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

Cristina Folgueira,1 Javier Carrión,1 Javier Moreno,2 Jose M. Saugar,3 Carmen Cañavate,3 Jose M. Requena1*

1Center for Molecular Biology (CSIC-UAM), Autonomous University of Madrid, Cantoblanco, Spain. 2Biological Research Center (CSIC), Madrid, Spain. 3WHO Collaborating Centre for Leishmaniasis, National Centre for Microbiology (ISCIII), Majadahonda, Spain

Received 23 February 2008 · Accepted 28 April 2008

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 (Δhsp70-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 Δhsp70-II mutant cultures. An accumulation of cells in the G2/M phase in cultures of the Δhsp70-II mutant was determined by flow cytometry. Furthermore, Δhsp70-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, Δhsp70-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, intracelluar 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-
tects cells from the deleterious effects of misfolded and aggregated proteins by hindering misfolding of proteins, disagggregating 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 digenic 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 the 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 Δhsp70-II null mutant (Δhsp70-II::NEO/Δhsp70-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 Δhsp70-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′-GGGATATCATGACATTTGAGGCGCCATG-3′) and 3′-Lito (5′-GGAAAGTTT TTGATGCAGCCTTCTGACCTTGG-3′) and the pBcH70lI clone [18] as template. The EcoRV–HindIII-cut PCR fragment was used to replace the cat ORF in the plasmid pBcA70-Ib, yielding pBc70-II clone. The pBcA70-Ib plasmid, a derivative of pCAT3C6 [8], contains the 5′UTR (+upstream sequences) and 3′UTR (+downstream sequences) of HSP70-II. The NotI fragment (5′UTR-HSP70-3′UTR-II) of the pBc70-II clone was inserted into the NotI restriction site of plasmid pBac3, which contains the puromycin resistance gene. The resulting clone, p70I-Pac (sense), was used to transfect Δhsp70-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 × 10^⁵ 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 26°C 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 × 10⁶ cells/ml, and further cultivated at 26°C. 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 26°C 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 with 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 4°C for 1 h. The fixed cells were collected by centrifugation and resuspended in 500 μl of citrate buffer pH 7 (45 mM MgCl₂, 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 37°C 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 37°C/5% CO₂ with stationary-phase promastigotes at a protozoan:cell ratio of 5:1. After 2 h of incubation, non-internalized promastigotes were collected by centrifugation and resuspended in 1 × 10⁶/ 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 PBS, and placed onto glass microscopy slides. These preparations were fixed in methanol for 5 min, air dried for 24 h, stained with 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.

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**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 37°C/5% CO₂. Macrophage-associated CFSE fluorescence was determined by flow cytometry in a FACSCalibur flow 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 10⁷ 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 H₂SO₄, 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 Δhsp70-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 Δhsp70-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 × 10⁶ per ml) during stationary phase were lower than those of the parental strain (25–27 × 10⁶ per ml). Specifically, coincident with entry into stationary phase, the density of the Δhsp70-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 Δhsp70-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 Δhsp70-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 Δhsp70-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, Δhsp70-II mutant and HSP70-II re-expressor growth rates (A). For the mutant Δhsp70-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, Δhsp70-II mutant, and HSP70-II re-expressing promastigotes was determined by CFSE staining (see Methods).
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 \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) and wild-type promastigotes. However, the peaks of the fluorescence distribution were wider in the \( \Delta hsp70-II \) population than in the wild-type one. Again, the growth of \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) cultures were remarkably heterogeneous. This was documented by Giemsa staining of promastigotes from wild-type and \( \Delta hsp70-II \) cultures at different times (Fig. 2). In the \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) cultures at different times

![Fig. 2. Morphology of *Leishmania infantum* wild-type, \( \Delta hsp70-II \), 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.](image-url)
during growth and then analyzing cellular DNA content by flow cytometry (Table 1). The cell-cycle distribution showed that the percentage of cells in G2/M phase was higher in the \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) culture than in the wild-type culture, indicating a delay in the S phase of mutant cells.

**Reduced infectivity of the \( \Delta hsp70-II \) mutant in macrophages.** The continuous culture and in vitro passage of *Leishmania* leads to a gradual loss of infectivity. Thus, since generation of the \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) mutant maintained its infectivity. Nonetheless, the goal of this study was to evaluate the properties of the \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) promastigotes (Fig. 3). Although the percentage of “fluorescent” macrophages was comparable after these cells had been incubated with either \( \Delta hsp70-II \) (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 \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) mutant in BALB/c mice.** The ability of the \( \Delta hsp70-II \) 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 \( \Delta hsp70-II \) mutant, protozoans were recovered only in the spleens, and not in the livers. Based on the cell counts, the \( \Delta hsp70-II \) 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* (\( \Delta hsp70-

### Table 1. Cell cycle analysis of wild-type and \( \Delta hsp70-II \) promastigotes by flow cytometry. The values are the percentages of cells in the G1, S and G2/M stages of the cell cycle

<table>
<thead>
<tr>
<th>Percentage of cells harvested on the indicated day</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>80.63</td>
<td>81.24</td>
<td>83.53</td>
<td>84.93</td>
<td>78.72</td>
<td>77.57</td>
<td>76.42</td>
<td>81.88</td>
<td>81.86</td>
</tr>
<tr>
<td>S</td>
<td>3.74</td>
<td>4.18</td>
<td>3.48</td>
<td>2.89</td>
<td>4.99</td>
<td>6.61</td>
<td>7.02</td>
<td>3.5</td>
<td>3.63</td>
</tr>
<tr>
<td>( \Delta hsp70-II )</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>59.98</td>
<td>75.65</td>
<td>76.35</td>
<td>76.45</td>
<td>75.65</td>
<td>74.01</td>
<td>70.78</td>
<td>69.73</td>
<td>68.69</td>
</tr>
<tr>
<td>S</td>
<td>13.35</td>
<td>5.84</td>
<td>4.03</td>
<td>3.57</td>
<td>3.05</td>
<td>4.3</td>
<td>4.85</td>
<td>4.83</td>
<td>4.57</td>
</tr>
<tr>
<td>G2/M</td>
<td>23.28</td>
<td>16.29</td>
<td>18.07</td>
<td>18.16</td>
<td>20.03</td>
<td>19.89</td>
<td>21.66</td>
<td>22.8</td>
<td>26.18</td>
</tr>
</tbody>
</table>
mutant transfected with p70II-Pac). After re-introduction of the gene in the Δhsp70-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 Δhsp70-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 Δhsp70-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, *Δhsp70-II*, and re-expressor promastigotes to BALB/c mice. Groups of four mice were inoculated with either wild-type, *Δhsp70-II*, or re-expressor (*p70II-Pac transfected mutant*) promastigotes

<table>
<thead>
<tr>
<th></th>
<th>Spleen</th>
<th>Liver</th>
<th>DO_{60}^{b}</th>
<th>IgG_{2a}/IgG_{1}^{c}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>6.27 ± 0.51</td>
<td>7.85 ± 0.07</td>
<td>0.61 ± 0.09</td>
<td>0.39 ± 0.15</td>
</tr>
<tr>
<td><em>Δhsp70-II</em></td>
<td>2.66 ± 0.29</td>
<td>ND^{d}</td>
<td>0.27 ± 0.05</td>
<td>0.71 ± 0.16 (**y)</td>
</tr>
<tr>
<td><em>Δhsp70-II</em> (+<em>p70II-Pac</em>)</td>
<td>2.35 ± 0.51</td>
<td>ND</td>
<td>0.24 ± 0.04</td>
<td>0.85 ± 0.20 (**y)</td>
</tr>
</tbody>
</table>

*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.

*y*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 *Δhsp70-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 *Δhsp70-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* *Δhsp70-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 *Δhsp70-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 *Δhsp70-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 *Δhsp70-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 G1/M transition, since the percentage of mutant *Δhsp70-II* cells detected in the G1/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 G1/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 *Δhsp70-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 Δhsp70-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 repressor 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 Δcpa 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 Δhsp70-II mutant may be related, in part, to an impaired capacity for growth at the temperatures of the mammalian host. In fact, the Δhsp70-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 Δhsp70-II.

The Δhsp70-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 Δhsp70-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 Δhsp70-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.

Acknowledgements. This work was supported by grants from the Spanish Ministry of Science and Technology (BFU2006-08346) and the Fondo de Investigaciones Sanitarias (ISICII-RETIC RD06/0021/0008-FEDER and ISICII-RETIC-RD06/0021/0009-FEDER). Also, a grant from Fundación Ramón Areces (Madrid, Spain) is acknowledged.

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