Microreview

A touch of Zen: post-translational regulation of the Leishmania stress response

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Summary

Across bacterial, archaeal and eukaryotic kingdoms, heat shock proteins (HSPs) are defined as a class of highly conserved chaperone proteins that are rapidly induced in response to temperature increase through dedicated heat shock transcription factors. While this transcriptional response governs cellular adaptation of fungal, plant and animal cells to thermic shock and other forms of stress, early-branching eukaryotes of the kinetoplastid order, including trypanosomatid parasites, lack classical mechanisms of transcriptional regulation and show largely constitutive expression of HSPs, thus raising important questions on the function of HSPs in the absence of stress and the regulation of their chaperone activity in response to environmental adversity. Understanding parasite-specific mechanisms of stress-response regulation is especially relevant for protozoan parasites of the genus Leishmania that are adapted for survival inside highly toxic phagolysosomes of host macrophages causing various immuno-pathologies of leishmaniasis (Fig. 1). Leishmania encounters dramatic environmental changes during the infectious cycle, some of which have been shown to induce parasite differentiation and stage-specific expression of virulence determinants appropriate for survival in both insect and vertebrate hosts (Zilberstein and Shapira, 1994). In particular, after transmission to the host during a sand fly blood meal and uptake by macrophages, parasites are exposed to various forms of stress, including temperature shift from 26 to 37°C (for visceral species), host cell cytotoxic activities such as reactive oxidants produced during the phagocytic oxidative burst, low pH as well as digestive enzymes following the maturation of the parasite-harbouring phagosome to a fully acidified phagolysosome through vesicular fusion (Sibley, 2011). Acidic pH and high temperature encountered inside the host cells trigger the developmental transition to the amastigote stage, that is adapted for intracellular survival and proliferation in the hypoxic, glucose-deprived and hydrolytic environment of the phagolysosome (Rosenzweig et al., 2008).

Thus, Leishmania has evolved mechanisms of environmental sensing that are able to distinguish between the physiology of vector and host during the infectious cycle, and trigger stage differentiation that adapts parasite biology to extracellular or intracellular growth. Significantly, the differentiation signals are largely linked to environmental stress, raising important questions on the regulation of trypanosomatid heat shock proteins (HSPs) and chaperones by environmental cues, and their dual
role in signal transduction and stage differentiation on one hand, and protein folding and stress resistance on the other hand.

**Leishmania stress protein families**

In all organisms, the response to heat shock is regulated by dedicated signalling pathways resulting in increased expression of HSPs. These proteins are highly conserved along the evolutionary tree and show a wide distribution throughout various intracellular compartments. HSPs play crucial roles in folding, assembly of newly synthesized proteins, and are implicated in controlling protein trafficking, activity and degradation. They are classified into five major families on the basis of their molecular weight [HSP100, HSP90, HSP70, HSP40 and small HSP (sHSP)], which are conserved in trypanosomatids (Requena, 2012) and have been linked by pharmacological and genetic analyses to parasite stage differentiation, viability and intracellular survival. In the following, we briefly summarize the major conserved *Leishmania* HSPs family members, which have been discussed in more detail in a recent review (Clos and Hombach, 2015).

*Leishmania major* HSP100 (LmjF29.1270) shows cytoplasmic localization and is one of the few parasite proteins that show a significant increase in stage-specific abundance during axenic amastigotes differentiation (Hubel et al., 1995). It is expressed in early *Leishmania donovani* axenic stage development suggesting that it could play a role in amastigote protein synthesis (Krobtsch et al., 1998). While *HSP100* null mutant parasites were viable at both the promastigote and axenic amastigote stages, they failed to establish a productive intracellular infection, thus revealing an important function of this HSP in parasite virulence (Hubel et al., 1997), likely due to its critical role in exosome-dependent immune modulation (Silverman et al., 2010).

The *Leishmania* genomes encode up to 18 *HSP90* gene copies [also referred in the literature as HSP83, LmjF33.0312 to LmjF33.0365, (Zilka et al., 2001)]. HSP90 is considered as a regulatory HSP given the fact that its main function is chaperoning client proteins implicated in cellular signal transduction and cell cycle control. This adenosine triphosphate (ATP)-dependent protein is the centre of chaperone complexes known as ‘HSP90 foldosomes’ that are formed through interaction with other chaperones and co-chaperones [including HSP70, HSP40 or stress-induced protein 1 (Sli-1)], and are implicated in client protein folding (Rohl et al., 2013). The HSP90 interaction with Sli-1 via a conserved tetratricopeptide repeat (TPR) domain, and the presence of stage-specific foldosome complexes in *Leishmania* have been confirmed by immuno-precipitation and functional genetics analysis (Morales et al., 2010; Hombach et al., 2014).

Only little is known about the *Leishmania HSP70* protein family. Sixteen *HSP70* genes were reported in *Leishmania*. Aside from LmjF01.0640, all other HSP70 members were classified according to their sequence identity to known HSP70s from other species into cytoplasmic (LmjF28.2770, LmjF28.2780, LmjF28.2820, LmjF26.1240, LmjF18.1370), mitochondrial (LmjF30.2460, LmjF.30.2470, LmjF.30.2480, LmjF.30.2490, LmjF.30.2550), endoplasmic reticulum (ER; LmjF28.1200, LmjF.35.4710), and nuclear (LmjF.26.0900) HSP70 (Louw et al., 2010). Basic Local Alignment Search Tool (BLAST) searches using LmjF28.2770 as a query allowed us to identify two additional putative HSP70 proteins in the *L. major* genome database belonging to the Nucleotide Binding Domain (NBD) sugar kinase HSP70 actin superfamily (LmjF32.0190, LmjF.29.1240). In addition, LmjF.29.1240 contains a TPR domain suggesting its possible role as a co-chaperone (S. Drini et al., in preparation). HSP70 proteins are highly abundant in *Leishmania* promastigotes (Brandau et al., 1995), and interact with several co-chaperones, including Sli-1 and SGT (small glutamine-rich TPP; Ommen et al., 2010; Hombach et al., 2013), and presumably a large number of HSP40 family members (Folgueira and Requena, 2007).

Limited information is available for the *L. major* HSP40 and sHSP protein families. The HSP40 family comprises 66 different members identified by BLAST searches using published sequences of *Trypanosoma cruzi* HSP40s (Folgueira and Requena, 2007). These proteins regulate...
HSP70 ATP hydrolysis and are responsible for substrate specificity (Requena, 2012). Members of the shSP chaperone family, which are characterized by a molecular weight ranging from 12 to 43 kDa, are poorly conserved among eukaryotes, which are characterized by a molecular specificity (Requena, 2012). Members of the sHSP chapter are ATP hydrolysis and are responsible for substrate specificity (Requena, 2012). Members of the shSP chaperone family, which are characterized by a molecular weight ranging from 12 to 43 kDa, are poorly conserved in all organisms. In the L. major genome database, it has been reported that by sequence homology to Saccharomyces cerevisiae, only one gene has been found corresponding to HSP20 (LmJF29.2450). Recently, an additional small HSP, HSP23, was characterized and shown to be critical for L. donovani survival at elevated temperatures, including intracellular persistence in macrophages (Hombach et al., 2014). More studies are needed for this class of protein in order to further elucidate its regulatory role in stress response (Folgueira and Requena, 2007).

### Post-transcriptional regulation of the Leishmania stress response

Across all major eukaryotes, the cellular response to stress is regulated on transcriptional levels through trans-acting nuclear factors termed heat shock factors (HSFs) that act as master regulators of stress-induced gene expression through binding to defined cis-acting heat shock element inside promoters or enhancers of HSP genes (Bjork and Sistonen, 2010) (Fig. 2). However, this canonical regulatory mechanism of the stress response acting on transcriptional levels does not apply to Leishmania and related trypanosomatids. First, in contrast to most eukaryotes, there are no classical transcription factors encoded in the Leishmania genome and gene expression occurs by highly parasite-specific mechanisms involving poly-cistronic transcription (Das et al., 2008; Daniels et al., 2010) and generation of mature mRNAs through a trans-splicing reaction that is linked to polyadenylation (Michaeli, 2011). Second, as a result of this unique expression strategy, the entire genome is largely constitutively expressed and HSP gene transcription is not enhanced upon exposure to stress in contrast to other eukaryotes (Brandau et al., 1995; Quijada et al., 1997), a phenomenon that is illustrated by recent transcriptome analyses of L. donovani exposed to different forms of stress (Saxena et al., 2007; Lahav et al., 2011; Fig. S1A).

However, the steady-state level of various HSP transcripts is regulated by post-transcriptional mechanisms, including RNA stability and translational control. For example, the 3′UTR of Leishmania and Trypanosoma brucei HSP70 confers increased transcript stability under stress conditions (Lee, 1998; Quijada et al., 2000), as does the 3′UTR of Leishmania HSP90 that is also required for preferential translation during stress (Zilka et al., 2001; David et al., 2010). This regulation relies on the interaction of trans-acting RNA-binding proteins that recognize cis-acting elements [reviewed in Kramer and Carrington (2011)]. In T. brucei for example, the zinc finger protein ZC3H11 has been identified as a master regulator of trypanosome chaperone mRNA stability through its binding to a common AUU nucleotide consensuss repeat motif inside the 3′UTR of HSP70 and other stress protein encoding genes (Droll et al., 2013). However, post-transcriptional regulation may have only little or no effect on stress protein abundance. In fact, no change of the steady-state level of the major HSPs was observed in L. donovani in response to environmental stress, in contrast to most other proteins that are less efficiently translated (Brandau et al., 1995; Rosenzweig et al., 2008; Morales et al., 2010; and Fig. S1B).

![Fig. 2. Model of stress-response regulation in Leishmania at post-translational level by phosphorylation. Most eukaryotes regulate stress protein abundance on transcriptional levels through dedicated trans-acting HSFs that are inactivated in the absence of stress by binding to HSP70 and HSP90, but activated under stress conditions thus enhancing stress protein production by binding to cis-acting heat shock elements. In Leishmania, the constitutive high expression of HSPs, the absence of trans-acting factors (TFs), and the coordinated phosphorylation of HSPs in response to stress at parasite-specific residues support a model of a reciprocal regulatory mechanism where the activity of stress kinases (SKs) is regulated by interaction with HSPs, and vice versa, HSP activities are regulated by phosphorylation through SKs.](image-url)
Post-translational regulation of trypanosomatid stress proteins

Analysis of L. donovani promastigote and axenic amastigote-enriched phosphoprotein fractions by two-dimensional (2D) electrophoresis identified a surprising twofold increase in global phosphoprotein abundance in number of HSPs and chaperones and revealed a dimensional (2D) electrophoresis identified a surprising amastigote-enriched phosphoprotein fractions by two-dimensional difference gel electrophoresis (2D-DiGE) analysis using the same extracts showed statistically significant differences in abundance for 38% of the detected phosphoproteins (corresponding to 318 out of 831 analysed spots) with a highly significant enrichment of chaperone function in the axenic amastigote phosphoproteome (Morales et al., 2010). These results were confirmed by recent systems-level studies investigating the L. donovani stage-specific phosphoproteome by shotgun phosphopeptide analysis and isobaric tags for relative and absolute quantification followed by Liquid chromatography-tandem mass spectrometry (iTRAQ LC-MS/MS) (Tsigankov et al., 2013; 2014; Fig. S1C). Cluster analysis of phosphorylation site conservation between Leishmania and human revealed a series of phosphorylation sites that are unique to certain Leishmania chaperones and HSPs despite their high conservation to respective human orthologs, including S13 in the TPR protein LinJ.27.2340, S14 in the putative DNAJ HSP LinJ.27.2350, or S433 and S604 in the HSP70 family member LinJ.28.2950 (Hem et al., 2010) at the time of this study only the Leishmania infantum genome database was available for interrogation with L. donovani proteomics data.

The coordinated, stress-induced phosphorylation of major Leishmania HSPs and chaperones at parasite-specific sites suggests a model of parasite stress protein regulation at post-translational levels and attributes potential important regulatory function to these unique residues. This possibility has been genetically investigated for the TPR-domain proteins Sti-1 and CyP40. L. donovani cyp40−/− null mutants were viable in culture, but failed to establish intracellular infection suggesting important virulence function of this co-chaperone (Yau et al., 2014). Null mutant analysis of Sti-1 demonstrated the essential nature of this co-chaperone for L. donovani promastigote and axenic amastigote viability, which was strictly dependent on the presence of two phosphorylation sites at S15 and S481 as demonstrated by 'plasmid shuffle' analysis (Morales et al., 2010). Sti-1 phosphorylation was correlated to the formation of amastigote-specific foldosome complexes containing ribosomal client proteins (Morales et al., 2010), suggesting that this post-translational modification may alter chaperone interactions or regulate the dynamic turnover of foldosome complexes. In contrast, despite the dramatic increase in phosphorylation of CyP40 in axenic amastigotes on a single serine residue at S274, cyp40−/− parasites complemented with an S/A phosphorylation site mutated form of CyP40 fully restored WT phenotype, thus ruling out a role of CyP40 phosphorylation in intracellular parasite survival in vitro. These examples illustrate that the relevance of stage-specific phosphorylation for chaperone function needs to be experimentally investigated for each single identified phosphorylation site.

Together, these reports point to a novel post-translational mechanism of stress-response regulation in Leishmania. By analogy to stress regulation through HSFs in other eukaryotes, the absence of these factors in trypanosomatids may have been compensated for by the evolution of protein kinases that regulate chaperone function. Similar to the tethering of HSFs by HSP70 and HSP90 in higher eukaryotes, Leishmania chaperones may regulate the activity of protein kinases that are released and activated during environmentally induced stage differentiation. In turn, these kinases may enhance chaperone activities and their affinity for unfolded proteins by HSP phosphorylation that sustains stress-induced amastigote differentiation (Fig. 2). This model of post-translational regulation of parasite differentiation is further supported by recent observations linking protein phosphatase to stage differentiation in Leishmania and T. brucei (Szoor et al., 2010; Naderer et al., 2011).

Putative Leishmania stress kinome

The post-translational model of Leishmania stress protein regulation raises the question on the nature of the corresponding protein kinases that recognize parasite HSPs and chaperones as substrates. In higher eukaryotes, exposure of cells to stress leads to a major change in gene expression, with an increase in the transcription of genes implicated in stress response such as those encoding HSPs. This change is mainly due to activation of transcription factors by phosphorylation through stress-activated protein kinases (SAPKs), key stress-signalling molecules and members of the mitogen-activated protein kinase (MAPK) family that sense and respond to extracellular environmental changes and are characterized by TGY, TGP or TQY amino acid motifs inside their activation loop (Duch et al., 2012). Only two of the 15 Leishmania MAPKs homologues, MPK4 and MPK12 are revealed as putative SAPK-like kinase by their TQY motif (Wiese, 2007). There is no published experimental data confirming that MPK12 is a SAPK-like kinase, except for its relatively high sequence identity to p38 (44%; Wiese, 2007). In contrast, MPK4 has been first linked to stress sensing using a transgenic approach that revealed increased phosphorylation and activity of this kinase.
during axenic amastigote differentiation (Morales et al., 2007). Subsequent null mutant analysis established essential functions in parasite viability, which was restored in mpk4−/− parasites expressing a kinase mutant with attenuated activity (Dacher et al., 2014). However, these mutants showed defects in pH-induced differentiation of procyclic to metacyclic parasites, which caused attenuation of intracellular survival, thus linking MPK4 to parasite pH sensing and/or tolerance.

Two other Leishmania MAPks, MPK10 and MPK7, have been linked to stress-regulated parasite differentiation and growth. Transgenic expression of MPK10 in L. donovani revealed a transient increase in kinase activity during the first 48 h of axenic amastigote differentiation, which is regulated by an auto-inhibitory domain that is essential for axenic amastigote viability (Cayla et al., 2014). Transgenic expression of MPK7, on the other hand, presents a defect in intracellular and axenic amastigote growth likely due to an enhanced endogenous stress response as judged by over-phosphorylation of the translation initiation factor eIF2α and substantial reduction in de novo protein biosynthesis (Morales et al., 2010; Lahav et al., 2011). eIF2α is the substrate of a stress-responsive kinase called PERK [protein kinase RNA (PKR)-like ER kinase (Cloutier et al., 2012)]. In Leishmania infantum, PERK phosphorylates eIF2α in response to ER stress (Chow et al., 2011; Cloutier et al., 2012). Impairing PERK leads to a defect in intracellular amastigote differentiation (Chow et al., 2011), which provides a further link between Leishmania stress signalling and parasite differentiation.

Finally, one other protein kinase involved in stress response in mammals and conserved in Leishmania was experimentally investigated in these parasites. Target of rapamycin (TOR) kinases are master regulators that adapt protein synthesis and cell cycle to environmental conditions (Laplanle and Sabatini, 2009). In L. major, TOR3 is essential for intracellular survival likely due to its implication in the parasite response to hypo-osmotic stress and glucose starvation (Madeira da Silva and Beverley, 2010).

Despite the publication of the TriTryp kinome (Naula et al., 2005), the identification of Leishmania stress kinases by bio-informatics prediction is rendered difficult due to the evolution of parasite-specific aspects in kinase regulation and functions. Thus discovery of novel stress kinases relies on functional approaches. One possible approach is represented by mining existing phosphoproteomics data. Tsingakov et al. recently identified protein kinases that are phosphorylated early during L. donovani axenic amastigote differentiation, and thus may be directly involved in parasite stress sensing and signalling (Tsigankov et al., 2014), including a MAPK (LinJ.05.0390), an ortholog of mammalian AKT1 (LinJ.25.2450) (Konishi et al., 1997; Sato et al., 2000; Koren et al., 2010), a Ca2+/calmodulin dependent protein kinase (LinJ.35.1070), and a CK1 (LmjF35.1010); the latter having been shown to be essential for intracellular survival (Rachidi et al., 2014), and thus being a prime candidate for future functional analysis.

Conclusion

Leishmania amastigote differentiation is triggered by stress signals encountered inside the fully acidified phagolysosomes of host macrophages, including low pH and high temperature. The emphasis of Leishmania stress protein regulation on post-translational mechanisms, such as phosphorylation, likely represents an evolutionary adaptation of trypanosomatid parasites to absence of transcriptional control, as well as to its intracellular lifestyle that depends on stress-induced, adaptive differentiation. This possibility is supported by the observations that inhibition of HSP90 ATPase activity by geldanamycin or inhibition of cyclophilin co-chaperones by cyclosporine A causes spontaneous conversion of promastigotes into amastigote-like forms in the absence of pH and temperature shock (Wiesigl and Clos, 2001; Yau et al., 2010). In contrast, inhibition of stress protein function in axenic amastigotes is lethal, demonstrating a dual role of Leishmania stress proteins in cellular signalling required to maintain the promastigote differentiation state, and in stress resistance required for amastigote survival (Yau et al., 2010). In conclusion, Leishmania not only evolved parasite-specific mechanisms of HSP gene regulation, but also parasite-specific HSP functions linking the activity of stress proteins to stage differentiation and stage-specific survival (Krobitsch and Clos, 1999; Hombach et al., 2014).

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**Supporting information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Fig. S1.** Abundance of stress protein transcripts (A), and stress proteins (B), and phospho-peptides (C) during axenic amastigote differentiation (data from Lahav et al., 2011; Tsigankov et al., 2014).