expect the impact on the differentiation processes to be a feature of Hsp100 in other *Leishmania* species as well.

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REFERENCES

1. **Bates, P. A.** 1993. Axenic amastigote culture of *Leishmania* amastigotes. Parasitol. Today **9:**143–146.
2. **Behin, R., J. Mauel, and B. Sordat.** 1979. *Leishmania major*: pathogenicity and in vitro macrophage function in strains of inbred mice. Exp. Parasitol. **8:**81–91.
3. **Brandau, S., A. Dresel, and J. Clos.** 1995. High constitutive levels of heat-shock proteins in human-pathogenic parasites of the genus *Leishmania*. Biochem. J. **310:**225–232.

3a. **Brandau, S.** Unpublished data.

3b. **Clos, J.** Unpublished data.

4. **Cruz, A., and S. M. Beverley.** 1990. Gene replacement in parasitic protozoa. Nature **348:**171–173.
5. **Doyle, P. S., J. C. Engel, P. F. P. Pimenta, P. P. Da Silva, and D. M. Dwyer.** 1991. *Leishmania donovani*: long-term culture of axenic amastigotes at 37°C. Exp. Parasitol. **73:**326–334.
6. **Freedman, D. J., and S. M. Beverley.** 1993. Two more independent selectable markers for stable transfection of *Leishmania*. Mol. Biochem. Parasitol. **62:**37–44.
7. **Gottesmann, S., C. Squires, E. Pichersky, M. Carrington, M. Hobbs, J. S. Mattick, B. Dalrymple, H. Kuramitsu, T. Shiroza, T. Foster, W. P. Clark, B. Ross, C. L. Squires, and M. R. Maurizi.** 1990. Conservation of the regulatory subunit for the Clp ATP-dependent protease in prokaryotes and eukaryotes. Proc. Natl. Acad. Sci. USA **87:**3513–3517.
8. **Gupta, N., N. Goyal, R. Kumar, A. K. Agrawal, P. K. Seth, and A. K. Rastogi.** 1996. Membrane characterization of amastigote-like forms of *Leishmania donovani* Trop. Med. Int. Health **1:**495–502.
9. **Hamann, L., R. Nickel, and E. Tannich.** 1995. Transfection and continuous expression of heterologous genes in the protozoan parasite Entamoeba histolytica. Proc. Natl. Acad. Sci. USA **92:**8975–8979.
10. **Handman, E., R. Ceredig, and G. F. Mitchell.** 1979. Murine cutaneous leishmaniasis: disease patterns in intact and nude mice of various genotypes and examination of some differences between normal and infected macrophages. Aust. J. Exp. Biol. Med. Sci. **57:**9–29.
11. **Hill, J. O.** 1986. Pathophysiology of experimental leishmaniasis: pattern of development of metastatic disease in the susceptible host. Infect. Immun. **52:**364–369.
12. **Howard, J. G.** 1985. Host immunity to leishmaniasis, p. 139–162. *In* K.-P. Chang and R. S. Bray (ed.), Leishmaniasis. Elsevier Science Publishers, Amsterdam, The Netherlands.
13. **Hübel, A., S. Brandau, A. Dresel, and J. Clos.** 1995. A member of the ClpB family of stress proteins is expressed during heat shock in *Leishmania* spp. Mol. Biochem. Parasitol. **70:**107–118.
14. **Hübel, A., and J. Clos.** 1996. The genomic organization of the *HSP83* gene locus is conserved in three *Leishmania* species. Exp. Parasitol. **82:**225–228.
15. **Hunter, K. W., C. L. Cook, and E. G. Hayunga.** 1984. Leishmanial differentiation in vitro: induction of heat shock proteins. Biochem. Biophys. Res. Commun. **125:**755–760.
16. **Joshi, M., D. M. Dwyer, and H. L. Nakhasi.** 1993. Cloning and characterisation of differentially expressed genes from in vitro-grown 'amastigotes' of *Leishmania donovani*. Mol. Biochem. Parasitol. **58:**345–354.
17. **Kapler, G. M., C. M. Coburn, and S. M. Beverley.** 1990. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol. Cell. Biol. **10:**1084–1094.

17a. **Krobitsch, S., and J. Clos.** Unpublished data.

17b. **Krobitsch, S., et al.** Unpublished data.

18. **Laban, A., and D. F. Wirth.** 1989. Transfection of *Leishmania enriettii* and expression of chloramphenicol acetyltransferase gene. Proc. Natl. Acad. Sci. USA **86:**9119–9123.
19. **Landry, J., P. Chretien, H. Lambert, E. Hickey, and L. A. Weber.** 1989. Heat shock resistance conferred by expression of the human HSP27 gene in rodent cells. J. Cell Biol. **109:**7–15.
20. **Lavoie, J. N., G. Gingras-Breton, R. M. Tanguay, and J. Landry.** 1993. Induction of Chinese hamster HSP27 gene expression in mouse cells confers resistance to heat shock. J. Biol. Chem. **268:**3420–3429.
21. **Lawrence, F., and M. Robert-Gero.** 1985. Induction of heat shock and stress proteins in promastigotes of three Leishmania species. Proc. Natl. Acad. Sci. USA **82:**4414–4417.

21a. **Lee, M. G.-S., B. L. Atkinson, S. H. Giannini, and L. H. T. van der Ploeg.** 1988. Structure and expression of the *HSP70* gene family of *Leishmania major*. Nucleic Acids Res. **16:**9567–9585.

22. **Li, G. C., L. G. Li, Y. K. Liu, J. Y. Mak, L. L. Chen, and W. M. Lee.** 1991. Thermal response of rat fibroblasts stably transfected with the human 70-kDa heat shock protein-encoding gene. Proc. Natl. Acad. Sci. USA **88:**1681–1685.
23. **Lindquist, S.** 1992. Heat-shock proteins and stress tolerance in microorganisms. Curr. Opin. Genet. Dev. **2:**748–755.
24. **Mann, H. B., and D. R. Whitney.** 1947. On a test of whether one of two random variables is stochastically larger than the other. Ann. Math. Stat. **18:**50–60.
25. **Parsell, D. A., and S. Lindquist.** 1994. Heat shock proteins and stress tolerance, p. 457–494. *In* R. I. Morimoto, A. Tissières, and C. Georgopoulos (ed.), The biology of heat shock proteins and molecular chaperones. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
26. **Parsell, D. A., Y. Sanchez, J. D. Stitzel, and S. Lindquist.** 1991. Hsp104 is a highly conserved protein with two essential nucleotide-binding sites. Nature **353:**270–273.
27. **Parsell, D. A., A. S. Kowal, M. A. Singer, and S. Lindquist.** 1994. Protein disaggregation mediated by heat-shock protein Hsp104. Nature **372:**475–478.
28. **Parsell, D. A., A. S. Kowal, and S. Lindquist.** 1994. Saccharomyces cerevisiae Hsp104 protein. J. Biol. Chem. **269:**4480–4487.
29. **Polla, B. S.** 1991. Heat shock proteins in host-parasite interactions. Parasitol. Today **7:**A38–A41.
30. **Polson, A., B. von Wechmar, and M. H. V. van Regenmortel.** 1980. Isolation of viral IgY antibodies from yolks of immunized hens. Immunol. Commun. **9:**475–493.
31. **Polson, A., T. Coetzer, J. Kruger, E. von Maltzahn, and K. J. van der Merwe.** 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunized hens. Immunol. Invest. **14:**323–327.
32. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **Sanchez, Y., J. Taulien, K. A. Borkovich, and S. Lindquist.** 1992. Hsp104 is required for tolerance to many forms of stress. EMBO J. **11:**2357–2364.
34. **Sanchez, Y., and S. L. Lindquist.** 1990. Hsp104 required for induced thermotolerance. Science **248:**1112–1115.
35. **Segovia, M., J. M. Artero, E. Mellado, and M. L. Chance.** 1992. Effects of long-term in vitro cultivation on the virulence of cloned lines of *Leishmania major* promastigotes. Ann. Trop. Med. Parasitol. **86:**347–354.
36. **Shapira, M., and E. Pinelli.** 1989. Heat-shock protein 83 of *Leishmania mexicana amazonensis* is an abundant cytoplasmic protein with a tandemly repeated genomic arrangement. Eur. J. Biochem. **185:**231–236.
37. **Smejkal, R. M., R. Wolff, and J. G. Olenick.** 1988. *Leishmania braziliensis panamensis*: increased infectivity resulting from heat shock. Exp. Parasitol. **65:**1–9.
38. **Solomon, J. M., J. M. Rossi, K. Golic, T. McGarry, and S. Lindquist.** 1991. Changes in Hsp70 alter thermotolerance and heat-shock regulation in *Drosophila*. New Biol. **3:**1106–1120.
39. **Squires, C., and C. L. Squires.** 1992. The Clp proteins: proteolysis regulators or molecular chaperones? J. Bacteriol. **174:**1081–1085.
40. **van der Ploeg, L. H. T., S. H. Giannini, and C. R. Cantor.** 1985. Heat shock genes: regulatory role for differentiation in parasitic protozoa. Science **228:**1443–1446.
41. **Vogel, J. L., D. A. Parsell, and S. Lindquist.** 1995. Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation. Curr. Biol. **5:**306–317.
42. **Young, D. B., A. Mehlert, and D. F. Smith.** 1990. Stress proteins and infectious diseases, p. 131–166. *In* R. I. Morimoto, A. Tissières, and C. Georgopoulos (ed.), Stress proteins in biology and medicine. Cold Spring Harbor Laboratory Press, Plainview, N.Y.
43. **Zilberstein, D., and M. Shapira.** 1994. The role of pH and temperature in the development of Leishmania parasites. Annu. Rev. Microbiol. **48:**449–470.