High constitutive levels of heat-shock proteins in human-pathogenic parasites of the genus *Leishmania*

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We have analysed the transcription of three heat-shock genes, *HSP70*, *HSP83* and *ClpB*, in the protozoan parasite *Leishmania*. All three heat-shock genes are transcribed constitutively and not heat-inducibly. However, we find that two major heat-shock proteins, HSP70 and HSP83, are synthesized at elevated rates during heat stress. We conclude that the cellular stress response in *Leishmaniae* is regulated exclusively on a post-transcriptional level much in contrast with all other eukaryotes examined so far.

The induced synthesis of HSP70 and HSP83, however, does not increase the steady-state level of either protein significantly. This is compensated by high constitutive levels of both proteins: HSP70 and HSP83 make up 2.1 % and 2.8 %, respectively, of the total protein in unstressed *Leishmania* promastigotes. Also, HSP70 is a strictly cytoplasmic protein in *Leishmania* and does not relocate into the nucleus during heat stress, as it does in other eukaryotes examined in the past.

INTRODUCTION

All organisms examined so far respond to elevated environmental temperatures and certain chemical stresses with the increased synthesis of a subset of chaperone proteins which are known as heat-shock proteins (HSPs) or stress proteins. In higher eukaryotes such as mammals and fruitflies, this cellular stress response is accomplished by an up to 100-fold transcriptional activation of the heat-shock genes [1,2]. This transcriptional induction is mediated by pre-existing negatively regulated transcription factors ([3–6], reviewed in [7]). The strongly induced synthesis of HSPs and a concomitant decrease in overall gene expression reflect the electrophoretic pattern of cellular proteins: distinct bands which represent the major HSPs become prominent and are easily identifiable [8]. A rapid and highly regulated cellular stress response is thought to be essential for free-living organisms, since environmental stresses can vary considerably in quality and severity.

The life cycle of many protozoan parasites, i.e. *Leishmaniae* and *Trypanosomae*, includes a transmission from poikilothermic phlebotomine insects into homeothermic mammals. This is considered a heat stress and a chemical stress situation as well. The genus *Leishmania* belongs to the family Trypanosomatidae and includes several species which cause the various forms of leishmaniasis and together afflict more than 12 million humans worldwide. Three stages of differentiation can be observed during the life cycle of *Leishmaniae*. They are transmitted into mammals as slender, flagellated, metacyclic promastigotes and phagocytized by skin macrophages. Within the phagosome they transform into round aflagellated amastigotes and proliferate. After rupture of the infected macrophage, the amastigotes are either phagocytized by other macrophages or ingested by a feeding sandfly. In the fly's digestive tract they transform into flagellated non-infective procyclic promastigotes [9]. They proliferate until they reach a stationary growth phase. At that point they can undergo differentiation into the infective metacyclic form [10], which can be transmitted to the next host when the sandfly takes a blood meal. Infections with *Leishmaniae* can either be localized (*L. major* and *L. tropica*), leading to ulceric skin lesions at the site of transmission, or they can be generalized, as in the fatal Kala Azar or visceral leishmaniasis, which is caused by the pro-

liferation of *L. donovani* in the tissue macrophages of internal organs such as liver, spleen and bone marrow.

Leishmania promastigotes can be cultured axenically in various liquid media at 25 °C. Metabolic labelling experiments showed that a temperature up-shift to 35 °C, which mimics transmission into a mammalian host, induces the synthesis of several defined HSPs [11]. Under such conditions one can also observe elevated HSP mRNA levels [12,13]. Lawrence and Robert-Gero [14] claimed that the expression of HSPs observed under heat stress in *L. major* promastigotes depends on RNA synthesis *de novo*. Also, DNA sequences reminiscent of eukaryotic heat-shock-responsive elements (HSEs) were found in the intergenic regions of heat-shock genes in *L. major* and in *Trypanosoma brucei* [12,15], although data on the functional significance of such sequences have not been reported. It was therefore speculated that *Leishmaniae* increase the synthesis of stress proteins in response to the stresses encountered during the transmission into a human host and that this stress response is regulated on a transcriptional level, much as in higher eukaryotes [16,17].

This view is now questioned. Miller [18] showed that non-stressed *L. mexicana amazonensis* promastigotes contain sufficient mRNA for the synthesis of large amounts of HSPs. The increase in HSP83 mRNA observed in this species during heat stress [19] was recently attributed to an increased mRNA half-life rather than induced RNA synthesis [20]. Moreover, metabolic labelling experiments with [³⁵S]methionine [11,14] can only indicate relative protein-synthesis rates and do not reflect intracellular levels of particular proteins. To establish the parameters of the heat-shock response in *Leishmania*, we analysed the regulation of two major heat-shock genes, *HSP70* [12] and *HSP83* (A. Hübel and J. Clos, unpublished work) in two Old World *Leishmania* species, *L. major* and *L. donovani*. We find differences between the cellular stress response in *Leishmaniae* as compared with any other eukaryotes examined to date.

MATERIALS AND METHODS

Leishmania strains and cultivation

The *Leishmania* strains MHOM/SU/73/5ASKH (*L. major*) and MHOM/IN/80/DD8 (*L. donovani*) were obtained from David

Evans, London School of Hygiene and Tropical Medicine. The promastigotes were grown in Hepes-buffered RPMI with 15% fetal-calf serum and 20 µg/ml gentamycin. All analyses were carried out with exponentially growing cultures $[(5-8) \times 10^6 \text{ ml}^{-1}]$.

Plasmids and bacterial strains

Plasmid propagation was carried out with DH5-alpha cells (Life Technologies). Expression of recombinant genes was performed in *Escherichia coli* strain BL21(DE3) [21].

The plasmid pLhsp70-1 (clone 11 [12]) was a gift from M. G.-S. Lee. The plasmid pBS10Rb.1, which contains the *L. major* gp63 gene [21a], was a gift from W. R. McMaster. The plasmid pLmACTrs [22], which contains the *L. major* actin gene, was a gift from D. F. Smith.

The *L. major* HSP83 gene was isolated in our laboratory (A. Hübel and J. Clos, unpublished work) and by others [22a]. The plasmid pLmHSP83-6.2 contains two complete gene repeats with coding and non-coding sequences. The open reading frame (ORF) was amplified enzymically by using the primers Nde-83 (5'-GCA CAT ATG ACG GAG ACG TTC GCG-3') and 83-TGA (5'-GGG AAT TCA GTC CAC CTG CTC CAT G-3'). The primers introduce *NdeI* and *EcoRI* sites at the 5'- and 3'-ends, respectively, which were used to subclone the ORF into pJC40 [23] to yield the plasmid pHSP83-40. The ORF of the *L. major* HSP70 gene was amplified with the primers Nde-70 (5'-G GGA ATT CAT ATG ACA TTC GAC GGC G-3') and 70-TAA (5'-CTCGGGATCC TTA GTC GAC CTC CTC GAC-3') and also subcloned into pJC40, by using the *NdeI* and *BamHI* sites introduced with the primers (pHSP70-40).

The plasmid pLmClpB-BamHI includes 5' flanking sequences and the 5' half of the *ClpB* gene from *L. major* [24] subcloned into pBluescript II KS+ (STRATAGENE).

Bacterial expression of *Leishmania* HSPs HSP70 and HSP83 and production of anti-HSP antibodies

The plasmids pHSP70-40 and pHSP83-40 were expressed and their products purified by a published protocol [23,24]. The purified gene products ($\geq 90\%$ purity by silver stain) were used to immunize laying hens. Eggs were collected before immunization and ≥ 2 weeks after the first boost. The IgY was purified from egg yolk as described [25,26].

Non-radioactive detection of nucleic acids

Digoxigenin labelling and Northern analysis were performed as described [24]. The hybridization filters were probed with Anti-Digoxigenin-AP (1:2000; Boehringer Mannheim) in Dulbecco's phosphate-buffered saline (DPBS, Gibco) with 0.2% iBlock (Tropix) and 0.02% Tween-20. Blots were developed with the chemoluminescence substrate CSPD (Tropix) according to the manufacturer's recommendations. An X-ray film was exposed for up to 5 h.

Nuclear run-on analysis

Nuclei from exponential-phase promastigotes were prepared by detergent lysis essentially as described in [27]. Cells were washed once with ice-cold DPBS, suspended at 0 °C in lysis buffer (10 mM Tris/HCl, pH 8.0, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM spermidine, 0.1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol, 0.1 mM phenylmethanesulphonyl fluoride and 0.5% Triton X-100) and kept on ice for 10 min. The crude nuclei were then pelleted at 6000 rev./min for 10 min in a Beckman J2-21 centrifuge (JA 20 rotor) at 4 °C. The nuclei were washed once in lysis buffer without detergent and resuspended in an equal volume of

2× nuclei storage buffer (100 mM Tris/HCl, pH 8.3, 80% glycerol, 10 mM MgCl₂ and 0.2 mM EDTA). They were stored frozen at -70 °C in 100 µl batches ($\sim 10^9$ nuclei).

A 100 µl portion of thawed nuclei was mixed with an equal volume of nuclear run-on buffer (8 mM MgCl₂, 80 mM Tris/HCl, pH 7.5, 50 mM NaCl, 50 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 4 mM ATP, 2 mM CTP, 2 mM GTP, 10 µM UTP, 10 mM phosphocreatine, 20 units/ml creatine kinase, 40 units of RNasin, 2 mM dithiothreitol) and 0.1 mCi of [α -³²P]UTP (400 Ci/mmol; Amersham) and incubated for 10 min at 37 °C. The radiolabelled nascent RNA was isolated by acidic guanidinium thiocyanate/phenol/chloroform extraction [28]. A 5 µg sample of each DNA probe to be tested was denatured in 0.4 M NaOH and applied on to a nylon membrane (Quiagen) in a vacuum slot-blot apparatus (Life Technologies). The membrane strips were then subjected to hybridization with the purified radiolabelled RNA in a solution consisting of 5× SSC, 5× Denhardt's solution, 0.05% SDS, 0.5 mM EDTA and 100 µg of denatured fish sperm DNA for 48 h at 65 °C. Subsequently the filters were washed at room temperature for 10 min in 6× SSC/1% SDS, followed by two washes at 65 °C in 2× SSC/1% SDS and a final wash at 65 °C in 1× SSC/1% SDS. X-ray film was exposed for 12 h to 2 days at -70 °C.

Separation of nuclei and cytoplasmic fractions from *Leishmania* lysates

Exponential-phase *L. major* promastigotes were incubated for 16 h at 25 °C or 35 °C. Parasites were collected by centrifugation and washed twice with DPBS. They were resuspended in lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, 0.1 mM phenylmethanesulphonyl fluoride, 0.5% Triton X-100, 5 mM dithiothreitol) and incubated on ice for 10 min. Subsequently the lysates were centrifuged at 4 °C for 10 min at 4000 g. The cytoplasmic fraction remained in the supernatant and the nuclei in the pellet. The nuclei were washed once in lysis buffer. The integrity of the isolated nuclei was verified in nuclear run-on transcription assays.

Western-blot analysis

SDS/PAGE and Western Transfer were performed as described in [29], except that a poly(vinylidene difluoride) membrane (Pall Biosystems Support) was used. The membrane was treated with blocking buffer [0.25% iBlock (Tropix) and 0.1% Tween 20 in DPBS], with IgY in blocking buffer, and with anti-(chicken IgG)-alkaline phosphatase conjugate (Dianova; 1:3000 in blocking buffer). Blots were stained with Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate.

Protein quantification

The total protein of *Leishmania* lysates and the solutions of recombinant HSP70 (rHSP70) and HSP83 (rHSP83), respectively, were quantified with commercially available protein assays (Bio-Rad Protein Assay, Bio-Rad; Micro BCA Protein Assay Reagent Kit, Pierce) by following the manufacturer's instructions. *Leishmania* lysates were additionally quantified by the biuret method [30] and by measuring the A_{214} .

Immunoprecipitation

Leishmania cultures were transferred to methionine-free RPMI culture medium (25 °C). Then 1×10^7 cells were labelled with 50 µCi/ml [³⁵S]methionine for 2 h at either 25 °C or 37 °C. Cells were harvested by centrifugation and lysed in 50 µl of solubilizing buffer (50 mM Tris/HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-

Figure 1 Northern-blot analysis of HSP mRNA in *L. major*

Exponentially growing *L. major* promastigotes were incubated either for 30 min at the temperatures indicated above the lanes (a, b) or at 35 °C for various lengths of time as indicated (d). Poly(A)⁺ RNA (1 µg) from each culture was analysed by denaturing agarose electrophoresis and Northern-blot analysis. Filters were probed with digoxigenin-labelled HSP83 DNA (a) or HSP70 DNA (b, d). Digoxigenin-labelled α-tubulin DNA (α-T) was included in the hybridization reactions to provide an internal standard. Blots were developed with alkaline phosphatase-conjugated anti-digoxigenin antibodies and the chemoluminescence reagent CSPD. The chemoluminographs in (a) and (b) were quantified by densitometric scanning and the normalized relative signal strengths are depicted in (c).

40, and 2.5 µg/ml each of aprotinin, leupeptin, pepstatin and antipain). Lysates were centrifuged at 13000 g for 15 min at 4 °C, and soluble proteins were incubated with 2 µl (HSP83) or 6 µl (HSP70) of antiserum for 1 h at 4 °C. A 35 µl portion of Protein A-Sepharose slurry was preincubated with anti-chicken IgG and added. The slurry was further incubated at 4 °C for 2 h. The immunoabsorbent was centrifuged and washed three times in washing buffer A (10 mM Tris/HCl, pH 7.5, 150 mM NaCl, 0.2% Nonidet P-40, 2 mM EDTA), twice in washing buffer B (as above, except with 500 mM NaCl) and once in washing buffer C (10 mM Tris/HCl, pH 7.5). For analysis on SDS/PAGE, 25 µl of SDS sample buffer [29] was added and the samples were heated for 5 min at 95 °C.

Immunocytochemical analysis

Leishmania promastigotes were cultured at 25 °C or 35 °C, for 2.5 h, washed with three changes of DPBS, applied to glass slides and air-dried. After fixation in chilled (−20 °C) methanol for 20 s, the slides were washed twice in DPBS and incubated with

Figure 2 Nuclear run-on analysis

Nuclei from promastigotes of *L. major* and *L. donovani* were prepared from cultures grown at 25 °C or 15 min after a temperature up-shift to 37 °C. The nuclei were incubated in the presence of [³²P]UTP to allow elongation of nascent RNAs. The radiolabelled RNA was used to probe *L. major* gene samples as specified on the right, which had been immobilized by slot-blot technique. The signal intensities as seen in the autoradiograph indicate the transcription rates occurring on the respective genes before the isolation of the nuclei.

blocking buffer [0.2% iBlock (Tropix), 0.1% Triton X-100, 0.02% Tween 20 in DPBS] for 45 min at room temperature. Anti-HSP70 (1:200) and anti-HSP83 (1:300) antibodies diluted in blocking buffer were applied on to the slides and incubated at 35 °C for 1 h. After three washes in DPBS with 0.1% Triton X-100, the slides were incubated with anti-chicken IgG fluorescein conjugate (Dianova; 1:50 dilution in blocking buffer) for 1 h at 35 °C. Slides were washed with three changes of DPBS and mounted.

RESULTS

Transcription in *Leishmania* is not affected by a heat shock

Our first objective was to investigate whether HSP mRNA synthesis is induced at all during heat stress in *Leishmania*. A Northern-blot analysis of *L. major* polyA⁺ RNA showed a marginal increase in both HSP70 and HSP83 mRNAs when the parasites were incubated at elevated temperatures (Figures 1a and 1b). Densitometric scanning of the blots revealed a heat-induced increase of less than 50% for the HSP70 mRNA and a less than 20% increase for the HSP83 mRNA (Figure 1c). The levels of the *ClpB* mRNA, which encodes a 100 kDa HSP, also increase moderately during heat stress [24]. The increase in HSP70 mRNA is transient and disappears after 1 h of sustained (Figure 1d). Moreover, the observed increases in heat-shock HSP mRNAs in no way compare with the induction observed in higher eukaryotes. We therefore assessed the RNA synthesis rates on a number of *Leishmania* genes before and during a heat shock.

Figure 2 shows the results of a nuclear run-on transcription experiment. Intact nuclei from *L. major* and *L. donovani* were isolated from promastigotes incubated at 25 °C or from promastigotes 15 min after a temperature up-shift to 37 °C. The nuclei were incubated in the presence of [³²P]UTP to allow elongation of transcripts. The radiolabelled nascent RNAs were extracted from the incubation mixes and used to probe

Leishmania gene fragments immobilized on a nylon membrane. As suspected, there was no heat-induced increase of RNA synthesis on the *HSP70* and *HSP83* gene loci of either *Leishmania* species. However, transcription rates on the major heat-shock genes, *HSP70* and *HSP83*, compare with the transcription rates on the genes encoding actin and gp63, both abundant proteins in *Leishmania* promastigotes. *HSP70* and *HSP83* genes are therefore transcribed at a constitutively high level, but are obviously not induced during a heat shock.

We also observe a constitutive non-heat-inducible RNA synthesis for the single *ClpB* gene, which encodes a 100 kDa heat-inducible protein, HSP100 [24]. This protein is barely detectable in unstressed promastigotes of *Leishmania*, but increases steadily during a heat stress. Its synthesis must therefore be under tight post-transcriptional control.

We observed no decrease in transcription under heat stress either on the actin gene or on the gene encoding the major surface protein gp63. The general decrease in signal strength observed for the heat-shocked *L. major* nuclei (Figure 2, second row) is probably due to the fact that this species does not tolerate 37 °C very well. At any rate, the heat-shock response in *Leishmania* obviously does not involve a transcriptional activation of the heat-shock genes.

HSP70 and HSP83 are stable and highly abundant proteins in non-shocked *Leishmania* promastigotes

As reported previously [11,14], *Leishmania* promastigotes subjected to a temperature up-shift increase the synthesis of several

proteins in pulse-labelling experiments, namely a 70 kDa and a 90 kDa species (Figure 3a, lanes 1 and 2). This induction of synthesis is transient, however, and ceases after 4–6 h of sustained heat shock (lanes 5 and 6). However, when we analysed *Leishmania donovani* proteins by SDS/PAGE and silver staining, we did not observe the appearance or a significant increase of any protein bands in this size range (Figure 3b). We therefore raised antibodies against recombinantly expressed leishmanial HSP70 and HSP83 and analysed the steady-state levels of both proteins by immunoblot analysis. We obtained strong basal signals for both HSPs in samples from *L. donovani* promastigotes cultivated at 25 °C (Figure 3c, lane 1). These levels increase only marginally in the course of a 24 h incubation at 37 °C (lanes 2 and 3). To ascertain whether the heat-induced protein bands in the metabolic labelling experiments (Figure 3a) were indeed the products of the known *HSP70* and *HSP83* genes, respectively, we performed a combination of metabolic labelling and immunoprecipitation to confirm their identity (Figure 3d). The signal strengths were quantified by PhosphorImager analysis. In promastigotes of *L. donovani* kept at 25 °C we detect low relative synthesis rates for both HSP70 and HSP83 (lanes 1 and 3). Heat stress at 37 °C, however, results in a 6-fold and 8-fold increase, respectively, in the relative synthesis rates of HSP70 and HSP83 (lanes 2 and 4). The different signal intensities that we observe for HSP83 and HSP70 are probably due to the 1.5-fold higher methionine content of HSP83. We obtained comparable results (not shown) when we analysed *L. major* promastigotes.

We therefore had to reconcile two sets of seemingly contradictory results; although the metabolic labelling and the immunoprecipitation experiments indicate a marked induction

Figure 3 Analysis of HSP synthesis

(a) Metabolic labelling with [^{35}S]methionine. *L. donovani* promastigotes were incubated either at 25 °C (lanes 1 and 7) or for the indicated lengths of time at 37 °C (lanes 2–6). [^{35}S]Methionine was added and incubation was continued for 1 h. Cells were harvested by centrifugation, dissolved in SDS sample buffer and subjected to SDS/PAGE (7.5% gel) and Western transfer. The protein bands on the membrane were revealed by autoradiography. The arrows indicate the positions of two apparent heat-shock proteins of 90 kDa and 70 kDa. The positions and sizes of marker proteins (kDa) are indicated on the right. (b) SDS/PAGE and silver staining. *L. donovani* promastigotes grown at 25 °C were subjected to a 37 °C heat shock for the indicated lengths of time, harvested by centrifugation, subjected to SDS/PAGE (7.5% gel) and revealed by silver staining. (c) As in (b), but gel was subjected to Western transfer. The membrane was probed with anti-HSP83 antibodies and anti-HSP70 antibodies. The positions and sizes of marker proteins (kDa) are indicated. (d) Immunoprecipitation. *L. donovani* promastigotes were labelled with [^{35}S]methionine for 2 h at either 25 °C (lanes 1 and 3) or 37 °C (lanes 2 and 4). Cells were harvested and lysed, and the cellular proteins were immunoprecipitated either with anti-HSP70 antibodies (left lanes) or with anti-HSP83 antibodies (right lanes). Precipitates were subjected to SDS/PAGE (7.5% gel). To reveal bands and quantify signals, the dried gel was scanned on a Molecular Dynamics PhosphorImager.

Figure 4 Pulse-chase metabolic labelling

L. donovani promastigotes were incubated with [35 S]methionine for 1 h at either 25 °C (lane 1) or 37 °C (lane 2). The 37 °C culture was washed twice with fresh medium without [35 S]methionine and split: half of the culture was shifted back to 25 °C (lanes 3–5), the other half was further incubated at 37 °C (lanes 6–8). Samples were taken after 3 h (lanes 3 and 6), 6 h (lanes 4 and 7) and 24 h (lanes 5 and 8). The samples were subjected to SDS/PAGE (7.5% gels), Western transfer and autoradiography. The arrows indicate the bands which represent HSP83 and HSP70 respectively.

Figure 5 Calibrated Western-blot analysis

For each lane 2×10^5 *L. major* promastigotes grown at 25 °C were dissolved in SDS sample buffer and mixed with 0 (lane 5), 5 ng (lane 1), 10 ng (lane 2), 20 ng (lane 3) and 40 ng (lane 4) of recombinant (r) *L. major* HSP70 (a) or *L. major* HSP83 (b). The proteins were subjected to SDS/PAGE on a 7.5% gel. The recombinant proteins each carry 20-amino-acid N-terminal histidine tails and thus migrate more slowly in the gel. In lane 5 no recombinant protein was added. After Western transfer the membranes were probed either with anti-HSP70 antibodies (a) or anti-HSP83 antibodies (b). Arrows refer to recombinant HSP70 (rHSP70) and promastigote HSP70 (a) and to recombinant HSP83 (rHSP83) and promastigote HSP83 (b). We estimate 30 ng of HSP70 and 40 ng of HSP83 for 2×10^5 promastigotes.

of HSP synthesis, the silver staining and the Western blots showed little increase in the steady-state levels of either HSP83 or HSP70. There were two possible explanations to reconcile these data: (i) the increased HSP synthesis could coincide with an accelerated degradation of pre-existing HSPs, or (ii) the steady-

Figure 6 Coomassie Blue banding pattern versus immunoblot analysis

Total protein of *L. major* promastigotes (lanes 1 and 3) and of *L. donovani* promastigotes (lanes 2 and 4) was subjected to SDS/PAGE and Western blotting. The membrane was stained either with Coomassie Brilliant Blue (a) or with a mixture of anti-HSP70 and anti-HSP83 antibodies (b). The arrows indicate the bands representing HSP83 and HSP70 respectively. The positions and sizes of marker proteins (kDa) are indicated on the left.

state levels of HSPs at 25 °C could be so high as to make the increased synthesis insignificant compared with the overall pool of HSPs. The following pulse-chase labelling experiment argues against the first possibility. HSP synthesis was induced in *L. donovani* promastigotes at 37 °C for 1 h in the presence of [35 S]methionine. The labelled amino acid was removed and the promastigotes were cultivated at normal temperature (25 °C) or elevated temperature (37 °C) for up to 24 h. The parasites were collected, dissolved in SDS sample buffer, run on SDS/PAGE, and the bands were revealed by autoradiography. The result, shown in Figure 4, indicates that the HSP70 and HSP83 proteins which were newly synthesized during the initial 1 h heat shock are stable for more than 24 h both at 25 °C and at 37 °C, and thus have an unusually long half-life. Therefore an elevated turnover of HSPs during heat stress can be ruled out.

To test the second hypothesis, we calibrated the Western-blot analysis with purified recombinant serially diluted HSPs. Total *L. major* protein from 2×10^5 promastigotes was mixed with the diluted standards, subjected to SDS/PAGE and Western blotting and stained with anti-HSP70 (Figure 5a) and anti-HSP83 (Figure 5b) antibodies. The recombinant proteins migrate more slowly and can be resolved from their respective natural counterparts on SDS/PAGE. This is due to a 20-amino-acid N-terminal histidine tail, which is contributed by the bacterial expression vector pJC40 [23]. Lanes which showed identical staining intensity of recombinant and natural HSPs were selected. As a result of this calibrated Western-blot analysis (Figure 5), we calculate that

Figure 7 Indirect immunofluorescence

L. major promastigotes were grown at 25 °C or subjected to a 2.5 h heat shock at 35 °C, and stained with anti-HSP70 antibodies (upper panels) or anti-HSP83 antibodies (lower panels). The antibodies were revealed by indirect immunofluorescence. Micrographs were taken at 600-fold magnification under UV light.

HSP70 and HSP83 amount to 30 ng and 40 ng, respectively, in 2×10^5 promastigotes. We determined the total cellular protein as 700 ng in 10^5 promastigotes, which is in good agreement with previously published data [31]. This means that HSP70 and HSP83 represent 2.1% and 2.8%, respectively, of the total protein of non-shocked *L. major* promastigotes. Identical experiments performed with *L. donovani* promastigote protein yield identical numerical values. We calculate that there are 1.3×10^6 molecules of HSP70 and 1.5×10^6 molecules of HSP83 per *Leishmania* cell. The antibodies that we used were raised against denatured recombinant HSPs. If different affinities interfered with the quantification, we assume that the affinity of the antibodies will be higher for the recombinant proteins. Therefore our calibration allows a conservative estimate.

The view that HSP70 and HSP83 are both highly abundant proteins is further supported by our finding that both proteins form prominent bands in the electrophoretic polypeptide pattern of *Leishmania* promastigotes. Figure 6 shows lanes from the same Western-blot membrane stained with either Coomassie Blue or a mixture of anti-HSP70 and anti-HSP83 antibodies. The antibody-stained bands co-migrate with two prominent bands in the Coomassie Blue stain (arrows). Although, taken alone, the separation on a one-dimensional gel is no proof for the identity of protein bands, we consider that this result nicely illustrates and corroborates the data obtained in the quantitative Western-blot analysis shown in Figure 5.

In summary, we conclude that *Leishmaniae* increase the synthesis of both HSP70 and HSP83 significantly, but transiently, in response to a heat stress. This induction of HSP synthesis is regulated at a post-transcriptional level. However, both proteins are stable and form unusually large intracellular pools, which are not altered significantly by the relative increase in synthesis *de novo*.

Figure 8 Subcellular distribution of HSP70 and HSP83

(a) Equivalent amounts of whole cells (W), nuclei (N) or cytoplasm (C) from *L. major* promastigotes incubated at 25 °C (lanes 1–3) or 35 °C (lanes 4–6) were dissolved in SDS sample buffer and subjected to SDS/PAGE and immunoblot analysis with anti-HSP70 antibodies. (b) As in (a), but whole cell protein is not shown and the blot was probed with anti-HSP83 antibodies.

HSP70 does not enter the nucleus during a heat shock

An interesting feature of the 70 kDa chaperones of higher eukaryotes such as *Drosophila* and mammals is their rapid relocalization into the nucleus upon heat stress [32,33]. However, when we analysed the subcellular localization of leishmanial HSP70 by indirect immunofluorescence, we found the protein to be predominantly cytoplasmic. The nucleus apparently does not stain with the anti-HSP70 antibodies (Figure 7). This distribution did not change during a heat stress. As reported previously [19], HSP83 is also a cytoplasmic protein.

We could substantiate this finding by biochemical fractionation of non-shocked and heat-shocked *Leishmania* promastigotes (Figure 8a). All the HSP70 is recovered with the cytoplasmic fraction of the parasites, whereas the nuclei do not contain

detectable levels of this chaperone protein. As before, a heat stress did not alter this clear intracellular distribution. As expected, we obtained identical results for HSP83 (Figure 8b). We conclude that, in contrast with its homologues in higher eukaryotes, leishmanial HSP70 does not relocate into the nucleus during a heat stress.

DISCUSSION

We have performed an analysis of the heat-shock response in *Leishmania* and have discovered several features which set these protozoans apart from the well-investigated higher eukaryotes. Firstly, the parasites do not induce the transcription of their heat-shock genes during a heat stress: the synthesis of all stress proteins appears to be regulated post-transcriptionally. Secondly, the stress-induced synthesis of two major HSPs, HSP70 and HSP83, is not accompanied by a significant increase in the steady-state level of either protein. This is due to unusually high intracellular pools of both proteins in the non-stressed parasite.

All experiments shown were performed with promastigotes from exponential-phase cultures (procyclic promastigotes) which are non-infective. However, our findings also apply to the infective stage of the parasite, the metacyclic promastigote. Parasites of this stage isolated from stationary growth-phase cultures [39,40] showed neither increased pools nor induced synthesis rates of HSPs compared with the procyclic promastigotes (S. Brandau and S. Becker, unpublished work).

The heat-inducible and constitutive 70 kDa chaperones of mammals and insects relocate into the nucleus early during a stress and associate with nuclear structures [32,33]. Therefore, a third feature which sets the *Leishmaniae* apart from the other eukaryotes is that their heat-inducible 70 kDa chaperone, HSP70, has an exclusively cytoplasmic distribution. Another HSP70 family member in *L. major*, cp70.4, which is expressed constitutively shares this cytoplasmic localization [34]. Our results argue against previously published work [19] in which a portion of HSP70 was found with the nuclear fraction of heat-shocked *L. mexicana amazonensis* promastigotes. In that work the authors could only monitor the distribution of metabolically labelled 70 kDa proteins, which may have included other protein species than HSP70. On the other hand, we cannot exclude the possibility that the HSP70 proteins of New World and Old World *Leishmaniae* behave differently in this regard.

Based on their finding that transcription of the *L. mexicana amazonensis* HSP83 genes is not heat-inducible, Argaman et al. [20] recently proposed a post-transcriptional regulation of HSP synthesis. Using two different *Leishmania* species, *L. major* and *L. donovani*, and three *Leishmania* stress genes we can now generalize their finding and corroborate the hypothesis that the heat-shock response in the *Leishmaniae* is regulated on a post-transcriptional level. This is quite unusual, since the factors and cognate DNA sequences involved in the regulation of heat-shock gene transcription constitute one of the most highly conserved transcriptional control systems in eukaryotic organisms [7,35].

Earlier results with [³⁵S]methionine incorporation assays had indicated elevated synthesis rates for some HSPs during heat stress [11,14]. We could substantiate these findings with our immunoprecipitation experiments. These elevated relative synthesis rates, however, do not result in significantly increased intracellular levels, due to the large constitutive pool of HSPs. The high intracellular concentrations and the long half-life of the HSPs provide for a very slow heat-shock response in *Leishmania*. This may even be a general feature of kinetoplastid protozoa. A member of the same family, *Trypanosoma cruzi*, shows high

constitutive levels of a 60 kDa HSP which do not increase during a heat shock [36].

The maintenance of large intracellular pools of both HSP70 and HSP83 during the rapid proliferation of the parasite should necessitate high rates of constitutive synthesis. This is underscored by our finding that the transcription rates of the heat-shock RNAs compare with RNA synthesis rates on major housekeeping genes.

The slow stress response may also explain our failure to observe induced thermotolerance (for review see [37]) after application of short sub-lethal heat shocks (S. Brandau and J. Clos, unpublished work). We have, however, recently identified a novel leishmanial heat shock protein which belongs to the ClpB/HSP100 family [24]. This protein is barely detectable in non-shocked promastigotes of *Leishmania*, but is synthesized continuously under heat stress ([24]; S. Brandau and S. Becker, unpublished work). It remains to be seen whether this HSP100 affords any additional protection which may be required for survival in the human host.

It is an interesting problem how *Leishmaniae* can proliferate while burdened with large pools of stress proteins. *Drosophila* cells which overexpress HSP70 at normal temperatures cease growing, and when they resume growth, HSP70 is sequestered into granules [38]. When *Drosophila* embryos recover from heat stress, HSP70 is sequestered into granules shortly after the cessation of stress [37]. Immuno-electron microscopy may reveal whether HSP70 is sequestered in specific compartments of the unshocked *Leishmania promastigote*.

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REFERENCES

- Lindquist, S. (1986) Annu. Rev. Biochem. **55**, 1151–1191
- Nover, L. (1991) Heat Shock Response, CRC Press, Boca Raton, FL
- Zimarino, V. and Wu, C. (1987) Nature (London) **327**, 727–730
- Clos, J., Westwood, J. T., Becker, P. B., Wilson, S., Lambert, K. and Wu, C. (1990) Cell **63**, 1085–1097
- Rabindran, S. K., Giorgi, G., Clos, J. and Wu, C. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 6906–6910
- Rabindran, S. K., Haroun, R. I., Clos, J., Wisniewski, J. and Wu, C. (1993) Science **259**, 230–234
- Wu, C., Clos, J., Giorgi, G., Haroun, R. I., Kim, S.-J., Rabindran, S. K., Westwood, J. T., Wisniewski, J. and Yim, G. (1994) in The Biology of Heat Shock Proteins and Molecular Chaperones (Morimoto, R. I., Tissières, A. and Georgopoulos, C., eds.), pp. 395–416, Cold Spring Harbor Laboratory Press, Plainview, NY
- Tissières, A., Mitchell, H. K. and Tracy, V. M. (1974) J. Mol. Biol. **84**, 389–398
- Cheng, T. C. (1986) General Parasitology, pp. 121–127, Academic Press, Orlando
- Sacks, D. L. (1989) Exp. Parasitol. **69**, 100–103
- Hunter, K. W., Cook, C. L. and Hayunga, E. G. (1984) Biochem. Biophys. Res. Commun. **125**, 755–760
- Lee, M. G.-S., Atkinson, B. L., Giannini, S. H. and van der Ploeg, L. H. T. (1988) Nucleic Acids Res. **16**, 9567–9585
- Shapira, M. and Pedraza, G. (1990) Mol. Biochem. Parasitol. **42**, 247–256
- Lawrence, F. and Robert-Gero, M. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 4414–4417
- Glass, D. J., Polvere, R. I. and van der Ploeg, L. H. T. (1986) Mol. Cell. Biol. **6**, 4657–4666
- Newport, G., Culpepper, J. and Agabian, N. (1988) Parasitol. Today **4**, 306–312
- Polla, B. (1991) Immunol. Today **12**, A38–A41
- Miller, J. S. (1988) Mol. Biochem. Parasitol. **30**, 175–184
- Shapira, M. and Pinelli, E. (1989) Eur. J. Biochem. **185**, 231–236

- 20 Argaman, M., Aly, R. and Shapira, M. (1994) *Mol. Biochem. Parasitol.* **64**, 95–110
- 21 Studier, F. W. and Moffatt, B. A. (1986) *J. Mol. Biol.* **189**, 113–130
- 21a Button, L. L., Russell, D. G., Klein, H. L., Medina-Acosta, E., Kares, R. E. and McMaster, W. R. (1989) *Mol. Biochem. Parasitol.* **32**, 271–284
- 22 Searle, S., Campos, A. J. R., Coulson, M. R., Spithill, T. W. and Smith, D. F. (1989) *Nucleic Acids Res.* **17**, 5081–5095
- 22a Radi, A., Argaman, M. and Shapira, M. (1995) *Exp. Parasitol.* **80**, 159–162
- 23 Clos, J. and Brandau, S. (1994) *Protein Expression Purif.* **5**, 133–137
- 24 Hübel, A., Brandau, S., Dresel, A. and Clos, J. (1995) *Mol. Biochem. Parasitol.* **70**, 107–118
- 25 Polson, A., von Wechmar, B. and van Regenmortel, M. H. V. (1980) *Immunol. Commun.* **9**, 475–493
- 26 Polson, A., Coetzer, T., Kruger, J., von Maltzahn, E. and van der Merwe, K. J. (1985) *Immunol. Invest.* **14**, 323–327
- 27 Bhaumik, M., Das, S. and Adhya, S. (1991) *Parasitology* **103**, 197–205
- 28 Chomczynski, P. and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- 29 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning*, Cold Spring Harbor Laboratory Press, Plainview, NY
- 30 Gornall, A. G., Bardawill, C. J. and David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766
- 31 Bahr, V., Stierhof, Y.-D., Ilg, T., Demar, M., Quinten, M. and Overath, P. (1993) *Mol. Biochem. Parasitol.* **58**, 107–122
- 32 Velazquez, J. M. and Lindquist, S. (1984) *Cell* **36**, 655–662
- 33 Ohtsuka, K. and Laszlo, A. (1992) *Exp. Cell Res.* **202**, 507–518
- 34 Searle, S. and Smith, D. F. (1993) *Exp. Parasitol.* **77**, 43–52
- 35 Fernandes, M., O'Brien, T. and Lis, J. T. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissières, A. and Georgopoulos, C., eds.), pp. 375–393, Cold Spring Harbor Laboratory Press, Plainview, NY
- 36 Sullivan, M. A., Olson, C. L., Winquist, A. G. and Engman, D. M. (1994) *Mol. Biochem. Parasitol.* **68**, 197–208
- 37 Parsell, D. A. and Lindquist, S. (1994) in *The Biology of Heat Shock Proteins and Molecular Chaperones* (Morimoto, R. I., Tissières, A. and Georgopoulos, C., eds.), pp. 457–494, Cold Spring Harbor Laboratory Press, Plainview, NY
- 38 Feder, J. H., Rossi, J. M., Solomon, J. and Lindquist, S. (1992) *Genes Dev.* **6**, 1402–1413
- 39 Sacks, D. L., Hieny, S. and Sher, A. (1985) *J. Immunol.* **135**, 564–569
- 40 Howard, M. K., Sayers, G. and Miles, M. A. (1987) *Exp. Parasitol.* **64**, 147–156