Cell Stress & Chaperones (1999) 4 (3), 191–198

Article no. casac.1998.0149

A novel role for 100 kD heat shock proteins in the parasite *Leishmania donovani*

Sylvia Krobitsch,[1] and Joachim Clos[1,2]

[1]Leishmania Unit, Bernhard Nocht Institute for Tropical Medicine, 20359 Hamburg, and [2]Department of Biochemistry and Molecular Biology, Institute of Food Chemistry and Biochemistry, University of Hamburg, Hamburg, Germany

Abstract Heat shock proteins of the 100 kD family have been known to confer general stress tolerance in yeast and plants. Several protozoan parasites possess genes for Hsp100 proteins. In *Leishmania* species the protein is expressed under heat stress and during the mammalian stage, the amastigote. We show here that replacement of the *clpB* gene which encodes Hsp100 does not affect thermotolerance or general viability in *Leishmania donovani* insect stages (promastigotes) nor in axenically cultured mammalian stages (amastigotes). However, its expression is required for normal development of the parasite inside mammalian host cells. Hsp100 appears to function as an antagonist of amastigote-to-promastigote differentiation and a promoter of full amastigote development.

INTRODUCTION

Eukaryotic as well as prokaryotic cells rely on the synthesis of heat inducible chaperone proteins, the heat shock proteins, for general and inducible thermotolerance. The phenomenon of inducible thermotolerance can be observed in yeast, insect cells, and mammalian cells alike. The effector molecules responsible for inducible thermotolerance vary, however. In insect cells, e.g. *Drosophila*, Schneider line cells synthesis of Hsp70 during a mild heat shock imparts thermotolerance during a subsequent exposure to non-permissive temperature while heat-induced expression of Hsp70 and small Hsps confers added stress protection to mammalian cells (reviewed in: Parsell and Lindquist 1993; Parsell and Lindquist 1994). In yeast, inducible stress tolerance depends on the expression of a 104 kD Hsp which belongs to the ClpB family of stress proteins (Sanchez and Lindquist 1990; Sanchez et al. 1992; Parsell et al. 1991). Other members of the ClpB protein family also confer stress tolerance to plants and bacteria (Lee et al. 1994; Eriksson and Clarke 1996). The functions of Hsp100/ClpB proteins appear to lie in the resolubilization of heat-denatured, aggregated polypeptides (Schirmer et al. 1996; Laskowska et al. 1996). By this they aid in the repair of stress-damaged protein complexes such as splicing complexes (Vogel et al. 1995).

Many parasitic organisms have to adapt to drastically different environments during their often complex life cycles (Newport 1988). This adaptation is evident from the morphological and biochemical differentiation of parasites when they undergo stage conversion. Protozoan parasites of the genus *Leishmania* have a digenic life cycle: they proliferate as flagellated, elongated promastigotes in the gut of sandflies and as intracellular, aflagellated amastigotes inside mammalian macrophages. Transmission from the poikilothermic insect vector into a mammalian host includes an increase of ambient temperature by more than 10°C. This temperature upshift is a key trigger for the promastigote–to–amastigote differentiation (Zilberstein and Shapira 1994; Saar et al. 1998).

The proliferation of the amastigotes in the macrophages and the destruction of these host cells is causative for the pathology of *Leishmania* infections, e.g. limited, self-healing cutaneous lesion caused by *L. major* and the visceralizing, fatal Kala Azar caused by *L. donovani*. One evident difference between these two *Leishmania* species is their different thermotolerance behaviour. *L. major* can only tolerate temperatures up to 35°C in keeping with its tropism for mammalian skin;

Received 2 March 1999; Revised 29 April 1999; Accepted 29 April 1999
Available on-line 17 August 1999

Correspondence to: J. Clos, Bernhard Nocht Institute, Bernhard Nocht St. 74, 20359 Hamburg, Germany. Tel.: +49 40 428 184 81; Fax.: +49 40 428 184 00; E-mail clos@bni.uni-hamburg.de

L. donovani in contrast will proliferate at temperatures above 37°C which correlates with its viscerotropic pathology (Callahan et al. 1996).

Leishmania species express a 100 kD Hsp, Hsp100, under heat stress only (Hübel et al. 1995). Replacement of the corresponding *clpB* gene in *L. major* had a moderate effect on the thermotolerance of this species reducing viability at 35°C (Hübel et al. 1997). The gene replacement also greatly affected the virulence of this parasite in laboratory animals. We, therefore, investigated whether diverged structure or function of Hsp100 may be responsible for the different permissive temperature ranges of *L. major* and *L. donovani*. Under this assumption a lack of Hsp100 should lower the temperature tolerance of *L. donovani* to that of *L. major* or below. We, therefore, performed a *clpB* gene replacement in *L. donovani* and analyzed the phenotype of the resulting mutants.

MATERIALS AND METHODS

Parasite culture and strains

L. donovani strain MHOM/SD/??/Lo8 (=Lo8 strain), a gift from D. Zilberstein, was used throughout our analyses. The *L. donovani clpB* gene replacement strains TF13, *ΔclpB 13/1*, *ΔclpB 13/2*, and *ΔclpB 13/3* have been described before (Krobitsch et al. 1998). The construction of these strains by homologous recombination and their verification is detailed elsewhere (Krobitsch and Clos, manuscript submitted).

Promastigotes of *L. donovani* were cultivated in M199 medium supplemented with 25% fetal calf serum and 20 μg/ml gentamycin. For cultivation under acidic conditions the medium was buffered by addition of MES buffer (pH 5.0) to 40 mM and pH was adjusted to 5.5. Cell density was routinely determined using a CASY cell counter (Schärfe Systems).

For animal passage 4×10^8 Lo8 strain promastigotes were injected intraperitoneally into BALB/c mice. After 8 weeks of incubation the animals were sacrificed, parasites were grown as promastigotes from spleen tissue and stored frozen in liquid nitrogen.

For in vitro amastigote development frozen promastigotes fresh from a passage through mice were brought back into liquid culture and, within 2 weeks, subjected to the stage differentiation regimen: cells were first heat shocked for 24 h at 37°C, harvested by mild centrifugation and resuspended in fresh medium at pH 5.5. Cultivation was continued at 37°C for 5 days and stage development was monitored by microscopy and by SDS-PAGE and immunoblot using antibodies against the amastigote-specific A2 protein (Charest and Matlashewski 1994; Charest et al. 1996; Zhang and Matlashewski 1997).

For thermotolerance experiments cultures were synchronized for at least 1 week. Cells were seeded at 5×10^5 per ml and incubated at the respective temperature. Aliquots of the cultures were counted daily using a CASY cell counter (Schärfe Systems).

In vitro infections

Peritoneal macrophages from BALB/c mice were seeded into sterile chamber slides (NUNC) at $1–2 \times 10^6$ cells per ml in M199 medium supplemented with 25% fetal calf serum, 40 mM HEPES, pH 7.4, 10 μg/ml heme, 100 μM adenine, and 1,2 μg/ml 6-biopterin. After allowing 1 h at 37°C for adhesion of the cells an equal number of *L. donovani* culture forms was added. After 4 h at 37°C the supernatant was removed and the cells were washed once with fresh medium. After addition of fresh medium the infected macrophages were incubated for 1 or 2 days. Cells were then fixed with ice-cold methanol, stained with Giemsa and analyzed microscopically.

Electron microscopy

Samples were prepared and transmission electron micrographs were taken according to Reynolds' procedure (Reynolds 1963) on a Philipps CM-10 transmission electron microscope.

Imaging

Immunoblots were digitalized on a Umax S6E flatbed scanner. Microscopic images on 35 mm slides were scanned using a Nikon Coolscan LS-20E film scanner with Silver Fast software. Digital images were cropped and juxtaposed using Adobe Photoshop software, Version 5.0. Line drawings were generated and combined with halftone images using Claris Draw, Version 1.0dv3 and CricketGraph III.

RESULTS

Temperature tolerance of *L. donovani ΔclpB* mutants

We tested three *ΔclpB* strains of *L. donovani*, the heterozygous gene replacement mutant, and the wild type for survival at 25°C and at a temperature, 37°C, which represents the upper limit for proliferation in vitro. The outcome of this analysis is represented in Figure 1. At 25°C (Fig. 1A) no effect on general viability nor proliferative capacity can be observed with either parasite strain. Proliferation at 37°C is slowed considerably both for wild type and for the mutant *L. donovani* strains. However, no significant difference can be observed owing to the lack of Hsp100 expression in the mutants (Fig. 1B).

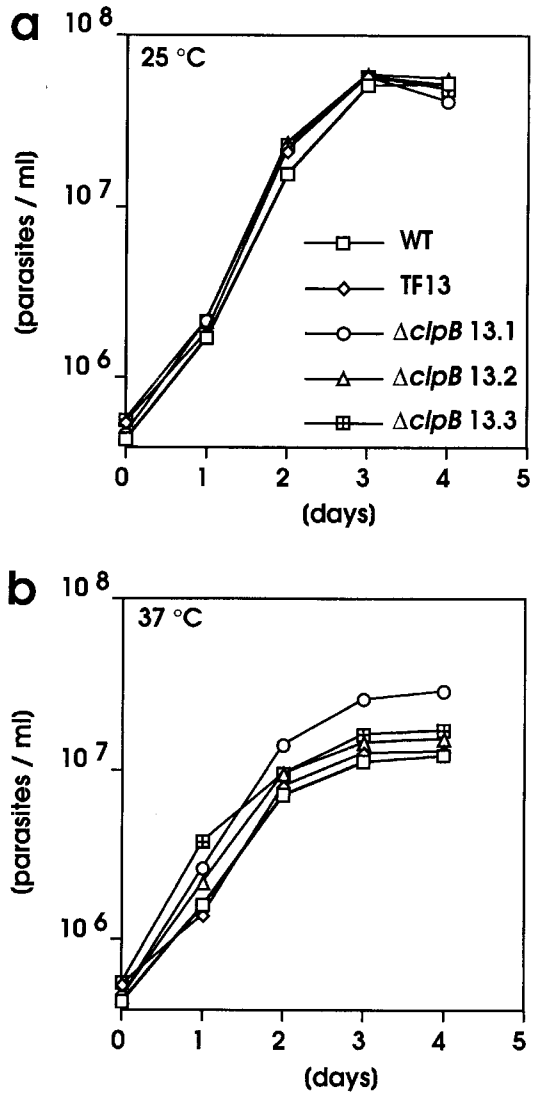

Fig. 1 Kinetic of culture density. *L. donovani* promastigotes of a wild type strain (Lo8), a heterozygous *clpB* gene replacement strain (TF 13), and of three double *clpB* gene replacement strains (*ΔclpB* 13.1, *ΔclpB* 13.2, and *ΔclpB* 13.3) were seeded in liquid culture at 1 × 10^6 cells/ml and incubated at 25°C (panel a) or 37°C (panel b). Cell density was monitored daily. The panels show representative results from a series of five independent experiments.

We also tested wild type and mutant parasites for inducible thermotolerance: promastigotes were exposed to a mild heat shock, 24 h at 33°C, known to induce Hsp100 synthesis in wild type promastigotes, and then challenged at 40°C. The pretreatment did not alter the survival at 40°C, nor could we observe any differences between the wild type and mutant promastigotes (data not shown). We conclude that Hsp100 does not confer general stress tolerance in *Leishmania donovani*, induced or uninduced. In addition, Hsp100 is not responsible for the different thermotolerance ranges of *L. major* and *L. donovani* since the *L. donovani* gene replacement strains retain a superior thermotolerance over the *L. major ΔclpB* mutants, which do not tolerate temperatures of 35°C and higher (Hübel et al. 1997).

Amastigote to promastigote differentiation

We had previously shown that *ΔclpB* mutants of *L. donovani* can form viable axenic amastigote stages in vitro induced by heat treatment in an acidic medium (Krobitsch et al. 1998). These axenic amastigote-like forms cannot be distinguished by scanning electron microscopic analysis from the axenic amastigotes derived from wild type promastigotes. However, the expression of at least one amastigote stage-specific protein family, the A2 gene products (Charest and Matlashewski 1994; Charest et al. 1996) is impaired in the mutant amastigotes. It therefore seemed as if the mutants were not fully committed to the amastigote stage.

To test this possibility, we analyzed the in vitro amastigote-to-promastigote stage differentiation of wild type vs mutant *L. donovani*. Fully differentiated axenic amastigote-like forms of wild type *L. donovani* and of two *ΔclpB* strains were collected and resuspended in standard medium at neutral pH and incubated at 25°C. While proliferation of axenic amastigotes is very limited (~2 days per generation), promastigote cultures of *L. donovani* under our cultivation conditions can double in 6 to 8 h (data not shown). Proliferation is, therefore, a good indicator for amastigote-to-promastigote differentiation.

Starting with the wild-type axenic amastigotes, an onset of proliferation cannot be observed before day 2 after the change of culture conditions (Fig. 2a). In contrast, proliferation of the mutants starts without noticeable delay and they reach a stationary growth phase more than 1 day ahead of the wild type. After the onset, rates of proliferation are indistinguishable between wild type and mutants. It therefore seems as if the mutants can switch to the promastigote stage faster.

We also distinguished between the two parameters which govern differentiation, temperature and pH of the medium. Fully differentiated amastigote-like forms of wild type and *ΔclpB* mutants were either shifted to 25°C and pH 7.0, to 37°C and pH 7.0, or to 25°C and pH 5.5. Outgrowth of promastigotes from these cultures was monitored after 3 days. Under all conditions outgrowth of *ΔclpB* mutants exceeded the outgrowth of wild-type promastigotes by more than one order of magnitude (Figure 2b). In addition, the experiment shows that elevated temperature (37°C) is the dominant parameter which stabilizes the amastigote stage. Under heat stress and neutral pH only one strain, *ΔclpB 13.2*, showed limited promastigote growth while the others remained as axenic amastigotes. At 25°C and pH 5.5, both *ΔclpB*

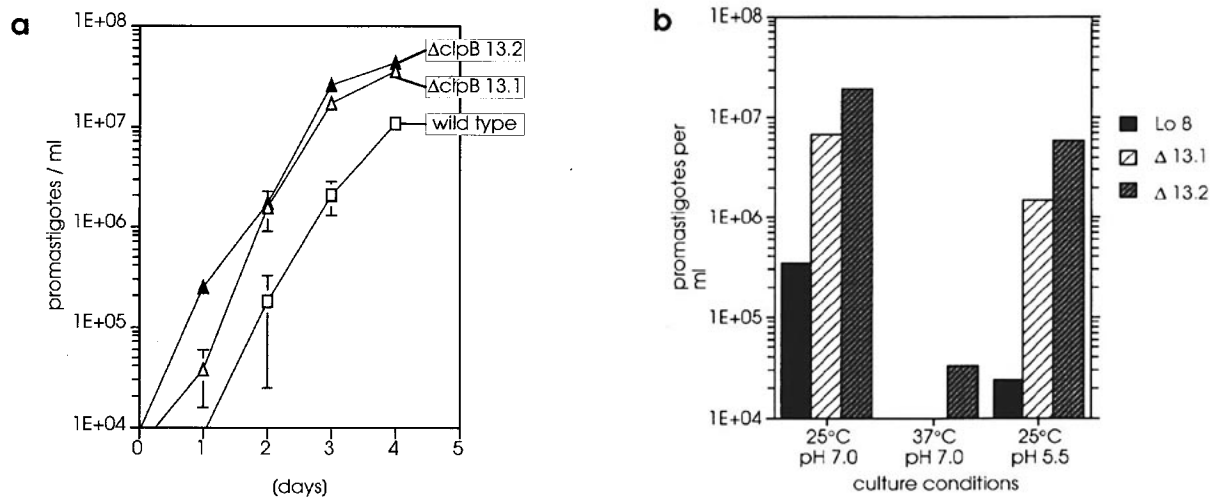

Fig. 2 (a) Kinetic of promastigote outgrowth from axenic amastigote cultures. Axenic amastigotes were derived from wild type *L. donovani* (Lo8) and from two *ΔclpB* gene replacement strains. On day 0 the axenic amastigotes were harvested by sedimentation and 5×10^7 cells per ml were seeded in neutral medium (pH 7.0) and incubated at 25°C to promote differentiation toward the promastigote stage. Promastigotes were counted daily using a hemocytometer. Results are given as means of four independent cultures. Standard deviation is indicated. Cell counts are at a logarithmic scale. No wild type promastigotes were observed at day 1. (b) Promastigote outgrowth under various culture conditions. Axenic amastigotes of the same strains as in (a) were seeded in growth media at pH 7.0 or 5.5 and incubated at either 25°C or 37°C. Promastigote cell counts were taken at day 3 after the seed and are represented as a bars at a logarithmic scale.

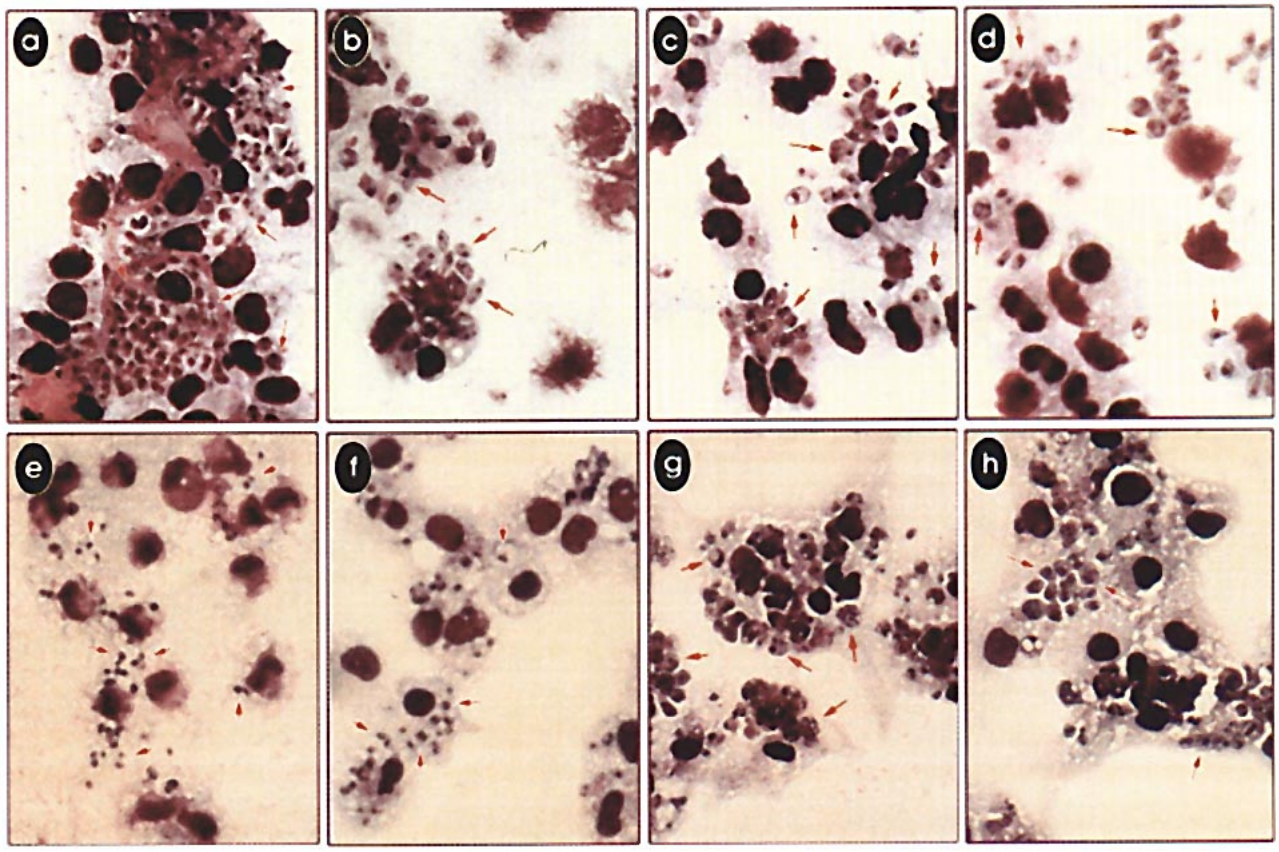

Fig. 3 In vitro infection of mouse peritoneal macrophages with *L. donovani* wild type and with *ΔclpB* gene replacement mutant strains 13/2 and 13/3. Panels a, e, f: wild type; panels b, c: strain 13/2; panels d, g, h: strain 13/3. Panels a-d: infection with stationary phase promastigotes; panels e-h: infection with axenic amastigotes. Infected macrophages were fixed 2 days after infection and stained with Giemsa. Arrows point at intracellular parasites.

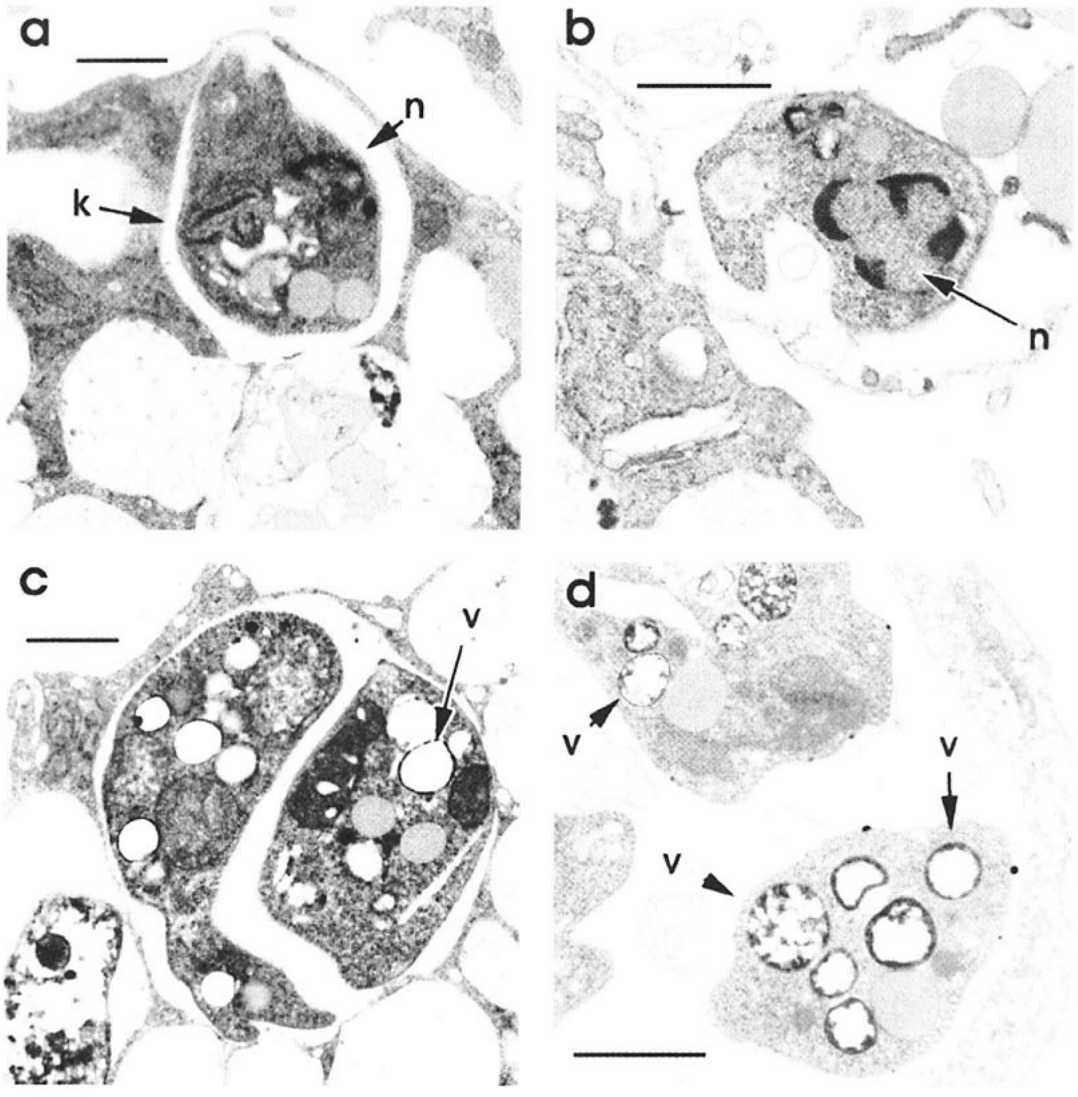

Fig. 4 In vitro infection of macrophages with axenic amastigotes of *L. donovani* wild type (panels a, b) and with mutant strain 13/3 (panels c, d). Two days post-infection macrophages were harvested, fixed and analyzed by transmission electron microscopy. The images show typical intracellular forms of *Leishmania parasites*. The nuclei (n), kinetoplasts (k), and the vacuoles (v) observed in the mutants are pointed out. The bars represent 1 μm.

mutants proliferate almost as rapidly as at neutral pH. Outgrowth of the wild-type promastigotes, in contrast, is delayed to an even greater extent at acidic pH compared with neutral pH. Since Hsp100 is a very stable protein in *Leishmania* with a half life of approximately 36 h (Brandau 1996); its levels do not drop immediately after a temperature shift to 25°C (data not shown). The different kinetics of promastigote differentiation in wild-type *L. donovani* and in *ΔclpB* strains imply therefore that Hsp100 is an antagonist of promastigote differentiation. The results also show that the negative effect of elevated temperature on promastigote development does not solely depend on Hsp100.

The morphology of *ΔclpB* mutants in infected macrophages

Axenic amastigotes resemble true amastigotes from infected tissue in virtually all morphological and biochemical features tested to date (Doyle et al. 1991; Joshi 1993; Gupta et al. 1996; Ellis et al. 1998; Saar et al. 1998). However, the environment in axenic culture is certainly different from the phagosome of macrophages. The viability of axenic amastigotes, therefore, cannot be a true measure for the viability of intracellular amastigotes. For *L. donovani* there is no convenient animal infection model available. The infection in mice does not resemble the human infection, and the infection in the

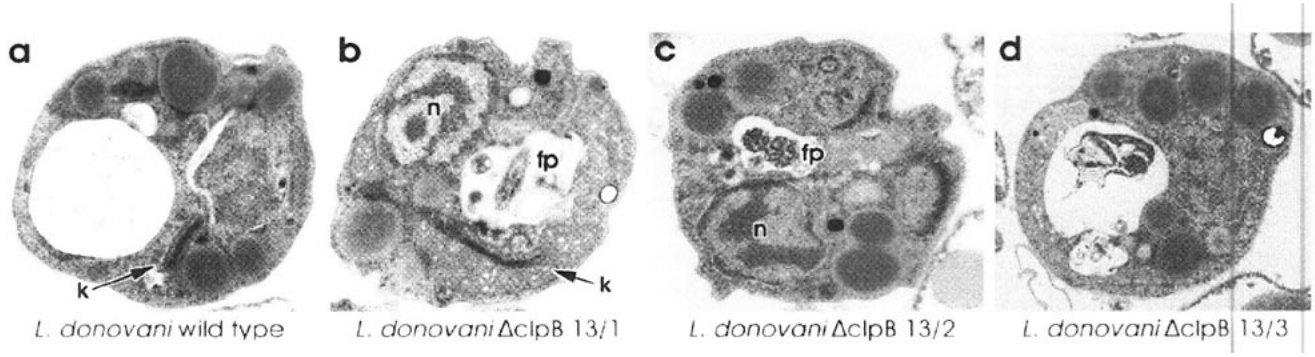

Fig. 5 Transmission electron microscopy of axenic amastigote stages of *L. donovani* wild type (a), and the *ΔclpB* strains 13/1 (b), 13/2 (c), and 13/3 (d). The nuclei (n), kinetoplasts (k), and flagellar pockets (fp) are pointed out where appropriate.

Syrian hamster takes months to develop. Moreover, visceral leishmaniasis does not show an overt clinical picture with which one could analyze the kinetic of an infection. For each data pair in a kinetic one animal would have to be sacrificed and individual differences in innate immunity could compromise the results.

However, in vitro infection systems with peritoneal macrophages can be used to analyze the proliferation of parasites in infected host cells for up to 2 days. Isolated peritoneal macrophages remain capable of phagocytosis for only a few hours after isolation, thus, excluding spread of a *Leishmania* infection in the culture.

We isolated macrophages from BALB/c mice and infected them with *L. donovani* promastigotes at a 1:1 cellular ratio. After 2 days adherent cells were fixed, stained with Giemsa and viewed by light microscopy. Infection with wild type *L. donovani* results in high numbers of typical, small Leishman bodies in the infected macrophages (Fig. 3a). Infection with mutant 13.2 (Fig. 3 b,c) or mutant 13.3 (Fig. 3d) also results in intracellular parasites. Their size, however, far exceeds the size of the wild-type amastigotes. Moreover, we observe that the mutant parasites develop vacuoles absent from wild-type amastigotes.

We also performed the experiment using axenic amastigotes of *L. donovani* wild type (Fig. 3 e,f) or *ΔclpB* 13.3 (Fig. 3 g,h) instead of promastigotes. While parasite counts are almost equal in macrophages infected with wild type and mutant parasites we again observe aberrant size and vacuolization with the mutants.

The size of subcellular structures in the Leishmaniae is at the limits of resolution for light microscopy. We therefore repeated the in vitro infection with axenic amastigotes of wild type and the *ΔclpB* 13.1 strain. Infected macrophages were fixed after 2 days and subjected to electron microscopic imaging (Fig. 4). Again, the mutant parasites (Fig. 4 c,d) displayed an aberrant morphology when compared with wild-type amastigotes (Fig. 4 a,b). In most instances we observed a loss of subcellular structures and, again, vacuolization.

The aberrant morphology of the mutants could have been missed in our earlier analyses of axenic amastigotes by scanning electron microscopy (Krobitsch et al. 1998). We therefore analyzed axenic amastigote stages of wild type *L. donovani* and of three *ΔclpB* mutants by transmission electron microscopy. Figure 5 shows the result: no abnormalities are observed with the mutant cells (b–d) compared with wild type (a).

We conclude that the lack of Hsp100 does not impair the viability or morphological appearance of axenically cultured *L. donovani* promastigotes or amastigotes, but has a significant impact on the integrity of intracellular stages. Together with its role in the expression of amastigote-specific proteins (Krobitsch et al. 1998) this implies a specific function for Hsp100 in the expression of proteins which are required solely for intracellular survival of *Leishmania* parasites in macrophages.

DISCUSSION

Leishmania Hsp 100 is not a mediator of cellular stress tolerance

Unlike the other members of the Hsp100/ClpB family of stress proteins, *Leishmania* Hsp100 has no major impact on thermotolerance. We observed a minor effect of *ΔclpB* gene replacement on the thermotolerance of *L. major* (Hübel et al. 1997), but the relatively moderate reduction of thermotolerance observed can hardly account for the severe reduction of virulence we observed in the *ΔclpB* strains. In *L. donovani* the replacement of the clpB gene did not affect thermotolerance at all. Therefore, Hsp100 cannot be responsible for the different thermotolerance profiles of *L. major* and *L. donovani*.

How can we explain the different results with *L. major* and *L. donovani*? *L. donovani* has a higher basal thermotolerance independent of Hsp100 expression. Thus, the temperature window in which Hsp100 confers tolerance to *L. major*, 33°–37°C, must be covered by other factors.

For example, *L. donovani* reportedly has 14 copies of the *hsp70* gene (MacFarlane et al. 1990) while only 4 were shown to exist in *L. major* (Lee et al. 1988). As shown in *Saccharomyces cerevisiae*, Hsp104 and Hsp70 may fill in for each other to a limited extent (Parsell and Lindquist 1994). This ability may be more pronounced in *L. donovani* due to the higher gene copy number although we do not observe any upregulation of Hsp70 concentration in the *ΔclpB* strains (not shown).

Hsp100 as an antagonist of promastigote development

Our experiments with the amastigote-to-promastigote development show that the balance between both stages in vitro is influenced by Hsp100. Its absence tilts the balance towards promastigote development, or rather, away from the amastigote stage. This is further indicated by the finding that Hsp100 synthesis is upregulated all through the amastigote stage where the protein can become even more abundant than Hsp70 (Hübel et al. 1997).

How can a putative chaperone participate in the stage differentiation? The underlying principle of Hsp100 proteins appears to be their ability to dissolve protein aggregates (Parsell et al. 1994; Schirmer et al. 1996). One may therefore hypothesize that Hsp100 helps to maintain or bring factors involved in the signalling pathways which govern stage development in(to) a soluble, functional state. Alternatively, Hsp100 may function in dissolving factor/inhibitor complexes involved in cell cycle control or stage differentiation. In particular, the clustering of Hsp100 alongside the cytoplasmic membrane may hint at interactions with cytoplasmic domains of membrane transporters or signal receptors. A direct effect on the components of the cytoskeleton could also be envisioned, although it does not appear to be likely: the structural integrity of axenically cultured amastigotes argues against defects in the cytoskeleton assembly.

We have also tried to detect proteins the solubility of which may be compromised in *ΔclpB* mutants. After ultracentrifugation of lysates from metabolically labeled wild type and mutant cells precipitated proteins were analyzed by SDS-PAGE and autoradiography. The results, though sometimes encouraging, have been too unreliable so far to warrant further characterization of precipitated protein species (J. Clos, unpublished results).

Hsp100 is crucial for intracellular stages of *L. donovani*

The most prominent phenotypical effects of *clpB* gene replacement are observed with parasites either in experimental host animals (Hübel et al 1997) or in isolated macrophages (this paper). In addition, the experiments with *L. major ΔclpB* mutants showed a selective pressure on the expression of Hsp100 during passage in susceptible mice (Hübel et al. 1997). It therefore appears as if the proteins whose expression is dependent on Hsp100 are required mostly in the intracellular environment of the macrophage, i.e. the phagolysosome.

The A2 family of amastigote-specific proteins is one example for Hsp100-dependent expression. We do not know as yet what function this protein family has. However, suppression of A2 protein expression by antisense RNA underscored the importance of this protein family for the virulence of *L. donovani* both in vivo and in vitro (Zhang and Matlashewski 1997). The *ΔclpB* mutants show a reduced expression of the A2 proteins as well (Krobitsch et al. 1998). The results, however, may not be compatible. A2 expression is reduced by only 80% in *ΔclpB* mutants compared to >94% in the antisense RNA expressing strains (Zhang and Matlashewski 1997). Moreover, the lack of A2 proteins in the antisense expression strains did not impair infectivity in an in vitro macrophage culture, but rather the intracellular proliferation of the parasites, which we did not monitor.

The development of axenic amastigote culture techniques for *L. donovani* may allow the identification of other stage-specific protein species. It will be interesting to observe whether such proteins are part of the same, Hsp100-dependent, regulon.

ACKNOWLEDGEMENTS

We thank C. Schmetz for expert technical assistance with electron microscopy and A. Macdonald for technical assistance. The work was supported by the Deutsche Forschungsgemeinschaft, Grant C1120/2–1.

REFERENCES

Brandau S (1996). Investigation of the synthesis and subcellular localization of heat shock proteins in leishmanias. In Thesis, Faculty of Biology (Hamburg: University of Hamburg).

Callahan HL, Portal IF, Bensinger SJ and Grogl M (1996) Leishmania spp: temperature sensitivity of promastigotes in vitro as a model for tropism in vivo. *Exp. Parasitol.* **84**, 400–9.

Charest H and Matlashewski G (1994) Developmental gene expression in *Leishmania donovani*: differential cloning and analysis of an amastigote-stage-specific gene. *Mol. Cell. Biol.* **14**, 2975–2984.

Charest H, Zhang W-W and Matlashewski G (1996) The developmental expression of *Leishmania donovani* A2 amastigote-specific genes is post-transcriptionally mediated and involves elements located in the 3′-untranslated region. *J. Biol. Chem.* **271**, 17081–17090.

Doyle PS, Engel JC, Pimenta PF, da Silva PP and Dwyer DM (1991) Leishmania donovani: long-term culture of axenic amastigotes at 37 degrees *C. Exp. Parasitol.* **73**, 326–34.

Ellis SL, Shakarian AM and Dwyer DM (1998) Leishmania: amastigotes synthesize conserved secretory acid phosphatases during human infection. *Exp. Parasitol.* **89**, 161–8.

Eriksson MJ and Clarke AK (1996) The heat shock protein ClpB mediates the development of thermotolerance in the cyanobacterium Synechococcus sp. strain PCC 7942. *J. Bacteriol.* **178**, 4839–46.

Gupta N, Goyal N, Kumar R, Agrawal AK, Seth PK and Rastogi AK (1996) Membrane characterization of amastigote-like forms of *Leishmania donovani. Trop. Med. Int. Health* **1**, 495–502.

Hübel A, Brandau S, Dresel A and Clos J (1995) A member of the ClpB family of stress proteins is expressed during heat shock in Leishmania spp. *Mol. Biochem. Parasitol.* **70**, 107–18.

Hübel A, Krobitsch S, Hoerauf A and Clos J (1997) Leishmania major Hsp100 is required chiefly in the mammalian stage of the parasite. *Mol. Cell. Biol.* **17**, 5987–95.

Joshi M, Dwyer DM and Nakhasi HL (1993) Cloning and characterization of differentially expressed genes from in vitro-grown 'amastigotes' of *Leishmania donovani. Mol. Biochem. Parasitol.* **58**, 345–354.

Krobitsch S, Brandau S, Hoyer C, Schmetz C, Hübel A and Clos J (1998) *Leishmania donovani* heat shock protein 100: characterization and function in amastigote stage differentiation. *J. Biol. Chem.* **273**, 6488–6494.

Laskowska E, Kuczynska-Wisnik D, Skorko-Glonek J and Taylor A (1996) Degradation by proteases Lon, Clp and HtrA, of Escherichia coli proteins aggregated in vivo by heat shock; HtrA protease action in vivo and in vitro. *Mol. Microbiol.* **22**, 555–71.

Lee MG, Atkinson BL, Giannini SH and Van der Ploeg LH (1988). Structure and expression of the hsp 70 gene family of Leishmania major [published erratum appears in Nucleic Acids Res 1988 Dec 9;16(23):11400–1]. *Nucleic. Acids. Res.* **16**, 9567–9585.

Lee YR, Nagao RT and Key JL (1994) A soybean 101-kD heat shock protein complements a yeast HSP104 deletion mutant in acquiring thermotolerance. *Plant. Cell.* **6**, 1889–1897.

MacFarlane J, Blaxter ML, Bishop RP, Miles MA and Kelly JM (1990) Identification and characterisation of a *Leishmania donovani* antigen belonging to the 70-kDa heat-shock protein family. *Eur. J. Biochem.* **190**, 377–384.

Newport G, Culpepper J and Agabian N (1988) Parasite heat-shock proteins. *Parasitology Today* **4**, 306–312.

Parsell DA, Kowal AS, Singer MA and Lindquist S (1994) Protein disaggregation mediated by heat-shock protein Hsp104. *Nature* **372**, 475–478.

Parsell DA and Lindquist S (1993) The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**, 437–96.

Parsell DA and Lindquist S (1994) Heat shock proteins and stress tolerance. In The biology of heat shock proteins and molecular chaperones, R.I. Morimoto, Tissières, A. and Georgopoulos, C., ed. (Plainview, New York: Cold Spring Harbor Laboratory Press), pp. 457–494.

Parsell DA, Sanchez Y, Stitzel JD and Lindquist S (1991) Hsp104 is a highly conserved protein with two essential nucleotide-biding sites. *Nature* **353**, 270–273.

Reynolds ES (1963) The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. Cell Biol.* **17**, 208–212.

Saar Y, Ransford A, Waldman E, Mazareb S, Amin-Spector S, Plumblee J, Turco SJ and Zilberstein D (1998) Characterization of developmentally-regulated activities in axenic amastigotes of Leishmania donovani. *Mol. Biochem. Parasitol.* **95**, 9–20.

Sanchez Y and Lindquist SL (1990) Hsp104 required for induced thermotolerance. *Science* **248**, 1112–1115.

Sanchez Y, Taulien J, Borkovich KA and Lindquist S (1992) Hsp104 is required for tolerance to many forms of stress. *EMBO. J.* **11**, 2357–2364.

Schirmer EC, Glover JR, Singer MA, Lindquist S (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. *Trends. Biochem. Sci.* **21**, 289–296.

Vogel JL, Parsell DA and Lindquist S (1995) Heat-shock proteins Hsp104 and Hsp70 reactivate mRNA splicing after heat inactivation. *Curr. Biol.* **5**, 306–317.

Zhang WW and Matlashewski G (1997) Loss of virulence in Leishmania donovani deficient in an amastigote-specific protein, A2. *Proc Natl Acad Sci* U S A **94**, 8807–8811.

Zilberstein D and Shapira M (1994) The role of pH and temperature in the development of Leishmania parasites. *Annu. Rev. Microbiol.* **48**, 449–470.