Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis

Intracellular protozoan parasites of humans: the role of molecular chaperones in development and pathogenesis

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ABSTRACT

Certain kinetoplastid (*Leishmania* spp. and *Tryapnosoma cruzi*) and apicomplexan parasites (*Plasmodium falciparum* and *Toxoplasma gondii*) are capable of invading human cells as part of their pathology. These parasites appear to have evolved a relatively expanded or diverse complement of genes encoding molecular chaperones. The gene families encoding heat shock protein 90 (Hsp90) and heat shock protein 70 (Hsp70) chaperones show significant expansion and diversity (especially for *Leishmania* spp. and *T. cruzi*), and in particular the Hsp40 family appears to be an extreme example of phylogenetic radiation. In general, Hsp40 proteins act as co-chaperones of Hsp70 chaperones, forming protein folding pathways that integrate with Hsp90 to ensure proteostasis in the cell. It is tempting to speculate that the diverse environmental insults that these parasites endure have resulted in the evolutionary selection of a diverse and expanded chaperone network. Hsp90 is involved in development and growth of all of these intracellular parasites, and so far represents the strongest candidate as a target for chemotherapeutic interventions. While there have been some excellent studies on the molecular and cell biology of Hsp70 proteins, relatively little is known about the biological function of Hsp70-Hsp40 interactions in these intracellular parasites. This review focuses on intracellular protozoan parasites of humans, and provides a critique of the role of heat shock proteins in development and pathogenesis, especially the molecular chaperones Hsp90, Hsp70 and Hsp40.

INTRODUCTION

Intracellular parasites, by their nature, survive and multiply in a potentially hostile environment, the cells of their host. Even after they have adapted to this environment, parasites are forced to leave the now relatively safe haven of the host animal to seek "new pastures", and to spread to further hosts. Often, this transmission cycle is carried out within the body of arthropod vectors, although some parasites, such as *Toxoplasma gondii*, endure direct exposure to the external environment in their quest for a new host. Even once within the host or vector, parasite migration brings these organisms into contact with changing conditions. Whether transmitted by an insect vector, or directly, it is clear that the life cycle of these parasites entails multiple changes of environmental conditions, including temperature, pH, oxidative stress, as

well as desiccation. Add to this the threat of clearance by the host immune system, and it becomes clear that, for these parasites, staying alive is a constant challenge. To ensure survival and propagation under these harsh extremes, the parasites have evolved numerous mechanisms to counter these conditions, including antigenic variation and modulation of host cells. Nevertheless, environmental changes are an integral part of the life cycle of these parasites. Studies on model systems have, over the past few decades, revealed an important role for proteins of the molecular chaperone class in allowing cells to survive, and adapt to, changing conditions.

Increasing genomic sequencing data has revealed many novelties that seem to be specific to an intracellular life cycle, including a tendency for intracellular organisms (whether symbionts, bacteria or parasites) to undergo genome reduction, simplifying or indeed losing many processes common to non-intracellular organisms [1-3]. Whilst some of this can be explained by increased nutrient availability (with the host cell providing much of what is needed in an easy to access form, e.g. amino acids), it has also been suggested that this strategy produces “more bang for the buck”, that is, allowing the production of more infectious units per unit of metabolic effort. It is thus even more surprising to note that, despite this genome reduction, many intracellular pathogens still contain a large, indeed sometimes expanded, complement of molecular chaperones. Although studies on the importance of molecular chaperones in an intracellular life cycle are still very much in their infancy, the data so far supports an important role for this class of proteins in not just intracellular survival and differentiation of these parasites, but also their virulence.

MOLECULAR CHAPERONES

Molecular chaperones act as quality control factors in the cell, facilitating the correct folding and assembly of other proteins, or the degradation of proteins misfolded beyond repair [4]. They are therefore vital for ensuring that the structural integrity of the cellular protein machinery is maintained under normal physiological conditions, but especially under conditions of cell stress or disease states. However, molecular chaperones are more than quality control factors; they are also responsible for regulating protein conformational state, thereby holding proteins (especially signalling proteins) in a primed state that is readily activated according to a particular signal (e.g. post-translational modification, or ligand binding). The cellular functions

of some of the major molecular chaperones (e.g. heat shock protein 70 [Hsp70] and heat shock protein 90 [Hsp90]) are regulated by a cohort of co-chaperone proteins (e.g. Hsp70/Hsp90 organizing protein [Hop], and heat shock protein 40 [Hsp40]). Several different co-chaperone-regulated protein folding pathways are conserved from prokaryotic to eukaryotes, and are interconnected to form a well organized chaperone network within the cell [5, 6]. More recently, a new role for molecular chaperones as signal transducers has been recognised and this role is now generally regarded as 'chaperokine' function (reviewed by [7]). It is thought that chaperokines, especially Hsp70, are capable of modulating immune cells by binding to their cell surfaces.

Hsp90 is a highly abundant molecular chaperone, and in eukaryotic systems normally occurs as five different isoforms; two cytosolic (inducible Hsp90α and constitutive Hsp90β), an endoplasmic reticulum (ER) localized glucose regulated protein 94 (Grp94), a mitochondrial tumour necrosis factor receptor-associated protein 1 (TRAP1), and a membrane-associated HSP90N [8]. There are over 300 different Hsp90 client proteins, consisting mainly of transcription factors and kinases, including certain oncogenic proteins (androgen/estrogen receptors and proto-oncogenic protein kinases) and prion proteins. The Hsp70/Hsp90-organizing protein (Hop), also known as stress-inducible protein 1 (STI1), coordinates the functional cooperation between Hsp70 and Hsp90, so as to ensure efficient delivery of these client proteins from Hsp70 to Hsp90 (reviewed in [9]). Hsp70 in partnership with its co-chaperone Hsp40 is the prototypical molecular chaperone machine involved in ensuring protein homeostasis in the cell, and including the capture and delivery of client proteins to Hsp90. There are numerous different Hsp70s (13 members in humans) and an even great number of Hsp40s (49 members in humans; [8, 10]). The interaction of Hsp40s with their partner Hsp70s is dependent on their signature J domain (reviewed in [11]), and a number of key residues required for general binding and specificity have been identified [12, 13]. Hsp40s have been categorized into four classes (Types I-IV) based on the presence or absence of functional domains in addition to the J domain (Types I-III) or J-like domain (Type IV). Many of the Types I and II Hsp40 proteins are capable of binding substrates and targeting them to an Hsp70, while the Type III proteins are highly specialized serving mainly to recruit an Hsp70 to a specific location [11]. The Type IV Hsp40s have a J-like domain in which the highly conserved histidine-proline-aspartic acid (HPD) motif is corrupted [14].

Indeed, Hsp40s potentially confer specificity on the Hsp70-Hsp40 chaperone machinery through their ability to deliver a defined range of protein substrates to specific partner Hsp70s, or by concentrating Hsp70s in the vicinity of a substrate [11, 15, 16]. A particular Hsp70 potentially interacts with more than one Hsp40, with each Hsp40 processing a specific set of substrates.

The Hsp70-Hsp40 chaperone machinery processes at least 15% of all proteins synthesized in the cell, and a subset of these protein substrates are ultimately destined to be Hsp90 client proteins. The diversity of substrate proteins processed through the Hsp70-Hsp40 and Hsp90 chaperone pathways reflects their involvement in a variety of fundamental cellular processes such as proliferation, differentiation, development, the stress response, and pathogenesis [17, 18]. It is well established that Hsp90, Hsp70 and their co-chaperones are over-expressed in different human diseases, especially most cancers where they contribute to cancer progression and metastasis [19, 20]. Hsp90 is involved in the morphological development of multicellular organisms, and has been proposed to act as a molecular capacitor of morphological evolution [21, 22]. There are reviews on the stress response of protozoans [23], and the heat shock proteins of kinetoplastids [24] and apicomplexa [14, 25, 26]. Recent experimental evidence has indicated that molecular chaperones are involved in the development and pathogenesis of infectious diseases caused by protozoan parasites of humans. Although many chaperones have been shown to be recognised by immune sera from infected individuals, for the purposes of this review we shall not discuss these proteins as potential vaccine candidates. This review rather focuses on intracellular protozoan parasites of humans, and provides a critique of the role of heat shock proteins in development and pathogenesis, especially the molecular chaperones Hsp90, Hsp70 and their associated co-chaperones.

KINETOPLASTID PARASITES

Parasites of the class Kinetoplastida are characterised by their possession of the kinetoplast, a mass of DNA found within the single mitochondrion. Included in this class are several important human pathogens, which cause Chagas disease (*Trypanosoma cruzi*), sleeping sickness (*Trypanosoma brucei* spp.) and leishmanasis (*Leishmania* spp.). These parasites cycle between arthropod vectors and the human host, encountering numerous changes in environment, many of which are

accompanied by stage differentiation of the parasite. Unusually, regulation of protein expression in these parasites takes place almost exclusively at the post-transcriptional level [27], thus all reactions to environmental change must be regulated by post-translational mechanisms. In this section we shall concentrate our attention on the intracellular *Leishmania* spp. and *T. cruzi*.

***Leishmania* spp.**

Introduction

Parasites of the genus *Leishmania* cause leishmaniasis in a variety of vertebrate hosts. *Leishmania* are spread by the bite of an infected sandfly, which transmits the promastigote stage of the parasite into the human skin, where they then invade host macrophages. Within macrophages, the parasite differentiates into the amastigote stage, and multiplies. Depending on the host immune response, and particular species of parasite, the disease can manifest itself in different ways, each with its own specific health risks, ranging from the mild cutaneous, to the potentially lethal visceral form [28]. *Leishmania* spp. are commonly found in tropical and sub-tropical regions. Amongst parasitic diseases, only malaria kills more people than leishmaniasis, with the disease afflicting an estimated 500 000 people annually [29, 30]. As the interplay of host immune response and parasite strain often determines the seriousness of disease manifestation, a growing problem is co-infection with *Leishmania* and HIV [31]. Additionally, increasing resistance to commonly used drugs such as pentavalent antimony is a cause for alarm, and new treatment strategies will be needed to keep the parasite, and thus the disease, in check [32, 33].

In common with other kinetoplastid parasites, *Leishmania* spp. undergo a complex life cycle involving progression from an insect host (promastigote stages, with an anterior flagellum) to a mammalian host (amastigote stages) and back again [34]. During this transition the parasites experience an increase in ambient temperatures. In the past decade, numerous studies have revealed that heat shock proteins induced by this temperature shift play various important roles in the parasite's survival, virulence and proliferation. In this section we shall detail how molecular chaperones are intimately involved in a variety of processes related to parasite development and pathogenesis.

Hsp90 family

Early reports suggested multiple copies of the *HSP90* gene, with more recent data based on genomic sequencing revealing up to 17 gene copies in *L. major*, which are arranged into tandem clusters and encode essentially identical proteins [24, 35] (Table 1). Multiple copies of *HSP90* are common amongst *Leishmania* spp., and seem to allow for high synthesis levels of the encoded proteins in an organism that relies on post-transcriptional regulation [36]. Indeed, in support of this hypothesis it has been estimated that Hsp90 constitutes 2.8% of the entire protein content of *L. donovani* promastigotes, and that absolute levels of Hsp90 increase in parasites subjected to a rise in temperature [37]. More importantly, LmHsp90 appears to play a pivotal role in stage differentiation from promastigote to amastigote. Treatment of promastigote stage parasites with the Hsp90 inactivating agent geldanamycin (GA) induced synthesis of amastigote specific proteins including A2 [38, 39]. Additionally, the morphological changes of the parasites upon GA treatment was highly similar to those induced by heat shock, which itself has also been shown to induce differentiation. Although long-term treatment of parasites with GA led to growth arrest, occasionally spontaneous escape mutants could be isolated which proliferated normally. Genotypic analysis of these mutants revealed amplification of the *HSP90* locus and an increased level of Hsp90 [38]. Taken together, the above data suggest that removal of active Hsp90 by either heat shock induced sequestration or inactivation by GA is a trigger for stage differentiation, and is a fascinating example of a parasite that apparently uses its chaperone complement as a cellular thermometer to sense the environment and control developmental processes.

In addition to the multi-copy Hsp90 family, *Leishmania* also encode a single glucose regulated protein 94 kDa (Grp94) protein (Table 1). Originally cloned from *Leishmania infantum*, the protein localises to the ER and is required for synthesis of phosphoglycans including lipophosphoglycan (LPG) which in turn is implicated in *Leishmania* virulence [40-43]. Lack of LPG synthesis by inactivation of *GRP94* attenuates virulence but does not otherwise appear to affect parasite viability [44]. Thus, it appears that, in contrast to other model systems, the activity of *Leishmania* Grp94 is focused largely on parasite virulence, with little or no influence on viability.

In recent decades it has become clear that Hsp90 associates with numerous co-chaperones that help to regulate protein function and association into multi-chaperone complexes. A recent study identified a plethora of potential *Leishmania* Hsp90 co-chaperones by *in silico* searches. So far, only two of these, Hop/STI1 and small glutamine-rich tetratricopeptide repeat protein (SGT) have been characterised in detail [45, 46]. As both of these co-chaperones interact with multiple chaperone partners, we discuss their role in parasite viability in later sections.

Hsp70 family

As for *HSP90*, *Leishmania* contain multiple copies of *HSP70*, with the absolute copy number varying between strains (*L. major* Hsp70 proteins listed in Table 1). Although early data suggested a *L. major* complement of 5 *HSP70* genes encoding cytoplasmic proteins, 4 of which were arranged in tandem [47], later analyses revealed a further 2 gene copies [24, 48]. Additionally, *L. major* encodes 5 putative mitochondrial Hsp70s, the genes of 4 of which are arranged in a tandem array [24]. Cytosolic Hsp70 represents 2.1% of the total protein content of *L. major* promastigotes, and heat shock has been shown to increase Hsp70 abundance [37].

Upon being taken up by macrophages, parasites are exposed not only to heat shock, but also to various macrophage defence mechanisms including release of reactive oxygen species, all of which heighten parasite oxidative stress. Experiments demonstrate that heat shock treatment of *Leishmania chagasi* (mimicking transmission to the vertebrate host), leads to heightened parasite resistance to this stress. Hsp70 may be involved in this, as further experiments could demonstrate that over-expression of LcHsp70 in promastigotes leads to increased resistance to macrophage induced oxidative stress. This would appear to be a parasite survival strategy to guarantee survival in a hostile environment, within the professionally phagocytic macrophage [49].

In addition to a role in parasite survival in a natural situation, Hsp70 has also been implicated in drug resistance. Induced over-expression of *L. tarentolae* Hsp70 was associated with a significantly increased resistance to pentavalent antimony [50]. Although the authors of this study concluded that this is likely to be an indirect effect,

their data strongly support a link between Hsp70 levels and the metal resistance phenotype observed.

In common with many organisms, *Leishmania* encode a protein homologous to glucose regulated protein 78 kDa/immunoblobulin binding protein (Grp78/BiP) which has been localised to the ER in *L. donovani*, and most likely is involved in translocation of secretory proteins into the ER lumen, and protein quality control within this compartment [51]. Although the role of BiP itself in parasite virulence has, to our knowledge, not been determined, a recent study demonstrated both co-localisation and direct interaction of *L. donovani* BiP with the virulence associated protein A2, and could also show that ectopic expression of A2 in *L. major* (which lack endogenous A2) increased parasite survival after heat shock [52]. Thus, A2 itself is potentially a stress-response protein, although evidence for a direct molecular role of A2 as such is still lacking.

Although a recent study identified 66 putative Hsp40 proteins in *Leishmania*, none of these have been studied at a molecular level [24] (Table 1). Given the importance of Hsp70 in stress response and virulence, it is likely that Hsp40 proteins may, due to their essential co-chaperone effect on their partner Hsp70s, also be required for parasite virulence. Further studies will be required to investigate the Hsp70/Hsp40 partnerships.

Further chaperones, co-chaperones and multi-chaperone complexes

The gene encoding *L. major* Hsp100 (LmHsp100) was first cloned in 1994 [35]. Early studies demonstrated that, following transfer from the sandfly to the mammalian host, Hsp100 production is induced and persists. Indeed, the presence of Hsp100 appears to be required for synthesis of amastigote stage proteins, including the amastigote specific protein A2 [53] (see also the following sections on Hsp90 influence on A2 synthesis, and the role of A2/BiP interactions in the stress response). Although inactivation of the *HSP100* gene does not lead to a block in differentiation from the promastigote to amastigote stage, knock-out parasites have decreased virulence and are unable to proliferate in host macrophages [54]. Repeated mouse infection cycles using these deletion mutants eventually led to the isolation of escape mutants with increase virulence, although wild type virulence could not be fully re-established [55].

Later studies, using cosmid complementation to select for escape mutants in the Δ*hsp100* background isolated a 46kDa protein that was responsible for this phenotypic reversion [56]. Although this protein has not been characterised in any great detail, it does not appear to be a molecular chaperone and thus is unlikely to actually complement the Δ*hsp100* mutation in terms of function, but rather compensates for the lack of Hsp100 (or downstream effects) by another mechanism [56]. Although, in other systems, the loss of Hsp100 can be partially compensated for by higher levels of Hsp70, this could not be shown in the spontaneous escape mutants [55]. Unusually, LmHsp100 does not seem to form homohexameric complexes, usually required for the function of the caseinolytic peptidase B protein homolog/heat shock protein 104 (ClpB/Hsp104) superfamily to which this protein belongs, but rather trimeric complexes [53]. Taken together, these data suggest that LmHsp100 carries out a highly specialised (although until this point poorly defined) and important function, and is essential for wild type virulence.

Calreticulin, a specialized ER chaperone, regulates protein disulphide isomerase (PDI) and the 57kDa ER protein (Erp57) function, helps in folding of newly synthesised glycoproteins, and also has a role in protein quality control. Over-expression of a truncated (thus potentially dominant negative) form of calreticulin in *L. donovani* leads to decreased secretion of acid phosphatases (one of the major secreted glycoproteins), and a lower survival rate in macrophages. Interrupting the calreticulin system may thus lead to incorrect trafficking, or incorrect folding of proteins secreted to the macrophage and which may be essential for modulation of macrophage defence mechanisms [57].

For optimal function and/or correct regulation, many chaperones require the presence of co-chaperones. An *in silico* analysis has previously identified numerous putative co-chaperones [24]; however the function of only two have been studied in any detail. Ommen *et al.* identified an atypical small glutamine-rich tetratricopeptide repeat protein (SGT) [45]. In other systems, SGT has been demonstrated to interact with both Hsp70 and Hsp90, likely through the tetratricopeptide repeat (TPR) domains. SGT from *L. donovani* was demonstrated to interact (either directly or indirectly) with LdHop, LdHip and LdHsp70. Although a strong association with LdHsp90 could not be directly observed, immunofluoresence experiments suggested at least co-

localisation. To study the function of SGT, the authors attempted gene disruption. Initial experiments were not successful, however the presence of an add back copy of the gene allowed the endogenous gene loci to be deleted, indicative of an important function for this protein [45]. Further studies will be required to identify the exact role of this protein in modulating chaperone complex formation.

A recent study addressed the formation of heat shock protein complexes in *L. donovani*, and was able to show evidence for stage specific complex formation [46]. This study also identified several LdSTI1/Hop containing complexes, thus implicating a central role for this protein in assembly of functional multi-chaperone complexes. To investigate how this complex formation may be regulated, the authors attempted to inactivate *sti1*. Direct gene inactivation was not achieved; however the addition of an episomal *sti1* copy eventually allowed gene disruption, suggesting an essential function for LdSTI1. To gain further insight into this observation, mutant forms of LdSTI1, lacking potential phosphorylation sites were expressed in the parasites, and tested for their ability to complement the gene inactivation. This analysis revealed that two putative phosphorylation sites were essential for LdSTI1 function and hence *L. donovani* viability [46]. The authors suggest that, in the absence of a transcriptional control of protein levels, the parasites may use phosphorylation to regulate levels of functional protein, and thus assembly of multi-chaperone complexes.

Trypanosoma cruzi

Introduction

T. cruzi is transmitted by triatomine insect vectors, and is the causative organism of Chaga's disease (also called American trypanosomiasis) in humans, with approximately 17 million people infected [58]. In the insect gut, epimastigotes replicate and differentiate into metacyclic trypomastigotes, the infective form of *T. cruzi* that is transmitted to the human host [59]. This stage enters the bloodstream of the human host and invades a variety of cell types, where it enters the cytosol and differentiates into amastigotes. Several lines of experimental evidence suggest that *T. cruzi* invades by triggering the recruitment and fusion of lysosomes at the plasma membrane, which facilitates entry of the parasite into the cell [60]. Within an hour, the parasite induces lysis of the lysosomal membrane, and enters the cytosol.

Intracellular amastigotes differentiate into trypomastigotes that are released into the blood and invade other cells or are taken up by the insect vector to continue the cycle. Therefore, as for other kinetoplastids, drastic environmental changes accompany dramatic developmental changes in the parasite. Interestingly, unlike for *L. donovani*, the developmental stages of the *T. cruzi* life cycle do not correlate with the stages of temperature change, suggesting that stage differentiation does not require a heat stress trigger [61]. Therefore, the heat shock encountered when moving from the insect vector (~26^{o}C) to the human host (~37^{o}C) occurs prior to the differentiation of trypomastigotes to amastigotes. While *T. cruzi* stage development does not appear to be temperature-dependent *per se*, the levels of heat shock proteins appear to increase in response to both temperature increases and developmental transitions. It is well established that *T. cruzi* responds to heat shock by increasing the synthesis of a range of different heat shock proteins, including Hsp100, Hsp90, Hsp70 and Hsp60 [62, 63]. Furthermore, proteomics analyses of developmental stages of *T. cruzi* have shown increased synthesis of Hsp90, Hsp70 and Hsp60 [64] In particular, for the transition from trypomastigote to the amastigote stage, in addition to the increase in heat shock proteins, there is an almost exclusive increase in proteins involved in ER to Golgi trafficking [65]. These changes in heat shock protein levels may be a requirement for developmental progression and/or represent a mechanism for adaptation to stress. However, before an understanding of the biological role of these proteins can be determined, the specific isoforms need to be identified, and their molecular chaperone properties elucidated.

Hsp90 family

A *T. cruzi* gene encoding the major cytosolic Hsp90 (also called Hsp83) has been characterized [66], and a recent analysis of the genome revealed the presence of six homologous genes [24] (Table 1). Three genes encoding Grp94, and two genes encoding TRAP1 have also been identified (Table 1). There is also a gene encoding a *T. cruzi* Hop, suggesting that the Hsp90-Hop-Hsp70 chaperone machinery is functional in this parasite. The Hsp90 inhibitor, GA, has been shown to cause growth arrest in *T. cruzi* [61], as it has for the other parasitic protozoa, *L. donovani* [38], *P. falciparum* [67], and *T. gondii* [68]. However, various doses of GA manifest disparate effects on the different parasite species. In *L. donovani* the elevated temperature encountered during the transmission from a sand fly to a mammalian host triggers

stage progression from the promastigote to the amastigote stage [38]. Treatment of *L. donovani* with low doses of GA mimics this stage differentiation, while treatment with high doses causes growth arrest of the promastigote. Treatment of *T. cruzi* with GA does not trigger stage development, which is consistent with the finding that the progression of the natural life cycle is not correlated with temperature change [61]. Treatment of *T. cruzi* with GA causes growth arrest and prevents trypomastigote-to-epimastigote differentiation, suggesting that Hsp90 is important for the maturation of proteins involved in epimastigote differentiation.

Hsp70 family

The *T. cruzi* Hsp70 complement contains representatives of all the major isoforms found in higher eukaryotes, as well as a number of unusual isoforms (Table 1). If partial gene sequences are included, *T. cruzi* potentially encodes 28 Hsp70 proteins [24]. However, if these partial gene sequences are excluded, there are only 11 genes encoding full-length Hsp70 proteins (Table 1). The cytosolic Hsp70.4 isoform, closely related to the canonical cytosolic Hsp70, was found to be highly enriched in amastigotes, and undetectable in trypomatigotes [65]. In contrast, the Hsp70-like isoform, Hsp70.a, was found to be expressed exclusively in trypomastigotes. Therefore, Hsp70.4 and Hsp70.a are potentially important in *T. cruzi* stage development. Interestingly, the *T. cruzi* Hsp70.4 was identified on the basis of sequence similarity to *L. major* Hsp70.4; however its gene is one of those that remains to be full annotated on the genome.

Hsp40 family and Hsp70-Hsp40 partnerships

T. cruzi possesses a relatively large Hsp40 complement of 67 proteins [24] (Table 1). Very few of these Hsp40s have been biochemically characterised, and very little is known about potential Hsp70-Hsp40 partnerships. Five cytoplasmic *T. cruzi* Hsp40 proteins have been biochemically characterized (Tcj1, a type III Hsp40; Tcj2, Tcj3, and Tcj4, all type I Hsp40s; and Tcj6, a type II Hsp40 [69, 70], as well as a mitochondrial Hsp40 (TcDJ, a type III Hsp40) [71]. Tcj2 mRNA levels increase under heat shock [69], and it is able to stimulate the ATPase activity of the major cytosolic, heat inducible Hsp70 [72]. Furthermore, it can functionally substitute for the essential yeast Hsp40, Ydj1 [72]. Tcj6 can functionally substitute for the yeast Hsp40, Sis1, and associates with ribosomes [70]. Therefore, Tcj2 may functionally interact with

TcHsp70 *in vivo*, and is potentially an essential protein, especially under stress conditions, while Tcj6 may play an important role in protein synthesis. TcDJ1 is highly upregulated in epimastigote compared to metacyclic trypomastigotes, suggesting that it is developmentally regulated, and potentially involved in mitochondrial biosynthetic pathways. A more in-depth and systematic analysis of *T. cruzi* Hsp70-Hsp40 partnerships is needed.

T. cruzi and Leishmania spp. versus T. brucei – being inside or outside of a cell

A thorough comparison of the chaperone machineries of the intracellular kinetoplastids to that of their extracellular relative (*T. brucei*) would provide valuable insights into the biology of these parasites. However, as for the intracellular kinetoplastid parasites, the role of molecular chaperones in the life cycle of *T. brucei* is poorly understood. *T. brucei* appears to have a reduced chaperone complement (with genes encoding 12 Hsp70s, 3 Hsp90s, and 65 Hsp40s) compared to *T. cruzi* and *Leishmania* spp.; however, the higher number of genes encoding chaperones in the intracellular parasites appears to be the result of gene duplication so that the actual number of unique family members are similar to *T. brucei* [21]. The *T. brucei* Hsp70 proteins have been extensively studied (especially cytosolic TbHsp70), with many isoforms expressed in both the insect and mammalian stages of the life cycle [73, 74]. In contrast, very few *T. brucei* Hsp40 proteins have been biochemically characterized (e.g. Tbj1; [75]). Using RNA interference (RNAi) knockdown studies, certain *T. brucei* Hsp70 and Hsp40 proteins (especially ER chaperones; e.g. TbBiP) have been implicated in protein secretion, glycosylation (including variant surface glycoprotein (VSG) presentation), and cell viability [76, 77]. The requirement for high levels of VSG on the surface of *T. brucei* potentially represents an adaptation to survival in an extracellular environment that places a unique burden on its chaperone machinery.

APICOMPLEXAN PARASITES

Apicomplexan parasites are characterized by the apical complex containing secretory organelles and microtubular structures involved in the attachment and penetration of host cells. Most apicomplexan also host an essential organelle, the apicoplast. The apicoplast appears to be a modified chloroplast containing plastid DNA. It has been implicated in the lipid metabolism, heme and amino acid synthesis. The group includes important human pathogens like *Plasmodium ssp.* (causing malaria) and

Toxoplasma spp. (causing toxoplasmosis). Apicomplexans infect both invertebrates and vertebrates, but their mode of transmission varies: some are transmitted by insects, while other are transmitted in faeces of an infected host or when a predator eats infected prey.

Plasmodium spp.

Introduction

The malaria parasite *Plasmodium* spp. belongs to the group of apicomplexan protists. Five different *Plasmodium* species cause malaria in humans, of which *Plasmodium falciparum* is the most deadly. Several hundreds of million people contract malaria and almost 1 million human lives are lost each year due to this disease [78-80].

The parasite's complex life cycle starts with the inoculation into the blood stream of the human host by the bite of an infected mosquito. From here the parasite gets to the liver, where it invades liver-cells. After a time of transformation and replication merozoites are released into the blood stream again. Merozoites are the forms that invade red blood cells. Within a red blood cell the merozoites grow and divide. After 48-72 hours – depending on the species – the host cell ruptures and 8-32 new merozoites are released to invade uninfected red blood cells [81]. The infection within the human body is maintained by this cycle of invasion, multiplication and egress. This is also the stage of infection responsible for the characteristic symptoms of a malaria infection like periodic fever episodes, anemia and deterioration of vital organs. Some of the parasite cells differentiate into gametocytes, which can develop further, when taken up by an anopheles mosquito [82]. The sexual processes take place in the midgut of the mosquito. After another transformation and multiplication step the parasites end up in the salivary glands of the mosquito, ready to be transferred into another human during the mosquito's next blood feed [83].

Outline of chaperone networks in *Plasmodium*

Most of the molecular data on human malaria parasites stems from studies on *P. falciparum*, as it can be readily maintained and propagated in the laboratory, its genome sequence has been known since 2002, and it is particularly virulent. Both the

interactions of the plasmodium protein network and its underlying genome sequence diverge from those of other eukaryotes [84]. This divergence is also reflected in peculiarities in the chaperone system. The genome of *P. falciparum* encodes ~95 chaperones and co-chaperones, which accounts for roughly 2% of the total number of genes. The protein family of Hsp70 and Hsp90 are fairly conserved, with 6 (PfHsp70-1/PF08_0154; PfHsp70-2/PFI0875w; PfHsp70-3/PF11_0351; PfHsp70-x/MAL7P1.228; PfHsp70-y/MAL13P1.540; and PfHsp70-z/PF07_0033) [26] and 4 orthologues (PF07_0029; PFL1070c; PF11_0188 and PF14_0417) [25], respectively (Table 1). The genome does not encode homologues of calnexin and calreticulin, which are specialized chaperones involved in the folding of N-glycosylated proteins [85]. This is not surprising given the absence of a protein N-glycosylation pathway in the parasite [86]. The PfHsp40 family with 44 members [14, 25, 87], however, shows a massive radiation (in comparison to the 22 Hsp40 proteins in *Saccharomyces cerevisiae* [15]).

Many interactions are based on indirect evidence like yeast two hybrid screen or *in silico* placement by homology. Hard experimental evidence is relatively scarce. Naturally most of the knowledge is derived from the asexual (blood) stages, since sexual (mosquito) stages are more cumbersome to access. Nonetheless the multitude and importance of cellular processes chaperones are involved in become more and more obvious.

Development

The life-cycle of the malaria parasite *Plasmodium* spp. is characterized by the change between the poikilothermic insect vector, the mosquito, and the human host. In both the mosquito and the human there are stages of massive replication (sporogony in the mosquito mid gut and schizogony in the human liver and red blood cells), which puts major demands on the synthesis and transport of cellular components to maintain cellular homeostasis. Both PfHsp70 and PfHsp90 are essential for the development of *P. falciparum* as studies involving specific inhibitors have shown [67, 88, 89]. Specific pyrimidinones, which have an effect on Hsp70, inhibit parasite growth [89]. Likewise, upon treatment with geldanamycin, a benzoquinone ansamycin antibiotic that interferes with Hsp90 function, the development of the parasite is arrested in ring stages [67].

DNA metabolism

During the phases of multiplication, the parasite must provide effective means to control protein synthesis and DNA replication. The *P. falciparum* homologue of the Hsp90 co-chaperone p23 (PF14_0510), has been shown in yeast two hybrid experiments to interact with the *P. falciparum* DNA topisomerase II (PF14_0316) and the chromosome associated protein PFD0685c [90]. Recently, a direct interaction with PfHsp90, chaperone activity and suppression of PfHsp90 ATPase activity was shown for Pfp23 *in vitro* [91].

One of the characteristics of apicomplexan is the presence of an essential organelle, the apicoplast. This apicoplast, which is related to chloroplast of plants, contains its own circular genome. However, similar to other organelles like mitochondria, some apicoplast genes have been transferred to the nucleus. A nuclear encoded PfHsp40 protein (Pfj1; PFD0462w) was found to interact with the origin of replication of the circular apicoplast genome, and a role for this Hsp40 in replication or repair of the apicoplast genome was suggested [92].

Adaptation to the human host

Inside the human body the parasite encounters two events involving temperature change: when initially being injected by the mosquito into the human body, and during the fever response of the human host. In addition to the required changes in metabolism, this temperature difference must have a major impact on the parasite. It is believed that the parasite uses these fever peaks as environmental cue for synchronization. An orchestrated, coordinated release of invasive forms, the merozoites, puts an excessive demand on the immune system in a short timeframe. This leads to the successful evasion of the immune system by some of the parasites, which once inside the cell, are again out of the reach of the immune system. Both PfHsp90 and PfHsp70 have been shown to be induced and translocated to the nucleus upon exposure of the parasite to 41^{o}C [25, 93]. Pavithra et al. also observed acceleration parasite maturation upon heat shock [93]. Further evidence for an important role of PfHsp70 in providing thermotolerance was obtained by heterologously expressing PfHsp70 in a thermosensitive *E. coli* DnaK-null mutant. PfHsp70 could restore the ability of this *E. coli* strain to withstand thermal stress [94].

In an attempt to compile a proteomic profile of clinical *Plasmodium* isolates several highly abundant chaperones were identified: PfHsp90 (PF07_0029), PfHsp70-1 (PF08_0054), PfHsp70-x (MAL7P1.228) and two putatively exported PfHsp40 proteins (RESA/PFA0110w; and PF11_0509) were abundant enough in ring stages to be identified [95]. The parasites have to endure the fever episodes while being in the ring stage. Complementary to this proteomic analysis, Oakley et al. compared parasites kept at 37^{o}C to parasites incubated at 41^{o}C for 2 h by microarray analysis [96]. The analysis of this heat shock on the genome wide expression level of proteins revealed an up-regulation of not only PfHsp90 and PfHsp70 (2.4 and 5.3-fold respectively), but also eight PfHsp40 proteins.

Another PfHsp40 protein implicated in protection of the parasite during febrile episodes is RESA (PFA0100c), the ring-infected erythrocyte surface antigen. It is probably the best-studied PfHsp40 in *Plasmodium*. After invasion and release of dense granule contents into the newly formed parasitophorous vacuole (PV), this molecule is transferred to the red blood cell membrane skeleton to stabilize spectrin against thermally-induced denaturation and dissociation [97]. It has been reported to play a role in protecting the red blood cell membrane during febrile episodes and red blood cells infected with RESA deficient parasites are more susceptible to heat-induced vesiculation and show rigidity during febrile episodes [98, 99]. RESA is found in all field strains examined so far, but can be disrupted in *in vitro* culture, indicating an important role in the host.

Heme metabolism

The malaria parasite derives nutrients from the digestion of hemoglobin. At the same time by-products have to be detoxified. Falcipain-2, a cysteine protease involved in hemoglobin metabolism, showed in yeast two hybrid experiments interactions with PfHop (PF14_0324), the Hsp90/Hsp70 co-chaperone. Both Hsp70 and Hsp90 have been found in association with ferriprotoporphyrin, a product in the heme-metabolism [100], which indicates an important involvement of these chaperones in the acquisition of nutrients.

Gametocytogenesis

The maturation of blood-stream parasites into forms that can be taken up by the mosquito and the exflagellation of male gametes are mainly triggered by a drop in temperature [101, 102]. Although the molecular mechanism for this is unknown, it is reasonable to assume that chaperones play a role in perceiving the temperature signal or responding to it.

Pathogenesis

Chromatin-remodeling

Apicomplexan parasites seem to have an unusual reliance on epigenetic mechanisms, since there is an apparent paucity in transcription factors [103, 104]. Furthermore, virulence mechanisms like antigenic variation, that are essential for the survival of *Plasmodium* in the host, are tightly linked to chromatin-remodelling in *Plasmodium* [105]. Parasite specific chaperones involved in these processes might therefore provide a good avenue for intervention. PfHsp90 has been identified as being part of a complex involved in chromatin remodelling [84]. Other components of this complex include a histone chaperone (nucleosome assembly protein/PFI0930c) [106], and PFL0625c an annotated translation initiation factor that is involved in histone acetylation in yeast [107].

Drug-resistance

Despite several (initially) very effective drugs, *Plasmodium* infections continue to be a problem of enormous proportions. This is due to the swift ability of the parasite to develop mechanisms, which eliminate or compensate for the drug action. Hsp90 may be involved in the development of drug resistance in the malaria parasite: one of the yeast two hybrid interactions for PfHsp90 is with PF10_0242, which shows homologies to a PgP-like ABC transporter. In the human system an interaction of human Hsp90 and a PgP-like ABC transporter was shown, which lead to an increase in drug resistance [108]. In addition, Hsp90 has been implicated in the development of drug resistance in fungi by acting as an “evolutionary” capacitor [109]. One of the Hsp90 co-chaperones of the Hsp100 family is the chloroquine resistance gene Cg4 (PF07_0033), which was implicated in chloroquine resistance [110]. Chloroquine,

once the golden bullet in the fight against malaria, has lost its effectiveness due to the spread of the resistance. Given the constant arms race of producing anti-malarial drug, which are then neutralized by a drug resistance mechanism, understanding the development of this process is crucial and might provide us with important insights into the biology of pathogens.

Membrane modification/Protein export

Probably the best experimental data on the involvement of chaperones in pathogenesis is the remodelling of the host red blood cell. The reintroduction of an active protein transport machinery in a cell that has lost its nucleus and ceased all protein synthesis and transport, is one of the most remarkable features of malaria parasites. This is achieved by exporting (depending on the *Plasmodium* species) around 3-8% of the total number of gene products beyond the parasite's own confines into the red blood cell cytoplasm. A typical exported protein contains a recessed N-terminal signal sequence that allows entry into the ER. The default pathway from here is beyond the parasite's plasma membrane to the PV [111]. Most of the proteins destined for the red blood cell, enter the red blood cell cytosol via a translocon [112]. Such proteins characteristically carry permutations of the pentameric sequence RxLxE towards the N-terminus of the protein [113, 114]. Once inside the red blood cells, parasite proteins are located either in the cytosol, in parasite induced membranous structures like Maurer's clefts, or at the red blood cell plasma membrane.

Along this transport pathway there is plenty of potential for chaperone involvement: the parasite ER contains the Hsp70 homolog PfBiP (PFI0875w) [115]. One of the confirmed components of the translocon is PfHsp101 (PF11_0175). In one of the models for the translocon mechanism, the polypeptide chain has to be dragged through the translocon and a strong candidate for providing the catalytic energy required for this action is human Hsp70. It is remarkable that the only chaperones containing the export signature sequence are PfHsp40 family members. PfHsp70 on the other hand does not contain any export sequence and does not enter the red blood cell [116]. Since this parasite-derived partner of PfHsp40 proteins is missing, it is a very attractive possibility that they functionally interact with human Hsp70 chaperones. Noteworthy is also that in *Plasmodium* species infecting mice, only one Hsp40 protein contains the export sequence. *Plasmodium* species infecting humans

like *P. vivax* and *P. knowlesi* contain 2, but the most virulent *P. falciparum* contains 18 PfHsp40 proteins with an export sequence [117]. Of these 18, three are of the type II class, containing all necessary domains to interact with Hsp70, whereas 15 belong to the type III/IV class, which might have more specialized *Plasmodium*-specific functions [14, 87].

It has been hypothesized that the expansion of this exported family is linked to the increased virulence seen in *P. falciparum* [87]. The major virulence factor in *P. falciparum* is the erythrocyte membrane protein (PfEMP1). PfEMP1 is encoded by a family of ~ 60 genes, but at any one time only one of these antigenically distinct copies is expressed in a parasite cell. Erythrocyte membrane exposed PfEMP1 acts as a ligand, by which infected red blood cells adhere to receptors on blood vessel endothelium. This process of cytoadhesion prevents the infected red blood cell from being flushed into the spleen, where it would be removed from circulation and destroyed [118]. Protrusions of the erythrocyte plasma membrane, referred to as knobs, are formed to aid in the display of PfEMP1. MESA (mature parasite-infected erythrocyte surface antigen, PFE0040c; also called PfEMP2), an exported PfHsp40, might provide the structural link to the red blood cell cytoskeleton [119]. Recently a gene knock-out screen revealed an exported PfHsp40 protein that was essential for knob formation [120]. Disruption of PF10_0381 leads to a loss in cytoadherence. However, it is still unclear whether this PfHsp40 protein is a component of the knob complex itself or whether it is involved in the delivery of other components to this structure. Knock-out studies have also indicated that only one of the three exported PfHsp40 type II proteins is essential for the survival of the parasites (PFA0660w) [120]. Two of these exported PfHsp40 proteins were found to be associated with cholesterol containing membranes [121]. They seem to be localized in punctuate, highly mobile structures, termed J-dots. It was suggested that these structures play a role in the trafficking of parasite-derived proteins through the erythrocyte cytosol.

Deciphering the functions and interactions of the *Plasmodium* chaperone system will be a challenging and exciting exercise. Given the already apparent peculiarities of this system it is hoped that these studies will reveal targets that can be exploited in the fight against this disease [122, 123].

Toxoplasma gondii

Introduction

Toxoplasma gondii is an apicocomplexan parasite, which has two main life stages: the sexual stage (coccidia like) largely occurs in species of the Felidae family (domestic and wild cats), which constitutes the primary host of the parasite. The asexual stage takes place in other warm-blooded species, such as mammals and birds. *T. gondii* causes opportunistic infection in immunocompromised hosts such as HIV/AIDS subjects. When the parasite invades its host, a PV that surrounds a slow replicating form of the parasite (the bradyzoite) develops. The development of the vacuole protects the parasite, hiding it from the immune system. Once in the vacuole, the parasite multiplies, consequently, the host cell bursts releasing tachyzoites. As opposed to bradyzoites, tachyzoites are motile, and hence are easily accessed by the host immune response. The development of toxoplasmic encephalitis is closely linked to the switch from the latent bradyzoite stage to the tachyzoite stage that is characterized by rapid replication [124, 125]. The most acute form of the infection causes toxoplasmic encephalitis which may lead to fatal outcomes. In addition, the development of toxoplasmosis in humans has been associated with behavioural change, characterized by hallucinations and increased reckless contact [126]. Most of the drugs in current use are not effective in clearing the parasite from cysts and therefore it is important to understand the physiological processes that govern the development of the parasite towards development of effective drugs.

The role of heat shock proteins in the development and pathogenesis of *T. gondii*

How *T. gondii* develops from the bradyzoite form to the tachyzoite stage remains largely unknown, but it is evident that stress is capable of regulating the development of the parasite. Stress conditions have been shown to promote conversion of the parasite population towards the bradyzoite stage in vitro [125, 127, 128]. For *T. gondii* to undergo stage transition, it is important that its rate of replication be reduced. Physiological stress, and nitric oxide (NO), an important molecule produced by innate immune cells, both seem to facilitate differentiation of *T. gondii* by reducing the rate of parasite replication [129]. Coincidentally, heat shock proteins are upregulated during stressful conditions and it is not surprising that they have been implicated in the development and pathogenesis of *T. gondii* [68, 130, 131]. One of

745 the earliest pieces of evidence linking heat shock proteins to the development of *T.*
746 *gondii* was due to the production of a bradyzoite antigen by *T. gondii* cultured on
747 murine macrophages isolated from the bone marrow [129]. The production of this
748 antigen was found to correlate with the production of nitric oxide by the macrophages
749 [129] and it was later deciphered that the antigenic structure belongs to a small heat
750 shock protein, BAG1/hsp30 [132].
751
752 The successful development of *T. gondii* in host cells lies in the swing of balance
753 between the host immune response against the parasite's capability to evade the
754 hostile reaction of the host [133]. During a primary infection by *T. gondii*, host cells
755 are stimulated to release of interleukin 12 (IL-12) and interferon-gamma (IFN-γ) as
756 part of the innate immune response and the combined effect of these two cytokines
757 are crucial to host survival [133]. It is also thought that the release of IFN-γ slows
758 down the replication of tachyzoites, promoting their transition to bradyzoites [129]. It
759 was further suggested by Gross et al. that IFN-γ triggers NO-based immune response,
760 thus slowing down the replication of tachyzoites, facilitating their conversion to
761 bradyzoites [134]. The mechanism by which Hsp70 regulates differentiation in *T.*
762 *gondii* is not very clear. Weiss et al. proposed that cytosolic *T. gondii* Hsp70
763 (TgHsp70) was expressed in response to host inflammatory responses following an
764 infection [130]. In this study, it was further noticed that the expression of TgHsp70
765 was only observed in infected mice that were challenged by lethal doses. Challenge of
766 the mice by non-lethal doses of the parasite did not result in expression of TgHsp70.
767 Thus, it was argued that expression of TgHsp70 serves as a warning for danger during
768 the development of lethal toxoplasmosis [135]. Interestingly, human Hsp60 is also
769 thought to serve as a danger warning system to the innate immune response [136].
770 The production of nitric oxide (NO) is thought to be important in the elimination of
771 tachyzoites through its effect on mitochondrial and nuclear localized enzymes [137].
772 However, the action of NO is only beneficial to host cells in acute infections stages as
773 its prolonged action is thought to halt replication of tachyzoites, thus promoting their
774 transition to the relatively dormant bradyzoites [129], which seek refuge in cysts, thus
775 promoting a chronic condition [133]. It is thought that the expression of TgHsp70
776 during a primary infection is thought to promote subsequent tolerance to NO [138],
777 thus TgHsp70 is capable of promoting parasite development and pathogenesis.
778 Conflicting observations have been reported with regard to the role of TgHsp70 on

779 the stage transition of *T. gondii* parasites. Weiss et al. proposed that conditions that
780 favour Hsp70 production are associated with the development of *T. gondii* into the
781 bradyzoite stage of development [130]. However, evidence from an independent study
782 reported that high levels of Hsp70 protein were linked to the conversion of the
783 parasite from bradyzoites to tachyzoites [131]. Although the role of Hsp70 in the
784 development of *T. gondii* is controversial, it is clear that the protein plays an
785 important role in this process.
786
787 It has been suggested that TgHsp70 is capable of suppressing host immune response
788 by inhibiting NO release by macrophages particularly during the development of
789 acute toxoplasmosis [135]. The suppression of host immune response is deemed to
790 serve the primary purpose of reducing host tissue damage, inadvertently facilitating
791 parasite invasion. Furthermore, it has been reported that infection of mice by *T. gondii*
792 led to the production of antibodies to TgHsp70 that cross-reacted with mouse Hsp70
793 [139]. Furthermore, TgHsp70 was reported to induce the proliferation of murine B
794 cells isolated from both uninfected- and *T. gondii*-infected mice [140]. The fact that *T.*
795 *gondii*-infected mice are capable of producing anti-TgHsp70 antibodies that cross-
796 react with mouse Hsp70 is thought to promote a self immune response by the host,
797 leading to deleterious consequences [139]. On the contrary, TgHsp70 is thought to
798 promote the maturation of dendritic cells [141], thus priming them to activate the
799 adaptive immune response. Therefore, the mechanism in which TgHsp70 modulates
800 the development and pathogenesis of the parasite as well as the host immune response
801 is still enshrouded in controversy. Nonetheless, the contrasting views on the role of
802 TgHsp70 may largely be due to its ability to adjust its role relative to subtle changes
803 in the environment during parasite development. The study by Weiss et al. further
804 proposed that inhibition of heat shock protein synthesis using the flavonoid quercetin
805 suppressed transition into the bradyzoite stage, and in contrast, indomethacin, a
806 compound which is known to enhance heat shock protein synthesis promoted the
807 transition of the parasites to bradyzoites [130]. This is further evidence suggesting a
808 role for heat shock proteins in the development of *T. gondii*. Furthermore, it appears
809 that TgHsp70 plays particularly important role at the host-parasite interface. For
810 example, *T. gondii* cells growing *in vitro* or in infected immunocompromised mice
811 barely expressed Hsp70 protein and expression of this protein was only significantly

noted in parasites isolated from infected immunocompetent mice [142]. This suggests that TgHsp70 is expressed as part of the parasite defence mechanism.

Role of Hsp90 in the development of *T. gondii*

A homologue of Hsp90 from *T. gondii* has been described and this protein shares higher homology with Hsp90 isoforms from other apicocomplexan families compared to its relation with Hsp90 proteins from *Trypanosoma cruzi* and *Leishmania* species [143] (Table 1). Exposure of *T. gondii* to the Hsp90 inhibitor, GA, resulted in reduced growth of tachyzoites [143]. TgHsp90 was reported to be resident in the cytosol of tachyzoites [143]; however, another study proposed that TgHsp90 occurs in the cytosol of tachyzoites, and that in bradyzoites the protein is localized to both the nucleus and cytosol [68]. Furthermore, it was observed that TgHsp90 was heat inducible and that its expression was more enhanced at the bradyzoite stage [68]. This suggests that the expression and localization of TgHsp90 are both regulated by the development of the parasite. In their study, Echeverria et al. further observed that GA inhibited both the transition of the parasite from tachyzoites to bradyzoites and vice versa, intimating that TgHsp90 is important for attainment of both life stages of the parasite in spite of its differential localization at the two development stages [68]. It has further been suggested that TgHsp90 is secreted to the exterior surroundings by extracellular tachyzoites prior to their invasion of host cells [143]. The possible secretion of TgHsp90 is in contrast to TgHsp70 which seems restricted to the cytosol. However, TgHsp90 could not be located in the PV in infected cells [143]. Nonetheless, the proposed export of TgHsp90 to the exterior by tachyzoites could facilitate modulation of its exported client proteins that may be involved in invasion. Indeed inhibition of TgHsp90 resulted in decreased invasion capability by the tachyzoites, making the chaperone an attractive drug target [143].

Chaperone networks and co-chaperones from *T. gondii*

A regulatory element that is sensitive to stressful pH conditions was located upstream of the *T. gondii* Hsp70 gene [144]. However, there was no evidence of the presence of a similar element within the loci of the Hsp60 and Hsp90 genes, suggesting that regulation of Hsp70 may be distinct [144]. Nonetheless, it is conceivable that chaperone networks and chaperone-co-chaperone partnerships exist in this parasite. An Hsp60 homologue from *T. gondii* has been described as localized to the

mitochondrion and a role for this protein in the development of the parasite has been suggested [145]. In addition, at least five members of the small heat shock family, all possessing the α-crystalline signature motif of this group have been identified on the *T. gondii* genome [146]. Members of this family were found to localize to distinct sub-cellular compartments and most of them were constitutively expressed, with the exception of two that were expressed at unique growth stages; TgHsp28 was reportedly expressed at the tachyzoite stage [146] whilst, TgHsp30/Bag1 was expressed only at the bradyzoite stage [132, 147] This further suggests stage specific roles for this group of chaperones, further highlighting a possible universal role of molecular chaperones in the differentiation of *T. gondii*.

According to a model by Ma et al. [144], molecular chaperones regulate the activity of their client proteins which in turn may have a direct role in differentiation and apoptosis. However, it is evident that molecular chaperones from *T.gondii*, especially TgHsp70 and TgHsp90 operate both as canonical chaperones (regulating protein folding) as well as chaperokines [7]. Irrespective of which of the two functions ('chaperone' and 'chaperokine') may be more influential in differentiation, it would be important to understand how these two prominent chaperones are regulated by their co-chaperone partners. Co-immunoprecipitation studies have identified the possible existence of a Hip-Hsp70-Hsp90 and a p23-Hsp90 complex in *T. gondii* [148]. This is the first line of evidence suggesting the possible interaction of TgHsp70 and TgHsp90 with possible co-chaperone partners. A recent study based on genomic data observed that none of the 19 obligate parasites studied had all ten of the most common Hsp90 co-chaperones present in their system, and it was further suggested that Hsp90 co-chaperones displayed flexible, disparate distribution across species of parasites [149]. This suggests that TgHsp90 could be regulated by a unique suite of co-chaperone partners, an attribute that renders it an attractive drug target.

CONCLUSIONS AND FUTURE PERSPECTIVES

There is growing body of evidence that chaperones play an important role in the life cycle of a wide variety of important human pathogens. Amongst intracellular parasites, kinetoplastids appear to have an expanded complement of genes encoding Hsp90, Hsp70 and Hsp40 isoforms, and the Hsp40 family appears to be an extreme example of evolutionary radiation of a gene family. In contrast, the apicomplexan

parasite *P. falciparum*, appears to have evolved a minimal set of genes encoding Hsp90 and Hsp70, but maintains an expanded and diverse family of genes encoding Hsp40 isoforms. In addition, a disproportionately high percentage of the *P. falciparum* genome is dedicated to chaperone-encoding genes. It is tempting to speculate that the diverse environmental insults that intracellular parasites endure has resulted in the evolutionary selection of diverse and expanded chaperone networks. Furthermore, there is strong evidence that these parasites require the services of certain chaperones for successful development and pathogenesis. Hsp90 in particular is involved in development and growth of all of these intracellular parasites, and represents the strongest drug target candidate yet. Future studies should focus on developing suitable inhibitors specific for Hsp90, which can be developed into lead compounds for anti-parasitic drug development. The GA analogue, 17-AAG, is in phase II clinical trials as an anti-cancer drug, and represents an obvious start point for anti-parasitic drug development.

Interestingly, most of the research conducted on the role of molecular chaperones in the life cycle of *T. gondii* has focussed on their chaperokine function, where they act as signal transducers. The role of *T. gondii* Hsp70 as a chaperokine has received particular attention, and this protein is thought to regulate the development of the parasite directly, as well as indirectly by modulating the host immune system. Given the fact that molecular chaperones from parasites may be capable of regulating host immune responses, it is important to understand how this process is regulated by co-chaperone factors. It is conceivable that heat shock proteins from other species, apart from *T. gondii* may also act as chaperokines. Therefore, future studies should focus on the role of molecular chaperones both as facilitators of protein folding and as signal transducers.

A number of Hsp40 isoforms, especially in the case of *P. falciparum*, have been shown to be essential proteins, or important for pathogenesis. Although the molecular details of the Hsp70-Hsp40 chaperone interactions and pathways in other parasites remain to be elucidated, this partnership represents an emerging drug target. In particular, despite their high diversity, copy number, and potential importance in parasite survival, very little is known about the Hsp40 proteins of all of the parasites here reviewed. It is imperative that these Hsp40 proteins and their interactions with

914 partner proteins, especially Hsp70, are fully elucidated. The interaction of certain
915 exported *P. falciparum* Hsp40 proteins with human Hsp70 and the translocon
916 machinery involved in protein export represents a fascinating host-parasite interface
917 for further investigation. To sum up, although much remains to be experimentally
918 examined, the study of the diversity of chaperone function in intracellular parasites
919 so far proves once more the adage that " [nature]….*works like a tinkerer-a tinkerer*
920 *who does not know exactly what he is going to produce but uses whatever he finds*
921 *around him whether it be pieces of string, fragments of wood, or old cardboards; in*
922 *short it works like a tinkerer who uses everything at his disposal to produce some*
923 *kind of workable object"* [150].
924
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