Chapter 2

Review of Literature
2.A. Introduction

2.A.1. Classification of Leishmania

Leishmania spp. are classified as a large complex of vector borne Kinetoplastids within the Trypanosomatidae family. Kinetoplastids are a wide-spread group of flagellate protozoa distinguished by the presence of extra-nuclear DNA containing granules, located in the mitochondrion in close proximity to the flagellar pocket. These were called kinetoplasts (Fernandes et al., 1999; McCann et al., 1999) based on the erroneous assumption that they were involved in cell movement. The DNA in these structures is organised into a concatenated mass of maxi and mini-circles, carrying genes for editing RNA and homologues of mitochondrial proteins (Sturm and Simpson, 1990; Maslov et al., 1998; Shlomai, 2004). Traditionally, Kinetoplastids are divided into the biflagellate Bodonidae and uniflagellate Trypanosomatidae.

Members of the Bodonidae are characterised by a well defined cytostome. Bodo, a typical genus of this family, has several free-living species that feed on bacteria in aquatic, marine or terrestrial environments (Diamant, 1990; Poynton, 2001). Members of the Trypanosomatidae, in contrast, have reduced or absent cytostomes, with feeding being entirely by absorption. They are parasitic on a wide variety of animal, plant and insect hosts. Typical life cycles of these members are complex involving more than one host, and multiple morphological transitions. Distinguishing cellular features include a single emergent flagellum that may in some cases form an undulating membrane; a single discoid and highly branched mitochondrion; a glycosome (Michels, 2006); and a cytoskeleton composed of cortical microtubules. Three genera cause human diseases: Trypanosoma brucei complex (African sleeping sickness), Trypanosoma cruzi (Chagas' disease), and Leishmania spp. (Leishmaniasis). All of these are parasites of the blood and/or tissues of the human host and are transmitted by arthropod vectors.
2.A.2. Impact of Leishmania Parasites as Pathogens

Leishmania infections are major health problems in large parts of the world (Figure 2.1). According to the WHO (1990), more than 12 million humans are infected with one of the pathogenic Leishmania species, over 2 million new infections occur each year, and some 400 million people are at risk (Desjeux, 2004). Mortality rates can vary, though in untreated cases of visceral Leishmaniasis, fatality is above 95%.

In spite of the existence of a variety of control strategies, case numbers of these infections have not diminished significantly in recent years, and are even increasing in certain countries. The problem is compounded by the emergence of treatment resistant Leishmania strains, and more importantly, the impact of Leishmania as an opportunistic pathogen in AIDS patients. In addition, first line therapeutics – the pentavalent antimonials - are toxic, and due to repeated courses of treatment in therapy-resistant cases, are themselves responsible for a case fatality of 4% in certain endemic countries (Clos and Krobitsch, 1999).

2.A.3. Clinical Manifestations of Leishmaniasis

Leishmania have a wide range of clinical manifestations that depend upon the nature of the infecting parasite species, host responses, and poorly understood host-parasite-vector interactions.

(a) Cutaneous Leishmaniasis (CL) – The most common form of the disease, it is characterized by generally benign, painless, non-pruritic and self healing lesions that may take between a few weeks to many months to cure (Figure 2. A). Primary lesions, at the site of the sand-fly vector bite, may be of papular or nodular nature. In some cases, satellite lesions occur in the vicinity of the original lesion (Modabber, 2007). Often, secondary infections by bacteria are associated with these lesions.

(b) Diffuse Cutaneous Leishmaniasis (DCL) and Leishmaniasis Recidivans – These are rare manifestations of Cutaneous Leishmaniasis. DCL is characterized by disseminated nodular lesions that tend to be scaly and non-ulcerated, and resemble lepromatous leprosy. Recivida is associated with a chronic recurrence of nodular lesions or a rash characterized by hypersensitivity. Cure of both of these is difficult.
Figure 2.1. *Leishmania* infections are major health problems in large parts of the world. According to the WHO (1990), more than 12 million humans are infected with one of the pathogenic *Leishmania* species, over 2 million new infections occur each year, and some 400 million people are at risk (http://www.who.int/csr/resources/figure23.gif).
Figure 2.2. Typical presentation of Cutaneous (A), Muco-cutaneous (B) and Visceral (C) Leishmaniasis. Depending upon the nature of the infecting species, host responses and certain poorly understood host-parasite-vector interactions, *Leishmania* spp. have a wide range of clinical manifestations. Cutaneous leishmaniasis, the most common form of the disease, is characterized by self-healing skin lesions. In muco-cutaneous leishmaniasis, simple skin lesions metastasize to the mucosae of the nose and mouth. Visceral leishmaniasis is a more generalized infection of the reticuloendothelial system involving the spleen, liver, bone marrow and lymph nodes (http://www.wehi.edu.au/facweb/faculty/emanuela_handman/images/visceralMucoLesion).
(c) Mucocutaneous Leishmaniasis (MCL) – This occurs in a small proportion (< 5%) of patients with simple cutaneous Leishmaniasis. It is primarily caused by members of the *L. braziliensis* complex. The disease begins as simple skin lesions that metastasize through the blood-stream or lymphatic, particularly to the mucosae of the nose and mouth (Figure 2.2. B). Expression of this form of the disease can occur several years after the primary lesion. Unless treated properly, it can lead to severe pathology and deformity (Modabber, 2007).

(d) Visceral Leishmaniasis (VL) – This is the most serious form of the disease and can be fatal if untreated. It is characterized by a generalized infection of the reticuloendothelial system (RES) involving the spleen, liver, bone marrow and lymph nodes. The disease is associated with fever, spleno- and hepatomegaly, and enlarged lymph nodes and tonsils (Figure 2.2. C). In chronic stages, patients often exhibit a wasting syndrome in spite of good appetite. Other possible manifestations include edema, anemia, leucopenia, monocytes, lymphocytes, and thrombocytopenia. Inadequate treatment can lead to the cutaneous disease known as post kala-azar Dermal Leishmaniasis, which is characterized by nodular lesions similar to that in DCL. However, in contrast to DCL, this post kala-azar is easily curable.

### 2.A.4. Infecting *Leishmania* Species and their Geographical Distribution

Infections in humans are caused by about 21 of the 30 *Leishmania* species that infect mammals. These include the *L. donovani* complex with 3 species (*L. donovani, L. infantum*, and *L. chagasi*); the *L. mexicana* complex with 3 main species (*L. mexicana, L. amazonensis*, and *L. venezuelensis*); *L. tropica*; *L. major*; *L. aethiopica*; and the subgenus *Viannia* with 4 main species (*L. (V.) braziliensis, L. (V.) guyanensis, L.(V.) panamensis*, and *L.(V.) peruviana*). Most species, though morphologically indistinguishable, can be differentiated based on their geographical distribution, isoenzyme analysis, molecular methods, or monoclonal antibodies. A list of *Leishmania* spp. along with their typical clinical manifestations and geographical distributions are summarized in Table 2.3.
<table>
<thead>
<tr>
<th>SPECIES</th>
<th>CLINICAL MANIFESTATIONS</th>
<th>GEOGRAPHICAL DISTRIBUTION</th>
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<tbody>
<tr>
<td><em>L. mexicana</em> complex</td>
<td>CL and rare cases of MCL and DCL.</td>
<td>Central America; northern and central parts of S. America; very rare in southern USA.</td>
</tr>
<tr>
<td><em>L. braziliensis</em> complex</td>
<td>CL with some cases developing MCL later.</td>
<td>Central America and various parts of S. America.</td>
</tr>
<tr>
<td><em>L. major</em></td>
<td>CL</td>
<td>Northern and central Africa; Middle East; southern Asia.</td>
</tr>
<tr>
<td><em>L. tropica</em></td>
<td>CL and rare cases of Recidiva</td>
<td>Middle East and southern Asia.</td>
</tr>
<tr>
<td><em>L. aethiopica</em></td>
<td>CL and rare cases of DCL</td>
<td>Ethiopia.</td>
</tr>
<tr>
<td><em>L. donovani</em></td>
<td>VL with rare cases of post kala-azar CL</td>
<td>East Africa; sub-Sahara; southern Asia including India and Iran.</td>
</tr>
<tr>
<td><em>L. infantum</em></td>
<td>CL or VL depending on strain</td>
<td>North Africa and southern Europe.</td>
</tr>
<tr>
<td><em>L. chagasi</em></td>
<td>VL and some atypical CL</td>
<td>Foci in Brazil, Venezuela and Colombia; isolated cases throughout Southern and Central America.</td>
</tr>
</tbody>
</table>

Table 2.3. Most *Leishmania* species can be identified by their clinical manifestations and geographical distributions. Human infections are caused by 21 of the 30 *Leishmania* species that infect mammals. Though morphologically indistinguishable, most of these species are identifiable by the clinical manifestations – varying from cutaneous Leishmaniasis (CL), muco-cutaneous Leishmaniasis (MCL), diffuse cutaneous Leishmaniasis (DCL) to visceral Leishmaniasis (VL) - and geographical distributions, typical to them.
2.A.5. *Leishmania* Life Cycle

Though associated with variable clinical manifestations, all *Leishmania* species have dimorphic life cycles with distinct modes of transmission, depicted in Figure 2.4.

In one stage of their life cycle, they reside within the digestive tract of their insect vectors – female sand flies from the *Phlebotomus* or *Lutzomyia* genera – as elongated, flagellate and highly motile forms called promastigotes (Chang and Dwyer, 1976). These may enter the skin tissue of mammals from which an infected fly takes its blood meal. The promastigotes are phagocytosed by tissue macrophages. They escape lysis in the phagolysosome of these macrophages to differentiate into rounded, non-flagellate and consequently non-motile forms called amastigotes (Chang and Dwyer, 1976; Burchmore and Barrett, 2001).

Proliferation of amastigotes as obligate intracellular parasites, by some unknown mechanisms, results in destruction of infected macrophages. Released amastigotes are phagocytosed by other macrophages, bloodstream monocytes, or dendritic cells (Bogdan and Rollinghoff, 1998), resulting in the spread of infection. Infections by *L. major* and *L. tropica* are limited to the draining lymph node. In contrast, *L. donovani* and *L. infantum* spread, in time, into the entire reticulo-endothelial system, *e.g.*, spleen, liver and bone marrow. Overt effects like lesions in cutaneous infections and splenomegaly or hepatomegaly in visceral infections are caused by the local or generalized depletion of macrophages. This, in addition to the fact that *Leishmania* skews the immune response in a TH2-dependent direction (Bogdan and Rollinghoff, 1998), promotes opportunistic infections, especially by intracellular pathogens like viruses.

When a feeding sand fly ingests and destroys infected macrophages from the peripheral blood, amastigotes are released into the lumen of the fly's gut. Within 24 hours, these transform into promastigotes - called procyclins - that attach themselves to the gut epithelium and proliferate till they reach the stationary phase of growth - called metacyclics. At this stage, a change in surface molecules enables the promastigotes to detach from the gut epithelium and spread to the mouthpart of the fly. The cycle may start afresh when this infected sand fly takes its next blood meal.
Figure 2.4. *Leishmania* spp. have a distinct dimorphic life cycle. Metacyclic promastigotes of *Leishmania* spp. are injected into the skin when an infected sandfly vector bites the mammalian host. These forms are efficiently opsonised by serum components and phagocytosed by macrophages. The parasites complete differentiation into amastigote forms in the phagolysosomes of the infected macrophages. They multiply rapidly and on lysis of the infected cell are disseminated to infect scavenging macrophages in the vicinity. When a sandfly takes its next blood meal, infected macrophages are lyosed in the fly midgut to release the parasites. These transform into promastigotes, which attach to the wall of the midgut (Sacks and Noben-Trauth, 2002).
2.A.6. Treatment

Since no vaccines against Leishmaniasis are currently available, treatment of this disease relies solely on chemotherapy. The first line of therapy involves the use of pentavalent antimonials, pro-drugs that are reduced by glutathione to an active trivalent species in a reaction catalyzed by thiol-dependent-reductase (Mishra et al., 2007). Though usually effective, treatment with these compounds requires parenteral (intramuscular or intravenous injections) administration and continues over a long duration (20 - 28 days). In addition, drug resistant strains have emerged in several regions endemic to the disease (like India), leading to increased toxicity and failures in treatment (Berman, 2003).

These disadvantages have led to the use of other compounds like amphotericin B, pentamidine, paromomycin, allopurinol etc for therapy. Amphotericin B, an antifungal macrolide polyene, is currently the drug with the highest cure rate. It causes parasite cell lysis by acting on membrane sterols. Though effective, this line of treatment also has the same disadvantages as the first line of therapy, in addition to being more expensive. Lipid formulations of this drug, though prohibitively expensive in developing countries, involve much lower doses and fewer side effects, and are known to be very effective against visceral Leishmaniasis. The other antifungals that are in use, like ketoconazole, fluconazole and terbinafine, are less effective (Mishra et al., 2007).

Recently, many anti-cancer alkylphosphocholines have been found to be effective as membrane synthetic ether-lipid analogues. In addition, many of them are administered orally. Among the most promising of these are miltefosine (hexadecylphosphocholine), edelfosine and ilmofosine (Mishra et al., 2007). Miltefosine, a recently registered oral drug that is still in Stage IV clinical trials, is proving to highly effective in the treatment of visceral Leishmaniasis. Apart from being easy to administer, it is both less toxic and less expensive.
2.B. Molecular Biology of Thermoregulation

2.B.1. Heat Shock

Exposure of cells to potentially damaging stress factors such as growth at elevated temperatures or heat shock triggers a variety of signalling pathways, some of which facilitate cell survival while others initiate cell death programs. Under these conditions, the ultimate fate of the cell may either be the development of thermo-tolerance and recovery from stress-associated damage if survival pathways prevail; or cell death, if death pathways prevail (Gabai and Sherman, 2002). The paradoxical activation of both pro- and anti-apoptotic signalling events in response to the same stimulus averts the onset and persistence of a pathological state by ensuring that neither aberrant cellular survival nor inappropriate cell death occurs (Beere, 2004; Beere, 2005).


In most cell types, exposure to non-extreme temperatures is associated with apoptosis (Gabai and Sherman, 2002). The most common form of apoptosis is via an intrinsic caspase-dependent pathway involving loss of mitochondrial membrane potential. However, both caspase-independent and Fas ligand - Fas receptor mediated extrinsic pathways are also known to be associated with heat-induced apoptosis. In some cell types, exposure to moderate heat shock causes a form of death, distinct from apoptosis. Called clonogenic or reproductive death, cells die after several divisions and are unable to form colonies (Gabai and Sherman, 2002).

Since heat shock is proteotoxic in nature, the extensive denaturation and aggregation of a variety of intracellular proteins – especially those forming the nuclear matrix and cytoskeleton (Nguyen, 1989; Kampinga, 1993; Lepock et al., 1993) – may be an important factor in determining cell fate. However, there is no data that directly links damage of cellular proteins to cell death. In contrast, heat shock is known to trigger death pathways mainly by activation of the stress related c-Jun NH2-terminal kinase (JNK) (Verheij et al., 1996); and survival pathways by the activation of Akt and extra-cellular signal-regulated kinases ERK
The relative extent of activation of the two pathways is known to be vital in determining fate of cells under heat stress.

Activation of the stress-inducible JNK signalling pathway mediates both pro- and anti-apoptotic effects depending on the type and duration of the damaging signal as well as cell type. Active JNK influences intrinsic death pathways by interacting with one or more BCL-2 family members (Srivastava et al., 1999; Yamamoto et al., 1999; Kharbanda et al., 2000; Tournier et al., 2000) to enhance stress-induced efflux of cytochrome c from the mitochondria and activate a cascade of caspases, including caspases-9 and caspases-3 (Li et al., 2000; Mosser et al., 2000; Samali et al., 2001). In addition, it activates the extrinsic death pathway by regulating stress-induced expression of Fas ligand (Faris et al., 1998; Sreedhar et al., 2000).

Survival kinases, Akt or ERK, may interfere with heat-induced apoptosis and promote cell survival in several ways. These include inhibition of JNK, pro-survival regulation of BCI-2 family members, inactivation of the pro-apoptotic fork-head transcription factor FKHRL1, and increased activation of the anti-apoptotic factor NF-KB (Datta et al., 1999; Kennedy et al., 1999; Cross et al., 2000; Levresse et al., 2000). In addition, ERK can interfere with the Fas induced activation of caspase-8 (Erhardt et al., 1999) and the cytochrome c induced activation of caspase-3 (Tran et al., 2001).

JNK, Akt and ERK kinases also have vital roles in both the caspase-independent and clonogenic forms of cell death induced by heat shock. For instance, inhibition of JNK protects against a caspase-independent form of cell death in human fibroblasts exposed to severe heat shock (Gabai et al., 2000). In contrast, inhibition of ERK or Akt induced a caspase independent form of apoptotic death in almost the entire fibroblast population on exposure to a much milder and non-toxic form of heat shock (Gabai et al., 2000). Similarly, by poorly understood mechanisms, heat induced clonogenic cell death is reduced on inhibition of JNK (Zanke et al., 1996), and increased on inhibition of Akt (Ma et al., 2001).
2.8.3. Heat Shock Response

One of the first heat shock associated survival pathways to be discovered in cells was the Heat Shock Response. Also called the cellular stress response, it is defined as the process of induction of a conserved family of stress related proteins (Lindquist and Craig, 1988), known by association as the Heat Shock Proteins. These proteins provide protection from and facilitate cellular recovery after exposure to heat shock by chaperoning against proteo-toxic stress and/or forming a cellular network to modulate the coordinated, multi-step regulation of apoptotic signalling events (Beere, 2005).

2.8.4. Classification and Sub-cellular localisation of Heat Shock Proteins

Heat Shock Proteins, the mediators of the Cellular Stress Response, were initially discovered because of their association with temperature stress in cells (Pirkkala et al., 2001), but only certain members of this family are inducible in expression. A majority are expressed constitutively under normal growth conditions. Members of this family are broadly classified based on their size (in kDa) into Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp27 and small heat shock protein families. All members show distinct sub-cellular localisations, summarised in Table 2.5.

2.8.5. Structure of Heat Shock Proteins

All heat shock proteins, whether constitutive or inducible, have conserved multi-domain structures (Figure 2.6). At their amino terminal end, is a regulatory ATPase domain, with the differential ability to bind ATP and ADP. Also called the nucleotide binding domain (NBD) (Harrison et al., 1997), the sequence of this stretch is highly conserved across species, and shares a high degree of identity with other nucleotide binding proteins, like the cytoskeletal protein, actin. In their role as molecular chaperones, this domain is important to heat shock proteins as it provides the energy for the ATP driven process of protein folding (Mayer et al., 2000; Pellecchia et al., 2000). At the carboxy-terminal end of heat shock proteins is a catalytic domain with the ability to bind polypeptides (Zhu et al., 1996). Since the sequence of this domain, also called the peptide binding domain (PBD), is directly related to the polypeptide...
<table>
<thead>
<tr>
<th>FAMILY</th>
<th>MEMBERS</th>
<th>INTRACELLULAR LOCALISATION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Small Hsp's</td>
<td>Hsp10, GROES, Hsp16, α-crystallin, Hsp20, Hsp25</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Hsp40</td>
<td>Hsp40, DNAJ, SIS1</td>
<td>Cytosol</td>
</tr>
<tr>
<td>Hsp47</td>
<td>Hsp47</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Calreticulin</td>
<td>Calreticulin, Calnexin</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Hsp60</td>
<td>Hsp60, Hsp65, GROEL</td>
<td>Cytosol and Mitochondria</td>
</tr>
<tr>
<td>Hsp70</td>
<td>Hsp72, Hsc70 (Hsp73), Hsp110/SSE, DNAK, SSC1, SSQ1, ECM10, Grp78 (BiP), Grp170</td>
<td>Cytosol Mitochondria Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Hsp90</td>
<td>Hsc84, Hsp86, HTPG, Gp96 (Grp94, endoplasmin)</td>
<td>Cytosol Endoplasmic Reticulum</td>
</tr>
<tr>
<td>Hsp100</td>
<td>Hsp104, Hsp110, CLIP Proteins, Hsp78</td>
<td>Cytosol Mitochondria</td>
</tr>
</tbody>
</table>

**Figure 2.5. Classification and localisation of heat shock proteins.** Cellular heat shock proteins are broadly classified, based on their size in kDa, into several families. All families consist of both constitutively and inducible expressed members, and show distinct sub-cellular localisations.
Figure 2.6. Heat Shock Proteins have highly conserved multi-domain structures. Structures of the ATPase domain and the peptide-binding domain of Hsp70 shown representatively for E. coli DnaK, generated with the program MOLSCRIPT. The a-helical latch of the peptide binding domain is shown in yellow and a ball-and-stick model of the extended peptide substrate in pink. The amino acid sequence of the peptide is indicated in single-letter code (D, Asp; E, Glu; G, Gly; L, Leu; N, Asn; R, Arg; T, Thr; and V, Val). ATP indicates the position of the nucleotide binding site (Hartl, 1996).
pool of the cell, it is highly variable. In inducible members, it is further characterized by an extreme C-terminal tetra-trico-peptide or TPR motif, with a sequence that can vary from EEVD in members of the Hsp70 family to MEEVD in members of the Hsp90 family. Since inter-domain communication is mediated through this motif, the entire C-terminal domain of these proteins is essential for their role as chaperones as well as signaling modulators (Hartl and Hayer-Hartl, 2002). The relative length of the regulatory and catalytic domains of heat shock proteins vary not just between different members of the same family, but also between the same members from different species. Occasionally, a hinge domain, of variable length, separates these domains. Even in the absence of the hinge, though, the two domains are capable of independently folding into their functional conformations (Hartl and Hayer-Hartl, 2002).


Even on exposure to heat shock, the stoichiometry of cellular heat shock proteins – constitutive vs. inducible – is finely regulated. Regulation is at several levels – transcriptional, post transcriptional, translational as well as post translational. Due to the evolutionary significance of their function, the regulatory factors and pathways involved at each of these levels are highly conserved across species (Pirkkala et al., 2001). One of the best examples of this being that Heat Shock Transcription factors (HSF’S) (Morimoto, 1993; Voellmy, 1994) and Heat Shock Binding Proteins (like HSBP1) – key players of the transcriptional regulation of heat shock proteins - have homologues in almost all species studied. Their importance is underlined by the fact that transcriptional regulation is the primary cellular mechanism involved in Hsp control in almost all organisms studied.

HSF’s function mainly by binding to highly conserved cis-regulatory promoter elements, called Heat shock Promoter Elements or HSE’s, that are located in the TATA-box-proximal 5' flanking regions of heat shock genes. The occurrence of multiple HSE’s within a few hundred base pairs is a signature of most eukaryotic heat shock genes. HSE’s are characterized as multiple and inverse iterations of the penta-nucleotide motif 5'-nGAAAn-3'. Efficient binding to HSF’s requires at least three units: 5'- nGAAAnTTCnnGAAAn- 3' (Morimoto, 1998). This binding has two effects: one, HSF’s interact with components of the chromatin re-
modeling machinery to make the DNA more open to transcription; two, they interact with members of the basal transcription machinery like TBP which allow elongation of pre-initiated transcripts of Hsp genes by the stalled RNA pol II. The combined effect of these two functions results in elevated levels of cellular heat shock proteins. In contrast, HSBP’s prevent the enhanced synthesis of cellular heat shock proteins by binding to inactive HSF’s and displacing them from HSE’s (Satyal et al., 1998).


Heat shock interferes with protein folding and association, and therefore, adversely affects their function. A fraction of the constitutively expressed heat shock proteins that are not actively involved in chaperoning, sequester inactive HSF monomers (Ali et al., 1998; Zuo et al. 1998) in transient nuclear and/or cytosolic complexes (Morimoto, 1998). In response to the increase in cellular levels of misfolded, denatured and aggregated proteins, these Hsp-HSF complexes disassociate. A step-wise process (Figure 2.7) involving nuclear re-localization and the acquisition of both DNA binding competence and transcriptional activity by stress-inducible phosphorylation (Hensold et al., 1990; Jurivich et al., 1992; Sarge et al. 1993; Cotto et al. 1996; Kline and Morimoto, 1997) activate the freed HSF’s. The active HSF’s induce transcription of genes encoding the inducible Hsp’s, HSF’s, HSBP’s and inhibitory kinases. This process is reversed during the attenuation of the Stress Response. Inhibitory kinases phosphorylate HSF’s to make them inactive; the transcriptional activity of HSF’s is repressed by direct binding to Hsp’s (Abravaya et al. 1992; Baler et al. 1992; Mosser et al. 1993; Rabindran et al. 1994; Shi et al. 1998); and HSF trimers are negatively regulated by HSBP (Morimoto, 1998). These, in combination, cause disassociation of active HSF trimers into inert monomers. The negative feedback loop established in this way prevents the induction of Hsp’s once cells are returned to their original growth temperature.

2.B.8. Hsp Mediated Protection

Conventionally, the protective effect of induced heat shock proteins (Mizzen and Welch 1988; Huot et al. 1991; Jaattela et al. 1992; Mestril et al. 1994; Marber et al. 1995;
Figure 2.7. Regulation of the heat shock response in most organisms is primarily at the transcriptional level. Activation of the transcriptional regulator heat shock factor 1 (HSF1) is linked to the appearance of non-native proteins and the requirement for molecular chaperones (Hsp90, Hsp70, and Hdj1) to prevent the appearance of misfolded proteins. HSF1 exists in the control state in the cytoplasm or nucleus as an inert monomer (shown as intra-molecularly negatively regulated for DNA-binding and transcriptional activity) through transient interactions with chaperones such as Hsp90 and Hsp70. Activation of HSF1 is associated with a step-wise process, including re-localization to the nucleus and acquisition of a DNA binding competent state that is transcriptionally inert, stress-inducible phosphorylation associated with transcriptional activity, and inducible transcription of heat shock genes. During attenuation of the heat shock response, the transcriptional activity of HSF1 is repressed by direct binding of Hsp70 and Hdj-1, and the trimers are negatively regulated by HSF binding protein 1 (HSBP1), which binds both to the hydrophobic heptad repeat of HSF1 and to Hsp70. These events lead to the dissociation of HSF1 trimers and appearance of HSF1 inert monomers (Morimoto, 1993).
Mehlen et al. 1995; Plumier et al. 1995; Mosser et al. 1997) is attributed to their ability to enhance the efficiency of chaperoning and repair of heat labile cellular proteins (Lindquist, 1986; Lindquist and Craig, 1988; Gething and Sambrook, 1992; Parsell and Lindquist, 1993; Parsell et al., 1993; Nollen and Morimoto, 2002). According to a recently emerging theory, however, Hsp mediated cyto-protection is due to their ability to act as modulators of heat activated signaling pathways (Beere and Green, 2001; Parcellier et al., 2003; Beere, 2005). Though it is likely that Hsp mediated cyto-protection might be a collective effect of both these functions, several studies suggest that the relative importance of the two might vary in different cell types. These studies are based on the fact that the refolding ability of inducible heat shock proteins - mediated by their regulatory ATPase domains - is essential for their role as chaperones but dispensable in their role as signaling modulators. For instance, Hsp72 mutants lacking their ATPase domain protect fibroblasts from heat-induced killing. But in spite of their ability to inhibit JNK, these mutants fail to protect lymphoid cells from heat induced cell death (Gabai and Sherman, 2002).

2.8.9. Heat Shock Proteins as Molecular Chaperones

Briefly, molecular chaperones protect non-native protein chains from misfolding and aggregation, but do not contribute conformational information to the folding process (Ellis and Hemmingsen, 1989; Gething and Sambrook, 1992; Hartl, 1996). All heat shock proteins – constitutive and inducible – function as molecular chaperones. Due to its proteo-toxic nature, heat stress increases the need for such chaperoning. It is, in fact, this effect of heat shock that triggers the synthesis of inducible heat shock proteins in the cell (Morimoto, 1998).

(a) Chaperones and de novo Protein Folding

In the cytosol, a majority of small proteins (65-80%) fold rapidly in the presence of certain chaperones, like specialized Hsp70 proteins, that bind directly to ribosomes. Longer chains (10-20%) fold upon one or more cycles of ATP-driven binding and release from a second class of nascent chain binding chaperones, like the classical Hsp70 proteins, that do not directly associate with ribosomes (Deuerling et al., 1999; Teter et al., 1999; Thulasiraman et al., 1999). About 15 to 20% chains fold in a reaction assisted by Hsp70 - Hsp40 complexes, and a fraction of these, consisting of many eukaryotic kinases and signal
transduction proteins, are subsequently transferred to Hsp90 for folding (Hartl and Hayer-Hartl, 2002).

Certain specialized heat shock proteins, like Bip/Grp78 and Grp94/gp96 - members of the Hsp70 and Hsp90 families, are known to be involved in protein folding in the unique environment of the endoplasmic reticulum (Kleizen and Braakman, 2004). While others like the Ssa1-4p from the Hsp70 family in yeast are involved in the many steps of protein transfer and folding in the mitochondria (Sass et al., 2003).

(b) Chaperones and the Cellular Cytoskeleton

Molecular chaperones have different but cooperative roles in the formation and function of the eukaryotic cytoskeleton composed of microtubules, microfilaments and intermediate filaments (Liang and MacRae, 1997). For example, Hsp70 protects the centrosome and probably intermediate filaments during heat shock, and like Hsp90, binds to microtubules (Brown et al., 1996).

(c) Chaperones, Misfolding and Protein Aggregation

All chaperones recognize the hydrophobic residues and/or unstructured backbone regions in their substrates. Chaperones, like Hsp70 and Hsp90, promote the refolding process through several cycles of substrate binding and release, regulated by their ATPase activity and cofactor proteins. This chaperone binding may not only block intermolecular aggregation directly by shielding the interactive surfaces of non-native polypeptides and unassembled protein subunits, but also may prevent or reverse intra-molecular misfolding (Hartl and Hayer-Hartl, 2002).

(d) Chaperones and Protein Degradation

Under a variety of in vivo conditions, despite the presence of chaperones and the proteolytic machinery, proteins may show misfolding and form aggregates. Certain chaperones, especially those from the Hsp70 and Hsp90 family, have the ability to ubiquitinate such misfolded substrates, through their interactions with ubiquitin ligases like CHIP, and direct them to the 26S proteasomes for degradation (McDonough and Patterson, 2003; Parcellier et al., 2003a).
2.B.10. Heat Activated Signaling Pathways in Hsp Regulation

Enhanced synthesis of heat shock proteins is mainly triggered by the proteo-toxic nature of exposure to elevated growth temperatures. However, heat shock activated signaling pathways can also modulate the expression and chaperone function of heat shock proteins.

The key mechanism involved in heat shock induced Hsp synthesis is the HSF1-mediated transcription of Hsp genes (McMillan et al., 1998). HSF activity is regulated by reversible phosphorylation at multiple sites (Knauf et al., 1996; Kline and Morimoto, 1997). Many kinases – including heat activated Akt, ERK and JNK – are involved in this regulation. For example, heat induced activation of Akt increases HSF1 activity possibly through inhibition of its negative regulator (Chu et al., 1996; Xavier et al., 2000) glycogen synthase kinase-3 (Bijur and Jope, 2000); whereas heat activated ERK and JNK decrease HSF1 activity by phosphorylation at distinct sites (Chu et al., 1996; He et al., 1998; Dai et al., 2000). Thus, it is likely that regulation of Hsp transcription by stress-activated signaling pathways may be important in influencing the balance between survival and death programs. In certain cases, heat activated kinases may also be involved in the post-transcriptional modulation of chaperone activity (Gabai and Sherman, 2002). For example, only over-expression of Hsp27 proteins with intact phosphorylation sites can protect cells against heat stress (Lavoie et al., 1995). Phosphorylation of Hsp27, at specific serine residues, is mediated by the strongly heat activated MAPKAP-2/3 kinase (Lavoie et al., 1995; Kyriakis and Avruch, 1996).


By their ability to modulate heat activated signaling pathways, inducible heat shock proteins provide an important cellular feedback loop. Though members of all heat shock proteins families are involved in this modulation, Hsp72 and Hsp90 seem to have the most important and best characterized roles. Modulation by these proteins occurs at several different levels of the heat activated death pathway:

1. Hsp72 prevents release of cytochrome c from the mitochondria (Mosser et al., 2000; Steel et al., 2004), either directly or indirectly by interfering with components upstream of mitochondrial membrane permeabilisation. It suppresses caspase-8 mediated cleavage and
activation of Bid by a mechanism independent of its ATPase domain (Gabai et al., 2002). In contrast, its ability to prevent Bax translocation into the mitochondria is dependent on both its ATPase and peptide binding domains (Gotoh et al., 2004). It binds to Apaf-1 and prevents its oligomerisation with cytochrome c (Beere et al., 2000; Pandey et al., 2000), thus inhibiting the formation of a functional apoptosome and inhibiting caspase-9 activation in vitro (Zou et al., 1997; Zou et al., 1999). In addition, though not known to prevent the release of AIF (a factor important in caspase independent cell death) from the mitochondria, both Hsp72 and Hsp90 can bind to this molecule to prevent its nuclear translocation and pro-apoptotic signaling (Ravagnan et al., 2001; Gurubuxani et al., 2003).

2. Hsp72 negatively regulates JNK activity in cells exposed to heat shock by stimulating JNK phosphatase, an enzyme that inactivates JNK (Gabai and Sherman, 2002). Several studies indicate that Hsp72 mediated protection against apoptosis is due to this ability of the protein, which is independent of its ATPase domain. For example, over-expression of Hsp72 dramatically reduces heat-induced activation of JNK (Gabai et al., 1997; Mosser et al., 1997) to inhibit caspase-dependent apoptosis in non-transformed cells (Gabai et al., 1997), caspase-independent apoptosis (Gabai et al., 2000) in fibroblasts (Gabai and Yaglom; et al., 2000), and heat induced clonogenic death (Zanke et al., 1996) in certain cell lines.

3. Hsp72 mediated inhibition of JNK is also responsible for the phenomenon of acquired stress tolerance, where mild heat shock followed by a recovery period makes cells resistant to severe heat shock as well as other forms of cellular stress. This is because mild heat shock rapidly and transiently activates JNK (and survival kinases) to a level that is insufficient to turn on the apoptotic process, and induces slow (within several hours) accumulation of Hsp72. The accumulated Hsp72 suppresses activation of JNK in cells exposed to severe stress, preventing apoptosis (Gabai and Sherman, 2002).

4. Hsp72 suppresses heat-induced activation of other MAPK's, p38 (Gabai et al., 1997) and ERK (Song et al., 2001). Since p38 and ERK have protective roles, this ability of Hsp72 inhibits cell survival pathways. However, in vivo, their inhibition of JNK induced death pathways seems more important in determining cell fate (Gabai and Sherman, 2002). Hsp72 mediated inhibition of ERK is mediated by binding to Bag-1 protein, an upstream component of the ERK signaling pathway (Song et al., 2001). Over-expression of Bag-1 negates the Hsp72
mediated inhibition of ERK (Song et al., 2001), protecting cells from heat stress (Takayama et al., 1997; Yang et al., 2000). In addition, Bag-1 also inhibits the ATPase activity of Hsp72, blocking their ability to refold proteins (Takayama et al., 1997; Nollen et al., 2000). This seems to suggest that in protection against apoptosis, the ability of Hsp's to refold proteins may not be as essential as its ability to modulate signaling pathways.

5. Antisense for Hsp72 induces a caspase-independent form of cell death that is attributed to the ability of this protein to maintain the integrity of lysosomal membranes, preventing cathepsin release into the cytosol (Nylandsted et al., 2004). In certain cases, like tumour cell lines, Hsp72 is known to inhibit apoptosis even at a stage downstream of caspases (Jaattela et al., 1998).

6. Hsp90-Cdc37 complexes promote cell survival under heat stress by aiding in maintenance of stable and active Akt through inhibition of its PPA2 mediated de-phosphorylation (Sato et al., 2000; Basso et al., 2002). In addition, Hsp90 directly binds to and stabilizes RIP1 (Devin et al., 2000; Zhang et al., 2000), and in complexes with cdc37 interacts with IKK complexes (Chen et al., 2002; Park et al., 2003; Parcellier et al., 2003; Septanova et al., 1996; Kimura et al., 1997; Lewis et al., 2000). Both of these enhance cell survival by increased activation of NF-kB.

7. Although the role of DAXX in mediating Fas-induced death is controversial, some survival-promoting effects of Hsp90 have been linked to their ability to regulate the recruitment of DAXX (Yang et al., 1997) and ASK1 (Park et al., 2002) to the Fas signaling complex.


Though the heat shock response was discovered in cells exposed to heat stress, it is known to be triggered in response to a wide variety of signals (Lis and Wu 1993; Morimoto 1993; Wu 1995), broadly classified into three categories, and summarized in Figure 2.8:

(a) Environmental Stress Factors – which apart from heat shock (Mosser et al., 1997; Mosser et al., 2000), include exposure to amino acid analogues, oxygen free radicals, transition heavy metals, inhibitors of energy metabolism etc.
Figure 2.8. Signals Inducing the Heat Shock Response. Heat shock response, involving the activation of HSF and binding to HSE, results in the elevated expression of Hsp's such as Hsp70. This response is activated in cells during a variety of conditions, which include environmental and physiological stress, many patho-physiological states, as well as non-stressful conditions, like cell growth and development (Morimoto, 1993).
(b) Pathophysiological States - which include fever and inflammation, neuro-hormonal stress, hypertrophy, oxidant injury, ischemia, anti-neoplastic chemicals, viral and bacterial infections, neuronal injury, aging, tissue injury and repair (Leppä and Sistonen, 1997) etc.

(c) Non-stress Conditions – which include cell cycle, growth factors, development and differentiation, oncogenes and proto-oncogenes (Morimoto, 1993) etc.

2.8.13. Inducible Heat Shock Proteins in Host-Pathogen Interactions

Constitutive Hsp’s are known to be immuno-dominant antigens; the main targets of host immune response in a variety of infections; and are involved in many autoimmune conditions (Zügel and Kaufmann, 1999). However, the role of inducible Hsp’s these interactions are not so well characterized. An increasing body of work has shown that during these interactions, not only do pathogens induce the conserved heat shock response and manipulate it to their own advantage, but also parasite heat shock proteins assume a variety of roles (Jaattela, 1990; Kaufmann, 1991; Steinhoff, 1991; Steinhoff, 1994), some of which are non-canonical. Some examples of these are:

1. *Leishmania pifanoi* amastigotes induce host Hsp32 to enhance heme-degradation and avoid macrophage generated super oxides (Pham *et al*., 2005).

2. Induction of Hsp70 in *Toxoplasma gondii* is important in conversion of the rapidly multiplying tachyzoite form to the chronically infective bradyzoite form. This helps the parasite evade immune system mediated clearance in immuno-competent individuals (Weiss *et al*., 1998; Böhne *et al*., 1999; Weiss and Kim, 2000).

3. Induction of parasite Hsp70, in the presence of certain immunosuppressive cytokines, is an important molecular event in reactivation of *Toxoplasma gondii* infections during certain immuno-deficient conditions, like AIDS (Weiss *et al*., 1998).

4. Prolonged expression of Hsp60, in contrast to the short term expression of Hsp70, in the bacteria *Brucella abortus*, a bovine facultative intracellular pathogen, allows enhanced survival in the host and uninterrupted synthesis of parasite proteins even under acute stress (Roop *et al*., 1992).
5. An immuno-dominant 47kDa heat stable breakdown product of inducible Hsp90 proteins, expressed in cell wall of Candida albicans, binds to host proteins interfering with its structure or function. This leads to increased mortality of infected mice and impaired renal function in sub-lethally challenged mice (Cowen et al., 2006).

6. At high temperatures, levels of htpG-like gene in Vibrio cholerae increase. This reduces expression levels of toxR that enhances transcription of genes encoding cholera entero-toxin and other virulence determinants. As a result, there is increased expression of several genes important in early stages of the infection cycle of the bacteria in the host intestine (Parsot and Mekalanos, 1990).

7. A secretary 66kDa homologue of Salmonella typhimurium, in dimeric or polymeric forms, is responsible for binding of the bacteria to the intestinal mucus (Ensgraber and Loos, 1992).

8. Purified Hsp from L. pneumophila, E. coli, M. tuberculosis, Mycobacterium leprae, and Mycobacterium bovis have been shown to directly induce the production of cytokines in macrophages (Retzlaff et al., 1994).

9. A 58kDa heat shock protein, distinct from Hsp60, is expressed on the surface of Staphylococcus aureus. This has been hypothesised to be vital in adherence and invasion of epithelial cells of the host (Dziewanowska et al., 2000).

10. A small Hsp called PVHsp28 has been reported to show metallo-protease activity in the human malaria parasite Plasmodium vivax. This is expressed in the erythrocyte stages of the parasite as a 55kDa protein that is processed to a 28kDa mature form. Due to its ability to remain active at temperatures as high as 55°C, this protein plays multiple roles in the parasite, especially during its transfer from mosquitoes to humans and during malarial fever (Fakruddin et al., 1997; Fakruddin et al., 2000).


There are only a handful of reports linking inducible heat shock proteins in a cell with the development and differentiation, but significantly, these reports are from a wide variety of organisms. Some examples of these are:
1. Pattern of Hsp induction during the heat shock response of fungi, like S. cerevisiae and N. crassa, varies with their development. Induced Hsp34 and Hsp38, for instance, is believed to have key roles during transition of fungi from their vegetative to differentiation stage (Kurtz and Lindquist, 1984; Atkinson et al., 1993; Heikkila, 1993).

2. Differentiation events such as seed formation in many plants are associated with the induction of a variety of small Hsp's with molecular sizes between 17-22kDa (Heikkila, 1993).

3. Induction of small heat shock proteins is observed during differentiation in a variety of invertebrates, birds and mammals. In Drosophila, for example, Hsp22, Hsp23, Hsp26 and Hsp27 are expressed in a tissue specific manner that has a key role in development (Wiess et al., 1998).

4. Hsp70 expression is induced during sporulation in Blatocladia emersonii; and is associated with hyphal branching and secretion in response to steroids in Achlya ambisexualis (Heikkila, 1993; Wiess et al., 1998).

5. In Histoplasma capsulatum, mitochondrial ATPase activity and Hsp70 induction have been correlated with the transition from mycelium to yeast phase (Patriarca et al., 1992). Similarly, Hsp70 expression has been associated with the asexual stages of P. cynomolgi (Eckert et al., 1992).

6. The heat shock response in Xenopus is not only responsible for affecting gene expression, but also for changes in cellular metabolism. For example, it interrupts oxidative phosphorylation to promote anaerobic glycolysis (Nickells and Browder, 1985).

2.B.15. Inducible Heat Shock Proteins and Evolvability

Recent studies suggest that positioned between the source of heritable variation (genotype) and its expression (phenotype), Hsp chaperone families act as "capacitors for morphological evolution" by buffering cryptic variations, allowing them to accumulate in phenotypically normal populations (Rutherford and Lindquist, 1998; Wagner et al., 1999). Chaperones can do this either directly by allowing correct folding of mutant polypeptides to mask their phenotypic effects; or indirectly by regulating signal transduction to buffer
expression of morphogenic variation in threshold traits. This is enhanced by the increased
dependence of mutant proteins on cellular chaperones; and the limiting nature of chaperone
mediated protein folding under severe protein stress. By stabilizing an active wild-type-like
conformation of a non-functional or partly functional protein, alterations covertly accumulate
until an environmental change induces their phenotypic expression, with significant
consequences in the evolution of populations and lineages (Rutherford and Lindquist, 1998;
Sangster et al., 2004).

2.8.16. Hsp70 and Hsp90 Proteins

Hsp70 and Hsp90 proteins are two of the most abundantly expressed and highly
conserved cellular proteins. Through their dual ability to act as chaperones and modulators of
signaling, they are vital in protection of cells against stress. In addition, they are actively
involved in a variety of non-stress associated cellular functions like development,
differentiation, pathogenesis and evolvability. However, they differ significantly in their cellular
expression patterns.

Expression of the Hsp90 gene is mainly constitutive, but is capable of increase on
induction of the heat shock response. In contrast, Hsp70 proteins are expressed in two
different forms in eukaryotic cells: cHSC73 is constitutive, while iHsp72 is highly stress-
inducible. Different genes encode the two proteins. The gene for cHSC73 is similar to any
other cellular RNA in containing intervening sequences that require post-transcriptional
screening (Brown et al., 1993). In contrast, the gene for iHsp72 lacks such sequences. Since
mRNA processing is compromised in cells exposed to heat shock (Mayrand and Pederson,
1983; Yost and Lindquist, 1986), it has been hypothesized that the high levels of iHsp72 under
stress may represent a way for cells to rapidly increase levels of this protein in the cytosol and
nucleus by bypassing the need for this processing. This is supported by the high degree of
sequence relatedness between the two proteins, with the only difference being in their extreme
c-terminal domains. Several studies, however, suggest that both proteins have distinct cellular
roles. For example, only increased iHsp72 (Li and Werb, 1982), but not cHSC73, is involved in
the cellular phenomenon of acquired thermo-tolerance (Gerner and Schneider, 1975; Henle
and Leeper, 1976; Mizzen and Welch, 1988). Similarly, over-expression of iHsp72 is known to
substantially reduce the kinetics of growth in at least some cells (Feder et al., 1992); while cHSC73, being constitutively expressed, has no such effect. Both proteins are independently capable of transient interactions with a variety of other cellular proteins. However, in cells under stress, they usually form relatively stable complexes, by an ATP-driven process, that translocate to the nucleus to mediate cyto-protection. The significance of this association is poorly understood (Brown et al., 1993).

2.C. Heat Shock in Leishmania Life Cycle

2. C. 1. Signals Triggering Parasite Differentiation

Transmission of the parasite, during its life cycle, from a poikilothermic arthropod vector to a homeothermic mammalian host involves exposure to two different extracellular environments: the ambient temperature increases from 23-26°C in sand flies to 37°C in mammals; the pH reduces from 6.8-7.2 in insect gut or mouthparts to 5.5 in the phagolysosome of macrophages.

The process of differentiation can be mimicked in vitro by shifting promastigotes from an insect like environment to an intra-lysosome like environment (Saar et al. 1998; Gupta et al. 2001; Somanna et al., 2002; Debrabant et al., 2004; Barak et al., 2005). Cultured promastigotes of L. mexicana and L. pifanoi are known to differentiate into axenic amastigotes merely upon an increase in incubation temperature to 34°C. In contrast, in vitro development of axenic amastigote-like forms of L. donovani and L. infantum require a treatment regimen involving 24 hour heat stress at 37°C followed by incubation at 37°C and pH 5.5 for 3 to 5 days (Zilberstein and Shapira, 1994). Dropping the growth temperature to 25°C and shifting the pH to 7.0 induces reversal of axenic amastigotes into promastigotes.

These suggest that the rise in temperature during transmission and the acidification of the phagolysosome are together both necessary and sufficient signals for the induction of
promastigote to amastigote stage conversion, and thus key factors for parasite persistence within their mammalian hosts (Clos and Krobitsch, 1999).

2.C.2. Temperature Tolerance and Tissue Tropism

Many studies suggest a close correlation between the temperature tolerance of *Leishmania* species and their tissue tropism within their mammalian hosts.

Strictly cutanotropic *Leishmania* species like *L. major*, *L. braziliensis* and *L. mexicana* do not tolerate temperatures in excess of 34-35°C *in vitro*, which is the maximal temperature of the human skin. Thus lesions resulting from these infections were conventionally treated by heat radiated from oil lamps or candles. In contrast, viscerotropic species of *L. donovani*, *L. infantum* (*L. chagasi*), and *L. tropica* (reported to cause a rare form of viscerotropic Leishmaniasis), tolerate temperatures well in excess of 37°C, which corresponds with the temperature range in the human abdominal cavity (Berman and Neva, 1981; Sacks et al., 1983; Callahan et al., 1996).

Whether this correlation reflects a causal relationship or not is debatable. A recent study tried developing a genetic screen to identify any genes that by conferring increased temperature tolerance could increase the viscerotropic potential of a cutanotropic species like *L. major*. Temperature sensitive *L. major* was transfected with the cosmid library of the genomic DNA of *L. donovani*. Recombinant parasites were subjected to a selection temperature of 37°C, and the survivors isolated were used for infections. Though, many species of cosmids and plasmids were found to confer a selective advantage in the competitive temperature tolerance selection screens, limited analysis of their sequences provided no clues to the nature or function of the isolated genes. In addition none of these were found to be capable of increasing the permissive temperature range of *L. major* to match that of *L. donovani* (Clos and Krobitsch, 1999).

2.C.3. Molecular Basis of Differentiation

Differentiation of the promastigote to amastigote forms in *Leishmania* is a complex process accompanied by a number of morphological and biochemical changes. For example,
parasites change shape from elongated to spherical, and lose most of their flagellum. They undergo a major shift in their metabolism, especially in the rate and pH optima for several processes, including DNA synthesis (Mukkada et al., 1985) and nutrient uptake (Mazareb et al., 1999).

Little is known about the molecular events that mediate differentiation. A recent DNA micro-array based expression profiling (Saxena et al., 2007) investigated the time course of changes in RNA abundance during the in vitro differentiation of the parasite in a host free system. This revealed that several hundred genes showed an ordered progression of transient or permanent up- and down-regulation during differentiation.

Analysis of the stage regulated genes revealed that those permanently down regulated were enriched for genes involved in cell growth, maintenance and cell motility. Changes in their expression occurred early in the differentiation process. In contrast, genes that were permanently up-regulated were under-represented in those controlling protein and other metabolism, but enriched for transporters, surface proteins and proteins of unknown function. Changes in their expression levels occurred late in the differentiation process where morphological transition of the parasite was essentially complete.

Several genes, including those encoding heat shock proteins, ubiquitin hydrolases, RNA binding proteins, protein kinases, a protein phosphatase, and a histone deacetylasel, showed transient changes in their expression during differentiation. In view of the nature of these proteins, it is likely that apart from determining protein and RNA turnover, mRNA abundance may be vital in influencing signaling pathways associated with differentiation (Saxena et al., 2007).


Heat shock proteins have key roles in cells exposed to elevated growth temperatures. Genes encoding the major heat shock proteins in many Leishmania species have been identified, cloned and analysed:

(a) Hsp90
Hsp90, the predominant *Leishmania* heat shock protein, is encoded by at least 5 gene copies arranged in tandem, and constitutes approximately 2.8% of the cellular protein content in unstressed parasites (Shapira and Pinelli, 1989; Brandau *et al.*, 1995; Hubel and Clos, 1996). It is localised to the cytoplasm, where it shows a more of less even distribution.

(b) Hsp70

Members of the Hsp70 family of proteins are also highly abundant and constitute over 2% of the cellular protein content in unstressed cells (Brandau *et al.*, 1995). Like their mammalian homologues, these proteins are of two types – constitutive and inducible. Inducible members are encoded by 4 (*L. major*) to 14 (*L. donovani*) gene copies (Lee *et al.*, 1988; Wallace *et al.*, 1992). In addition, at least three additional members with promastigote specific expression patterns have been identified, one of which shares its coding region with inducible heat shock genes (Searle *et al.*, 1989; Searle and Smith, 1993). All Hsp70 proteins are primarily cytosolic in location. Unlike mammalian and insect homologues, these proteins do not relocate to the nucleus upon heat stress.

(c) Hsp100

The *Leishmania* ClpB homologue of Hsp100, encoded by a single copy of the clpB gene, is the only heat shock protein identified so far that clearly shows a more or less stage specific expression pattern (Hübel *et al.*, 1995). Though negligible in promastigotes, this protein becomes abundant upon heat stress, during differentiation into axenic amastigotes and in true animal-derived amastigotes of *L. major* (Hübel *et al.*, 1997). Like its yeast and bacterial counterparts, the *Leishmania* protein forms oligomeric complexes both *in vitro* and *in vivo* (Krobitsch *et al.*, 1998). The protein is localised to the cytosol in close proximity to the cell membrane (Hübel *et al.*, 1995).

(d) Hsp60

A gene encoding an Hsp60 homologue, recently identified in *L. major*, is known to be immunogenic in South American Leishmaniasis patients (Rey-Ladino, 1997). This protein is localised to the mitochondria. More recently, two homologues of cpn60 genes with divergent sequences have been identified in *L. donovani*, one of which, called cpn60.1, is homologous to the *L. major* gene identified previously. The other, cpn60.2, encodes a peptide sequence with a
high degree of conservation with cpn60 sequence of Trypanosomes. The predominantly mitochondrial localisation and relatively high expression levels of cpn60.2 suggests that it the true Hsp60 homologue in *Leishmania*. In contrast, levels of the cpn60.1 protein are almost undetectable. The reason for these low levels of expression is not clearly understood, especially in view of the fact that its open reading frame, unlike a pseudogene, is uninterrupted.

2.C.5. Evidence for a Modified Stress Response in *Leishmania*

The increase in temperature during transmission of *Leishmania* sp. from sand flies to mammals that is vital for parasite differentiation constitutes a heat shock in other systems. Genes expressing heat shock proteins are known to show transient changes in expression during differentiation of the parasite – a molecular event associated with the induction of the heat shock response. Amastigotes show a decrease in protein metabolism and activation of certain signal transduction pathways (Saxena *et al.*, 2007) – both of which are changes known to involve inducible heat shock proteins as chaperones or signalling modulators. Change in temperature is a regular feature of the parasite life cycle; and temperature tolerance of the parasite is strongly implicated in determining its tissue tropism. In view of the fact that the temperature range of mammalian tissue is very limited, and under physiological conditions does not exceed 42°C, the parasite seems to have a survival advantage in that its stress response only needs to cope with a pre-defined temperature change unlike the wide temperature change encountered by free living micro-organisms. All of these, collectively, seem to indicate that the parasite may have evolved a stress response that is adapted to its specific needs (Clos and Krobitsch, 1999).

2.C.6. Distinctive Features of the Parasite Stress Response

Though homologues of all major mammalian heat shock proteins have been identified in *Leishmania*, the parasite stress response shows certain distinctive features:

1. There are no mechanisms for transcriptional regulation of the stress response.
This was indicated by the absence of heat shock transcription factors or HSF's in the parasite (Clos and Krobitsch, 1999). It was supported by the observation that there was no reduction in Hsp protein levels on treatment of Leishmania with the potent transcriptional inhibitor Actinomycin C1 (Clos et al., 1998). Nuclear run on assays confirmed these findings by showing that there was no heat induced increase in the transcriptional levels of 4 heat shock genes in three Leishmania species (Argaman et al., 1994; Brandau et al., 1995). Thus, Leishmania spp regulate their heat shock response, and probably their entire gene expression exclusively at the post-transcriptional level (Clayton, 2002).

2. Heat Stress is the only signal capable of inducing the stress response.

Results from several studies seem to suggest that the induction of the stress response in Leishmania is restricted to conditions of heat stress only. Many of the physiological and chemical stress factors known to induce this response in yeast or in metazoans failed to do so in Leishmania (Clos et al., 1998). This is unexpected, especially because at least one of these physiological stress factors of low pH is a key trigger for conversion of promastigotes of L. donovani into amastigotes (Zilberstein and Shapira, 1994). However, this observation seems to support the absence of regulated transcription mechanisms in the parasite.

3. Post transcriptional regulation of gene expression is primarily mediated by a 3' - untranslated (UTR) sequence (Charest et al. 1996).

In most organisms, gene expression levels are primarily determined by regulatory 5'UTR sequences. In contrast, regulation of expression in Leishmania heat shock genes is mainly through specific 3'UTR sequences (Aly et al., 1994), involved in the poly-adenylation of heat shock protein transcripts. These sequences not only determine the relative stability of the HSP RNA at 23°C and 37°C, but also influence their translational efficiencies by modulating their ability to bind to ribosomes (Quijada et al., 1997; Quijada et al., 2000; Zilka et al., 2001; Boucher et al., 2002).

These differences are mainly due to the unusual organization of Kinetoplastid genomes. Most genes in Leishmania and Trypanosoma (Graham, 1995; Myler et al., 1999; Martinez-Calvillo et al., 2003; Worthey et al., 2003; Martinez-Calvillo et al., 2004; Ivens et al., 2005), notably those encoding members of the HSP70 and HSP90 families, are organized into large, tandem clusters on the same DNA strand (Lee et al., 1988; Shapira and Pinelli, 1989).
RNA polymerase II mediated transcription results in the synthesis of poly-cistronic pre-RNA molecules, lacking introns. Mature mono-cistronic RNA molecules are generated by trans-splicing and poly-adenylation (Perry and Agabian, 1991; LeBowitz et al., 1993). As a result of this constitutive synthesis of transcripts, differential regulation of gene expression relies both on the relative stabilities and translational efficiencies of the mature transcripts (Beetham et al., 1997; Burchmore and Landfear, 1998; Wu et al., 2000; Brittingham et al., 2001; Papadopoulou, et al., 2003; Folgueira et al., 2005). For example, depending on the *Leishmania* species, there is a 1.5 – 5 fold induction of heat shock mRNA levels during heat stress (Lee et al., 1988; Shapira and Pedraza, 1990; Aly et al., 1994). Some studies attribute this increase to the enhanced stability of the transcribing RNA species under heat shock. Increased mRNA stability may facilitate up regulation of heat shock protein levels during an extended period of heat stress – for example, in the mammalian stage of the parasite life cycle. However, the rapid kinetics of the stress response observed in *in vitro* cultures suggests regulated translation as an additional means for short-term stress response (Clos and Krobitsch, 1999).

2.C.7. Emerging roles for Heat Shock Proteins in *Leishmania*

Many of the identified heat shock proteins have been associated with specific roles in the life cycle of *Leishmania* parasites. For example, Hsp90 is known to be essential for cell division in both *Leishmania sp.* and *Trypanosoma sp.* (Wiesgigl and Clos, 2001; Graefe et al., 2002). In *Leishmania sp.*, especially, Hsp90 proteins seem to have a key role in the rapid growth kinetics of the promastigotes stages. A transient increase in levels of this protein has been linked to survival in parasites given short exposures to mammalian temperatures. Given their conventional roles in other cells, this suggests that Hsp90 might have an important role in mediating a negative feedback loop that inhibits the heat activated stress response. In *Leishmania sp.*, but not in *Trypanosoma sp.*, the pharmacological abrogation of Hsp90 activity using inhibitors like geldanamycin and radicicol is known to trigger differentiation of promastigotes into amastigotes (Wiesgigl and Clos, 2001; Folgueira and Requena, 2007). This indicates that expression of the Hsp90 protein may be essential for survival and development of the promastigote forms of the parasite.
Inducible expression of certain members of the Hsp70 family have been associated with increased survival on exposure to toxic doses of hydrogen peroxide (Wilson, 1994; Miller, 2000), as well as increased resistance to potassium antimony tartarate or PAT (Brochu et al., 2004). In addition, increase in levels of these proteins has been observed in promastigotes exposed to short: 1 - 2 hours or chronic: 24 - 48 hours (Raina and Kaur, 2006) periods of heat shock at mammalian temperatures. In view of their conventional roles in other cell systems, it is likely that these Hsp70 proteins have a protective role in the cell, but the implications and mechanisms of their increase in the parasite have yet to be determined.

Hsp100 proteins are known to show a distinct stage specific expression pattern (Hübel et al., 1997). Knockdowns of the ClpB gene showed lower expression levels of certain amastigote specific A2 genes. In addition, they exhibited altered morphologies on differentiation into amastigotes (Krobitsch et al., 1998); and reduced persistence after in vivo infections of peritoneal macrophages (Krobitsch and Clos, 1999). These indicate a role for Hsp100 in not just the regulation of stage specific gene expression of the parasite, but also in ensuring structural integrity of amastigotes to enhance their in vivo persistence in macrophages.

In addition to their parasite-specific roles, many heat shock proteins are also important in determining and influencing host-parasite interactions. For example, levels of parasite Hsp70 have been shown to increase in infected macrophages (Rey-Ladino et al., 1993). Both Hsp70 and Hsp90 proteins are known to be strong B-cell mitogens (Rico et al., 2002). Hsp70 is an immuno-dominant parasite epitope (Rico et al., 1998; Rico et al., 1999) and peptides derived from it via antigen processing are known to play important roles in the immunology of Leishmania infections.

2.0. Bibliography


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