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Effects of the disruption of the *HSP70-II* gene on the growth, morphology, and virulence of *Leishmania infantum* promastigotes

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Summary. The 70-kDa heat shock protein (HSP70) is highly conserved among both prokaryotes and eukaryotes and plays essential roles in diverse cellular functions not only under stress but also under normal conditions. In the protozoan *Leishmania infantum*, the causative agent of visceral leishmaniasis, HSP70 is encoded by two *HSP70* genes. Here, we describe the phenotypic alterations of *HSP70-II*-deficient (Δ*hsp70-II*) promastigotes. The absence of *HSP70-II* caused a major alteration in growth as the promastigotes reached stationary phase. In addition, aberrant forms were frequently observed in Δ*hsp70-II* mutant cultures. An accumulation of cells in the G_2/M phase in cultures of the Δ*hsp70-II* mutant was determined by flow cytometry. Furthermore, Δ*hsp70-II* promastigotes showed a limited capacity of multiplication within macrophages, even though attachment to and uptake by macrophages did not differ significantly from the wild-type. Moreover, Δ*hsp70-II* was highly attenuated in BALB/c mouse experimental infections. In mutants re-expressing *HSP70-II*, the growth rate was restored, the normal morphology was recovered, and interactions with macrophages increased. However, promastigotes re-expressing *HSP70-II* did not recover their virulence. Overall, these data highlight the essential role played by *HSP70-II* expression in *Leishmania* virulence, pointing to this gene as a promising target for therapeutic interventions. [**Int Microbiol** 2008; 11(2):81-89]

Key words: *Leishmania infantum* · gene *HSP70-II* · gene deletion · infectivity · phenotypic alterations

Introduction

All living organisms respond to heat shock and other stresses by synthesizing a set of highly conserved proteins, the heat-shock proteins (HSPs) [14]. However, HSPs also play essential roles in non-stressed cells [6] as they are involved in the folding, assembly, intracellular localization, secretion, activation, and degradation of many proteins. Both HSP70 and HSP90 interact with many of the components of signaling pathways that regulate growth and development [11]. In this regard, the molecular relationships between HSPs and signaling proteins are critical for the correct functioning of those pathways. For example, the relative levels of these proteins are likely to be important, as deficient or excess HSPs have been shown to result in aberrant growth control, developmental malformations, and cell death [15].

HSP70 was initially discovered based on its highly increased expression in cells stressed by an increase in temperature or other conditions that lead to protein misfolding. Consistent with its biological relevance, HSP70 is the most conserved protein known to date and is present in all organisms [10]. Among its many important functions, HSP70 pro-

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tects cells from the deleterious effects of misfolded and aggregated proteins by hindering misfolding of proteins, disaggregating misfolded proteins, and presenting irreversibly damaged proteins to proteasomes for degradation [15,28].

Protozoa of the genus *Leishmania* are the causative agents of diseases known collectively as leishmaniasis, which currently affects about 12 million people worldwide at a rate of 1.5–2 million new cases each year [16]. These microorganisms have a digenetic life cycle, alternating between free-living, flagellated promastigotes in phlebotomine sand flies and obligate intracellular aflagellated amastigotes that multiply within the macrophages of a mammalian host. Transmission from the ectothermic poikilothermic insect vector into a mammalian host involves heat stress, which seems to act as signal for cellular differentiation [31]. In this context, the study of the heat-shock response and the regulatory mechanisms controlling *HSP* expression will further our understanding of the differentiation process in this protozoan. Accordingly, we characterized the *Leishmania HSP70* locus and identified regulatory mechanisms controlling HSP70 expression. In *Leishmania infantum*, the *HSP70* locus consists of six copies of the gene arranged tandemly head-to-tail [19]. The first five genes (*HSP70-I*) are identical, whereas gene 6 (*HSP70-II*), at the 3′-end of the cluster, differs in its 3′-UTR sequence. This genetic organization is conserved in the genome of many *Leishmania* species [7]. Although all six genes are transcribed at similar rates, the abundance of mRNA derived from *HSP70-I* increases following heat shock, whereas that of mRNA derived from *HSP70-II* remains unaffected. Moreover, the *HSP70-I* transcripts are associated with ribosomes at both normal and heat-shock temperatures, whereas *HSP70-II* transcripts are translated specifically during heat shock [8].

In this work, a *L. infantum HSP70-II* deletion mutant was used to study the role played by this gene in different biological functions of the protozoan. Our results showed that a lack of *HSP70-II* expression has a pleiotropic effect, influencing cell morphology, replication, and virulence.

Materials and methods

Leishmania strains and growth conditions. The Δ*hsp70-II* null mutant (Δ*hsp70-II*::*NEO*/Δ*hsp70-II*::*HYG*) is a cloned line that was generated by targeted deletion of both *HSP70-II* alleles in *L. infantum* strain BCN150 [8]. Promastigotes of both the *L. infantum* wild-type strain (MCAN/ES/96/ BCN150) and the Δ*hsp70-II* null mutant were cultured in vitro as described in [8]. To culture the mutants, 20 μg G418 (Roche Diagnostics, Mannheim, Germany)/ml, and 50 μg hygromycin B (Sigma-Aldrich, St. Louis, MO)/ml were added to the medium.

Plasmid constructs and transfection experiments. For gene add-back studies, the complete *HSP70* ORF was PCR-amplified using the primers 5′-HSP70 (5′-GGATATCATGACATTCGAAGGCGCCATCG-3′) and 3′-Lito (5′-GGAAGCTTTT AGTCGACCTCCTCGACCTTGG-3′) and the pBlsc70Li clone [18] as template. The *Eco*RV–*Hin*dIII-cut PCR fragment was used to replace the cat ORF in the plasmid pBcat70-IIbis, yielding pB70-II clone. The pBcat70-IIbis plasmid, a derivative of pCATC3′C6 [8], contains the 5′UTR (+upstream sequences) and 3′UTR (+downstream sequences) of *HSP70-II*. The *Not*I fragment (5′UTR-HSP70-3′UTR-II) of the pB70-II clone was inserted into the *Not*I restriction site of plasmid p5Pac3, which contains the puromycin resistance gene. The resulting clone, p70II-Pac(sense), was used to transfect Δ*hsp70-II* null promastigotes following a previously described method [8]. Transfectants were selected by growth in RPMI medium containing 20 μM puromycin (Calbiochem, La Jolla, CA).

Growth kinetics. Promastigotes were harvested from stationary-phase cultures and diluted to 1×10^6/ ml in 10 ml of fresh medium. At 24-h intervals, the cell density was determined by hemocytometer enumeration. For fluorescent staining, promastigotes were harvested, washed twice with phosphate-buffered saline (PBS), and resuspended to 1×10^7 cells/ml in PBS. CFSE (5,6-carboxyfluorescein diacetate succinimidyl ester; Invitrogen) was added to a final concentration of 5 μM and cells were incubated at 26ºC for 10 min in the dark. The staining reaction was stopped by the addition of 5 volumes of ice-cold culture medium. Finally, the promastigotes were washed twice with medium, resuspended at 1×10^6 cells/ml, and further cultivated at 26ºC. Mean CFSE fluorescence was determined immediately after staining and at 1, 2, 3, 4, and 7 days of culture by flow cytometry on a FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software.

Microscopic examination. Promastigotes cultured at 26ºC for 4, 7, 9, or 12 days were harvested, washed twice with PBS, and placed onto glass microscopy slides. These preparations were fixed in methanol for 5 min, air dried for 24 h, stained for 20 min in Giemsa's azure eosin methylene blue solution (Merck, Darmstadt, Germany), diluted 1/20 in distilled water, and then washed five times with distilled water. Images were taken using an Axioskop2 plus microscope and a Coolsnap FX color camera.

Flow cytometric cell cycle analysis. Four million promastigotes were harvested by centrifugation, washed twice with PBS, resuspended in 1 ml of fixative solution (30% PBS/70% methanol) and incubated at 4ºC for 1 h. The fixed cells were collected by centrifugation and resuspended in 500 μl of citrate buffer pH 7 (45 mM $MgCl_2$, 20 mM MOPS, 30 mM sodium citrate, and 0.1% Triton X-100) containing 20 μg RNase A (Roche Diagnostics)/ml and 50 μg propidium iodide (Sigma-Aldrich)/ml. The samples were incubated at 37ºC for 20 min in the dark after which fluorescence was measured by flow cytometry on a FACSCalibur flow cytometer.

In vitro macrophage invasion. Promonocytes of human histiocytic lymphoma U937 cell line were induced to differentiate into macrophages by the addition of 1×10^{-8} M phorbol myristate acetate (PMA) to the culture medium. These macrophages were subsequently incubated in RPMI culture medium at 37ºC/5% CO_2 with stationary-phase promastigotes at a protozoan:cell ratio of 5:1. After 2 h of incubation, non-internalized promastigotes were removed and the remaining cells were incubated in RPMI culture medium at 37ºC/5% CO_2 for up to 4 days. At days 1, 2, 3, and 4, the cells were fixed with methanol and stained with Giemsa as described above. At least 600 macrophages per slide were counted; the percentage of infected macrophages and the number of intracellular amastigotes per infected cell were determined.

Analysis of promastigote-macrophage interaction. To evaluate promastigote-macrophage interactions, the promastigotes were stained with CFSE (as described above) and then incubated with U937 macrophages (5:1 protozoan to macrophage multiplicity) for 24 h at 37ºC/5% CO_2. Macrophage-associated CFSE fluorescence was determined by flow cytometry in a FACSCalibur cytometer.

Experimental infection in BALB/c mice. Seven-week-old female BALB/c mice were purchased from Harlan Interfauna Ibérica (Barcelona, Spain). Groups of BALB/c mice (n = 4) were intravenously inoculated in the lateral tail vein with 10^7 late-stationary-phase promastigotes in 100 µl of PBS. The sera obtained from the mice were stored in 50 % glycerol at –20ºC. Four weeks after infection, parasitic burdens were evaluated in the spleens and livers by limiting dilution [3,4]. The reciprocal of the highest dilution that was positive for promastigote growth was considered to be the number of viable microorganisms per organ.

Analysis of antibody responses. *Leishmania* crude antigen was prepared from *L. infantum* promastigotes by incubating the microorganisms in lysis buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl pH 8, and 1 mM PMSF) for 15 min. The suspension, kept on ice, was sonicated until a decrease in viscosity was observed. The insoluble material was pelleted at 10,000 ×*g* for 5 min and the supernatant was immediately stored at –70ºC until use.

Serum samples were analyzed for specific antibodies against *Leishmania* total antigen by standard ELISA assay. Briefly, standard plates (NUNC A/S, Roskilde, Denmark) were coated overnight at 4ºC with 100 µl of *Leishmania* crude antigen (2 µg/ml in PBS). The mouse sera were assayed at 1:100 dilutions. As secondary antibodies, the following peroxidase-conjugates (Nordic Immunology Laboratories, Tilburg, Netherlands) were used: goat anti-mouse IgG (1:1000 dilution), goat anti-mouse IgG1 (1:1000 dilution), and goat anti-mouse IgG2a (1:1000 dilution). The peroxidase substrate was orthophenylenediamine dihydrochloride (DAKO A/S, Glostrup, Denmark). After 30 min, the reaction was stopped by the addition of 100 µl of 1 M H_2SO_4, and the absorbance was read at 450 nm.

Statistical analysis. The significance of the differences was examined by Student's *t*-test; $P < 0.05$ was considered statistically significant.

Results

Altered growth and morphology of the Δ*hsp70-II* mutant. *HSP70-II* knockout *L. infantum* promastigotes were obtained by double-targeted gene replacement of both alleles, as described elsewhere [8]. The deletion mutant Δ*hsp70-II* was viable, but its growth properties in axenic media differed from those of the parental strain, mainly during late-exponential phase (Fig. 1A). The densities of the deletion mutant (12–14 × 10^6 per ml) during stationary phase were lower than those of the parental strain (25–27 × 10^6 per ml). Specifically, coincident with entry into stationary phase, the density of the Δ*hsp70-II* culture gradually decreased, whereas the number of microorganisms in the wild-type culture was maintained (and even slightly increased). Similar growth curves were obtained when the Δ*hsp70-II* mutant was grown in the absence or presence of hygromycin B and G418 (Fig. 1A); thus, the growth difficulties were not due to the presence of the antibiotics.

Further evidence that disruption of *HSP70-II* expression was the specific cause of growth alterations in the Δ*hsp70-II* mutant was obtained by episomal re-expression of *HSP70-II* in the knockout. Re-expression of *HSP70-II* significantly rescued the growth defects of the Δ*hsp70-II* mutant (Fig. 1A).

In addition to determination of the culture growth kinetics by hemocytometer enumeration, cell proliferation rates were analyzed by flow cytometry (Fig. 1B). Cell division

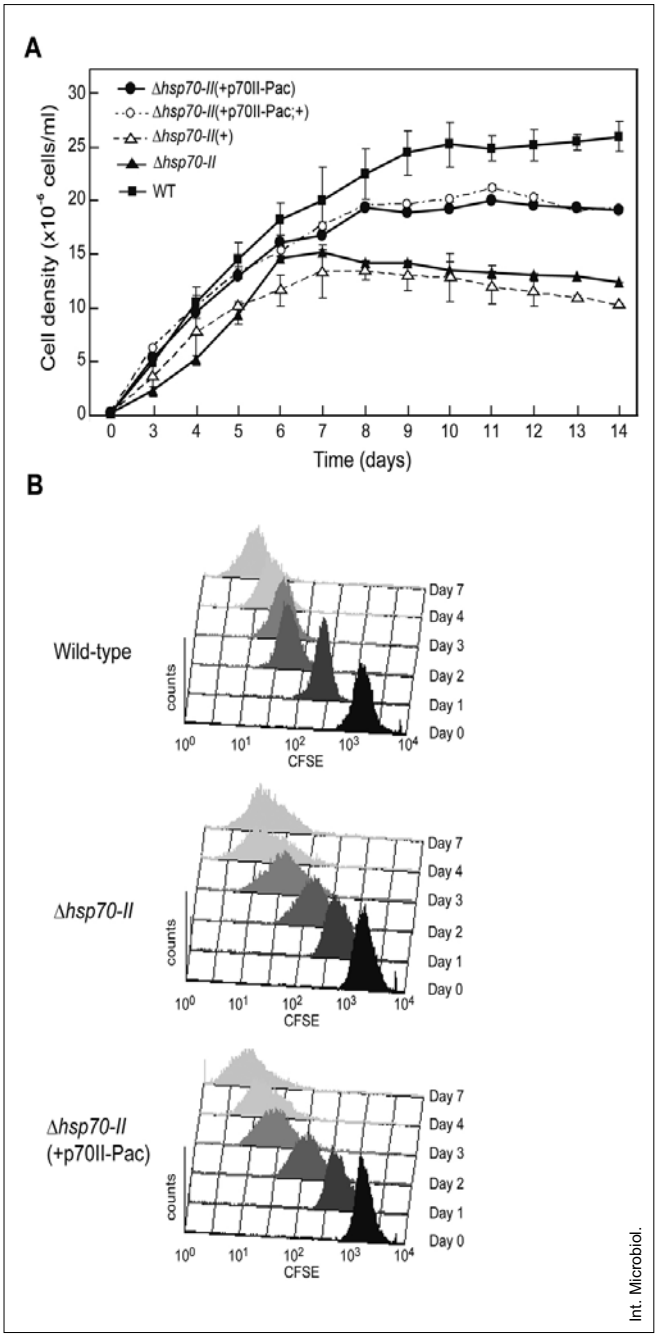

Fig. 1. *Leishmania infantum* wild-type, Δ*hsp70-II* mutant and *HSP70-II* re-expresser growth rates (**A**). For the mutant Δ*hsp70-II* and the *HSP70-II* re-expressing strain (p70II-Pac-transfected mutant) were cultured in the presence (+) or absence of selection antibiotics. Data represent the mean and standard deviation from at least three independent experiments. (**B**) Proliferation of wild-type, Δ*hsp70-II* mutant, and *HSP70-II* re-expressing promastigotes was determined by CFSE staining (see Methods).

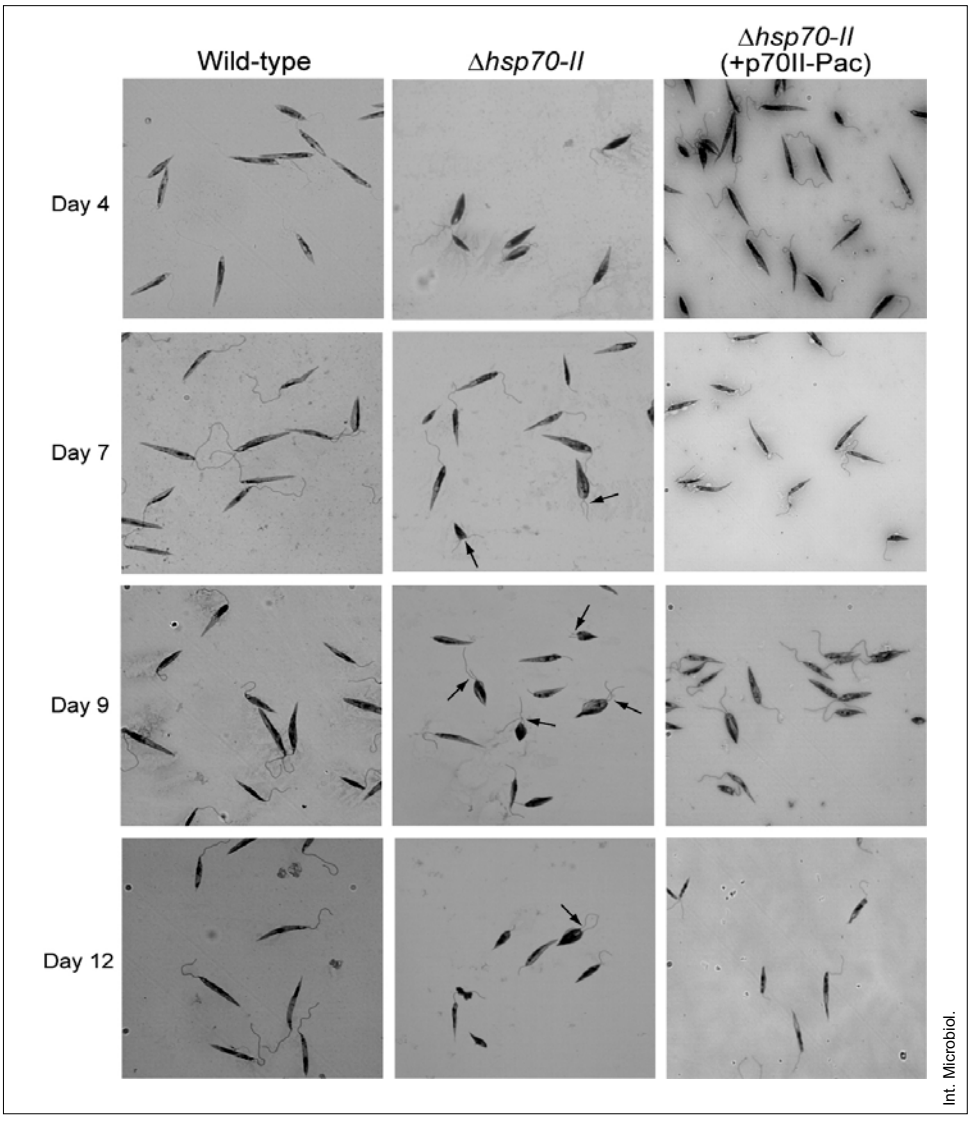

Fig. 2. Morphology of *Leishmania infantum* wild-type, Δ*hsp70-II*, and *HSP70-II* re-expressing promastigotes. At different points along the growth curve (cell densities are shown in Fig. 1A), promastigotes were harvested from the culture and stained with Giemsa. The morphology of the promastigotes was recorded using light microscopy. All images were taken under oil immersion of the preparations using a 63× objective. Arrows point to cells with two flagella.

was quantitatively measured with the stable intracytoplasmic dye CFSE. When a CFSE-stained cell divides, the dye is distributed among the daughter cells and fluorescence intensity per cell decreases accordingly. After CFSE staining of Δ*hsp70-II* and wild-type promastigotes, cultures were initiated at a starting density of 10^6 cells/ml. The CFSE fluorescence intensity was then determined at 0, 1, 2, 3, 4, and 7 days of culture. Remarkably, until day 4, there was a similar decrease in fluorescence intensity in the Δ*hsp70-II* and wild-type promastigotes. However, the peaks of the fluorescence distribution were wider in the Δ*hsp70-II* population than in the wild-type one. Again, the growth of Δ*hsp70-II* promastigotes expressing *HSP70-II* episomally was more homogeneous than growth of the mutant line (Fig. 1B).

Examination of the cultures under immersion microscopy revealed that the morphologies of the cells in the Δ*hsp70-II* cultures were remarkably heterogeneous. This was documented by Giemsa staining of promastigotes from wild-type and Δ*hsp70-II* cultures at different times (Fig. 2). In the Δ*hsp70-II* culture, a significant number of cells were smaller than those of wild-type promastigotes. In addition, rounded forms, promastigotes with two flagella, and dividing forms were more frequently observed in the Δ*hsp70-II* culture. The number of aberrant forms increased with time in culture. By contrast, morphological alterations were rare after the introduction of an episomal copy of *HSP70-II* into the mutant line (Fig. 2).

Effect of HSP70-II disruption on the cell cycle distribution. A cell cycle analysis of the mutant and wild-type cultures was carried out by harvesting promastigotes from either wild-type or Δ*hsp70-II* cultures at different times

Table 1. Cell cycle analysis of wild-type and Δ*hsp70-II* promastigotes by flow cytometry. The values are the percentages of cells in the G_1, S and G_2/M stages of the cell cycle

	Percentage of cells harvested on the indicated day								
	4	5	6	7	8	9	10	11	12
Wild-type									
G_1	80.63	81.24	83.53	84.93	78.72	77.57	76.42	81.88	81.86
S	3.74	4.18	3.48	2.89	4.99	6.61	7.02	3.5	3.63
G_2/M	14.61	11.99	11.28	11.33	12.49	12.79	13.9	13.08	13.16
Δ*hsp70-II*									
G_1	59.98	75.65	76.35	76.45	75.65	74.01	70.78	69.73	68.69
S	13.35	5.84	4.03	3.57	3.05	4.3	4.85	4.83	4.57
G_2/M	23.28	16.29	18.07	18.16	20.03	19.89	21.66	22.8	26.18

during growth and then analyzing cellular DNA content by flow cytometry (Table 1). The cell-cycle distribution showed that the percentage of cells in G_2/M phase was higher in the Δ*hsp70-II* cultures than in the wild-type cultures, a pattern that was maintained throughout the culture period. Similarly, the data showed that at days 4 and 5, the number of cells in S phase was higher in the Δ*hsp70-II* culture than in the wild-type culture, indicating a delay in the S phase of mutant cells.

Reduced infectivity of the Δ*hsp70-II* mutant in macrophages. The continuous culture and in vitro passage of *Leishmania* leads to a gradual loss of infectivity. Thus, since generation of the Δ*hsp70-II* mutant by double replacement required repeated transfection and drug selection steps [8], it could not be excluded that the lack of infectivity of the mutant line was a consequence of prolonged in vitro culturing. To address this possibility, BALB/c mice were inoculated with a high dose of mutant microorganisms. Interestingly, 28 days post-infection, *Leishmania* cells were recovered from the spleens of infected mice, which provided direct demonstration that the Δ*hsp70-II* mutant maintained its infectivity. Nonetheless, the goal of this study was to evaluate the properties of the Δ*hsp70-II* mutant, including infectivity, with respect to wild-type *L. infantum* promastigotes. In all subsequent infection experiments, promastigotes recently transformed from mouse-derived amastigotes were used.

Flow cytometry was used to analyze the interaction between U937 macrophages and Δ*hsp70-II* promastigotes (Fig. 3). Although the percentage of "fluorescent" macrophages was comparable after these cells had been incubated with either Δ*hsp70-II* (76.54%) or wild-type (92.22%) promastigotes, there were marked differences in the fluorescence intensities. The enhanced fluorescence shown by macrophages incubated with wild-type promastigotes was an indication that several protozoans were interacting with a single macrophage (Fig. 3B). Transfection of the Δ*hsp70-II* mutant with the *HSP70-II* expressing construct p70II-Pac greatly improved its ability to interact with macrophages (Figs. 3C,D).

Microscopic analysis of *Leishmania*-macrophage interactions after 1, 2, 3, and 4 days of protozoan-macrophage incubations showed that mutant and wild-type promastigotes infected similar numbers of macrophages, suggesting that the *HSP70-II* deficiency does not affect entry into macrophages. However, after 48 h, the number of amastigotes present in macrophages differed significantly between Δ*hsp70-II* and wild-type promastigotes, and the difference increased further on days 3 and 4. Moreover, re-expression of *HSP70-II* in the deletion mutant was not sufficient to rescue its limited capacity for multiplication inside macrophages (Fig. 3F).

Reduced infectivity of the Δ*hsp70-II* mutant in BALB/c mice. The ability of the Δ*hsp70-II* deletion mutant to cause visceral leishmaniasis in BALB/c mice was also investigated. Four weeks post-infection, large numbers of microorganisms were present in the liver and spleen of mice infected with wild-type *L. infantum* promastigotes (Table 2). However, in mice infected with the Δ*hsp70-II* mutant, protozoans were recovered only in the spleens, and not in the livers. Based on the cell counts, the Δ*hsp70-II* null mutant was determined to be around 1000-fold less virulent than the parental strain in the spleen of BALB/c mice and avirulent in the mouse liver.

Low numbers of protozoans were found also in the spleen of mice infected with cells re-expressing *HSP70-II* (Δ*hsp70-*

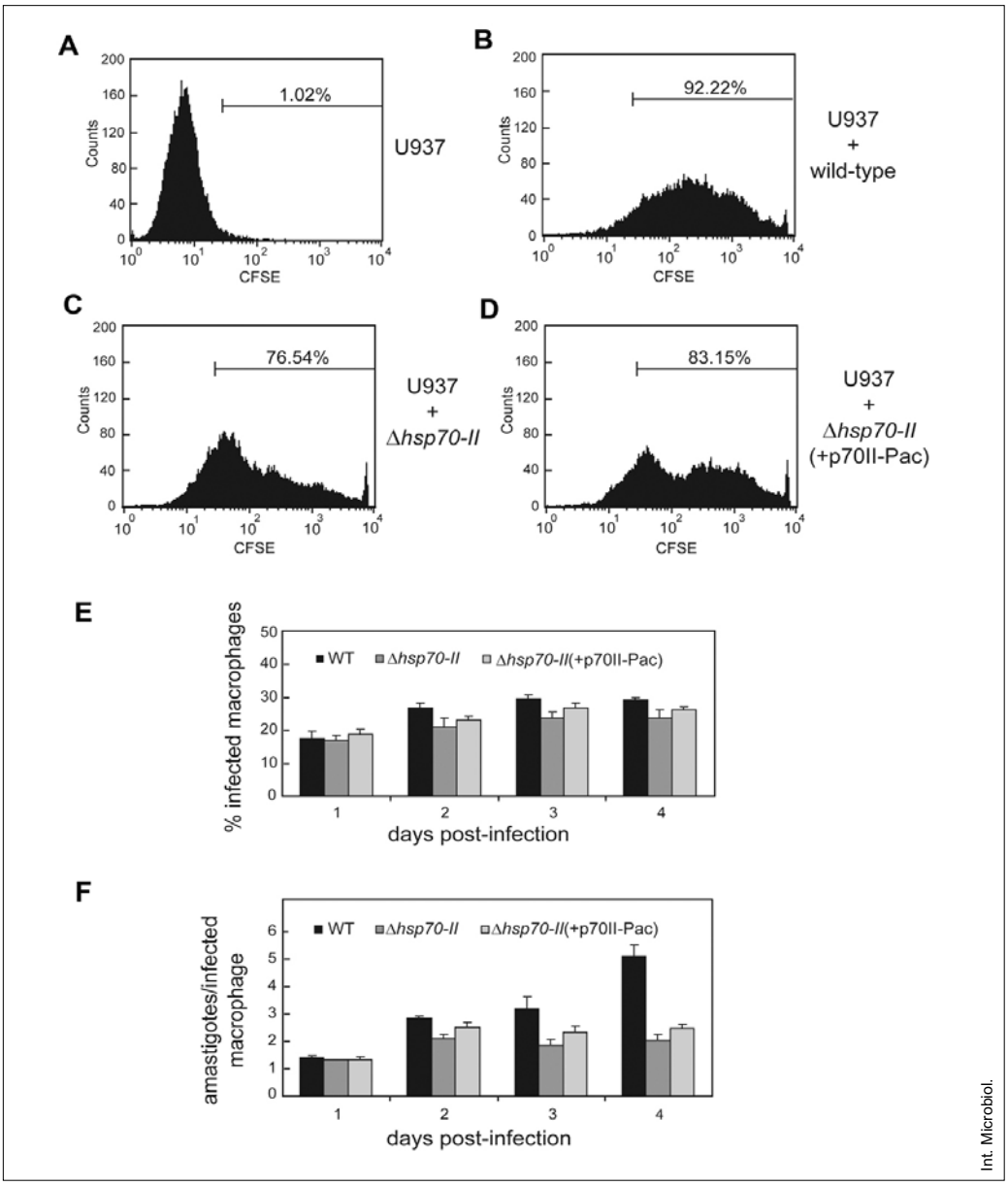

Fig. 3. Flow cytometric analysis of the interaction of *Leishmania infantum* wild-type, Δ*hsp70-II*, and *HSP70-II* re-expressing promastigotes with human U937 monocytic cells: (**A**) Non-infected macrophages; (**B**) macrophages infected by wild-type promastigotes; (**C**) macrophages infected by Δ*hsp70-II* promastigotes; (**D**) macrophages infected with *HSP70-II* re-expressing promastigotes (Δ*hsp70-II* mutant transfected with p70II-Pac). Data represent percentages of CFSE-labeled macrophages. At least 30,000 events (counts) were collected. (**E, F**) Macrophage infection rates of wild-type, Δ*hsp70-II*, and *HSP70-II* re-expressing promastigotes; (**E**) percentages of infected macrophages; (**F**) number of parasites per infected cell. Data are means and standard deviations from two experiments.

II mutant transfected with p70II-Pac). After re-introduction of the gene in the Δ*hsp70-II* mutant, the levels of accumulated *HSP70-II* mRNA were lower than those in wild-type promastigotes (data not shown). Analysis of the humoral response induced in the *Leishmania*-infected mice showed that animals infected with wild-type promastigotes had pronounced reactivity against *Leishmania* total antigen. Sera from mice infected with the Δ*hsp70-II* mutant (or the mutant transfected with p70II-Pac) also reacted positively against *Leishmania* antigens, but to a lesser extent than the sera from wild-type-infected animals. Analysis of specific IgG isotypes showed significantly higher IgG2a/IgG1 ratios in animals infected with the Δ*hsp70-II* mutant (or the re-expressing mutant), indicating that qualitative differences were obtained in the humoral response of mice infected with the wild-type vs. the mutant.

Table 2. Infectivity of *Leishmania infantum* wild-type, Δ*hsp70-II*, and re-expresser promastigotes to BALB/c mice. Groups of four mice were inoculated with either wild-type, Δ*hsp70-II*, or re-expresser (p70II-Pac transfected mutant) promastigotes

	Log (promastigotes/organ)[a]			
	Spleen	Liver	DO_{450}[b]	IgG_{2a}/IgG_1[c]
Wild-type	6.27 ± 0.51	7.85 ± 0.07	0.61 ± 0.09	0.39 ± 0.15
Δ*hsp70-II*	2.66 ± 0.29	ND[d]	0.27 ± 0.05	0.71 ± 0.16 (*)[e]
Δ*hsp70-II* (+p70II-Pac)	2.35 ± 0.51	ND	0.24 ± 0.04	0.85 ± 0.20 (**)[e]

[a]Four weeks after infection, parasitic burdens were determined by limiting dilution in both spleen and liver for each mouse. Data represent means plus standard deviations.
[b]The IgG antibody reactivity against *L. infantum* total antigen was determined in sera from mice at four weeks post-infection.
[c]The *Leishmania*-specific IgG1 and IgG2a isotypes were determined also by ELISA for individual sera. Data represent means (plus standard deviations) of the IgG2a/IgG1 ratio for each group of mice.
[d]ND, not detected.
[e]*P*-values of Student's *t*-test were calculated by comparison with the values found in sera from mice infected with wild-type promastigotes: *, 0.014; **, 0.005.

Discussion

This study was able to show that the lack of a functional *HSP70-II* gene in *Leishmania* has pleiotropic effects with respect to several biological characteristics of this protozoan. Microscopy (Fig. 2) confirmed that the frequency of cells with two flagella was higher in Δ*hsp70-II* cultures than in those of the wild-type. The increased number of aberrant forms with time in culture suggests that the function of *HSP70-II* is more relevant during stationary phase. By contrast, cultures of wild-type promastigotes consisted of cells more homogeneous in size, and aberrant forms were only rarely observed. Even though these findings suggest a cytokinesis-related problem in the Δ*hsp70-II* mutant, the differences in the cell cycle distribution of the mutant and wild-type promastigotes were not consistent with a specific block at any phase of the cell cycle. Similarly, complex phenotypes were observed after the deletion of *HSP70* genes in *Saccharomyces cerevisiae* [25] and *Drosophila melanogaster* [9]. In particular, *Ssa1* (one of the four cytosolic *HSP70* genes present in yeast) disruption in the human pathogen *Cryptococcus neoformans* resulted in alterations of multiple virulence-associated phenotypes, including reduced virulence in a mouse model [30].

In *Leishmania* Δ*hsp70-II* mutants a defect in growth was detected mainly as the promastigotes reached stationary phase (Fig. 1). In these cultures, the broader fluorescence peaks suggested that the mutant consisted of subpopulations with different division capabilities, including those in which division was accelerated. This would account for the decreased fluorescence intensity of the mutant line. In the stationary phase, the Δ*hsp70-II* mutant cultures reached only half the maximal cell density compared with that of the wild-type cultures. In addition, whereas the cell numbers of the wild-type cultures remained constant after reaching their maximum cellular density, the cell numbers in the Δ*hsp70-II* mutant cultures decreased soon after reaching the stationary phase. Similar behaviors have been reported for other *Leishmania* deletion mutants, such as *L. major* mutants lacking subunit 2 of serine palmitoyltransferase [29] or dihydroxyacetone phosphate acyltransferase [32], and an *L. mexicana* deletion mutant for the guanosine diphosphate-mannose pyrophosphorylase [24]. Cessation of growth at high cell densities has been shown to trigger the differentiation of *Leishmania* promastigotes into infective metacyclic forms [22]. Thus, the low-virulence phenotype of Δ*hsp70-II* mutant may have been in part due to an inability of these cells to differentiate into virulent metacyclic forms.

Our results also provide evidence that *HSP70-II* expression plays a role in controlling cell cycle progression at the G_2/M transition, since the percentage of mutant Δ*hsp70-II* cells detected in the G_2/M phase was higher than the percentage of wild-type promastigotes (Table 1). Likewise, a previous study showed that inhibition of HSP90 in *Leishmania* promastigotes by geldanamycin leads to cell cycle arrest, with an accumulation of cells in the G_2/M phase [26]. In many cellular processes, HSP70 and HSP90 work together, forming complexes that interact with signaling molecules and cell cycle regulators [11,17]. Furthermore, the observation that Δ*hsp70-II* promastigotes entered macrophages as efficiently as wild-type forms and the low numbers of

amastigotes present inside the cells (Fig. 3F) together suggest that *HSP70-II* is involved in the amastigote cell cycle and thus in amastigote replication. In addition, the lack of the *HSP70-II* protein product may delay *Leishmania* cell cycle progression in both developmental forms.

In the *L. major* database [www.genedb.org], the *HSP70-I* and *HSP70-II* genes that make up the *HSP70* locus in most *Leishmania* species [7] correspond to the entries LmjF28.2780 and LmjF28.2770, respectively. In the *L. infantum* database, the corresponding entries are LinJ28_V3.2960 (*HSP70-I*) and LinJ28_V3.3000 (*HSP70-II*). In both species, an identical protein is encoded by the two genes, with the only difference being in the strongly diverging sequences of the 3′-UTRs [19]. These sequences are involved in the differential regulation of the two *HSP70* genes [20]. In *L. infantum* promastigotes, *HSP70-I* mRNA is translated at normal and at heat shock temperatures, whereas *HSP70-II* mRNA is translated only in response to heat shock [8]. Steady-state levels of HSP70 are similar in Δ*hsp70-II* mutant and wild-type promastigotes [8]; therefore, the observed abnormalities of the mutant were most likely due to a lack of *HSP70-II* mRNA. These transcripts are probably used to produce new HSP70 at any moment and at specific locations within the cell. The importance of subcellular localization and temporal expression of mRNAs has been described for several genes in different organisms [12].

Add-back of *HSP70-II* to the mutant line restored normal growth as well as the wild-type morphology (Figs. 1 and 2) and enhanced interactions of the mutant with macrophages (Fig. 3D). However, neither in vitro nor in vivo infection experiments showed a recovery in virulence in the re-expresser line, perhaps because expression of *HSP70-II* from the rescue plasmid is different in promastigotes and amastigotes. Alternatively, it can be postulated that a fully functional *HSP70-II* is essential for amastigotes. Partial recovery of function by re-expression in *Leishmania* of a copy of a previously deleted gene has been described in other studies. For example, re-introduction of the gene encoding the CPA cysteine peptidase in an *L. infantum* Δ*cpa* mutant increased the in vitro infectivity of the cells but it did not improve the virulence of the mutant [5]. Similarly, the reduced infectivity to mice of a null mutant for the *ICP* gene, encoding another cysteine peptidase, was not restored by re-expressing the gene from either an episomally or a chromosomally integrated copy [2]. Given the temperature-dependent expression of *L. infantum HSP70-II* [8], the reduced virulence of the Δ*hsp70-II* mutant may be related, in part, to an impaired capacity for growth at the temperatures of the mammalian host. In fact, the Δ*hsp70-II* promastigotes are less tolerant to heat shock than wild-type cells (data not shown). These data indicate that key mechanisms regulating the expression of heat-shock genes remain to be determined but can perhaps be elucidated through the use of mutant lines such as Δ*hsp70-II*.

The Δ*hsp70-II* mutant was detected in the mice but was poorly proliferative, even when *HSP70-II* was re-introduced into the mutant. This finding provides a plausible explanation why episomal re-expression of *HSP70-II* was not sufficient to complement the reduced infectivity of the Δ*hsp70-II* mutant and suggests the potential usefulness of this mutant for the development of attenuated live vaccines to protect against the major infectious diseases caused by *Leishmania* and other protozoans [1]. In this regard, it should be noted that mice infected with Δ*hsp70-II* promastigotes had lower levels of *Leishmania*-specific antibodies but higher IgG2a/IgG1 ratios than mice infected with wild-type *L. infantum* promastigotes. This immunoglobulin pattern is associated with protective outcomes in *L. major* infections [27]. Nevertheless, before vaccine assays can be started, the spontaneous recovery of virulence by some attenuated *Leishmania* mutants (e.g., *lpg2* [23], *LmxPK4* [13], and *hsp100* [21] deletion mutants) makes it necessary to first be able to control the generation of escape variants, in which infectivity is restored.

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