Purification and characterization of TbHsp70.c, a novel Hsp70 from *Trypanosoma brucei*

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By

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ABSTRACT

One of Africa's neglected tropical diseases, African Trypanosomiasis, is not only fatal but also has a crippling impact on economic development. Heat shock proteins play a wide range of roles in the cell and they are required to assist the parasite as it moves from a cold blooded insect vector to a warm blooded mammalian host. The expression of heat shock proteins increases during these heat shock conditions, and this is considered to play a role in differentiation of these vector-borne parasites. Heat shock protein 70 (Hsp70) is an important molecular chaperone that is involved in protein homeostasis, Hsp40 acts as a co-chaperone and stimulates its intrinsically weak ATPase activity. *In silico* analysis of the *T. brucei* genome has revealed the existence of 12 Hsp70 proteins and 65 Hsp40 proteins to date. A novel Hsp70, TbHsp70.c, was recently identified in *T. brucei*. Different from the prototypical Hsp70, TbHsp70.c contains an acidic substrate binding domain and lacks the C-terminal EEVD motif. By implication the substrate range and mechanism by which the substrates are recognized may be novel. The ability of a Type I Hsp40, Tbj2, to function as a co-chaperone of TbHsp70.c was investigated. The main objective of this study was to biochemically characterize TbHsp70.c and its partnership with Tbj2 to further enhance our knowledge of parasite biology. TbHsp70.c and Tbj2 were heterologously expressed and purified and both proteins displayed chaperone activities in their ability to suppress aggregation of thermolabile MDH. TbHsp70.c also suppressed aggregation of rhodanese. ATPase assays revealed that the ATPase activity of TbHsp70.c was stimulated by Tbj2. The targeted inhibition of the function of heat shock proteins is emerging as a tool to combat disease. The small molecule modulators quercetin and methylene blue are known to inhibit the ATPase activity of Hsp70. However, methylene blue did not significantly inhibit the ATPase activity of TbHsp70.c; while quercetin, did inhibit the ATPase activity. *In vivo* heat stress experiments indicated an up-regulation of the expression levels of TbHsp70.c. RNA interference studies showed partial knockdown of TbHsp70.c with no detrimental effect on the parasite. Fluorescence microscopy studies of TbHsp70.c showed a probable cytoplasmic subcellular localization. In this study both TbHsp70.c and Tbj2 demonstrated chaperone activity and Tbj2 possibly functions as a co-chaperone of TbHsp70.c.

DECLARATION

I, Adélle Burger, declare that this is my own unaided work hereby submitted for the degree of Doctor of Philosophy of Rhodes University in the Faculty of Science. It has not been submitted for any degree for examination in any other university.

_____

Adélle Burger

Dated this ___ day of _____ _____ at _____.

DEDICATION

This thesis is lovingly dedicated to

My late father
Petri Burger (1950-2011)

PERSONAL ACKNOWLEDGEMENTS

My mother
Isabel Burger

My brother
Quinn Burger

Louise De Almeida

For your love and your encouragement

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LIST OF ABBREVIATIONS

~	Approximately
>	More than
α	Alpha
β	Beta
°C	Degrees Celsius
µ	Micro
µg	Microgram(s)
µl	Microlitre(s)
µM	Micromolar
A	Absorbance
A_{360}	Absorbance at 360nm
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
DNA	Deoxyribonucleic Acid
DnaK	Prokaryotic Hsp70
g	Gram(s)
His6	Hexahistidine tag
Kb	Kilo base pairs
kDa	Kilo Daltons
L	Litre(s)
m	Milli
M	Molar
mg	Milligram(s)
mM	Millimolar
ng	Nanogram(s)
Pi	Inorganic phosphate
PDB	Protein Data Bank
rpm	Revolutions per minute
w/v	Weight to volume ratio
g	Gravitational force

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CHAPTER 1

INTRODUCTION:

Literature Review

1.1. Kinetoplastids

Kinetoplastids are unicellular flagellate protozoans containing a unique structure within a single mitochondrion called the kinetoplast; a massive body of condensed mitochondrial DNA (Bastin *et al.,* 2000). Medical and ecological significance has resulted in intense scientific interest in the classification of kinetoplastids from the Class Trypanosomatidea (Domain: Eukarya, Kingdom: Protista, Phyulm: Euglenozoa) (Cavalier-Smith, 1981). Previously Euglenozoa was classified within Excavata (Cavalier-Smith, 2002). Due to characteristics unique to Phylum Euglenozoa, including nuclear genome organization, mitochondrial DNA organization, mitochondrial protein-import and pre-replication of nuclear DNA, Cavalier-Smith (2009) segregated Euglenozoa from infrakingdom Excavata. Taxonomic schemes revealed two primary kinetoplastid groups based on morphology: trypanosomatids, which are strictly uniflagellate parasites; and biflagellate bodonids (Vickerman, 1976). Order Kinetoplastida was proposed to be divided into the suborders of Prokinetoplastina, Metakinetoplastina and Trypanosomatina (Fig. 1.1), which contains Trypanosomatids (Moreira *et al.,* 2004). Being eukaryotic, kinetoplastids display characteristics such as a nucleus defined by a nuclear membrane, the endoplasmic reticulum, the Golgi apparatus, the endo-exocytosis system, the mitochondrion and other cellular organelles (Clayton *et al.,* 1995). The presence of a kinetoplast in the protozoan parasite trypanosome justifies its grouping into the order Kinetoplastida (Fig. 1.1) (Vickerman, 1976). Kinetoplastids are the only eukaryotes, however, that have organelles that may display specific and extreme features such as the presence of kinetoplast DNA, a flagellum and flagellar pocket, glycosomes, unique gene regulation and RNA-editing (Clayton *et al.,* 1995). The ability of kinetoplastids to adapt at the DNA, RNA and cellular organization levels are primarily due to the complex life cycle of trypanosomes, where the parasite needs to adapt to two different host environments (Clayton *et al.,* 1995).

1.2. Trypanosomatids

The first report of trypanosomatids in insects was established in 1851 by Burnett and Kent who documented *Leptomonas* and *Herpetomonas* as the first genera. A total of 350 named trypanosomatid species were listed in 1990; the number currently advances towards 400 (Podlipaev, 1990; Maslov *et al.,* 2012). Ultrastructural details of the trypanosomatids facilitated their taxonomy. Their relation to bodonids, ecto- and endoparasitic protists

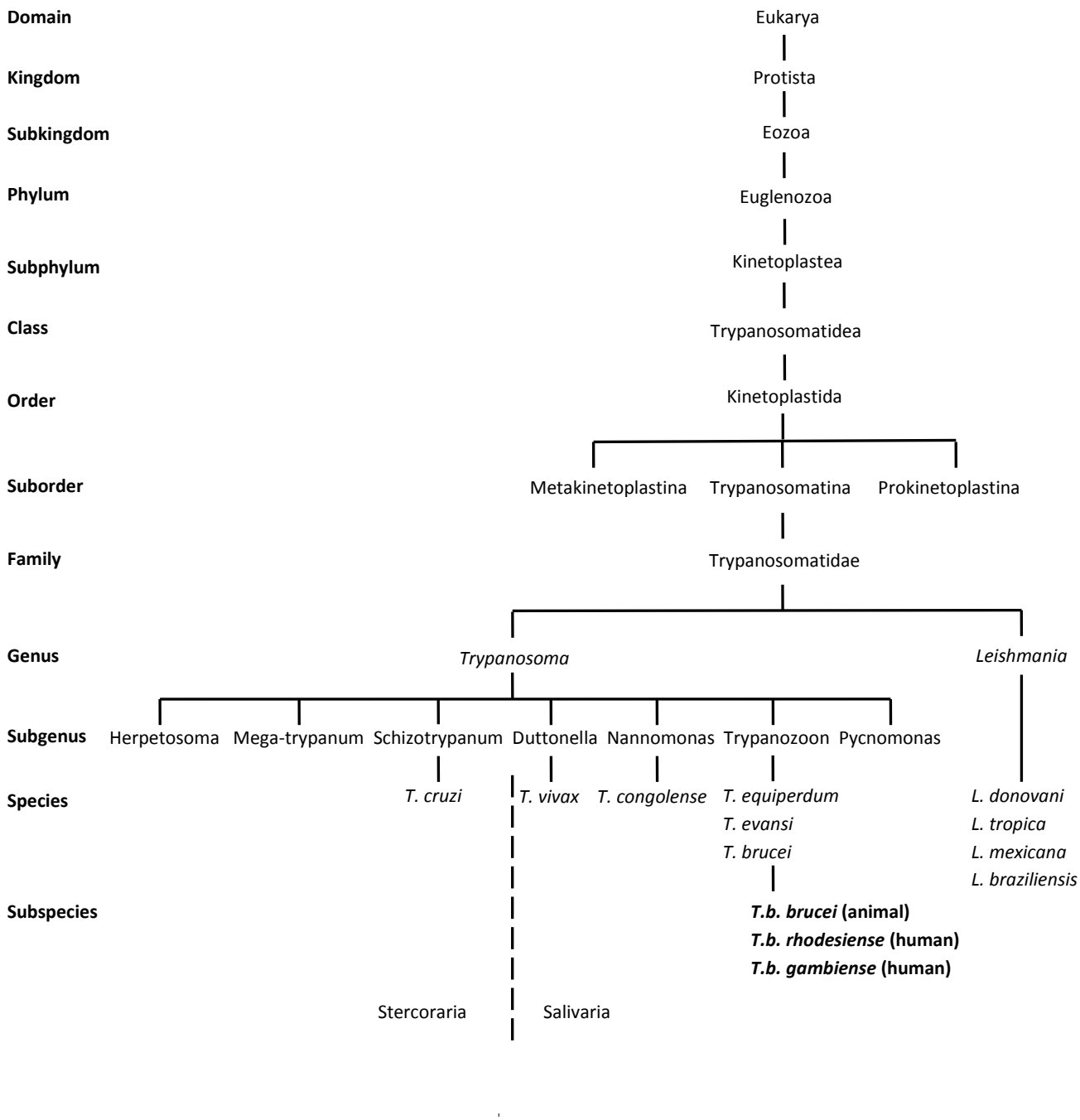


Figure 1.1. Classification of kinetoplastids
Genus *Trypanosoma* is grouped in the order Kinetoplastida and is divided into two primary groups of Stercoraria and Salivaria. The Stercoraria group includes Herpetosoma, Mega-trypanum and Schizotrypanum and Salivaria consists of Duttonella, Nannomonas, Trypanozoon and Pycnomonas. The species *T. cruzi* is grouped under the subgenus Schizotrypanum. *T. brucei* is grouped under Trypanozoon, and further divided into three subspecies of *T.b. brucei*, *T.b. rhodesiense* and *T.b. gambiense*. *Leishmania* from the Trypanosomatidae family is grouped under the Salivaria section and is divided into four species of *L. donovani*, *L. tropica*, *L. Mexicana* and *L. braziliensis* (adapted from Baral, 2010).

containing two flagellae, was realized due to the presence of a single flagellum in the trypanosomatids (Vickerman, 1976; Lukeš *et al.,* 2002). Furthermore, categorization under the taxon Kinetoplastea was as a result of the presence of the kinetoplast, and the paraxonemal rod justified classification of kinetoplastids under Euglenozoa (Fig. 1.1) (Simpson, 1997). The current rankless classification system, which concentrates on higher-order phylogenetic relationships, does not assign a specific taxonomic rank to Kinetoplastea or Euglenozoa (Adl *et al.,* 2012). Trypanosomatids parasitize all vertebrates, some invertebrates and some plants, making them highly successful organisms in terms of evolution (Vickerman, 1994; Vickerman, 2009). Trypanosomatids encompass the African trypanosomes, the American trypanosomes and leishmania species. The genera *Leishmania* and *Trypanosoma* are dixenous parasites in which its life cycle is divided between two host organisms (Maslov *et al.,* 2012). Classification of the trypanosomatid genera were primarily based on host interactions and morphological traits including cell shape and dimensions and positioning of the kinetoplast:flagellar pocket with respect to the nucleus (Hoare and Wallace, 1966; Svobodová *et al.*, 2007); biochemical characteristics as well as nutritional and ultrastructural traits were taken into consideration to differentiate trypanosomatid species (Levine *et al.,* 1980).

1.2.1. African Trypanosomiasis

African Trypanosomiasis (AT) is a disease giving rise to infection in both humans and animals. The etiological agent belongs to the genus *Trypanosoma,* an extracellularly blood- and tissue-borne unicellular parasitic protozoan. The trypanosome is transmitted to its mammalian host during a blood meal of the infected tsetse fly vector (*Glossina* sp.), which ensures the cyclical transmission of the parasite between numerous hosts. AT is predominantly distributed across sub-Saharan Africa, which correlates to the range of tsetse flies over an area of ~8 million km^2 (Molyneux *et al.,* 1996).

Human African Trypanosomiasis (HAT), also called African sleeping sickness, is caused by infection of two morphologically indistinguishable subspecies of *Trypanosoma brucei* endemic in Sub-Saharan Africa, *T. b. gambiense* and *T. b. rhodesiense* (Table 1.1). Their epidemiological features vary significantly yet both subspecies affect the central nervous system. *T. b. gambiense,* prominent in central and West Africa, gives rise to a chronic

infection with symptoms that may be dormant for months and even years and caused 97% of the reported cases from 1997 through to 2006 (Simarro *et al.,* 2008). *T. b. rhodesiense,* responsible for less than 10% of reported cases, occurs in southern and East Africa and causes an acute infection, which is severely virulent [World Health Organization (WHO), 2006]. In the first stage of HAT, the haemolymphatic phase, the parasites remain in the blood and lymph system, resulting in fever, headaches, joint pains and itching (WHO, 2006). In the second neurological phase, trypanosomes move to the cerebrospinal fluid which gives rise to symptoms such as confusion, disturbed sleep patterns, sensory disturbances, severe lethargy and coma; the symptoms for which HAT is renowned (WHO, 2006). Untreated, *T. b. rhodesiense* infection will result in death within months; *T. b. gambiense* within years (Brun *et al.,* 2009). Animals, domesticated and wild, have been identified as reservoirs for HAT infections (Njiokou *et al.,* 2006; Simo *et al.,* 2006).

African Trypanosomiasis is described as one of the neglected tropical diseases crippling economic development and causing death in Africa's poorest and most marginalized communities (WHO, 2000). The last surveillance was performed in 2006 with the number of cases of HAT between 50 000 and 70 000 (WHO, 2006). According to reports from WHO, African sleeping sickness ranks third after malaria and schistosomiasis as parasitic diseases that have an astronomical global impact (Cattand *et al.,* 2001). The incidence of HAT over the years has varied significantly depending on factors such as the ending of civil wars, as in Angola, strong commitments of WHO, national control programs, donors and nongovernment organizations (NGOs), and gratis provisions of antitrypanosomal drugs (Chappuis *et al.,* 2010). The 1920s and 1990s saw high incidence peaks; the 1960s and recent 2000 - 2009 saw a drop in the number of reported cases (Barret, 1999; Barret, 2006; Simarro *et al.,* 2008). The 1960s saw a radical decline in transmission, leading to the belief that HAT could be removed as a public health problem. However, a loss of interest in maintaining surveillance due to the rarity of reported cases was evident when re-emergence of the disease resulted in a very high incidence in the 1990s (Simarro *et al.,* 2011). NGOs were key players at this time in controlling HAT in the isolated and insecure regions (Simarro *et al.,* 2011). In 2004, there were 17 500 reported cases, however, due to areas without surveillance systems, the actual incidence was approximated at 50 000 – 70 000 cases (WHO, 2006). The isolated areas in the north-eastern Democratic Republic of Congo, including Doruma, Ango and Bili, have had no preventative measures against HAT taken over the last three decades; a control

program instigated by Médecins sans Frontières (MSF) in 2007 reported 3.4% disease prevalence and 60% of infected individuals in the first stage of the disease (Chappuis *et al.*, 2010). The project was abandoned in 2009 upon attack of the MSF team by rebels from the Lord's Resistance Army (Chappuis *et al.*, 2010). Restricted access to these remote regions in the Central African Republic and Democratic Republic of Congo leaves uncertainty about the actual incidence of HAT and those infected with the disease will have no access to care (Chappuis *et al.*, 2010). In 2009 the number of cases reported to WHO decreased to below 10 000 and the number of individuals screened during 2000 – 2009 increased as a result of screening by more health care facilities and improved surveillance in provinces other than the Democratic Republic of Congo (Simarro *et al.*, 2011).

The etiological agents *T. b. brucei* as well as *T. congolense* and *T. vivax* spp cause Nagana in wild and domestic animals; of particular economic importance, domesticated livestock (Table 1.1) (Vickerman, 1985). The infectious disease Nagana, a Zulu word meaning "to be in low or depressed spirits; powerless/useless", is also called Tsetse Fly Disease.

Table 1.1. Characteristics of the Tritryps (adapted from Stuart *et al.*, 2008)

Species	**Hosts**	**Vector**	**Disease**	**Disease profile in humans**	**Distribution**
Trypanosoma brucei brucei	Domestic livestock	Tsetse fly (*Glossina* spp)	Nagana	---	Sub-Saharan Africa
Trypanosoma brucei gambiense	Humans	Tsetse fly (*Glossina* spp)	Gambian sleeping sickness	Chronic	Central and West Africa
Trypanosoma brucei rhodesiense	Humans	Tsetse fly (*Glossina* spp.)	Rhodesian sleeping sickness	Acute	Southern and East Africa
Trypanosoma cruzi	Humans, domestic & wild animals	Reduviid bugs (*Triatominae* spp.)	Chagas' disease	Acute/Chronic	South and Central America
Leishmania major	Humans, domestic animals	Phlebotomine sandflies (*Phlebotomus*spp.)	Leishmaniasis	Acute	South and Central America, Europe, Africa, Asia

T. b. brucei is widespread in the arid and semi-arid regions of sub-Saharan Africa, and endemic in 36 African countries (WHO, 2006). Cattle that are infected with subacute, acute or chronic forms of the disease show symptoms such as intermittent fever, anemia, emaciation, hair loss, discharge from the eyes, occasional diarrhoea and paralysis which may result in death (WHO, 2006).

Treatment and diagnostics

African Trypanosomiasis affects neglected groups of people in remote and rural regions where health care systems have not yet been implemented thereby making it difficult to accurately estimate the number of cases (Matemba *et al.,* 2010). In 2009 the World Health Organization (WHO) reported less than 10 000 cases, in stark contrast to the annual reports in 1998 of 300 000 infected individuals (WHO, 1998; Aksoy, 2011). Factors influencing the continued problem of "sleeping sickness" is the fact that the available trypanosomal drugs (suramin, pentamidine, melarsoprol, eflornithine and nifurtimox) show very undesirable side effects (Gutteridge, 1985; Doua and Yapo, 1993): the drugs lack efficacy against all stages of HAT, the parasite develops a resistance to the drugs (Ross and Sutherland, 1997), and there is an inability of the drugs to eradicate all trypanosomal species and strains (Kubata *et al.,* 2004). For instance, elaminophenyl arsenical drug, melarsoprol, not only causes severe and lethal side effects, but started failing as a treatment against HAT (Stanghellini and Josenando, 2001; Brun *et al.,* 2003), which suggested the appearance of drug-resistant trypanosomes (reviewed by Cuervo *et al.,* 2010; Gehrig and Efferth, 2008). Due to the problems and difficulties of conventional drugs, and the lack of a vaccine against infections, an urgent need for new anti-trypanosomal drugs has developed as a lack of therapeutic intervention results in fatal HAT. Part of the solution to the problem was the development of a new treatment, a nifurtimox/eflornithine combination treatment (NECT), that allowed a drop in cost of drugs and delivery (WHO, 2009; Yun *et al.,* 2010). Fexinidazole was recently developed as an effective and easy to administer treatment against AT (Torreele *et al.,* 2010).

The parasite's complex biology has impeded the creation of vaccines; there are no prophylactic drugs; development of tests in the field enabling the stage of disease to be determined has been complicated (Molyneux *et al.,* 2010). The loop-mediated isothermal amplification (LAMP) method is a DNA-based diagnostic tool used in a field setting; parasites can be detected in infected humans as well as in tsetse fly populations and reservoir

animals (Njiru *et al.*, 2008; Wastling *et al.*, 2010). Diagnostic tools already in use for other diseases (tuberculosis, malaria, HIV) can be used as a platform for AT (Ndung'u *et al.*, 2010); originally developed for tuberculosis, the light-emitting diode (LED)-based fluorescence microscope is highly effective for AT diagnostics; the mini anion exchange centrifugation technique (mAECT) has improved diagnosis of infections by *T. b. gambiense* (Büscher *et al.*, 2009).

The sequenced *T. b. gambiense* genome (Jackson *et al.*, 2010), the tsetse fly (*Glossina morsitans morsitans)* genome (TDR, 2010) and transcriptome of *T. brucei* at single nucleotide resolution (Kolev *et al.*, 2010) will enable the development of new targets for diagnostics and treatments. Knowledge of the genome sequences of the remaining five tsetse species could enhance vector control through the development of species-specific attractants to bait and so reduce the vector populations (Omolo *et al.*, 2009; Rayaisse *et al.*, 2010). Many of the 500 protein kinases that have been identified have been implicated in disease processes (Manning *et al.*, 2002); they are well characterized drug targets, responsible for more than 30% of all drug discoveries (Doerig, 2004; Naula *et al.*, 2005; Weinmann and Metternich, 2005; Card *et al.*, 2009). Two isoforms of glycogen synthase kinase-3 (GSK-3) are present in *T. brucei*, *Tbru*GSK-3 short and *Tbru*GSK-3 long, homologous to human *Hs*GSK-3 (Ojo *et al.*, 2008). *Tbru*GSK-3 short was demonstrated to be essential to the survival of the cell and a target for anti-trypanosomal drug development (Ojo *et al.*, 2008) and two inhibitors, PF-04903528 and 0181276, showed a 7-fold selectivity for *Tbru*GSK-3 short over the human homologue (Oduor *et al.* 2012). Another kinase present in *T. brucei*, pyridoxal kinase (PdxK), necessary for the recovery of vitamin B6, was shown to be crucial for growth of the parasitic cell and its crystal structure was resolved (Jones *et al.*, 2012). PdxK has thus been established as a drug target against Trypanosomiasis and effective inhibitors need to be identified (reviewed by Phillips, 2012).

1.2.2. The cell biology of *T. brucei*

Trypanosomes possess a few very exclusive biochemical and morphological features including the presence of a single mitochondrion with a kinetoplast, a flagellum and flagellar pocket (Fig. 2) (Vickerman and Preston, 1976). The surface membranes of protozoal

kinetoplastids have been divided into the highly specialized morphological domains of the flagellar pocket, the flagellum and the pellicular plasma membrane (Balber, 1990).

Flagellum

A single flagellum emerges out from a specialized flask-shaped invagination of the plasma membrane called the flagellar pocket (Fig. 1.2) (Overath and Engstler, 2004). The paraflagellar rod, an atypical fibrous entity, attached to the flagellar axoneme, runs alongside the flagellum and plays a role in motility (Fig. 1.2) (Bastin *et al.,* 1998). The flagellum, present at all stages of the parasites life cycle, has been suggested to have numerous functions. This motility organelle allows forward movement of the parasite through wave-like pulses of the flagellar axoneme (Fig. 1.2) (Bastin *et al.,* 1999). The flagellum that functions to attach the parasite to the insect host endothelium (Vickerman, 1976), is also involved in cell signalling and chemotaxis (Fridberg *et al*., 2007), and facilitates morphogenesis and cell division (Kohl *et al.,* 2003; Kohl and Bastin, 2005). Trypanosomal protein trypanin also facilitates motility (Rupp and Porter, 2003); the presence of a microtubule-binding domain provides stability between the flagellum and sub-pellicular cytoskeleton association (the flagellum-attachment zone) thus imparting directional motility (Kohl *et al.,* 1999). A loss of trypanin function results in the parasite tumbling uncontrollably; disruption of the paraflagellar rod results in paralysis (Ersfeld and Gull, 2001; Kohl *et al.,* 2003). Cell division involves the daughter flagellum exactly tracking the parent flagellum allowing transferral of structural information from the old to new flagellum through cytotaxic inheritance (Moreira-Leite *et al.,* 2001). This process occurs through a mobile structure, the flagellar connector, which attaches three doublet microtubules of the old flagellum's axoneme to the tip of the daughter flagellum (Briggs *et al.,* 2004). The basal body forms the origin of the flagellum and is connected through the mitochondrial membrane to the mitochondrial genome (Ogbadoyi *et al.,* 2003).

Flagellar pocket

As the trypanosome transitions between various developmental stages, the cell architecture and positioning of the flagellar pocket relative to the nucleus changes (Fig. 1.3). The flagellar pocket is located either to the anterior of the nucleus (promastigote and epimastigote stages) or the posterior (trypomastigote stage). All of the endocytosis of larger nutrients and exocytosis trafficking occurs in the flagellar pocket. It is a highly differentiated region that

aids the internalization of host macromolecules whilst preventing the endocytic receptors from being accessed by the host (Webster and Russel, 1993).

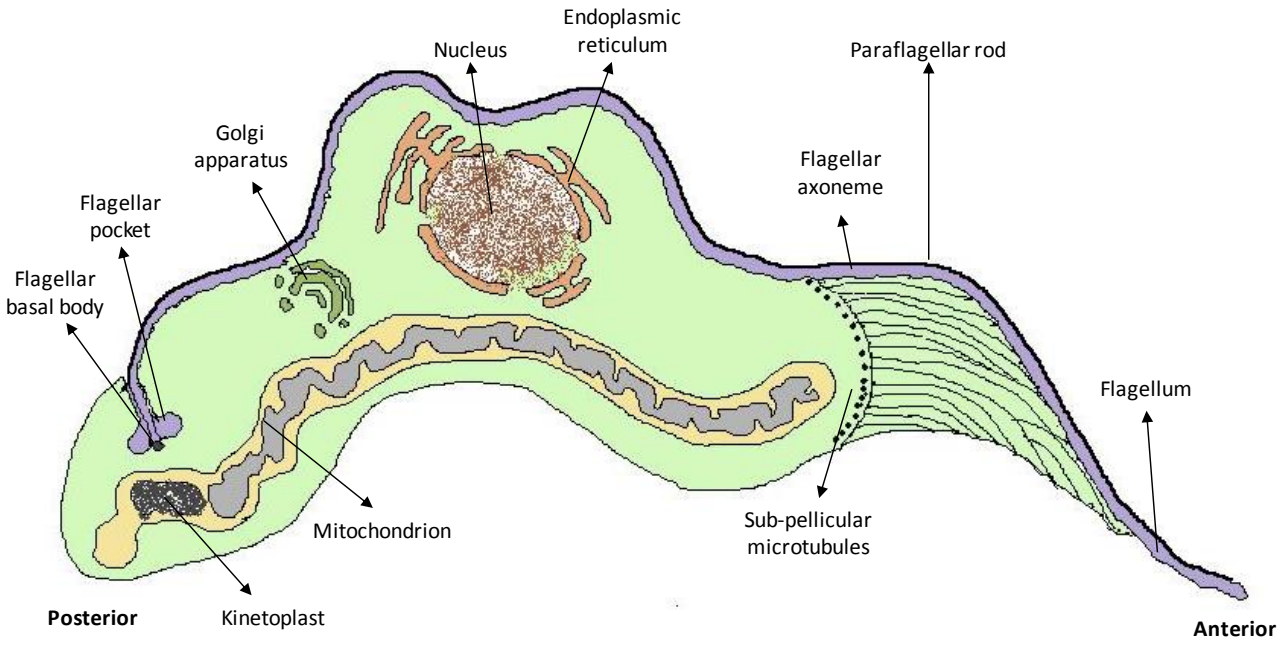


Figure 1.2. Schematic of the main structures and organelles of *T. brucei*.
Schematic showing organelles and structures of a bloodstream form trypanosome as would be observed in thin sections by transmission electron microscopy (adapted from Landfear and Ignatushchenko, 2001).

Mitochondrion

Trypanosomatid protozoa contain one large elongated mitochondrion per cell (Fig. 1.2), of which 10 – 20% of the total cellular DNA content is made up by a network of condensed mitochondrial DNA in the kinetoplast (Hajduk *et al.*, 1986). The kinetoplast and basal body are connected by a tripartite attachment complex that passes through the cell and mitochondrial membranes (Ogbadoyi *et al.*, 2003). The complex is made up of a series of filaments acting as guide ropes through which replication and segregation of the basal body and flagellum are connected to mitochondrial genome segregation (reviewed in Gull, 2003). The mitochondrial genome is comprised of two concatenated genetic elements, mini- and maxicircles (Englund *et al.*, 1982; Stuart, 1983). The highly numbered minicircles have an open conformation and are not supercoiled; maxicircles, less in number, encode the typical mitochondrial genes and are similar in sequence (Reviewed in Lukeš *et al.*, 2002). A notable trait of kDNA is the formation of a single planar network by the topological linkage of the

maxi- and minicircles (Ogbadoyi *et al.,* 2003). Many of the classical mitochondrial genes, however, are cryptogenes; processing of primary transcripts to functional mRNAs occurs through RNA editing (Stuart *et al.,* 2000). The translation system of *T. brucei* is unusual as mitochondrial transfer RNAs (tRNAs) are imported from the cytosol, due to the fact that no tRNA genes are encoded for by mitochondrial DNA (Salinas *et al.,* 2008). Along with edited mRNAs and imported tRNAs, the *T. brucei* ribosomal RNAs are amongst the shortest. Mammalian peroxisomes are membrane-bound cytoplasmic organelles, containing catalase and H_2O_2-producing oxidases, which compartmentalize different metabolic functions (reviewed in De Souza, 2002). Trypanosomatids possess functionally divergent organelles; the microbodies or peroxisomes were designated as the glycosomes of Trypanosomatids based on the results obtained by Opperdoes (Opperdoes, 1987; reviewed in Parsons, 2004). Energy generation is dependent on glycolytic reactions that occur within glycosomes located in the mitochondrion. As the glycosome has no genome, glycosomal proteins are coded for by nuclear genes, translated on free ribosomes and are taken up into the glycosome within 5 minutes of being synthesized (Opperdoes, 1987). The parasite resides extracellularly in the bloodstream and cerebrospinal fluid of its host, and so is continuously encased with low levels of glucose; the production of ATP occurs through glycolysis and the primary proteins present are glycolytic enzymes (Hart *et al.,* 1984). ATP is generated in a metabolic pathway unique to glycosomes, the Embden-Meyerhof segment of glycolysis. Enzymes hexokinase, glucose phosphate isomerise and phosphoglycerate kinase are present in the glycosomes but no net synthesis of ATP transcends within the glycosome; ATP is produced in the cytosol once pyruvate kinase passes the high energy Pi from phosphoenolpyruvate to ADP (reviewed in Parsons, 2004). Within the insect vector, conditions are different and glucose is not a luxury for the procyclic parasite. Blood is only available to the parasite every few days when the tsetse fly takes a bloodmeal and glucose is swiftly metabolized within the glycosomes. The glycosomes remain abundant, however, the glycolytic enzymes decrease in number and the induction of other enzymes allow for substrate-level phosphorylation which is essential to the survival of the parasite (Bochud-Allemann and Schneider, 2002; Coustou *et al*., 2003). Components of the Krebs cycle and the electron transport chain are present at the procyclic stage, as well as an atypical acetate:succinate CoA transferase and succinyl-CoA synthetase cycle in which ATP is generated by the conversion of acetyl coA to acetate (Bochud-Allemann and Schneider, 2002; Coustou *et al*., 2003). Many metabolic pathways occur in the glycosome, including peroxide metabolism, β oxidation of fatty acids, ether phospholipid

synthesis, carbon dioxide fixation, purine salvage, and *de novo* pyrimidine biosynthesis (Opperdoes, 1987).

Microtubulues

Microtubules are the primary constituents of the trypanosomatid cytoskeleton (reviewed in Gull, 1999). Extending from the anterior to the posterior axis of the cell, the network includes more than 100 microtubules, forming a stable sub-pellicular array that is highly cross-linked (Fig. 1.2) (Hertz-Fowler *et al.*, 2001). This dense corset not only supplies the cell with its form but is also coated with glycolipid proteins that shield the parasite against immune responses elicited by the host (Van der Ploeg, 1990). The distribution and biogenesis of organelles involved in secretory and endocytic pathways may be facilitated by the sub-pellicular microtubules, by either limiting exo- and endocytic roles to particular domains within the plasma membrane, or by functioning as cytoplasmic scaffolds for certain organelles or intermediates involved in transport (McConville *et al.*, 2002).

Gene expression

Trypanosomes are peculiar with regards to their gene expression, making them attractive models for investigating genome function and evolution (reviewed in Teixeira *et al.*, 2012). Most of their genomes are polycistronically transcribed (Martínez-Calvillo *et al.*, 2010), transcription of genes encoding for proteins is mediated by RNA polymerase I (Gunzl *et al.*, 2003), mature, capped mRNAs are produced through RNA trans-splicing (LeBowitz *et al.*, 1993) and mitochondrial genes transcribing mRNAs undergo thorough RNA editing (Hajduk *et al.*, 1993). In *T. brucei*, regulation of gene expression is post-transcriptional (Clayton and Shapira, 2007). Gene organization within the trypanosomatids involves directional clusters of densely packed genes in one strand separated by alterations in the coding strand (Teixeira *et al.*, 2012). Polycistronic pre-mRNAs are generated by the bi-direcional transcription between divergent gene clusters (Martínez-Calvillo *et al.*, 2003, 2004). Even though orthologues of the constituents of the RNA polymerase II complex have been recognized within the trypanosomal genome (Ivens *et al.*, 2005), no promoter, other than spliced leader promoter, is recognized by RNA polymerase II (Cribb and Serra, 2009; Cribb *et al.*, 2010).. Monocistronic transcripts are then generated upon coupled trans-splicing and poly-adenylation after polycistronic pre-mRNA has been produced (Teixeira *et al.*, 2012). Trans-splicing in trypanosomatids specifically refers to the same 39 nucleotides that cap every

mature mRNA, recognized by the leader sequence (Liang *et al.,* 2003). Intergenic regions contain polypyrimidine-rich tracts which direct the addition of spliced leader and poly-adenylation, generating mature mRNAs (LeBowitz *et al.,* 1993). Further uncommon features include enzymes connected to antioxidant metabolism (Olin-Sandoval *et al.,* 2010) and biosynthesis of glycosylphosphatidylinositol (GPI) (Lepesheva *et al.,* 2011; Koeller and Heise, 2011), both of which can be utilized for the development of drug targets.

1.2.3. The life cycle of *T. brucei*

Transmission of the trypanosome to the mammalian host occurs via the insect vector, the tsetse fly (*Glossina* spp). In contrast to Leishmania and *T. cruzi*, *T. brucei* are extracellular parasites right through their life cycle. The infective metacyclic trypomastigotes in the saliva of the tsetse fly are transmitted to the mammalian host when the fly takes a bloodmeal (Fig. 1.3). The infective form rapidly develops and differentiates into long slender bloodstream forms where they evade attack of the mammalian immune system via antigenic variation of their surface glycoprotein coat (Pays et al., 2004). The kinetoplast is positioned at the posterior end of the cell, and mitochondrial activity is lowered (Fig. 1.3) (Matthews, 2005). Differentiation to the short stumpy bloodstream form, the stage in which cell division is arrested, occurs once parasitemia has increased (Fig. 1.3).

Upon a tsetse bloodmeal, the trypanosomes transform into non-infective procyclic forms in the fly midgut. After active proliferation, trypanosomes migrate to and attach via the flagellum to the insect salivary gland and differentiate and proliferate as epimastigotes, where the kinetoplast is positioned anterior to the nucleus of the cell (Fig. 1.3). Differentiation into non-proliferative metacyclic trypomastigotes occurs when the cycle repeats itself.

The successful life cycle of *T. brucei* has been shown to be dependent on Clan CA cysteine proteases (Scory *et al.,* 1999; Mackey *et al.,* 2004). Proteases, catalysts of the enzymatic degradation of proteins, are ubiquitous in all and essential to many life forms. Clan CA cysteine proteases are required though out the life cycle of parasites. The dependency of the parasite on this family of proteases makes it an attractive drug target (Abdulla *et al.,* 2007; Doyle *et al.,* 2007; Dvorak *et al.,* 2008). Two papain family cysteine proteases are produced in *T. brucei* (BSF), namely rhodesain from *T. b. rhodesiense* (also called brucipain or

trypanopain from *T. b. brucei*) (Caffrey and Steverding, 2009) and *Trypanosoma brucei* cathepsin B (*Tb*CatB), cathepsin L- and B-like enzymes, respectively.

Rhodesain is accountable for the majority of the *T. b. rhodesiense* parasites' protease activity and enables crossing of the blood-brain barrier; *Tb*CatB is upregulated in BSFs (Mackey *et al.*, 2004; Abdulla *et al.*, 2008). Knockdown of *Tb*CatB was shown to rescue a *T. brucei*-infected mouse whereas rhodesain extended the survival of the mouse (Abdulla *et al.*, 2008).

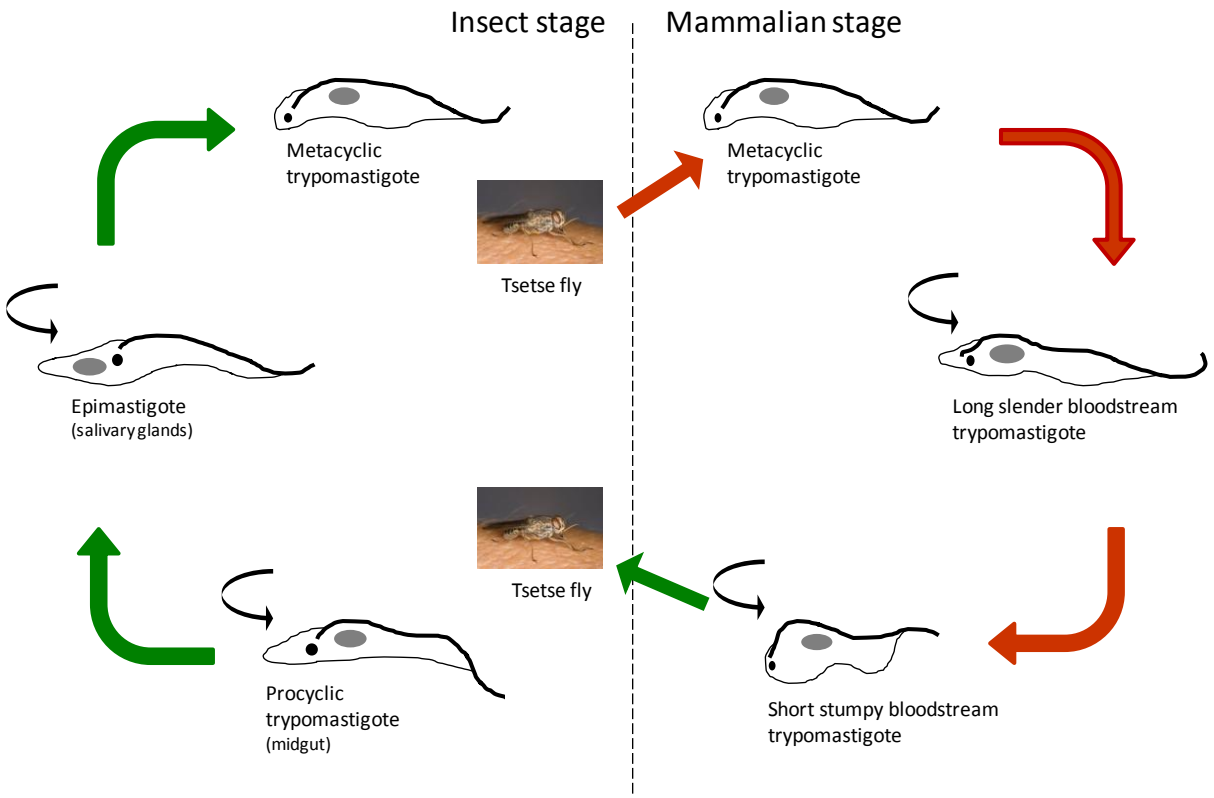


Figure 1.3. Schematic of the *T. brucei* life cycle transitioning between the insect vector and mammalian host.
Increased parasitemia in the mammalian host induces differentiation from the proliferating long slender bloodstream form to the short stumpy bloodstream form (proliferation is indicated by the half arrows). Trypanosomes are ingested by the tsetse fly during a bloodmeal and differentiate into proliferating procyclic trypomastigotes in the fly midgut. Cell division is arrested upon increased parasite numbers and differentiation to proliferative epimastigotes occurs in the salivary glands of the fly. Non-proliferative metacyclic trypomastigotes are formed, in preparation for transmission to a mammalian host via a tsetse fly bloodmeal (adapted from Matthews, 2005).

Inhibition of *Tb*CatB was thus proposed to be a promising target for cysteine protease inhibitors against African trypanosomiasis. The crystal *Tb*CatB·CA074 complex has been resolved (Kerr *et al.,* 2010). Recent findings have shown *Trypanosoma brucei* cathepsin L (*Tb*CatL), rather than *Tb*CatB, to be crucial to the survival of *T. brucei* and thus the more appropriate target for inhibition (Steverding *et al.,* 2012). However, some research is still focused on *Tb*CatB as a promising target, and the fully glycosylated precursor complex of *Tb*CatB has been resolved by a combination of *in vivo* crystallization and serial femtosecond crystallography (Redecke *et al.*, 2012).

T. brucei oligopeptidase B (*Tb*OPB), from the clan SC serine proteases, has been described as a pathogenic factor secreted by the parasite upon transmission of the disease; *Tb*OPB hydrolyses small substrates that have arginine or lysine residues, and has been implicated in African trypanosomiasis-related hormonal imbalances (Coetzer *et al.,* 2008). A prolyl oligopeptidase, *Tb*POP, from the same family, was shown to functionally replace *Tb*OPB in a Δ *opb* null mutant (Kangethe *et al.,* 2012). This finding suggests that development of effective drugs against HAT should entail more than one protease drug target (Kangethe *et al.*, 2012).

1.2.4. Parasite-host interactions

The parasitic protozoa have ensured their survival by developing various approaches to alter their immediate environment, to adjust host immune responses and to impede the host's anti-microbial activity (Corrales *et al.,* 2010). These processes are facilitated by materials secreted by the parasite (BurlMcGwire *et al.,* 2002; Santarem *et al.,* 2007). Trouble in differentiating between secreted proteins and molecules excreted by lysed, dead or dying parasites has resulted in a tedious process concerning the individual identification of secreted proteins (Corales *et al.,* 2010). Corales and colleagues (2010) developed a bioinformatics method in conjunction with functional tests that enabled the identification of 13 new extracellular secreted proteins. The noncellular extracellular matrix (ECM), found in every tissue and organ, provides the necessary scaffolding for cellular components and aids biochemical processes such as tissue morphogenesis, differentiation and homeostasis, and is generated by a heterogenous population of fibroblasts (Fries *et al.,* 1994; reviewed in Piña-Vázquez, *et al.,* 2012). In the first stage of Trypanosomiasis, *T. brucei* is observed in the hemolymphatic

system of its host, and reaches these tissues by secreting proteases, such as neutral metalloprotease, into the ECM; through the degradation of collagen, fibronectin and laminin, this protease allows parasite movement and migration (Huet *et al.,* 1992). De Sousa and colleagues (2010) gave evidence that the activity of this metalloproteinase can be inhibited by ethylenediaminetetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), phenanthroline and tetracycline. The highly conserved and main matrix metalloproteinase (MMP) in *T. brucei* is zinc metalloprotease GP63; it conducts various functions in different life cycle stages making it an attractive target for specific inhibitors (Bangs *et al.,* 2001). Once the parasite crosses the blood-brain barrier (BBB) the *T. brucei* secretion of metalloproteases and cell adhesion molecules facilitate the degradation of the BBB by altering components of the ECM (Hainard *et al.,* 2011). The modified ECM components have been shown to be useful as markers for early diagnosis of the second stage of Trypanosomiasis, which involves a more intricate treatment (Hainard *et al.,* 2011). Cysteine proteases, brucipain (*Tbr*CATL) and *Tb*CatB, are also involved in the process of movement across the BBB (McKerrow *et al.,* 1993; Grab *et al.,* 2009). The BBB is opened up by calcium activation signals induced by *Tbr*CATL, which permits the parasite to cross over (McKerrow *et al.,* 1993; Grab *et al.,* 2009). The *in vivo* upregulation of *Tb*CatB implies the involvement of this cysteine protease in parasite internalization. Protease-activated receptors (PARs) enhance permeability of the BBB once activated by cysteine proteases (McKerrow *et al.,* 1993; Grab *et al.,* 2009). The presence of PARs in a calcium-mediated signalling pathway thus permits parasites into the central nervous system (McKerrow *et al.,* 1993; Grab *et al.,* 2009).

Trypanosomes have developed a molecular mechanism called antigenic variation which enables them to evade the mammalian hosts' immune responses. Two different types of surface proteins cover trypanosomes during different life cycle stages. In the procyclic insect form, the parasite is covered by procyclin proteins; in the bloodstream form trypanosomes are covered by the variant-specific surface glycoprotein (VSG). Both of the non-infective procyclic and epimastigote forms have outer membranes that are enveloped in an invariant glycoprotein coat. Two forms of a protein, EP-procyclin and GPEET-procyclin, make up approximately 10^7 of this invariant glycoprotein coat. When the non-infective form of the parasite differentiates into the metacyclic infective form the EP- and GPEET-procyclin coat is substituted by 10^7 copies of a VSG, also fixed on the outside of the outer membrane via

GPI anchors. The VSG forms a 12 – 15 nm surface coat completely covering the parasite (Vickerman and Luckins, 1969). The VSG constitutes 10% of total protein content of the bloodstream parasite form and over 95% of externally exposed cell surface protein and so is the most copious surface protein in bloodstream trypanosomes (Vickerman and Luckins, 1969). Approximately five million VSG homodimers form the continuous layer that provides the parasite with a wall of protection against the host innate and specific immune effectors (Ziegelbauer and Overath, 1993). Each parasite is coated with a single VSG only, however, when the metacyclic population is considered as a whole in the tsetse fly, a total of 15 – 20 different VSG genes are expressed (Lenardo *et al.*, 1984; Turner *et al.*, 1998).

Antigenic variation describes African bloodstream trypanosomes rapidly switching from one VSG on their surface to another VSG at a rate of 10^{-2} to 10^{-7} switches/ doubling time of 5 -10 hours (Turner and Barry, 1989; Davies *et al.*, 1997; Turner *et al.*, 1997). In essence a chronic infection is maintained through antigenic variation that allows the populace of trypanosomes as a whole to remain a step ahead of the immune response because the only known role of the VSG is to provide a protective wall against the attack of the immune system on the other components of the outer membrane (Pays *et al.*, 1994). Most of the parasites express a homotype during the ascending phase of parasitemia which the host immune system recognizes and produces antibodies against (Black *et al.*, 1982). The major variable antigenic type (VAT) parasites are killed, causing parasitemia to go into a descending phase (Dempsey and Mansfield, 1983). However, during this process those parasites expressing a heterotype increase in number and one overgrows the rest, becoming a new homotype (Dempsey and Mansfield, 1983). This results in a new pulse of parasitemia, leading to a chronic infection. In this way antigenic variation centres on expression of the VSG, resulting in the ultimate wearing out of the host immune system (Dempsey and Mansfield, 1983).

Both humans and select primates have resistance to most trypanosomes due to the presence of trypanolytic factor (TLF) in their serum (Lugli *et al.*, 2004; Poelvoorde *et al.*, 2004). Trypanosome lytic factor (TLF) is a minor subclass of human serum high-density lipoprotein (HDL) capable of neutralizing the infectivity of trypanosomes (Hajduk *et al.*, 1989; Rifkin, 1991). *T. brucei brucei* is incapable of infecting man due to its susceptibility to TLF (Lugli *et al.*, 2004; Poelvoorde *et al.*, 2004). Both *T. brucei rhodesiense* and *T. brucei gambiense* are resistant to TLF, thereby causing sleeping sickness (Kieft *et al.*, 2010). The mechanism of

TLF resistance in *T. b. rhodesiense* involves the disruption of pore-forming activity through binding of serum resistance-associated (SRA) protein to the C-terminal α-helical domain of apolipoprotein L-1; SRA-expression in *T. b. brucei* would enable its resistance to TLF (Xong *et al.,* 1998; Oli *et al.,* 2006; Lecordier *et al.,* 2009). Apolipoprotein A-1 (apoA-1), apoL-1 and hatoglobin (Hp)-related protein (Hpr) are the major elements of TLF; Hpr and apoL-1 showing toxicity to trypanosomes (Shiflett *et al.,* 2005; Molina-Portela *et al.,* 2008). Hemoglobin (Hb) is a cofactor that facilitates the killing activity (Widener *et al.,* 2007). ApoL-1 is a membrane interactant, facilitating selective ion movement. The TLF/SRA protein complex has been suggested to inhibit the ApoL-1/membrane interaction, resulting in human serum resistance (Lecordier *et al.,* 2009). Death of the *T. b. brucei* parasite requires TLF (surface-bound) to be transported to the lysosome in order to be joined with the lysosomal membrane and activated (Hager *et al.,* 1994; Harrington *et al.,* 2009). Trafficking of TLF is promoted by Hpr/Hb bound to the *T. b. brucei* Hp/Hb receptor (Drain, *et al.,* 2001; Vanhollebeke *et al.,* 2008). The mechanism of resistance to TLF in *T. b. gambiense* has been suggested to be linked to the Hp/Hb receptor coding sequence (Kieft *et al.,* 2010). *T. b. brucei* is therefore popularly used in the laboratory in which to conduct research without posing a threat to researchers. Many biochemical and molecular studies have been conducted using the *T. b. brucei* Lister strain 427; Lister 427 is non-tsetse transmissible (Peacock *et al.,* 2008). It can thus be safely used to investigate human infections in laboratory and animal studies.

Trypanosoma brucei as a model organism

Protozoa have been selected as model organisms for animal-based experimentation not only because they are economically sensible and provide no ethical issues, but due to their diversity, abundance and versatility (Montagnes *et al.,* 2012).

In addition to its importance as the etiological agent of parasitic disease in man and livestock, *T. brucei* is also recognized as a model organism in which genetic, biochemical and cell biological approaches can be combined:

1. Its genome has been sequenced (Berriman *et al.,* 2005)
2. *T. brucei* is the most examined Trypanosoma (Verner *et al.,* 2010) due to the simplicity of its culturing and ease of genetic manipulation. Protocols have been developed in which organelles such as the nucleus, mitochondria, glycosomes and flagella can be purified.

3. It is compliant to approaches of forward and reverse genetics (Motyka and Englund, 2004)
4. The structured *T. brucei* cell is large enough to enable high resolution immunofluorescence and electron microscopy analyses

T. brucei offers a valuable system for investigations into cell biology, for features such as cell structure and morphology, organelle localization, cell division and protein transport (reviewed in Matthews, 2005). It has also been used as an experimental model for biological processes including antigenic variation (Rudenko, 2011), eukaryotic cilium and flagellum structure (Dawe *et al.*, 2007), glycosylphosphatidylinositol anchors (Ferguson, 1999) and RNA editing (Benne *et al.*, 1986). The development of stable cell lines expressing T7 RNA polymerase and the tetracycline repressor protein facilitated research of trypanosomal biology as well as experimentation within the tsetse fly (Wirtz *et al.*, 1999; Peacock *et al.*, 2005). Recently a single marker modular vector system was developed in which T7 polymerase and tetracycline repressor genes can be inserted into any of the trypanosomatids, allowing inducible transgene expression, inducible RNAi and inducible experimentation within the tsetse fly (Poon *et al.*, 2012). Many of the 8 131 predicted open reading frames of *T. brucei* have already been assigned functions (Berriman *et al.*, 2005; Subramaniam *et al.*, 2006) and *T. brucei* was amongst the first organisms in which RNA interference studies were demonstrated (Ngo *et al.*, 1998), a technique more efficient than knocking out target genes by homologous recombination. A first that involved *T. brucei* was high throughput screening of genes using a genomic RNAi library (Morris *et al.*, 2002).

The *T. brucei* parasite life cycle is intricate, involving both insect vector and mammalian host environments which results in varying temperatures, pH and metabolic pathways to which the parasite needs to adapt. The Hsp70 molecular chaperone family are most commonly synthesized in response to stress. Not only do the Hsp70s function as heat shock proteins but they also facilitate numerous cellular processes; co-chaperones are essential for the Hsp70s to perform these functions. The Hsp70-Hsp40 interaction may facilitate the translocation from insect vector to human host of *T. brucei.*

1.3. Molecular chaperones

The term "molecular chaperone" was first defined by Laskey in 1978 as a nuclear protein that determined the disassembly of nucleosomes during amphibian egg formation (Laskey *et al.,* 1978). The term was extended to include proteins that aid folding and assembly reactions (Ellis, 1987). Molecular chaperones typically associate with hydrophobic residues of proteins that are unfolded, serving to inhibit inappropriate molecular interactions (Schroder *et al.,* 1993; Parsell *et al.*, 1994).

Molecular chaperones are essential to the maintenance of cellular homeostasis by facilitating various functions including degradation of proteins, translocation, folding of co-translational products, refolding and solubilisation of misfolded proteins, amending protein aggregation, protein function regulation and protein complex assembly (Brodsky and McCracken, 1999; Eggers *et al.,* 1997; Hamman *et al.,* 1998). Chaperones stop hydrophobic surfaces from binding to each other by holding non-native unfolded intermediates thereby preventing the accumulation of misfolded proteins under cellular stress (Parsell *et al.,* 1994, Schroder *et al.,* 1993). When the cellular stress is removed, intermediates are degraded or refolded to prevent further aggregation from occurring (Parsell *et al.,* 1994, Schroder *et al.,* 1993).

The only known universal response to stress experienced by an organism is the production of stress proteins (Gross, 2004). The most commonly studied are the evolutionarily conserved families of genes encoding molecular chaperones, also known as heat shock proteins (Hsp) (Hartl, 1996). "Heat shock" infers elevated temperatures, but this class of proteins can be upregulated by any proteotoxic stress. Ritossa and colleagues were the first to discover genes responsive to heat stress in 1962 in *Drosophila* salivary glands when a "puffing pattern" in the polytene chromosomes was produced by an unknown protein upon accidentally increasing the incubation temperature. "Heat shock protein" was coined in 1974 upon discovery that those heat-induced gene products were 70 kDa and 27 kDa in size (Ritossa, 1962; Tissieres *et al.,* 1974). Hsps are either constitutively expressed (heat shock cognates, Hsc), and maintain cellular homeostasis, or are upregulated in response to external stimuli (heat shock proteins, Hsp) (Hartl and Hartl-Meyer, 2002). The most common stress inducing Hsp upregualtion is increased environmental temperatures, referred to as the heat shock response (HSR). In addition to hyperthermia, metabolic and exogenous stresses also include nutrient deficiencies; oxidative stresses; amino acid analogues; dehydration; osmotic

pressures; heavy metals and viral infections. (Corporeau and Auffret, 2003; Schill *et al.*, 2003; Chang, 2004; Hayward *et al.*, 2004; Todgham *et al.*, 2005; Schramm *et al.*, 2008). Stress-induced upregulation of Hsps facilitates management of the potentially deleterious consequences of the stresses by folding/refolding damaged polypeptides, inhibiting protein aggregation or self-association, and encouraging protein transport of targeted degraded proteins for their removal from the cell thereby preventing formation of cytotoxic aggregates (Young *et al.*, 2004; Mayer and Bukau, 2005; Brodsky and Chiosis, 2006; Schmitt *et al.*, 2006). The large and varied heat shock protein family is classified into major classes defined by molecular mass. These classes of Hsps include Hsp100 with molecular masses of 100 kDa, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps.

1.3.1. Hsp70 family of proteins

Hsp70 family members are present in all organisms; they are the most highly conserved heat shock protein family (Boorstein *et al.*, 1994). Numerous Hsp70 crystal structures have been resolved, including the substrate binding domains of human HSPA1L, HSPA2, HSPA5 and HSPA6 (Wisniewska *et al.*, 2010); *E. coli* full-length DnaK (Bertelson *et al.*, 2009); ATP-bound DnaK (Kityk *et al.*, 2012); bovine Hsc70 (Jiang *et al.*, 2005) and the substrate binding domain of yeast Hsp110, Sse1 (Liu and Hendrickson, 2007). Members of this protein family are present in most of the intracellular compartments, for instance in humans, Hsc70 and Hsp70 localize in the cytosol and nucleus, Grp78 is localized in the endoplasmic reticulum and MtHsp75 in the mitochondria; Hsc70, Grp78 and MtHsp75 are constitutively expressed, Hsp70 shows heat stress incubility (Wu *et al*, 1985; Milarski and Morimoto, 1989). Proteins forming part of the Hsp70 family are involved in various cellular processes such as protein folding and assembly of nascent polypeptides, refolding in the case of protein aggregation, protein translocation across membranes, ubiquitination and misfolded protein degradation, associations with signal transduction proteins and management of many regulatory proteins (Bukau and Horwich, 1998; Ryan and Pfanner, 2001; Young *et al.*, 2003; Pratt and Toft, 2003; Mayer and Bukau, 2005; Kabani and Martineau, 2008).

The highly conserved Hsp70 structure is made up of an ATPase domain at the N-terminus (45 kDa), and a substrate binding domain (SBD) (25 kDa) at the C-terminus, separated by a highly conserved hydrophobic linker region (Mayer and Bukau, 2005). The C-terminus is

further subdivided into a β-sandwich subdomain of 15 kDa with a peptide-binding cleft and a C-terminus lid (α-helical subdomain) that covers the peptide binding site (Fig. 1.4) (Mayer and Bukau, 2005). Present only in Hsp70 members of the eukaryotic cytosol is the EEVD motif at the C-terminus (Fig. 1.4). The EEVD motif is involved in the binding of Hsp70/Hsp90 organizing protein (Hop)/p60 and carboxyl terminus of Hsp70 interacting protein (CHIP), which are tetratricopeptide repeat (TPR) cofactors (Scheufler *et al.*, 2000). The EEVD motif has been shown to play regulatory roles in the functions of Hsp40/Hsp70 (Freeman *et al.*, 1995). It may even be possible that the EEVD motif ensures proficient transfer of non-native polypeptides to Hsp70 from its co-chaperone (Li *et al.*, 2006). In an attempt to identify its function, the disordered C-terminal region was suggested as a weak, secondary binding site for denatured substrates, specifically the DnaK residues 624-638, which contains the EEVKD motif (Smock *et al.*, 2011). The intrinsically disordered region (IDR) of the C-terminus may act as a flexible tether, keeping the refolded substrate released from the ATPase cycle in proximity of the chaperone through weak interaction with the EEVKD motif, thereby allowing a subsequent binding and release cycle (Smock *et al.*, 2011). Tompa and Csermely (2004) termed entropy transfer as the weak multivalent interactions occurring at an auxiliary binding site at an IDR that get exploited by chaperones.

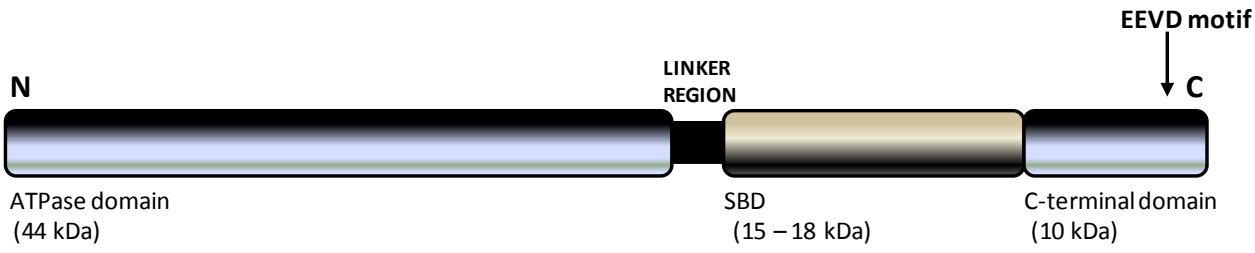


Figure 1.4. Schematic of Hsp70 domain organization.
The 44 kDa ATPase domain and 15 kDa substrate binding domain (SBD) are separated by the linker region. The C-terminal domain of cytosolic Hsp70 proteins typically contains a highly conserved EEVD motif.

This multivalent molecular identification by the conserved C-terminal residues may facilitate chaperoning of an assorted range of substrates. The EEVKD motif is absent freom *E. coli* HscA and only aids in the maturation of iron-sulphur cluster assembly protein IscA (Vickery and Cupp-Vickery, 2007). In contrast to the minimalistic functioning of HscA, SecB and

DnaJ are involved in shielding misfolded substrates from aggregation and degradation, as well as maintaining the unfolded substrates for transport to the secretory pathway (Randall and Hardy, 2002; Ullers *et al.,* 2004; Sakr *et al.,* 2010). Smock and colleagues (2011) suggest that the proposed mechanism may also apply to eukaryotic cytoplasmic Hsp70 proteins. The Hsp70 machinery does not function alone; an Hsp40 with a J-domain (Section 3.2) and a nucleotide exchange factor (NEF) are required as partners as they regulate the binding of Hsp70 to its client proteins. The ATPase domain of Hsp70 regulates the conformation of the SBD which interacts with a hydrophobic five residue segment of the substrate clients (Rudiger *et al.,* 1997). The allosteric Hsp70 cycle involves switches between the ATP- and ADP-bound states through ATP hydrolysis and substrate exchanges. The rapid bind- and release-rates of substrate binding occur in the ATP-bound state; the exchange reactions are slow in the ADP-bound state. The association with substrates occurs very rapidly and ATP hydrolysis is required to stabilize the interaction. Yet Hsp70 has a low basal ATPase activity making the changeover between the two states very slow. Hsp70 thus needs its partners to stimulate its ATPase activity to regulate capturing of client proteins, the J-domain; to dissociate ADP from its bound state with Hsp70, facilitating dissociation of client proteins, NEFs. The substrate binding to the SBD causes a 10-fold stimulation of the ATPase activity of Hsp70; the same degree of ATPase stimulation was observed for a truncated version of Hsp70 in which only the ATPase domain and linker region were present, suggesting the importance of the linker region in allostery and the ATPase domain function (Vogel *et al.,* 2006; Jiang *et al.,* 2007; Swain *et al.,* 2007).

Lobes I and II comprise the ATPase domain, each with subdomains IA, IB and IIA, IIB, respectively. All the subdomains engage in coordinating the substrate which binds to the base of the middle cleft formed between subdomains IB and IIB (Hurley, 1996). The reorientation of the subdomains causing substrate-dependent conformational changes is thought to be an inherent quality of all ATPase domains (Hurley, 1996). Significant flexibility in the ATPase subdomain reorientations have been demonstrated (Bhattacharya *et al.,* 2009; Woo *et al.,* 2009); a network of subdomain movements is responsible for the allosteric interactions in the ATPase domain of Hsp70 proteins (Zhang and Zuiderweg, 2004). The allosteric signal can occur in two directions. Disturbances in the substrate-binding site can either influence subdomain interfaces or a signal can spread to the substrate-binding site from the subdomain interface. This latter signal can monitor both substrate binding and ATPase activity

(Zhuravleva and Gierasch, 2012). Zhuravleva and Gierasch (2012) showed that the rotation of subdomain IIB and opening of the substrate-binding cleft was preferred when substrate was unbound; similar predictions were produced by elastic network modelling (ENM) and MD calculations (Woo *et al.,* 2009; Liu *et al.*, 2010). A two-way coupling pathway has been described between the linker region and bound ATP by Zhuravleva and Gierasch (2011). The binding of ATP, through subdomina reorientations, causes the exposure of a binding surface for the linker region. Binding of the linker region then stabilizes the active or "open" conformation of the ATPase domain, and the β-sheet of subdomain IIA joins the substrate binding site and the linker region. The orientation of the subdomain IIA β1-β2 turn that organizes the ATP γ-phosphate subsequently rotates. The ATP γ-phosphate and the linker region are required for the active conformation of the ATPase domain. The linker region thus functions as a switch: its association with the hydrophobic cleft, dependent on substrate binding, influences the conformation of the ATPase domain and monitors communication between the domains such that the SBD is brought close to the ATPase domain. Point mutations of both the linker region and residues within the hydrophobic cleft have been shown to result in reduced ATPase activity, and to hinder regulation provided by the SBD and co-chaperones (Vogel *et al.*, 2006; Jiang *et al.*, 2007; Liu and Hendrickson, 2007). It is probable that the mechanism of action of the regulation of the rate of ATP hydrolysis incurred by DnaJ occurs in a similar fashion as DnaJ binds to the hydrophobic cleft of the ATPase domain (Zhuravleva and Gierasch, 2012).

The evolutionary diversification of *Hsp70* genes is related to the versatile functioning of the proteins. Yet when the number of cellular Hsp70 proteins relative to Hsp40 proteins is considered, Hsp70s are far outnumbered (Craig *et al.*, 2006; Hageman and Kampinga, 2009). In mammalian cells, both the mitochondria and ER contain only one type of Hsp70, and four and six Hsp40 proteins, respectively (Craig *et al.*, 2006; Hageman and Kampinga, 2009). Humans have a total of 11 Hsp70 proteins, 13 NEFs and 41 Hsp40 proteins containing a J-domain which would suggest that Hsp40s may be the driving force in the functional diversity of the Hsp70 machinery (Kampinga and Craig, 2010).

Four different types of NEFs have been identified that facilitate the functioning of the eukaryotic Hsp70 cycle. They may all interact with the ATPase domain of Hsp70, but they differ in sequence as well as in the mechanism in which they destabilize substrate binding

(Cyr, 2008; Polier *et al.*, 2008; Schuermann *et al.*, 2008). NEFs GrpE and HspBPI/Bap have the sole purpose of functioning in substrate destabilization; they have no domains present other than that involved in nucleotide release. Only GrpE is present in mitochondria and bacteria, and facilitates resident Hsp70 proteins in roles ranging from polypeptide folding to translocation (Schroder *et al.*, 1993; Laloraya *et al.*, 1995). Similarly, cytosolic hHspBPI (scFes1) and hBap (scSls1p/scSillp) with the Hsp70s present in the ER play a role in protein refolding and protein degradation (Travers *et al.*, 2000; Tyson and Stirling, 2000).

The third type of NEF is structurally similar to Hsp70s. Hsp110 (hHSPH1-1/scSse1-2 and scLhs1) has an ATPase domain joined to a 9-stranded β-sandwich SBD by a linker region (Mukai *et al.*, 1993; Oh *et al.*, 1999). A few Hsp110s are able to bind substrate and prevent aggregation; Hsp110s can only function as "holdases" as the interaction cannot be modulated (Polier *et al.*, 2008). Under conditions of stress, stress inducible members Ssc2 in yeast and human HSPH1 may store client proteins before functioning with Hsp70 in refolding the partially denatured substrates (Mukai *et al.*, 1993; Dragovic *et al.*, 2006; Raviol *et al.*, 2006; Polier *et al.*, 2008; Schuermann *et al.*, 2008).

The last and most complex type of NEFs are the BAG proteins; all have a ~85 residue region that associates with the ATPase domain of Hsp70, the "Bag" domain, in common (Takayama and Reed, 2001). Yeast has only one Bag protein member, scSnl1, and humans have six members, hBAG-1, hBAG-2, hBAG-3 (CAIR-1/Bis), hBAG-4 (SODD), hBAG-5, and hBAG-6 (Scythe/BAT3) (Takayama and Reed, 2001), four of which have been experimentally demonstrated to associate with Hsp70 (BAG-1,2,3,6) (Takayama and Reed, 2001). Besides functioning as NEFs for Hsp70, hBAG-1 and hBAG-6 contain an ubiquitin-like domain (Ubi) which has been suggested to facilitate an interaction with the proteasome (Alberti *et al.*, 2002; Luders *et al.*, 2000). Two mechanisms for the degradation of unfolded polypeptides are present in all cells: the ubiquitin-proteasome pathway and lysosome-mediated autophagy (Agarraberes and Dice, 2001). Ubi is not present in hBAG-3, which has been associated with protein degradation independent of Hsp70, and so is likely to do so through autophagy as opposed to through the proteasome (Carra *et al.*, 2008; Carra *et al.*, 2009). The specific functioning of hBAG-3 as NEF for Hsp70 is still undefined (Carra *et al.*, 2009).

Hsp70 complexes often act together with other chaperone complexes, displaying a network of chaperone activity instead of physical interactions between the machineries. For instance, excessively unfolded polypeptides will be targeted by Hsp70s upstream prior to binding with chaperonins (Teter *et al.,* 1999; Kerner *et al.,* 2005). However, in some networks adapter proteins that act as physical bridges between the machineries do exist, as is the case with the Hsp70-Hsp90 complex. In this chaperone complex, essential for the maturation of hormone receptors and various transcription factors, substrates are transferred from Hsp70 to Hsp90 by a co-chaperone that interacts with both chaperones: a TPR-repeat protein, Hsp70/Hsp90 Organizing Protein (HOP) (Smith and Toft, 2008; Wandinger *et al.,* 2008). Hsp70 machines also form networks with small Hsps, all of which enhances Hsp70s multifunctionality (Haslbeck *et al.,* 2005; Liberek *et al.,* 2008). Various cofactors have been identified that participate in the ATP/ADP hydrolysis cycle of Hsp70. Hsp70-interacting protein (hHIP, p48) was shown to associate with and stabilize the ADP-bound state of Hsp70 (Hohfeld *et al.,* 1995; Prapapanich *et al.,* 1996; Ziegelhoffer *et al.,* 1996). HIP allows more substrate-binding time by competing with BAG-1 for binding to Hsp70 which slows down the substrate cycle (Ziegelhoffer *et al.,* 1996). Protein folding is enhanced when HIP concentrations are increased, both *in vitro* (Hohfeld *et al.,* 1995) and *in vivo* (Nollen *et al.,* 2001). HIP also plays a role in the assembly of Hsp70 into multiprotein complexes with Hsp90 (Nelson *et al.,* 2004). CHIP specifically targets protein clients of Hsp90 for degradation (Connell *et al.,* 2001; Meacham *et al.,* 2001; Xu *et al.,* 2002). CHIP displays chaperone activity in its ability to prevent protein aggregation (Rosser *et al.,* 2007), resulting in either folding (Kampinga *et al.,* 2003) or degradation (Rosser *et al.,* 2007). CHIP can initiate inhibition of Hsp40-stimulated ATPase activity of Hsp70 when bound to the ATP-bound state of Hsp70 (Ballinger *et al.,* 1999). CHIP thus appears to prevent Hsp70 binding its substrates and so encourages their degradation (Kampinga and Craig, 2010).

1.3.2. Hsp40 family of proteins

The Hsp40 proteins represent a large family that functions to facilitate the cellular activity of the Hsp70 proteins. Family members of Hsp70 and Hsp40 proteins are often co-localized enabling multiple Hsp40 members to associate with one Hsp70 member forming unique Hsp70-Hsp40 pairs that are involved in cellular processes at specific cellular locations (Caplan *et al.,* 1992; Ungermann *et al.,* 1994). Hsp40 proteins, being co-chaperones of Hsp70

proteins, are necessary for translation of proteins, folding and unfolding of polypeptides, protein translocation and degradation (Bukau and Horwich, 1998; Mayer and Bukau, 2005). In addition, Hsp40s may act as chaperones in their own rights, having formed interactions with and preventing aggregation of unfolded proteins, thereby displaying a holding-function (Stirling *et al.,* 2003). The Hsp40 proteins are present in all cellular organelles to facilitate Hsp70 ATPase activity and are essential in certain protein folding reactions (Cyr *et al.,* 1992). The major function of Hsp40s, however, involves regulation of ATP-dependent polypeptide binding by Hsp70 (Liberek *et al.,* 1991; Palleros *et al.,* 1993; Szabo *et al.,* 1994).

One of the most important and well characterised Hsp40 domains, the main site of interaction with Hsp70, is the J-domain, and is present in all members of the Hsp40 family (Fig. 1.5) (Huang *et al.,* 1998). J-proteins contain a very well-conserved 75 amino acid sequence, the J-domain, which is present at various locations within the Hsp40 protein (Caplan *et al.,* 1993). Four α-helices joined by loop regions comprise the J-domain (Pellechia *et al.,* 1996). The J-domain, first identified in *E. coli* DnaJ, has a well conserved signature feature, Histidine – Proline – Aspartic acid (HPD) tripeptide motif present in the loop region between the α-helices II and III (Qian *et al.,* 1996). Depending on which class the Hsp40 protein belongs to it may further contain a glycine-phenylalanine (G/F)-rich region and a cysteine/glycine-rich region that contains four motifs of CXXCXGXG that bears a resemblance to a zinc-finger domain, a glycine/methionine-rich region, a carboxy-terminal domain and a dimerization domain (Cheetham and Caplan, 1998). The zinc-finger domain is required for the interaction of Type I Hsp40 proteins with Hsp70 in functions of protein folding; zinc binding domain (ZBD) I and ZBDII are both necessary for protein folding, but only ZBDII is essential to the Hsp70-Hsp40 association in suppression of protein aggregation (Linke *et al.,* 2003; Fan *et al.,* 2005). The C-terminal domain of Type I and II Hsp40 proteins contain a peptide binding region that interacts with unfolded substrates (Sha *et al.,* 2000; Li *et al.,* 2003).

Hsp40 proteins are divided into four main classes based on the canonical prokaryotic protein, DnaJ. Type I Hsp40s contain all three canonical domains and are highly conserved, whereas Type II Hsp40s contain only the J-domain and the G/F-rich region (Fig. 1.5) (Cheetham and Caplan, 1998). Both Type I and some Type II Hsp40s have a peptide-binding region at the C-terminus which aids in the binding to non-native polypeptides, in turn allowing Hsp40 proteins to transport substrates to Hsp70 proteins for folding (Fig. 1.5) (Cyr *et al.,* 1994;

Bukau and Horwich, 1998; Johnson and Craig, 2001). The Type III Hsp40s, with respect to *E. coli* DnaJ, are poorly conserved due to possessing only the highly conserved J-domain (Cheetham and Caplan, 1998; Kelly, 1998). Conserved residues of the J-domain may modulate the affinity or specificity of the partnership of Hsp40/Hsp70 (Hennessy *et al.,* 2005; Garimella *et al.,* 2006). Unlike Types I and II Hsp40s where the J-domain occurs on the N-terminus, the J-domain of Type III Hsp40 can occur anywhere on its sequence (Cheetham and Caplan, 1998; Kelly, 1998). The canonical peptide-binding domain is also not present in Type III Hsp40s (Fig. 1.5) (Cyr *et al.,* 1994; Bukau and Horwich, 1998). The J-domain functions to facilitate the ability of Hsp70s to hydrolyze ATP and to mediate the Hsp70-Hsp40 interaction (Mayer and Bukau, 1998).

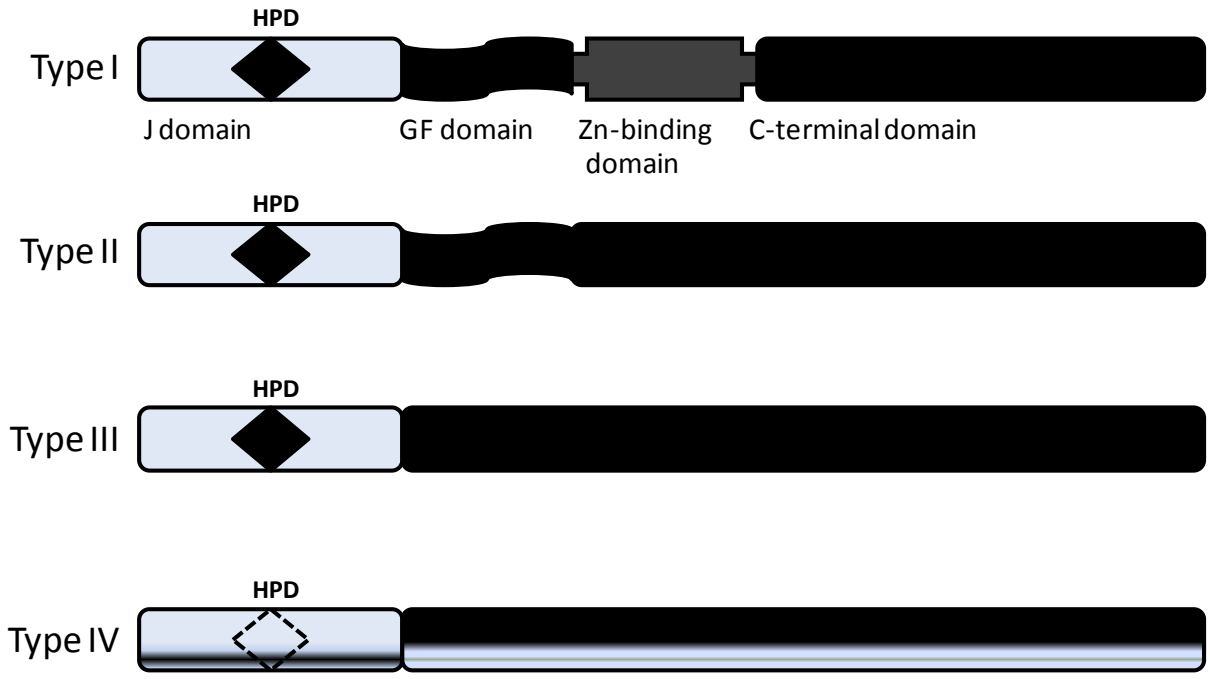


Figure 1.5. Schematic of the classification of Hsp40 proteins.
Hsp40 proteins are classified into four types according to the presence of a J-domain, GF domain and a zinc-binding domain (Cheetham and Caplan, 1998; Botha *et al.,* 2007). The HPD motif is typically present within the J-domain.

Very few of the Type III Hsp40s have been characterized because of their high levels of sequence diversity, and it has been suggested that they have specialized cellular functions distinct from those of Types I and II Hsp40s (Kelly, 1998; Genevaux *et al.,* 2001). Type IV

Hsp40s exhibit a compromised HPD motif (Fig. 1.5) (Botha *et al.*, 2007). Variations of residues within the HPD motif are known to disrupt the ability of Hsp40 to catalyse the ATP hydrolysis of Hsp70 (Mayer *et al.,* 1999; Genevaux *et al.,* 2001; Wittung-stafshede *et al.,* 2003).

1.3.3. The Hsp70/Hsp40 partnership

The complex that is formed between Hsp70 and polypeptide substrates is regulated by members of the Hsp40 family and nucleotide exchange factors. Hsp40 regulates the function of Hsp70 by three main mechanisms. The polypeptide-binding domain (PPD) of Hsp40 is required to bind and transport specific polypeptides to Hsp70; by doing so it regulates the functional specificity of Hsp70 (Cyr *et al.,* 1994; Cheetham and Caplan, 1998). The Hsp70-substrate association is then stabilized by Hsp40, which binds a site in the underside of the Hsp70 ATPase domain, thereby stimulating Hsp70's ability to hydrolyse ATP and stabilizing the Hsp70-ADP-substrate complex (Landry, 2003; Wittung-Stafshede *et al.,* 2003; Jiang *et al.,* 2007; Li *et al.,* 2009). The third mechanism involves different localizations of various Hsp40 family members within one cellular compartment, allowing the association of Hsp70 with different Hsp40s and thus different Hsp70-Hsp40 associations to bind unique polypeptides at the different cellular compartments (Brodsky and Schekman, 1993; Cyr and Neupert, 1996; Shen *et al.,* 2002). The cycling of the core machinery (Hsp70/J-domain/NEF) is conducive to productive folding owing to the fact that when the substrate is bound to the complex aggregation is prevented. Incomplete folding simply results in rebinding and release of substrate in reiterative cycles (Kampinga and Craig, 2010).

When polypeptides are misfolded, their generally buried hydrophobic residues become exposed which could result in adverse interactions with peptides, nucleic acids and other macromolecules. Hsp70 functions to resolve this by holding nascent peptides and protecting hydrophobic amino acids from undesirable interactions (Young *et al.,* 2004; Bukau *et al.,* 2006). Hsp70 protein activity occurs through cycles of interaction with the hydrophobic peptide segments of client proteins with its substrate binding domain (SBD) in an ATP-dependent manner, coupled to conformational changes (Fig. 1.6). The Hsp70 ATPase cycle switches between the low affinity of the ATP-bound state and rapid substrate-exchange rates, and the high affinity ADP-bound state with low substrate-exchange rates (Liberek *et al.,* 1991; Palleros *et al.,* 1993; McCarty *et al.,* 1995). The highly conserved J-domain of co-

chaperone Hsp40 is required to stimulate the ATPase activity of Hsp70 and results in Hsp70-ADP with a high affinity for substrate (Fig. 1.6). Once Hsp40 has presented the misfolded polypeptide to Hsp70, Hsp70 binds its short peptide regions with its exposed hydrophobic residues; the interaction is stabilized by both Hsp40 and Hip protein (Fig. 1.6). In order for the nucleotide binding cleft to be opened, the release of ADP is facilitated by a nucleotide-exchange factor, Bag-1 protein (Fig. 1.6). The ATPase cycle is completed when ATP binds the ATPase domain of Hsp70 resulting in a conformational change in the substrate binding domain and bound substrates are released (Brodsky and Bracher, 2007).

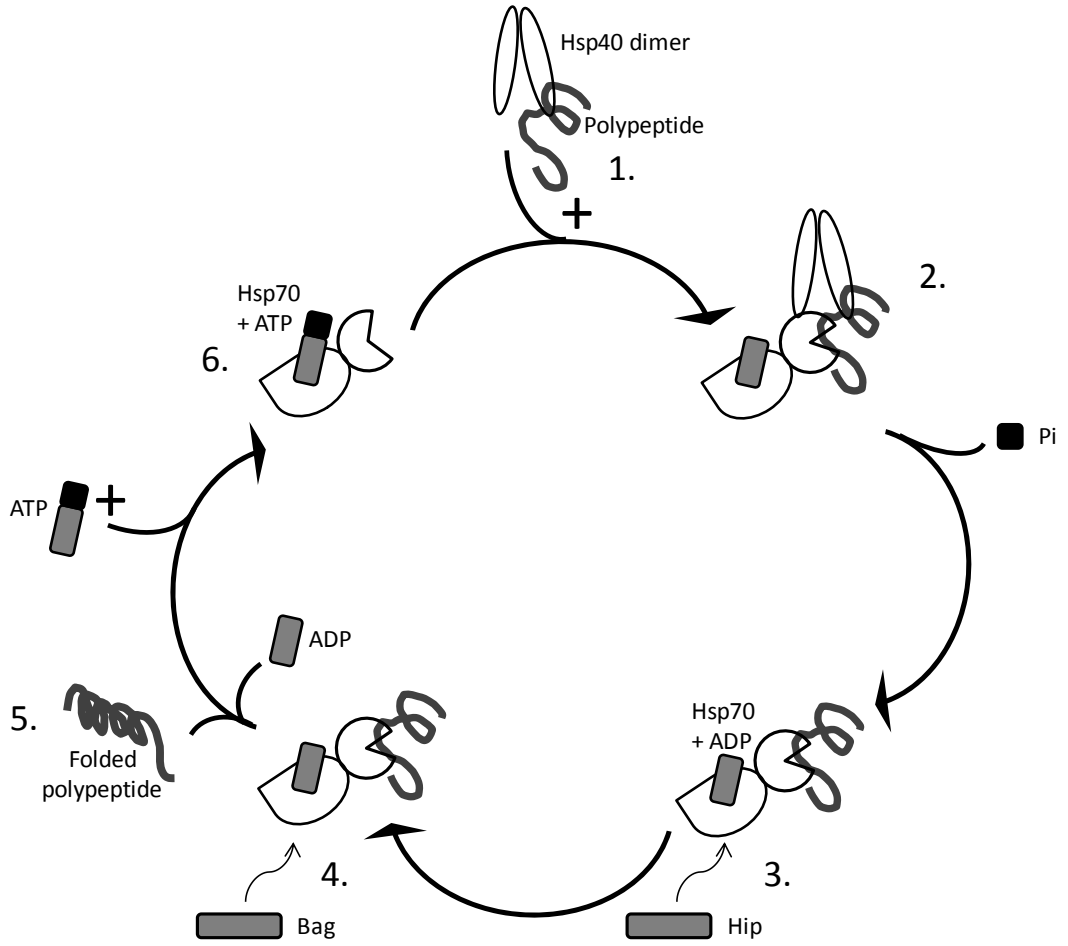


Figure 1.6. Schematic of the Hsp70 ATPase/folding cycle.
Hydrolysis and exchange of ATP mediates the Hsp70 folding cycle of substrate binding and release. (1) Dimeric Hsp40 recruits nascent or misfolded polypeptides to Hsp70. (2) The ATPase activity of Hsp70 is stimulated by the Hsp40 J-domain, resulting in the closed conformation of the Hsp70 nucleotide binding cleft, there with holding the polypeptide. (3) The ADP-bound Hsp70 complex is stabilized by the binding of Hip. (4) Nucleotide exchange is promoted by the binding of Bag. (5) Natively folded polypeptide is released upon the uptake of ATP. (6) The open conformation of ATP-bound Hsp70 is prepared for uptake of misfolded polypeptide (adapted from Meimaridou *et al.*, 2009).

Analysis of the open conformation of *E. coli* DnaK, the ATP-bound state, has recently revealed that the β-sheet and α-helical lid subdomains of the SBD are unattached from each other and interact with other regions of the SBD, the β-sheet subdomain displays an allosteric mechanism (Kityk *et al.,* 2012). The EEVD motif present at the end of the C-terminus of Hsp70 members is involved in binding to the tetratricopeptide repeat (TPR) domains of Hsp70/Hsp90 organizing protein (Hop) and CHIP (Scheufler *et al.,* 2000). The EEVD motif has been shown to play regulatory roles in the functions of Hsp40/Hsp70 such as altering the ATPase activity of Hsp70 and its ability to interact with substrates through deletion or mutation of the EEVD motif (Freeman *et al.,* 1995). It may even be possible that the EEVD motif ensures proficient transfer of non-native polypeptides to Hsp70 from its co-chaperone (Li *et al.,* 2006). Recent studies have shown that PfHop in *Plasmodium falciparum* co-localizes with PfHsp70 and PfHsp90 in the cytosol, and binding studies have confirmed its complex formation with PfHsp70 and PfHsp90 through the EEVD motif of PfHsp70 (Shonhai *et al.,* 2007) and its TPR domains (TPR1 and TPR2A) (Gítau *et al.,* 2012).

1.4. Hsp70 and Hsp40 proteins in kinetoplastids

The kinetoplastids have a complex life cycle, moving between biologically very different environments, the insect vector with a relatively cool temperature, and the mammalian host with higher temperatures. For instance, *T. brucei* proliferates and differentiates in the alimentary gut and salivary glands of the insect vector and grows in blood and tissue fluids in the mammalian host, thereby adapting to varying temperatures, pH, oxygen tension and nutrients (Jones *et al.,* 2008). These temperature changes result in increased production of Hsps (Maresca and Carratu, 1992).

The only Hsp40 proteins to have been characterized in kinetoplastids have been from *T. cruzi* and *T. brucei* (Tibbetts *et al.,* 1998; Edkins *et al.,* 2004; Louw *et al.,* 2010). Using predicted proteins with homologous J-domain sequences, BLAST searches were performed in the genome databases of the kinetoplastids. *T. cruzi* was discovered to have 67 Hsp40s, 66 in *L. major* and 65 in *T. brucei* (Folgueira and Requena, 2007). Most of the Hsp40s contain orthologues in the kinetoplastids and all those in *T. brucei* and *L. major* are encoded by single-copy genes; two genes encode for most of the Hsp40 proteins in *T. cruzi* (Folgueira and Requena, 2007). It has been suggested that the numerous Hsp40s in the kinetoplastids

have roles in cellular homeostasis that drove the diversification of the Hsp40 proteins (Folgueira and Requena, 2007).

Approximately 20 *Leishmania* species infect humans (Schonian *et al.,* 2010). New methods of species identification are necessary to allow correct identification and treatment (Requena *et al.,* 2012). A polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) approach was developed to allow identification of therapeutically applicable *Leishmania* species using *Hsp70* PCR amplification (Montalvo *et al.,* 2010). Due to the highly conserved coding region of the *Hsp70* gene, the untranslated regions (UTR) of the *Leishmania* species were considered a better target than the coding region and were used for species typing; the UTR of *Hsp70-1* genes was demonstrated to effectively serve as a molecular marker (Requena *et al.,* 2012).

Due to the evolutionary conservation of their sequences, genes encoding cytoplasmic Hsp70 proteins were amongst the first to be characterized in the kinetoplastids, hybridization with heterologous *Drosophila Hsp70* probes making cloning of these sequences possible (Glass *et al.,* 1986). *Hsp70* from *T. brucei* was initially thought to be a pseudogene due to the lack of the *Hsp70* 3´ end but was found to be active transcriptionally and was thus named the cognate *Hsp70* gene, revealing that temperature shifts do not influence the mRNA levels (Lee *et al.,* 1995). Another *Hsp70* gene was found in *L. major*, called *Hsp70.4* and via indirect immunofluorescence its localization was determined to be cytoplasmic (Searle and Smith, 1993).

To determine the origin of these cytoplasmic Hsp70s an evolutionary study was conducted. *L. major* Hsp70.4 and *T. brucei* atypical Hsp70.c were determined to form a monophyletic group found to be evolutionarily distant from *T. brucei* canonical Hsp70 (Table 2.4) (Simpson *et al.,* 2004). LmHsp70.4 and TbHsp70.c are however evolutionarily distinct clades – mutual divergence is thought to have occurred a long time ago (Simpson *et al.,* 2004). LmHsp70.4 and TbHsp70.c may be restricted to *Euglenozoa* - TbHsp70.c is only found in trypanosomatids and LmHsp70.4 is only found in bodonids (Simpson *et al.,* 2004). The physiological roles of these proteins remain to be investigated.

Two novel Hsp70 family members are Hsp70.a and Hsp70.b (Folgueira and Requena, 2007). Hsp70.a was found to be represented by LmjF01.0640 in *L. major* (Table 2.4) (Folgueira and Requena, 2007). The inferred sequence consists of 1112 amino acid residues and has a molecular mass of 117.5 kDa (Folgueira and Requena, 2007). The cellular location of Hsp70.a has not been determined. The Hsp70.b subfamily of proteins was found to be homologous with DnaK from proteobacteria and cyanobacteria (Folgueira and Requena, 2007). Hsp70.b proteins share a higher sequence homology with cyanobacterial DnaK than with Hsp70 members of the mitochondrial subfamily, and so the ancestor of kinetoplasts may very well have possessed chloroplasts (Hannaert *et al.*, 2003; Martin and Borst, 2003). As an example, both *Trypanosoma* and *Leishmania* have proteins that are more alike to plant and algae homologues than to eukaryotic homologues (Hannaert *et al.*, 2003).

The effect of stress on chaperones in kinetoplastids

Cellular stress such as heat shock typically results in a downregulation in expression of genes associated with growth, and an upregulation in those associated with the maintenance of protein structural integrity, such as heat shock proteins (reviewed in Schwede *et al.*, 2012). In response to heat shock trypanosomes have been reported to show a 50% drop in transcription rate; the steady state levels of *Hsp70* and *Hsp85* are either constant or enhanced (Muhich and Boothroyd, 1988; Muhich and Boothroyd, 1989; Muhich *et al.*, 1989; Lee, 1998). Belonging to the Hsp100 family of chaperone proteins, Hsp104 facilitates protein unfolding, disaggregation and degradation under conditions of stress (reviewed in Doyle and Wickner, 2009). This 869 amino acid protein has a sequence similarity of 76% to that of *T. brucei*, the gene structure of *Hsp104* has been described in *T. brucei* (Gottesman *et al.*, 1990) and only 23% sequence similarity with the human protein (Campos *et al.*, 2012). The effect of heat shock on the gene expression levels of *Hsp104* in *T. cruzi* epimastigotes was investigated and detected at low levels at normal temperatures and increased levels for both mRNA and protein during heat shock (Campos *et al.*, 2012). Studies on *T. cruzi* have connected the heat shock response with Hsp expression (Requena *et al.*, 1992; Olson *et al.*, 1994; Carvalho *et al.*, 1994; Fernandes *et al.*, 2005; Péres-Morales *et al.*, 2009). *T. cruzi* epimastigotes were subjected to heat stress at 42°C, and protein extracts analysed by 2-DE gel electrophoresis revealed that of the 521 ± 31 spots, peptide mass fingerprints for 24 spots were obtained (Péres-Morales *et al.*, 2012). Of these, 19 were found to be differentially abundant upon heat

shock and were functionally categorized, including metabolism, hypothetical proteins, protein synthesis, cell defence and cellular transport (Péres-Morales *et al.,* 2012).

1.4.1. Hsp70 and Hsp40 proteins in *T. brucei*

T. brucei has been shown to have 65 Hsp40 proteins and 12 Hsp70 proteins through *in silico* analysis, including orthologues in the Hsp70 family subdivisions (Folgueira and Requena, 2007). The cytoplasmic Hsp70s include a canonical Hsp70, TbHsp70 (Olson *et al.,* 1994); so-called canonical TbHsp70.4 with predicted cytoplasmic localization (Louw *et al.,* 2010); TbHsp110 (Andreasson *et al.,* 2008); and a new Hsp70, TbHsp70.c, also predicted to be cytoplasmic (Louw *et al.,* 2010a). The mitochondrial Hsp70s consist of mtTbHsp70A, mtTbHsp70B, mtTbHsp70C (Jones *et al.,* 2006; Vertommen *et al.,* 2008) and the endoplasmic reticulum Hsp70s include glucose regulated protein 78 (TbGrp78A and TbGrp78B), localized in the sub-pellicular membranes (Bangs *et al.,* 1993) and TbGrp170 predicted to localize in the ER (Louw *et al.,* 2010a). Novel TbHsp70.b has been predicted to be nuclear (Louw *et al.,* 2010a) but the localization of TbHsp70.a is unknown. Of particular interest to this research are the cytosolic Hsp70 proteins. Through proteomic analysis TbHsp70 and TbHsp110 were determined to be expressed in both the bloodstream and procyclic forms of the parasites life cycle stages (Jones *et al.,* 2006; Vertommen *et al.,* 2008); TbHsp70.4 and TbHsp70.c were also shown to be expressed in both forms of the parasite via high-throughput phenotyping (Alsford *et al.,* 2011). Canonical TbHsp70 has demonstrated upregulation in the absence of heat stress and so may be stimulated by various stresses (Van der Ploeg *et al.,* 1985). Even though TbHsp70 is closely related to human cytosolic Hsp70s it lacks the highly conserved EEVD motif (Louw *et al.,* 2010a). TbHsp70.4 is closely related to the canonical Hsp70s, but no functional characterization has been performed to date. TbHsp70.4 contains a slightly altered EDVD motif (Louw *et al.,* 2010a). The focus of this research is TbHsp70.c, a novel and divergent Hsp70 through lack of phylogenetically clustering with any major Hsp70 members (Louw *et al.,* 2010a). TbHsp70.c is present only within the Trypanosomatids and its amino acid sequence shows a lack of conservation through key differences in its SBD and the complete lack of an EEVD motif (Louw *et al.,* 2010a). The modifications or complete lack of an EEVD motif in the cytosolic Hsp70 proteins is rather unusual since it is required for association with TbHop, a protein essential for complex assembly of Hsp70 and Hsp90, involved in cellular protein folding (Odunuga *et al.,* 2004). By implication the mechanism of association of these chaperones is unique, and

may very well be required in parasite longevity. Of the 65 Hsp40s, 47 are Type III Hsp40 proteins (Louw *et al.*, 2010a). Only one of the *T. brucei* Hsp40 proteins, Tbj1, a Type III Hsp40 protein, has been expressed, purified and biochemically characterized to date (Louw *et al.*, 2010b). Type I Hsp40, Tbj2, has been shown experimentally to be cytoplasmic as well as essential to the survival of the parasite (Ludewig and Blatch, 2012).

1.4.2. Molecular chaperones as drug targets

Of late heat shock proteins have been emerging as prospective drug targets (reviewed in Pesce *et al.*, 2010; Shonhai *et al.*, 2010). Drugs have been discovered to cause cellular stress resulting in the induction of heat shock proteins, ultimately improving cytoprotection (Kim *et al.*, 2006; Meng *et al.*, 2011; Burcham *et al.*, 2012). Anti-heat shock protein drugs used in combination with current drugs could therefore synergistically improve the effectiveness of available drugs. Heat shock proteins are of importance to Trypanosoma as they are necessary when the parasite migrates from a cold environment in the insect vector to a warm one in the mammalian host (Maresca and Carratu, 1992; Jones *et al.*, 2008). The *T. brucei* genome has been shown to have 12 Hsp70s and 65 Hsp40s through *in silico* analysis (Louw *et al.*, 2010). The identity of a novel *T. brucei* protein, TbHsp70.c (Louw *et al.*, 2010), could expose unique characteristics of an Hsp70 protein via biochemical characterization. Functional analysis and Hsp70-Hsp40 interactions require characterization. A Type I PfHsp40 has been demonstrated *in vitro* to functionally interact with PfHsp70 (Botha *et al.*, 2011), Gítau and colleagues (2012) hypothesized that PfHsp40 may be a participant in the assembly of the PfHsp70-PfHop complex. An understanding of the degree of functional similarity between TbHsp70s and Hsp70s in other eukaryotic and prokaryotic systems as well as identifying and investigating essential TbHsp70s and their Hsp40 co-chaperones could lead to the treatment of Human African Trypanosomiasis through the development of new drug targets. It was found that TbHsp70 could be useful as a diagnostic antigen, yet the sensitivity and specificity were found to be insufficient. PfHsp70, PfHsp90 and PfHop have all been demonstrated to be upregulated in clinical malaria patients (Pallavi *et al.*, 2010), by implication they have a regulatory trigger in common and progression in the disease concides with their expression. *Plasmodium falciparum* Hsp70 proteins as potential anti-malarial drug targets have been reviewed by Shonhai *et al.* (2010) and Pesce *et al.*, (2010) and the features that make these proteins feasible as drug targets may very well be applicable to trypanosomal Hsp70 proteins.

1.5. Problem statement and motivation

Heat shock proteins have repeatedly demonstrated their ability to regulate cell differentiation and development: heat shock proteins have been implicated in the neural differentiation of murine embryonic carcinoma stem cells (Afzal *et al.,* 2011), Hsp90 is involved in human embryonal carcinoma cellular differentiation (Yamada *et al.,* 2000) and TbHsp70.c has been demonstrated to be essential to the parasite in differentiation from bloodstream to procyclic forms (Alsford *et al.,* 2011). However, many of the Hsp70s and Hsp40s have not been characterized. *T. brucei* Hsp70.c will be the focus of this study after bioinformatic analysis revealed a eukaryotic isoform that may represent a novel family of Hsp70 proteins. Atypically acidic residues are present in the substrate binding domain as well as in the substrate binding cavity of TbHsp70.c. By implication the substrate range and mechanism by which the substrates are recognized may be novel. Not only does the substrate binding domain differ from the prototypical Hsp70 protein but the C-terminal EEVD motif is missing from TbHsp70.c. The protein-protein interaction with co-chaperones is thus very likely unique. Interestingly, *T.b. brucei* shares a nucleotide identity of 99.2% with *T.b. gambiense*. Using a frequency histogram of percentage nucleotide identity it was shown that 86.4% of *T.b. gambiense* genes differ by less than 1% from their *T.b. brucei* orthologue. Knowledge obtained from studies performed on *T.b. brucei* could therefore be related to the human pathogen *T.b. gambiense*. A full biochemical characterization of TbHsp70.c will be performed to further understand the role of molecular chaperones and chaperone interactions, and the infectivity of this particular parasite. This research will be focused on unravelling novel biochemical characteristics of TbHsp70.c and its Hsp70-Hsp40 partnership. To date the TbHsp70.c co-chaperones have not been identified and the interaction of TbHsp70.c and its partner Hsp40s has not been elucidated.

1.6. Objectives

1.6.1. Broad objectives

1. To characterize the TbHsp70.c protein, specifically its abilities to act as a molecular chaperone.
2. To identify specific TbHsp70-TbHsp40 partnerships.

1.6.2. Specific objectives

In silico analysis

1. To perform extensive *in silico* analysis of TbHsp70.c. The amino acid sequences will be obtained and alignments of cytosolic TriTryps Hsp70s will be performed.
2. To establish the phylogenetic relationships between Hsp70 proteins from the TriTryps
3. TbHsp70.c will be homology modelled, and the modelling will be verified through various checks, including calculation of the root mean square deviation (RMSD) using PyMol, the *DOPE* (*Discrete Optimized Protein Energy*) *Z-score* using online software ProSA and Global Distance Test Total Score using the MetaMQAPII server.
4. Secondary structure prediction software will be used to compare the secondary structure of TbHsp70.c with that of other kinetoplastid Hsp70 proteins.
5. To perform composite analysis of TbHsp70.c and design anti-peptide antibodies.
6. To determine the level of conservation of the cytosolic J-proteins from TriTryps. The amino acid sequences will be obtained and multiple sequence alignments will be performed. The phylogenetic relationships between full length cytosolic Hsp40 proteins from the TriTryps will be established.

Expression and purification

1. To isolate the coding region of TbHsp70.c. Primers will be designed to allow PCR amplification of the coding region. The PCR products will be ligated into the cloning vector (pGEM-T Easy vector) and the transformed colonies will be screened. Ligations will be digested and the inserts ligated into the *E. coli* expression vector pQE80-L that is His-tagged at the N-terminals. The plasmid will be sequenced.
2. TcHsp70B, TbHsp70.c and Tbj2 will be expressed and purified in *E. coli* cells. The purification method will be optimized to ensure high purity of the proteins.
3. The oligomeric states of TbHsp70.c and Tbj2 will be determined using size-exclusion chromatography.

In vitro analysis

1. To investigate the ability of TbHsp70.c and Tbj2 to independently prevent misfolding of thermal MDH and rhodanese using aggregation suppression assays. The ability of

TbHsp70.c-Tbj2 in partnership to suppress protein aggregation will be investigated. TcHsp70B and Alfalfa Hsp70 will be included as controls.

2. To determine the steady state ATPase activity of TbHsp70.c.
3. To investigate the ability of Tbj2 to stimulate the ATPase activity of TbHsp70.c. TcHsp70B will be included as a control.
4. To determine the effect of small molecule modulators quercetin and methylene blue on the chaperone activity of TbHsp70.c and TbHsp70.c in partnership with Tbj2 using ATPase activity assays and aggregation suppression assays. TcHsp70B and Alfalfa Hsp70 will be included as controls.

In vivo analysis

1. The presence of TbHsp70.c will be probed in *T. brucei* cell lysates through western blot analysis using the peptide polyclonal antibodies specific against TbHsp70.c. Antibodies will be tested for specificity.
2. The effect of thermal stress on TbHsp70.c will be investigated at the protein level by heat shocking *T. brucei* 427 cells and using western analysis to detect for differences in protein expression levels.
3. Investigation of the ability of Tbj2 to bind TbHsp70.c using immunoprecipitation and pull down analysis.
4. RNA interference studies will require the single marker bloodstream form (SMB) of *T. b. brucei* and will include construction of interference plasmids by designing primers for PCR amplification of RNAi TbHsp70.c fragments and ligation into the RNAi vector $p2T7^{TAblu}$. Transfection of the trypanosomes will be followed by growth assays to monitor RNAi. The knockdown of TbHsp70.c will be assessed using western analysis.
5. The subcellular localization of TbHsp70.c will be determined using anti-TbHsp70.c peptide antibody and immunofluorescence staining and microscopy.
6. The effect of the cell cycle on the subcellular localization of TbHsp70.c will be determined using anti-TbHsp70.c peptide antibody and immunofluorescence staining and microscopy.

CHAPTER 2

BIOINFORMATIC ANALYSIS:

TbHsp70.c and its orthologues and homologues

2.1 Introduction

GeneDB, initially housing data for the kinetoplastids and *Schizosaccharomyces pombe* sequenced by the Pathogen Genomics group at the Wellcome Trust Sanger Institute (WTSI) (http://www.sanger.ac.uk), currently holds all pathogen genomes sequenced at the WTSI (Logan-Klumpler *et al.,* 2012). GeneDB is the largest multi-organism database of 41 organisms, including genomes of bacteria, protozoa (Table 2.1), helminth and arthropods; the common factor being the impact these organisms have on worldwide human health and economy (Logan-Klumpler *et al.,* 2012). To enable rapid and structured curation, data is stored in a multi-organism Chado database (Zhou *et al.,* 2006). Genomes within GeneDB are either proactively or reactively curated; proactive curation involves weekly amendments of annotations from both literature and user comments by the curators, whereas reactive curation relies predominantly on feedback from users. The genomes of *L. major, T. brucei 927* and *T. cruzi* have been proactively curated (Table 2.1). GeneDB frequently sends annotated and curated trypanosomatid genomes to TriTrypDB in collaboration with EuPathDB (Aslett *et al.,* 2010; http://www.tritrypdb.org). Relevant genomes are sent to PlasmoDB and SchistoDB in a similar fashion (Aurrecoechea *et al.,* 2009; http://www.plasmodb.org; (http://www.schistodb.net).

Table 2.1. Genomes from GeneDB and curation status (adapted from Logan-Klumpler *et al.,* 2012)

Parasite	Genome	Curation status
Leishmania major	Completed	Proactive
Trypanosoma brucei 927	Non-contiguous completed	Proactive
Trypanosome cruzi	Non-contiguous completed	proactive

Integrating highly-curated annotations from GeneDB with expression profiling data, proteomics results etc has proved challenging and so TriTrypDB was developed in 2009 as a collaborative effort between EuPathDB at the Universities of Pennsylvania and Georgia (Aurrecoechea *et al.,* 2007) and GeneDB at the WTSI and the Seattle Biomedical Research Institute (Aslett *et al.,* 2010). TriTrypDB thus effectively provides a current annotation, curation, and tools allowing queries against genomic scale datasets (Alsett *et al.,* 2010).

TriTryps and TbHsp70.c

The 11 megabase-sized diploid chromosomes of the *T. brucei* genome were sequenced by whole chromosome shotgun and bacterial artificial chromosome walking strategies, and contain 9068 predicted genes: approximately 900 pseudogenes and 1700 *T. brucei*-specific genes (Table 2.2) (Berriman *et al.,* 2005). More than 20% of the genome was discovered to encode subtelomeric genes (El-Sayed *et al.,* 2005) of which the bulk are specific to *T. brucei* and are involved with antigenic variation: 806 variant surface glycoprotein genes (Berriman *et al.,* 2005). The *T. cruzi* (El-Sayed *et al.,* 2005) and *L. major* (Ivens *et al.,* 2005) complete genomes have also been sequenced (Table 2.2), enabling comparative *in silico* analysis of the TriTryps, as was the case in which knowledge of the *T. brucei* chromosomes provided a structural basis for study of the *T. cruzi* chromosome organization (Weatherly *et al.,* 2009).

Table 2.2. TriTryps sequenced genomes published in 2005 (Berriman *et al.,* 2005; El-Sayed *et al.,* 2005; Ivens *et al.,* 2005).

Parasite	Chromosomes	Genome size (Mb)	Genes	Genes specific to parasite
Trypanosoma brucei	11, diploid	26	9068	1700
Trypanosoma cruzi	41, diploid	55	12 570	3736
Leishmania major	36, haploid	32.8	8272	910

In silico analysis revealed *T. brucei* to possess a complement of 65 Hsp40 proteins and 12 Hsp70 proteins (Table 2.3) (Folgueira and Requena, 2007). All the *T. brucei* Hsp70 proteins have orthologues in *T. cruzi* and *L. major*, and were grouped based on their sequence identities to Hsp70 proteins from other species that have been well characterized (Table 2.3) (Folgueira and Requena, 2007). Partial sequences are not included in the table. *T. cruzi* possesses 11 Hsp70 sequences, and *L. major* contains 14 sequences coding for Hsp70 proteins (Table 2.3) (Folgueira and Requena, 2007). TcHsp70B was shown to localize in the cytoplasm and in the nucleus upon induction of heat stress (Olson *et al.,* 1994). Subcellular localization of all the other TriTryps Hsp70 proteins was predicted using Wolf PSORT (Table 2.3) (Horton *et al.,* 2007; Louw *et al.,* 2010; wolfpsort.org).

Table 2.3. Complement of Hsp70 proteins within the TriTryps (adapted from Louw *et al.*, 2010; Folgueira and Requena, 2007).

	T. brucei		*T. cruzi*		*L. major*		
Subfamily	**Name**	**Gene locus**	**Name**	**Gene locus**	**Name**	**Gene locus**	**Predicted localization**
Hsp70	TbHsp70	Tb11.01.3110	TcHsp70A	Tc00.1047053511211.170	LmHsp70A	LmjF28.2770	Cytoplasm
	--	--	TcHsp70B	Tc00.1047053511211.160	LmHsp70B	LmjF28.2780	Cytoplasm
Hsp70.4	TbHsp70.4	Tb927.7.710	unknown	unknown	LmHsp70.4	LmjF26.1240	Cytoplasm
Hsp70.a	TbHsp70.a	Tb09.160.3090	TcHsp70.a	Tc00.1047053511585.70	LmHsp70.a	LmjF01.0640	Unknown
	--	--	TcHsp70.a	Tc00.1047053510155.70	--	--	Unknown
Hsp70.b	TbHsp70.b	Tb927.7.1030	TcHsp70.b	Tc00.1047053507513.50	LmHsp70.b	LmjF26.0900	Nucleoplasm
Hsp70.c	TbHsp70.c	Tb11.01.3080	TcHsp70.c	Tc00.1047053511211.220	LmHsp70.c	LmjF28.2820	Cytoplasm
BiP/Grp78	TbGrp78A	Tb11.02.5500	TcGrp78	Tc00.1047053506585.40	LmGrp78	LmjF28.1200	ER
	TbGrp78B	Tb11.02.5450	--	--	--	--	ER
Mitochondrial	mtTbHsp70A	Tb927.6.3740	mtTcHsp70	Tc00.1047053507029.30	mtLmHsp70A	LmjF30.2460	Mitochondria
	mtTbHsp70B	Tb927.6.3750	--	--	mtLmHsp70B	LmjF30.2470	Mitochondria
	mtTbHsp70C	Tb927.6.3800	--	--	mtLmHsp70C	LmjF30.2480	Mitochondria
	--	--	--	--	mtLmHsp70D	LmjF30.2490	Mitochondria
	--	--	--	--	mtLmHsp70E	LmjF30.2550	Mitochondria
Hsp110	TbHsp110	Tb10.389.0880	TcHsp110	Tc00.1047053507831.60	LmHsp110	LmjF18.1370	Cytoplasm
Grp170	TbGrp170	Tb09.211.1390	TcGrp170A	Tc00.1047053506885.440	LmGrp170	LmjF35.4710	ER
	--	--	TcGrp170B	Tc00.1047053508457.20	--	--	ER

Bioinformatic analysis revealed *T. brucei* Hsp70.c to be a eukaryotic isoform that may represent a novel family of Hsp70 proteins as it did not cluster phylogenetically with any of the other primary Hsp70 proteins (Louw *et al.*, 2010). *In silico* analysis of TbHsp70.c indicated the presence of atypical acidic residues in the substrate binding domain as well as in the substrate binding cavity of TbHsp70.c (Louw *et al.*, 2010). By implication the substrate range and mechanism by which the substrates are recognized may be novel. Not only does the substrate binding domain differ from the prototypical Hsp70 protein but the C-terminal EEVD motif is missing from TbHsp70.c. The protein-protein interaction with the co-chaperone Hsp70/Hsp90 organizing protein (Hop) is thus very likely unique or possibly absent. Using Wolf PSORT software TbHsp70.c was predicted to localize in the cytoplasm (Louw *et al.*, 2010). To date, TbHsp70.c has not been biochemically characterized and co-chaperones have not been identified. Of the 65 Hsp40s, only one of the Type III *T. brucei* Hsp40 proteins, Tbj1, has been expressed, purified and biochemically characterized to date (Louw *et al.*, 2010). No *in vitro* characterization of Tbj2 has been completed; *in vivo* analysis revealed that Tbj2 is essential to the survival of the cell (Alsford *et al.*, 2011). According to TriTrypDB (Aslett *et al*., 2010) TbHsp70.c is localized in the cytoplasm and previous studies have shown Tbj2 to localize in the cytoplasm (Ludewig, PhD Thesis, 2010).

In this chapter the aim to utilize available bioinformatic tools to infer the functioning of TbHsp70.c is presented. *In silico* knowledge will provide direction to the subsequent biochemical characterization of TbHsp70.c and its potential partner protein. Sequence similarity and antibody development was investigated. The bioinformatics tool composite analysis allows design of a peptide by selection of a region of the protein that meets predefined conditions. Antibodies can be produced by either purifying a target protein or inoculating a selected animal with the protein directly, or a peptide sequence can be engineered and antibody produced by immunizing an animal with the synthetic peptide (Bellstedt *et al.,* 1987). Should the purification of a protein not be feasible, due to either insufficient protein yields (<0.35 mg/ml) or difficulty in the purification process, antibody should be produced via peptide synthesis (Bellstedt *et al.,* 1987). Synthetic peptides are typically 10 to 25 amino acids in length, which is too small to induce an immune response in an animal that will produce sufficient yields of antibody. To compensate, a synthetic peptide is linked to a larger protein, Keyhole Limpet Hemocyanin (KLH), forming a conjugate used as an immunogen. Found in arthropods and molluscs, KLH is a copper-containing non-heme protein with high immunogenicity and many free amino groups which can be used for hapten coupling. Coupling, which occurs through a terminal cysteine residue on the synthetic peptide using sulfhydryl chemistry or amino groups, ensures controlled orientations of peptide onto carrier. To ensure successful immunization the peptide has to be of a high quality, which can be controlled in the design process. The process of designing a peptide involves various considerations such as the sequence, composition and length, all of which influence the assembly, purification and solubility of the antibody. The peptide sequence should be antigenic, hydrophilic and consist of secondary structures that allow solvent accessibility.

The specific objectives of the study included:

i. Comparison of the cytoplasmic TbHsp70 proteins to predicted cytoplasmic orthologues in *T. cruzi* and *L. major* using a multiple sequence alignment

ii. Assessment of the phylogeny of TbHsp70.c and putative orthologues

iii. Generation of a homology model of TbHsp70.c and validation of the model

iv. Prediction of the secondary structure of TbHsp70.c

v. Design and synthesis of TbHsp70.c peptide for polyclonal anti-TbHsp70.c peptide antibody production

2.2 Methods and materials

2.2.1 Conservation level of cytoplasmic Hsp70 and Hsp40 proteins from the TriTryps

Prior to protein sequence analysis using a multiple sequence alignment, trypanosomatid amino acid sequences were obtained from the GeneDB database (http://www.genedb.org/; Hertz-Fowler *et al*., 2004) and other Hsp70 protein sequences were retrieved from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/; Sayers *et al*., 2008), both sets of sequences in FASTA format. The accession numbers of all retrieved sequences are given in figures 1 and 2. The multiple sequence analysis was performed using MAFFT version 7 (Katoh *et al.*, 2005) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

2.2.2 Generation and confirmation of the accuracy of a TbHsp70.c homology model

The TbHsp70.c protein sequence, obtained from GeneDB database (www.genedb.org) was used as a query in a BLAST search to identify the best possible template sequence for homology modelling. Bovine Hsc70 was selected as it shared the highest sequence identity with TbHsp70.c. The template sequence and crystal structure of bovine Hsc70 (1YUW.pdb) (Jiang *et al.*, 2005) was obtained from the PDB database (http://www.rcsb.org/pdb/home/home.do), and aligned with TbHsp70.c using the ClustalW based alignment option. The alignment was saved in a PIR output format and used to construct 100 homology models using Modeller 9v3 software (http://www.salilab.org/modeller) (Eswar *et al.*, 2006). The resulting PDB files were viewed using PyMol (http://www.pymol.org) (DeLano, 2002). The model was validated through a series of checks including calculation of the root mean square deviation (RMSD) using PyMol, the *DOPE* (*Discrete Optimized Protein Energy*) *Z-score* using online software ProSA (Sippl, 1993) and Global Distance Test Total Score using the MetaMQAPII server (https://genesilico.pl/toolkit/unimod?method=MetaMQAPII) (Pawlowski, 2008).

2.2.3 Secondary structure prediction of TbHsp70.c

Prediction of the protein secondary structure was achieved by generating a multiple structure alignment with amino acid sequences retrieved from GeneDB (as in Section 2.2.1) in FASTA format using PROMALS3D (Pei *et al.* 2008). Input sequences were in FASTA format.

2.2.4 Composite analysis of TbHsp70.c and design of peptide for anti-peptide antibody production

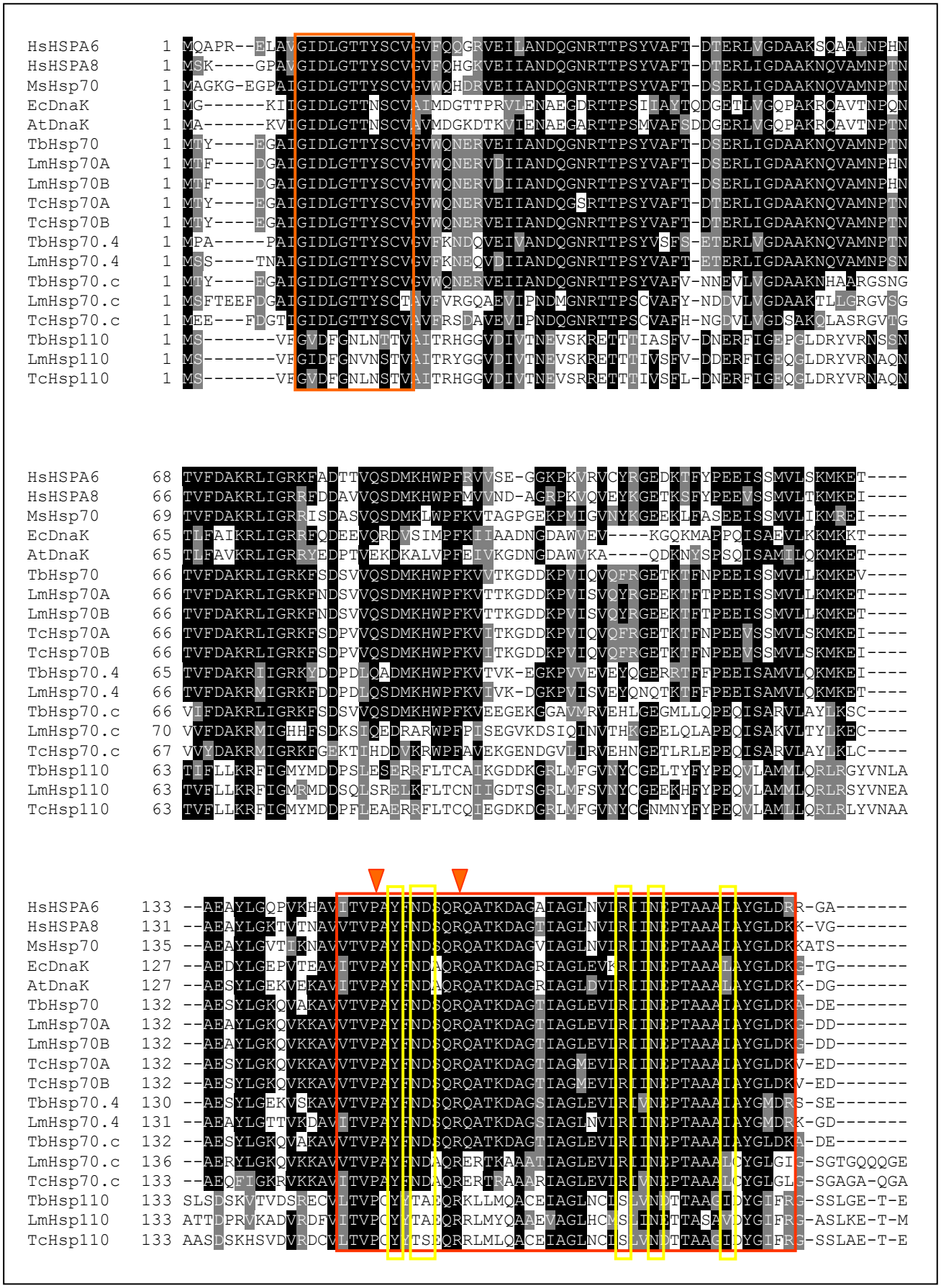
To facilitate the design of peptide for polyclonal anti-peptide antibody production specific against TbHsp70.c, the protein sequence composite analysis of Generunner http://www.generunner.net/) was used to locate antigenic regions within the amino acid sequence of TbHsp70.c. The composite analysis included considerations of antigenicity using Hopp-Woods and Jameson-wolf Index plots (Jameson and Wolf, 1988); surface exposure probability using the Emini plot (Emini *et al.*, 1985); secondary structure prediction using Garnier-Robson and Chou-Fasman plots (Garnier *et al.*, 1978; Chou and Fasman, 1978); charge density (calculated by Generunner using a sliding window calculation method; chain flexibility using the Karplus-Schultz plot (Karplus *et al.*, 1978) and transmembrane regions using Goldman and Eisenberg plots. An overall measure of antigenicity is achieved by the Jameson-Wolf Antigenic index which combines results from secondary structure predictions, Hopp-Woods, surface probability and chain flexibility (Jameson and Wolf, 1988). An antigenic region with the lowest similarity to any other *T. brucei* protein was assessed using BLAST before the peptide sequence (CQRGRGVTEGSGRPP) was submitted to the GenScript corporation (Piscataway, New Jersey, USA) to be synthesized. Once synthesized, GenScript used the peptide to produce polyclonal antibody against TbHsp70.c in rabbit.

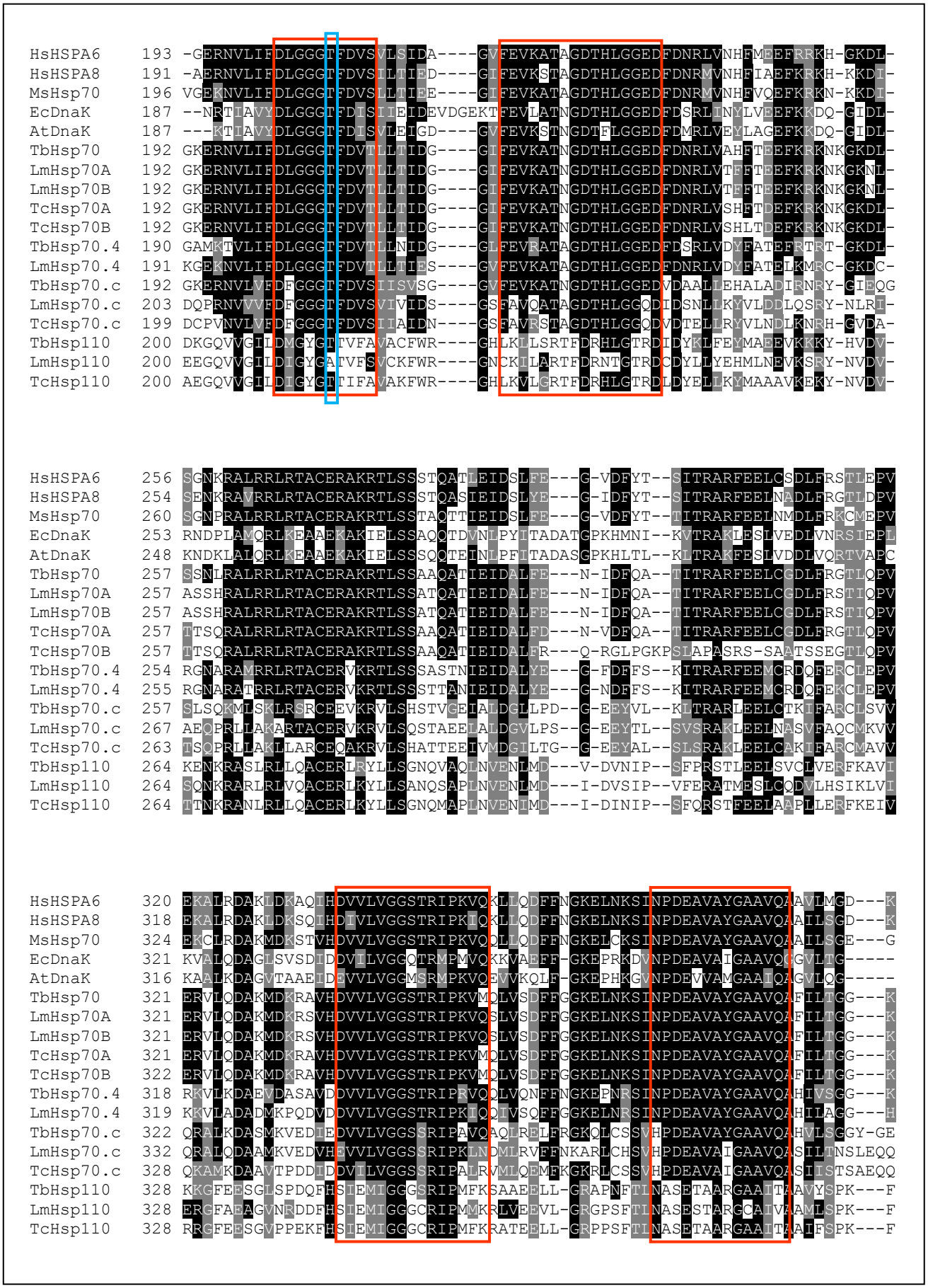
2.3 Results

2.3.1 Conservation level of cytoplasmic Hsp70 proteins from the TriTryps

A protein sequence alignment was performed on all the predicted cytoplasmic full length Hsp70 proteins from *T. brucei*, as detailed in Louw *et al.*, (2010) and their orthologues in *T. cruzi* and *L. major*, and compared with well characterized Hsp70s from eukaryotes (human and plant) and prokaryotes (Fig. 2.1, 2.2). A detailed analysis of the features of the ATPase domain (Fig. 2.1), substrate binding domain and C-terminal domain (Fig. 2.2) has been annotated on the figures.

There is generally a very high degree of conservation across both the ATPase and substrate binding domains than can be seen for the C-terminal domain for the aligned sequences (Fig. 2.1, 2.2). TbHsp70 shares a high sequence identity with reported orthologues LmHsp70A, LmHsp70B, TcHsp70A and TcHsp70B (85%, 85%, 86% and 86% respectively). TbHsp70 also shows a high pairwise alignment with the canonical Hsp70s from the eukaryotes, human Hsp70B and Hsc70, and plant Alfalfa Hsp70 (68%, 71% and 70%, respectively) suggesting that they could be homologues of TbHsp70. The prokaryotes *E. coli* and *A. bacterium* DnaK, however, do not share high sequence identities (45% and 49%, respectively) with TbHsp70. Pairwise alignment of TbHsp70.4 with HsHsp70B, HsHsc70, and Alfalfa Hsp70 (60%, 61% and 61%, respectively), although displaying a slightly lower similarity than seen for TbHsp70 with the eukaryotes, suggests homology. TbHsp70.c and its reported orthologues TcHsp70.c and LmHsp70.c share slightly lower sequence identities than expected; 54% and 60%, respectively, and 46%, 47% and 46% with HsHsp70B, HsHsc70, and Alfalfa Hsp70, respectively. The last *T. brucei* Hsp70 predicted to be cytoplasmic is Hsp110, sharing higher sequence identity with orthologue TcHsp110 (71%) than LmHsp110 (55%). TbHsp110 is reported to be closely related to the human Hsp110 homologue (Louw *et al.*, 2010). Through both the pairwise and multiple sequence alignments, conservation is observed to be maintained in particular groups of proteins: the sequences of eukaryotic HsHsp70B, HsHsc70 and MsHsp70 along with TbHsp70 and its orthologues, and the Hsp70.4 proteins all show similar trends forming one large group; the prokaryotic DnaK proteins, the Hsp110 proteins, and TbHsp70.c and its orthologues each form their own groups (Fig. 2.1, 2.2). The DnaK proteins show a degree of variation throughout the sequences, which is not unexpected due to their prokaryotic origin (Fig. 2.1, 2.2). Of the Hsp110 proteins and prokaryotic Hsp70s, the Hsp70.c proteins show a higher degree of sequence conservation to the canonical and well conserved Hsp70 proteins (Fig. 2.1, 2.2). As is often the case in molecular chaperones, the N-terminal ATPase domain is well conserved (Fig. 2.1) (Bork *et al.*, 1992; Shonhai *et al.*, 2007). The amino acids highlighted in orange boxes are highly conserved residues associated with heat shock proteins (Mount, 1985), and other than the Hsp110 proteins, show a high degree of conservation across the species aligned (Fig. 2.1). Residues previously determined to be essential to the functioning of DnaK (Vogel *et al.*, 2006), and conserved across all the species in this alignment, are those involved in the proline allosteric switch, and interdomain function, highlighted by orange arrows (Fig. 2.1). Amino acids that associate with DnaJ/Hsp40, within both the ATPase and substrate binding domains, yellow box (Gässler *et al.*, 1998; Suh *et al.*,


HsHSPA6 1 MQAPR--ELAVGIDLGTTYSCVGVFQQGRVEILANDQGNRTTPSYVAFT-DTERLVGDAAKSQAALNPHN
HsHSPA8 1 MSK----GPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFT-DTERLIGDAAKNQVAMNPTN
MsHsp70 1 MAGKG-EGPAIGIDLGTTYSCVGVWQHDRVEIIANDQGNRTTPSYVAFT-DSERLIGDAAKNQVAMNPTN
EcDnaK 1 MG------KIIGIDLGTTNSCVAIMDGTTPRVIENAEGDRTTPSIIAYTQDGETLVGQPAKRQAVTNPQN
AtDnaK 1 MA------KVIGIDLGTTNSCVAVMDGKDTKVIENAEGARTTPSMVAFSDDGERLVGQPAKRQAVTNPTN
TbHsp70 1 MTY----EGAIGIDLGTTYSCVGVWQNERVEIIANDQGNRTTPSYVAFT-DSERLIGDAAKNQVAMNPTN
LmHsp70A 1 MTF----DGAIGIDLGTTYSCVGVWQNERVDIIANDQGNRTTPSYVAFT-DSERLIGDAAKNQVAMNPHN
LmHsp70B 1 MTF----DGAIGIDLGTTYSCVGVWQNERVDIIANDQGNRTTPSYVAFT-DSERLIGDAAKNQVAMNPHN
TcHsp70A 1 MTY----EGAIGIDLGTTYSCVGVWQNERVEIIANDQGSRTTPSYVAFT-DTERLIGDAAKNQVAMNPTN
TcHsp70B 1 MTY----EGAIGIDLGTTYSCVGVWQNERVEIIANDQGNRTTPSYVAFT-DTERLIGDAAKNQVAMNPTN
TbHsp70.4 1 MPA-----PAIGIDLGTTYSCVGVFKNDQVEIVANDQGNRTTPSYVSFS-ETERLVGDAAKNQVAMNPTN
LmHsp70.4 1 MSS----TNAIGIDLGTTYSCVGVFKNEQVDIIANDQGNRTTPSYVAFT-ETERLIGDAAKNQVAMNPSN
TbHsp70.c 1 MTY----EGAIGIDLGTTYSCVGVWQNERVEIIANDQGNRTTPSYVAFV-NNEVLVGDAAKNHAARGSNG
LmHsp70.c 1 MSFTEEFDGAIGIDLGTTYSCTAVFVRGQAEVIPNDMGNRTTPSCVAFY-NDDVLVGDAAKTLLGRGVSG
TcHsp70.c 1 MEE---FDGTIGIDLGTTYSCVAVFRSDAVEVIPNDQGNRTTPSCVAFH-NGDVLVGDSAKQLASRGVTG
TbHsp110 1 MS-------VFGVDFGNLNTTVAITRHGGVDIVTNEVSKRETTTIASFV-DNERFIGEPGLDRYVRNSSN
LmHsp110 1 MS-------VFGIDFGNVNSTVAITRYGGVDIVTNEVSKRETTTIVSFV-DDERFIGEQGLDRYVRNAQN
TcHsp110 1 MS-------VFGVDFGNLNSTVAITRHGGVDIVTNEVSRRETTTIVSFL-DNERFIGEQGLDRYVRNAQN
HsHSPA6 68 TVFDAKRLIGRKFADTTVQSDMKHWPFRVVSE-GGKPKVRVCYRGEDKTFYPEEISSMVLSKMKETAEA
HsHSPA8 66 TVFDAKRLIGRRFDDAVVQSDMKHWPFMVVND-AGRPKVQVEYKGETKSFYPEEVSSMVLTKMKEIAEA
MsHsp70 69 TVFDAKRLIGRRISDASVQSDMKLWPFKVTAGPGEKPMIGVNYKGEEKLFASEEISSMVLIKMREIAEA
EcDnaK 65 TLFAIKRLIGRRFQDEEVQRDVSIMPFKIIAADNGDAWVEV----KGQKMAPPQISAEVLKKMKKTAEA
AtDnaK 65 TLFAVKRLIGRRYEDPTVEKDKALVPFEIVKGDNGDAWVKA----QDKNYSPSQISAMILQKMKETAEA
TbHsp70 66 TVFDAKRLIGRKFSDSVVQSDMKHWPFKVVTKGDDKPVIQVQFRGETKTFNPEEISSMVLLKMKEVAEA
LmHsp70A 66 TVFDAKRLIGRKFNDSVVQSDMKHWPFKVTTKGDDKPVISVQYRGEEKTFTPEEISSMVLLKMKETAEA
LmHsp70B 66 TVFDAKRLIGRKFNDSVVQSDMKHWPFKVTTKGDDKPVISVQYRGEEKTFTPEEISSMVLLKMKETAEA
TcHsp70A 66 TVFDAKRLIGRKFSDPVVQSDMKHWPFKVITKGDDKPVIQVQFRGETKTFNPEEVSSMVLSKMKEIAEA
TcHsp70B 66 TVFDAKRLIGRKFSDPVVQSDMKHWPFKVITKGDDKPVIQVQFRGETKTFNPEEVSSMVLSKMKEIAEA
TbHsp70.4 65 TVFDAKRIIGRKYDDPDLQADMKHWPFKVTVK-EGKPVVEVEYQGERRTFFPEEISAMVLQKMKEIAEA
LmHsp70.4 66 TVFDAKRMIGRKFDDPDLQSDMKHWPFKVIVK-DGKPVISVEYQNQTKTFFPEEISAMVLQKMKETAEA
TbHsp70.c 66 VIFDAKRLIGRKFSDSVVQSDMKHWPFKVEEGEKGGAVMRVEHLGEGMLLQPEQISARVLAYLKSCAEA
LmHsp70.c 70 VVFDAKRMIGHHFSDKSIQEDRARWPFPISEGVKDSIQINVTHKGEELQLAPEQISAKVLTYLKECAEA
TcHsp70.c 67 VVYDAKRMIGRKFGEKTIHDDVKRWPFAVEKGENDGVLIRVEHNGETLRLEPEQISARVLAYLKLCAEA
TbHsp110 63 TIFLLKRFIGMYMDDPSLESERRFLTCAIKGDDKGRLMFGVNYCGELTYFYPEQVLAMMLQRLRGYVNLA
LmHsp110 63 TVFLLKRFIGMRMDDSQLSRELKFLTCNIIGDTSGRLMFSVNYCGEEKHFYPEQVLAMMLQRLRSYVNEA
TcHsp110 63 TVFLLKRFIGMYMDDPFLEAERRFLTCQIEGDKDGRLMFGVNYCGNMNYFYPEQVLAMLLQRLRLYVNAA
HsHSPA6 133 --AEAYLGQPVKHAVITVPAYFNDSQRQATKDAGAIAGLNVLRIINEPTAAAIAYGLDRR-GA-------
HsHSPA8 131 --AEAYLGKTVTNAVVTVPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKK-VG-------
MsHsp70 135 --AEAYLGVTIKNAVVTVPAYFNDSQRQATKDAGVIAGLNVLRIINEPTAAAIAYGLDKKATS-------
EcDnaK 127 --AEDYLGEPVTEAVITVPAYFNDAQRQATKDAGRIAGLEVKRIINEPTAAALAYGLDKG-TG-------
AtDnaK 127 --AESYLGEKVEKAVITVPAYFNDAQRQATKDAGRIAGLDVIRIINEPTAAALAYGLDKK-DG-------
TbHsp70 132 --AESYLGKQVAKAVVTVPAYFNDSQRQATKDAGTIAGLEVIRIINEPTAAAIAYGLDKA-DE-------
LmHsp70A 132 --AEAYLGKQVKKAVVTVPAYFNDSQRQATKDAGTIAGLEVIRIINEPTAAAIAYGLDKG-DD-------
LmHsp70B 132 --AEAYLGKQVKKAVVTVPAYFNDSQRQATKDAGTIAGLEVIRIINEPTAAAIAYGLDKG-DD-------
TcHsp70A 132 --AESYLGKQVKKAVVTVPAYFNDSQRQATKDAGTIAGMEVIRIINEPTAAAIAYGLDKV-ED-------
TcHsp70B 132 --AESYLGKQVKKAVVTVPAYFNDSQRQATKDAGTIAGMEVIRIINEPTAAAIAYGLDKV-ED-------
TbHsp70.4 130 --AESYLGEKVSKAVVTVPAYFNDSQRQATKDAGSIAGLEVLRIVNEPTAAAIAYGMDRS-SE-------
LmHsp70.4 131 --AEAYLGTTVKDAVITVPAYFNDSQRQATKDAGSIAGLNVIRIINEPTAAAIAYGMDRK-GD-------
TbHsp70.c 132 --AESYLGKQVAKAVVTVPAYFNDSQRQATKDAGTIAGLEVIRIINEPTAAAIAYGLDKA-DE-------
LmHsp70.c 136 --AERYLGKQVKKAVVTVPAYFNDAQRERTKAAATIAGLEVIRIINEPTAAALCYGLGIG-SGTGQQQGE
TcHsp70.c 133 --AEQFIGKRVKKAVITVPAYFNDAQRERTRAAARIAGLEVIRIVNEPTAAALCYGLGLG-SGAGA-QGA
TbHsp110 133 SLSDSKVTVDSRECVLTVPCYKTAEQRKLLMQACEIAGLNCISLVNDTTAAGIDYGIFRG-SSLGE-T-E
LmHsp110 133 ATTDPRVKADVRDFVITVPCYKTAEQRRLMYQAAEVAGLHCVSLINETTASAVDYGIFRG-ASLKE-T-M
TcHsp110 133 AASDSKHSVDVRDCVLTVPCYKTSEQRRLMLQACEIAGLNCISLVNDTTAAGIDYGIFRG-SSLAE-T-E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MsHsp70 196 VGEKNVLIFDLGGGTFDVSLLTIEE----GIFEVKATAGDTHLGGEDFDNRMVNHFVQEFKRKN-KKDI-
EcDnaK 187 --NRTIAVYDLGGGTFDISIIEIDEVDGEKTFEVLATNGDTHLGGEDFDSRLINYLVEEFKKDQ-GIDL-
AtDnaK 187 ---KTIAVYDLGGGTFDISVLEIGD----GVFEVKSTNGDTFLGGEDFDMRLVEYLAGEFKKDQ-GIDL-
TbHsp70 192 GKERNVLIFDLGGGTFDVTLLTIDG----GIFEVKATNGDTHLGGEDFDNRLVAHFTEEFKRKNKGKDL-
LmHsp70A 192 GKERNVLIFDLGGGTFDVTLLTIDG----GIFEVKATNGDTHLGGEDFDNRLVTFFTEEFKRKNKGKNL-
LmHsp70B 192 GKERNVLIFDLGGGTFDVTLLTIDG----GIFEVKATNGDTHLGGEDFDNRLVTFFTEEFKRKNKGKNL-
TcHsp70A 192 GKERNVLIFDLGGGTFDVTLLTIDG----GIFEVKATNGDTHLGGEDFDNRLVSHFTDEFKRKNKGKDL-
TcHsp70B 192 GKERNVLIFDLGGGTFDVTLLTIDG----GIFEVKATNGDTHLGGEDFDNRLVSHLTDEFKRKNKGKDL-
TbHsp70.4 190 GAMKTVLIFDLGGGTFDVTLLNIDG----GLFEVRATAGDTHLGGEDFDSRLVDYFATEFRTRT-GKDL-
LmHsp70.4 191 KGEKNVLIFDLGGGTFDVTLLTIES----GVFEVKATAGDTHLGGEDFDNRLVDYFATELKMRC-GKDC-
TbHsp70.c 192 GKERNVLVFDFGGGTFDVSIISVSG----GVFEVKATNGDTHLGGEDVDAALLEHALADIRNRY-GIEQG
LmHsp70.c 203 DQPRNVVVFDFGGGTFDVSVIVIDS----GSFAVQATAGDTHLGGQDIDSNLLKYVLDDLQSRY-NLRI-
TcHsp70.c 199 DCPVNVLVFDFGGGTFDVSIIAIDN----GSFAVRSTAGDTHLGGQDVDTELLRYVLNDLKNRH-GVDA-
TbHsp110 200 DKGQVVGILDMGYGTTVFAVACFWR----GHLKLLSRTFDRHLGTRDIDYKLFEYMAEEVKKKY-HVDV-
LmHsp110 200 EEGQVVGILDIGYGATVFSVCKFWR----GNCKILARTFDRNTGTRDCDYLLYEHMLNEVKSRY-NVDV-
TcHsp110 200 AEGQVVGILDIGYGTTIFAVAKFWR----GHLKVLGRTFDRHLGTRDLDYELLKYMAAAVKEKY-NVDV-
HsHSPA6 256 SGNKRALRRLRTACERAKRTLSSSTQATLEIDSLFE---G-VDFYT--SITRARFEELCSDLFRSTLEPV
HsHSPA8 254 SENKRAVRRLRTACERAKRTLSSSTQASIEIDSLYE---G-IDFYT--SITRARFEELNADLFRGTLDPV
MsHsp70 260 SGNPRALRRLRTACERAKRTLSSTAQTTIEIDSLFE---G-VDFYT--TITRARFEELNMDLFRKCMEPV
EcDnaK 253 RNDPLAMQRLKEAAEKAKIELSSAQQTDVNLPYITADATGPKHMNI--KVTRAKLESLVEDLVNRSIEPL
AtDnaK 248 KNDKLALQRLKEAAEKAKIELSSSQQTEINLPFITADASGPKHLTL--KLTRAKFESLVDDLVQRTVAPC
TbHsp70 257 SSNLRALRRLRTACERAKRTLSSAAQATIEIDALFE---N-IDFQA--TITRARFEELCGDLFRGTIQPV
LmHsp70A 257 ASSHRALRRLRTACERAKRTLSSATQATIEIDALFE---N-IDFQA--TITRARFEELCGDLFRSTIQPV
LmHsp70B 257 ASSHRALRRLRTACERAKRTLSSATQATIEIDALFE---N-IDFQA--TITRARFEELCGDLFRSTIQPV
TcHsp70A 257 TTSQRALRRLRTACERAKRTLSSAAQATIEIDALFD---N-VDFQA--TITRARFEELCGDLFRGTIQPV
TcHsp70B 257 TTSQRALRRLRTACERAKRTLSSAAQATIEIDALFR---Q-RGLPGKPSLAPASRS-SAATSSEGTIQPV
TbHsp70.4 254 RGNARAMRRLRTACERVKRTLSSSASTNIEIDALYE---G-FDFFS--KITRARFEEMCRDQFERCLEPV
LmHsp70.4 255 RGNARATRRLRTACERVKRTLSSSTTANIEIDALYE---G-NDFFS--KITRARFEEMCRDQFEKCLEPV
TbHsp70.c 257 SLSQKMLSKLRSRCEEVKRVLSHSTVGEIALDGLLPD--G-EEYVL--KLTRARLEELCTKIFARCLSVV
LmHsp70.c 267 AEQPRLLAKARTACERVKRVLSQSTAEELALDGVLPS--G-EEYTL--SVSRAKLEELNASVFAQCMKVV
TcHsp70.c 263 TSQPRLLAKLLARCEQAKRVLSHATTEEIVMDGILTG--G-EEYAL--SLSRAKLEELCAKIFARCMAVV
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LmHsp110 264 SQNKRARLRLVQACERLKYLLSANQSAPLNVENLMD---I-DVSIP--VFERATMESLCQDVLHSIKLVI
TcHsp110 264 TTNKRANLRLLQACERLKYLLSGNQMAPLNVENLMD---I-DINIP--SFQRSTFEELAAPLLERFKEIV
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HsHSPA8 318 EKALRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQDFFNGKELNKSINPDEAVAYGAAVQAAILSGD---K
MsHsp70 324 EKCLRDAKMDKSTVHDVVLVGGSTRIPKVQQLLQDFFNGKELCKSINPDEAVAYGAAVQAAILSGE---G
EcDnaK 321 KVALQDAGLSVSDIDDVILVGGQTRMPMVQKKVAEFF-GKEPRKDVNPDEAVAIGAAVQGGVLTG-----
AtDnaK 316 KAALKDAGVTAAEIDEVVLVGGMSRMPKVQEVVKQLF-GKEPHKGVNPDEVVAMGAAIQAGVLQG-----
TbHsp70 321 ERVLQDAKMDKRAVHDVVLVGGSTRIPKVMQLVSDFFGGKELNKSINPDEAVAYGAAVQAFILIGG---K
LmHsp70A 321 ERVLQDAKMDKRSVHDVVLVGGSTRIPKVQSLVSDFFGGKELNKSINPDEAVAYGAAVQAFILIGG---K
LmHsp70B 321 ERVLQDAKMDKRSVHDVVLVGGSTRIPKVQSLVSDFFGGKELNKSINPDEAVAYGAAVQAFILIGG---K
TcHsp70A 321 ERVLQDAKMDKRAVHDVVLVGGSTRIPKVMQLVSDFFGGKELNKSINPDEAVAYGAAVQAFILIGG---K
TcHsp70B 322 ERVLQDAKMDKRAVHDVVLVGGSTRIPKVMQLVSDFFGGKELNKSINPDEAVAYGAAVQAFILIGG---K
TbHsp70.4 318 RKVLKDAEVDASAVDDVVLVGGSTRIPRVQQLVQNFFNGKEPNRSINPDEAVAYGAAVQAHIVSGG---K
LmHsp70.4 319 KKVLADADMKPQDVDDVVLVGGSTRIPKIQQIVSQFFGGKELNRSINPDEAVAYGAAVQAHILAGG---H
TbHsp70.c 322 QRALKDASMKVEDIEDVVLVGGSSRIPAVQAQLRELFRGKQLCSSVHPDEAVAYGAAVQAHVLSGGY-GE
LmHsp70.c 332 QRALQDAAMKVEDVHEVVLVGGSSRIPKLNDMLRVFFNKARLCHSVHPDEAVAIGAAVQASILTNSLEQQ
TcHsp70.c 328 QKAMKDAAVTPDDIDDVILVGGSSRIPALRVMLQEMFKGKRLCSSVHPDEAVAIGAAVQASIISTSAEQQ
TbHsp110 328 KKGFEESGLSPDQFHSIEMIGGGSRIPMFKSAAEELL-GRAPNFTLNASETAARGAAITAAVYSPK---F
LmHsp110 328 ERGFAEAGVNRDDFHSIEMIGGGCRIPMMKRLVEEVL-GRGPSFTLNASESTARGCAIVAAMLSPK---F
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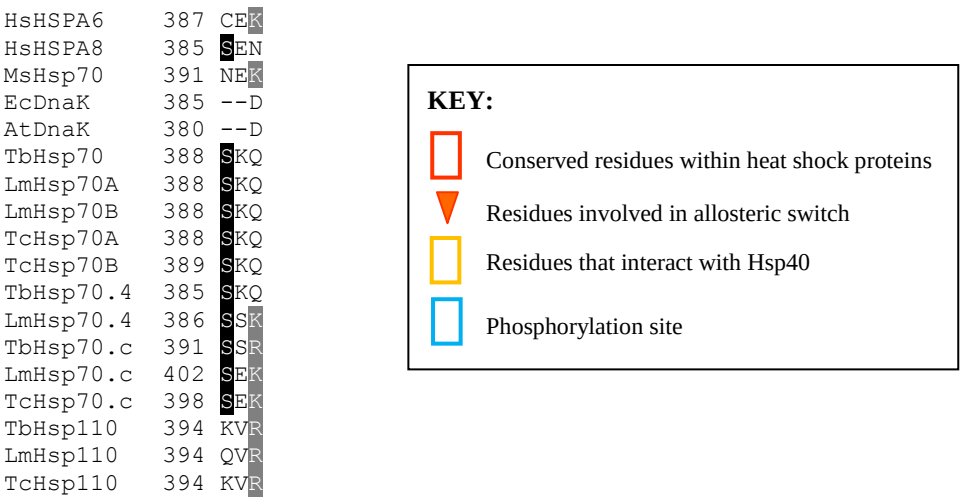
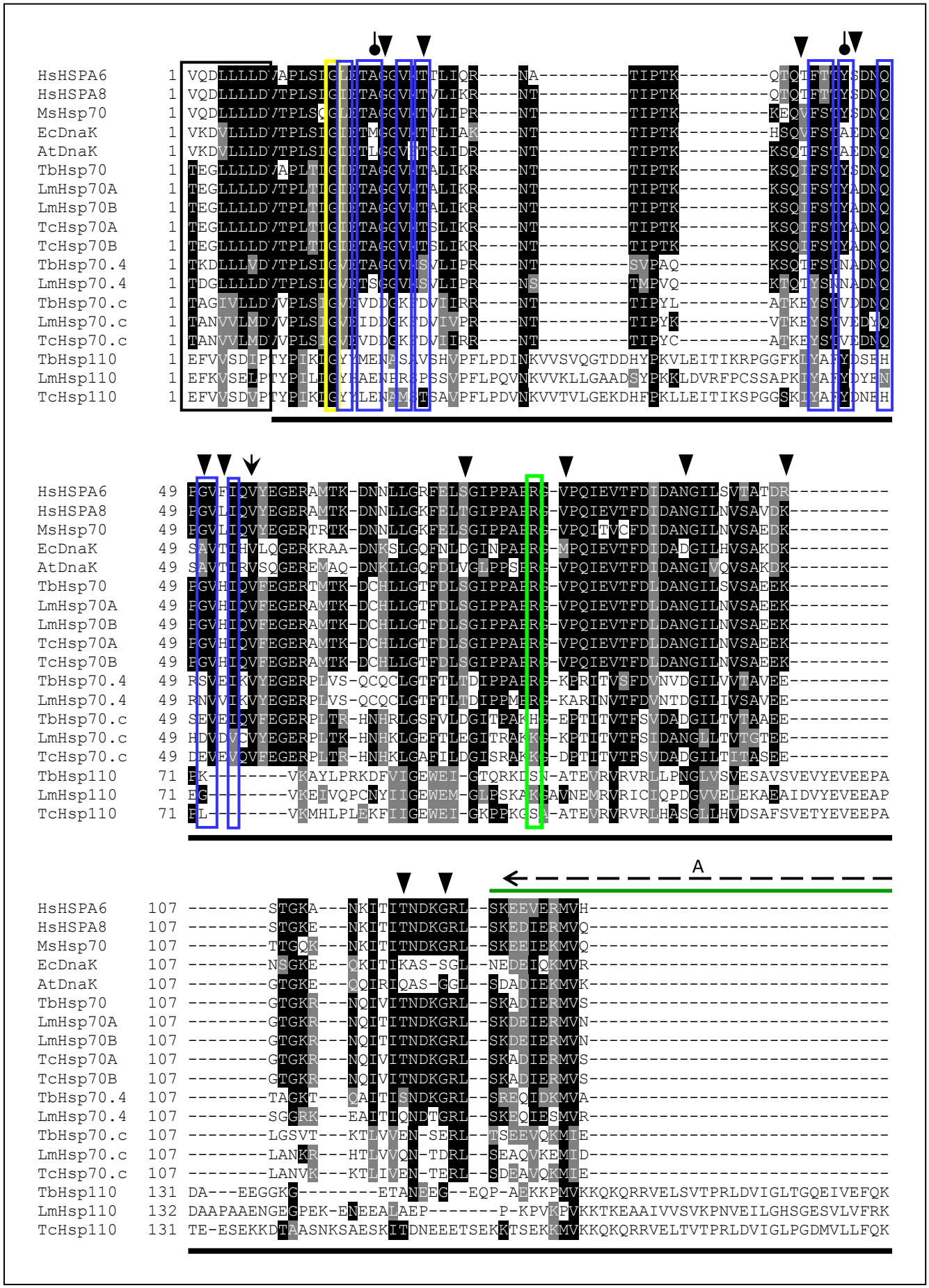


Figure 2.1. Multiple sequence alignment of predicted cytoplasmic Hsp70 proteins from the TriTryps and eukaryotic and prokaryotic canonical Hsp70s.
Multiple sequence alignment of ATPase domains of TriTryps Hsp70 proteins predicted to be cytoplasmic (Louw *et al.*, 2010) and well characterized eukaryotic and prokaryotic Hsp70 proteins. Amino acid accession numbers for the Hsp70 proteins were obtained from GeneDB (http://www.genedb.org/; Hertz-Fowler *et al.*, 2004) and NCBI (http://www.ncbi.nlm.nih.gov/; Sayers *et al.*, 2008). *HsHSPA6* – human Hsp70B (GenBank accession no.NP_002146.2); *HsHSPA8* – human Hsc70 (GenBank accession no. AAK17898.1); *MsHsp70 – Medicago sativa* Hsp70 (GenBank accession no. AAV98051.1); *EcDnaK* - *E. coli* DnaK (GenBank accession no. NP 285706.1); *AtDnaK – Agrobacterium tumefaciens* DnaK (GenBank accession no. AAR84665.1); *TbHsp70 – T. brucei* Hsp70 (GeneDB accession no. Tb11.01.3110); *LmHsp70A – L. major* Hsp70A (GeneDB accession no. LmjF.28.2770); *LmHsp70B – L. major* Hsp70B (GeneDB accession no. LmjF28.2780); *TcHsp70A – T. cruzi* Hsp70A (GeneDB accession no. Tc00.1047053511211.170); *TcHsp70B – T. cruzi* Hsp70B (GeneDB accession no. Tc00.1047053511211.170); *TbHsp70.4 – T. brucei* Hsp70.4 (GeneDB accession no. Tb927.7.710); *LmHsp70.4 – L. major* Hsp70.4 (GeneDB accession no. LmjF26.1240); *TbHsp70.c – T. brucei* Hsp70.c (GeneDB accession no. Tb11.01.3080); *TcHsp70.c – T. cruzi* Hsp70.c (GenBank accession no. Tc00.1047053511211.220); *LmHsp70.c – L. major* Hsp70.c (GenBank accession no. LmjF.28.2820); *TbHsp110 – T. brucei* Hsp110 (GeneDB accession no. Tb10.389.0880); *LmHsp110 – L. major* Hsp110 ((GeneDB accession no. LmjF18.1370); *TcHsp110 – T. cruzi* Hsp110 (GeneDB accession no. Tc00.1047053507831.60). MAFFT (Katoh *et al.*, 2005) Boxshade (http://www.ch.embnet.org/software/BOX_form.html) software was used to generate this alignment. Identical residues are white against a black background, and similar residues are against a grey background.

1998) and the DnaK/Hsp70 phosphorylation site, cyan box (McCarty and Walker, 1991) show no variation across any of the species in the alignment, except for the Hsp110 proteins (Fig. 2.1, 2.2). The linker region of TbHsp70 and its orthologues, as well as the Hsp70.4 proteins, shows a high degree of similarity to that of the well characterized eukaryotic and prokaryotic Hsp70s (Fig. 2.2). The conserved residues constituting the hydrophobic arch are replaced by acidic and aliphatic residues in the TriTryps Hsp70.c proteins, highlighted by black arrows (Fig. 2.2). The hydrophobic pocket represented by the valine residue shows no variation across the alignment, except in the Hsp110s; the loss in conservation may be an artefact of the multiple sequence


HsHSPA6
HsHSPA8
MsHsp70
EcDnaK
AtDnaK
TbHsp70
LmHsp70A
LmHsp70B
TcHsp70A
TcHsp70B
TbHsp70.4
LmHsp70.4
TbHsp70.c
LmHsp70.c
TcHsp70.c
TbHsp110
LmHsp110
TcHsp110
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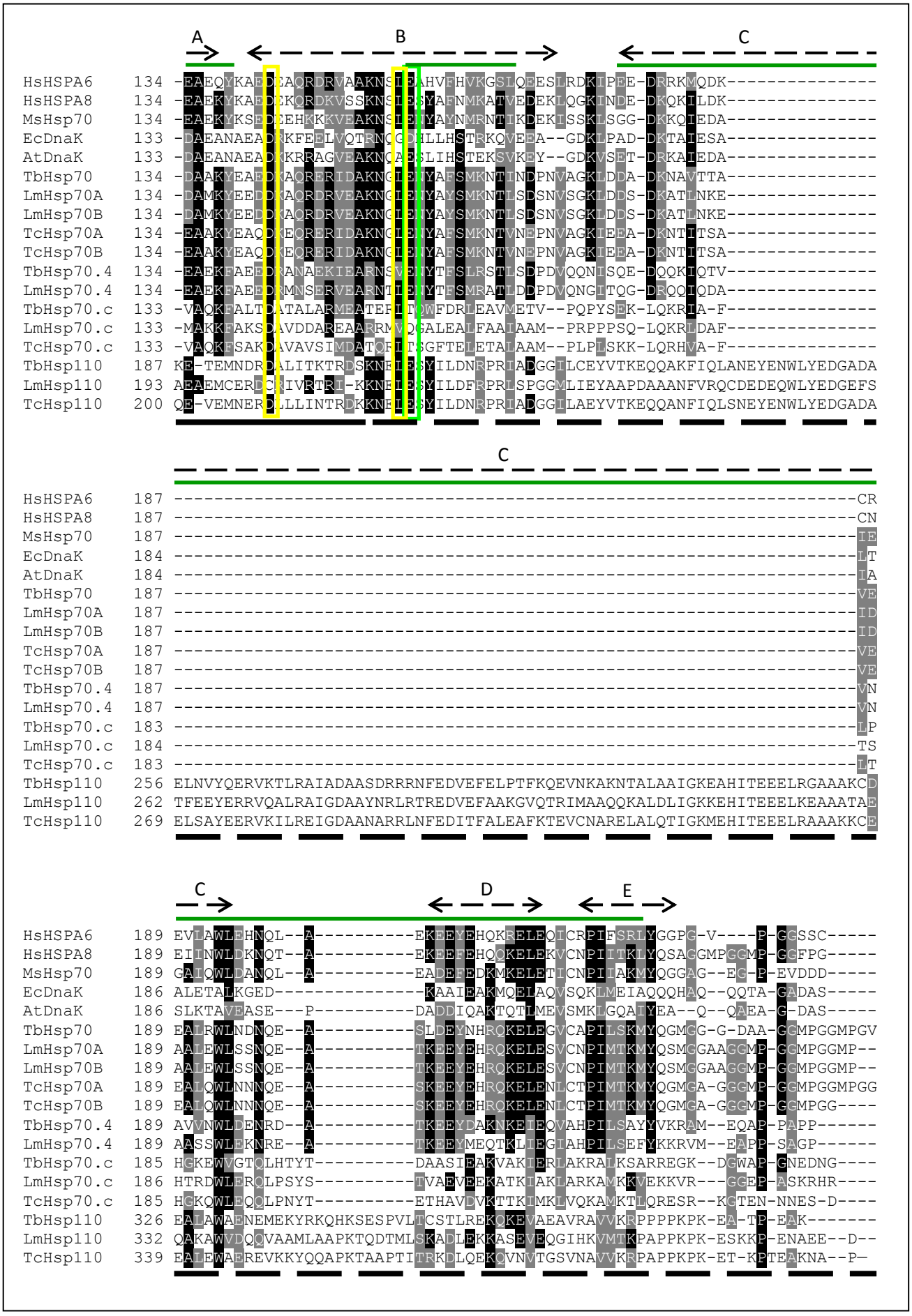
A
B
C
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HsHSPA8 134 -EAEKYKAEDKKQRDKVSSKNSLESYAFNMKATVEDEKLQGKINDE-DKQKILDK---------------
MsHsp70 134 -EAEKYKSEDEEHKKKVEAKNSLENYAYNMRNTIKDEKISSKLSGG-DKKQIEDA---------------
EcDnaK 133 -DAEANAEADRKFEELVQTRNQGDHLLHSTRKQVEEA--GDKLPAD-DKTAIESA---------------
AtDnaK 133 -DAEANAEADKKRRAGVEAKNQAESLIHSTEKSVKEY--GDKVSET-DRKAIEDA---------------
TbHsp70 134 -DAAKYEAEDKAQRERIDAKNGLENYAFSMKNTINDPNVAGKLDDA-DKNAVTTA---------------
LmHsp70A 134 -DAMKYEEDDKAQRDRVEAKNGLENYAYSMKNTLSDSNVSGKLDDS-DKATLNKE---------------
LmHsp70B 134 -DAMKYEEDDKAQRDRVEAKNGLENYAYSMKNTLSDSNVSGKLDDS-DKATLNKE---------------
TcHsp70A 134 -EAAKYEAQDKEQRERIDAKNGLENYAFSMKNTVNEPNVAGKIEEA-DKNTITSA---------------
TcHsp70B 134 -EAAKYEAQDKEQRERIDAKNGLENYAFSMKNTVNEPNVAGKIEEA-DKNTITSA---------------
TbHsp70.4 134 -EAEKFAEEDRANAEKIEARNSVENYTFSLRSTLSDPDVQQNISQE-DQQKIQTV---------------
LmHsp70.4 134 -EAEKFAEEDRMNSERVEARNTLENYTFSMRATLDDPDVQNGITQG-DRQQIQDA---------------
TbHsp70.c 133 -VAQKFALTDATALARMEATERLTQWFDRLEAVMETV--PQPYSEK-LQKRIA-F---------------
LmHsp70.c 133 -MAKKFAKSDAVDDAREAARRMVQGALEALFAALAAM--PRPPPSQ-LQKRLDAF---------------
TcHsp70.c 133 -VAQKFSAKDAVAVSIMDATQRLTSGFTELETALAAM--PLPLSKK-LQRHVA-F---------------
TbHsp110 187 KE-TEMNDRDALITKTRDSKNRLESYILDNRPRLADGGILCEYVTKEQQAKFIQLANEYENWLYEDGADA
LmHsp110 193 AEAEMCERDCRIVRTRI-KKNRLESYILDFRPRLSPGGMLIEYAAPDAAANFVRQCDEDEQWLYEDGEFS
TcHsp110 200 QE-VEMNERDLLLINTRDKKNRLESYILDNRPRLADGGILAEYVTKEQQANFIQLSNEYENWLYEDGADA
C
HsHSPA6 187 ----------CR
HsHSPA8 187 ----------CN
MsHsp70 187 ----------IE
EcDnaK 184 ----------LT
AtDnaK 184 ----------IA
TbHsp70 187 ----------VE
LmHsp70A 187 ----------ID
LmHsp70B 187 ----------ID
TcHsp70A 187 ----------VE
TcHsp70B 187 ----------VE
TbHsp70.4 187 ----------VN
LmHsp70.4 187 ----------VN
TbHsp70.c 183 ----------LP
LmHsp70.c 184 ----------TS
TcHsp70.c 183 ----------LT
TbHsp110 256 ELNVYQERVKTLRAIADAASDRRRNFEDVEFELPTFKQEVNKAKNTALAAIGKEAHITEEELRGAAAKCD
LmHsp110 262 TFEEYERRVQALRAIGDAAYNRLRTREDVEFAAKGVQTRIMAAQQKALDLIGKKEHITEEELKEAAATAE
TcHsp110 269 ELSAYEERVKILREIGDAANARRLNFEDITFALEAFKTEVCNARELALQTIGKMEHITEEELRAAAKKCE
C
D
E
HsHSPA6 189 EVLAWLEHNQL--A----------EKEEYEHQKRELEQICRPIFSRLYGGPG-V----P-GGSSC-----
HsHSPA8 189 EIINWLDKNQT--A----------EKEEFEHQQKELEKVCNPIITKLYQSAGGMPGGMP-GGFPG-----
MsHsp70 189 GAIQWLDANQL--A----------EADEFEDKMKELETICNPIIAKMYQGGAG--EG-P-EVDDD-----
EcDnaK 186 ALETALKGED----------------KAAIEAKMQELAQVSQKLMEIAQQQHAQ--QQTA-GADAS-----
AtDnaK 186 SLKTAVEASE---P----------DADDIQAKTQTLMEVSMKLGQAIYEA--Q--QAEA-G-DAS-----
TbHsp70 189 EALRWLNDNQE--A----------SLDEYNHRQKELEGVCAPILSKMYQGMGG-G-DAA-GGMPGGMPGV
LmHsp70A 189 AALEWLSSNQE--A----------TKEEYEHRQKELESVCNPIMTKMYQSMGGAAGGMP-GGMPGGMP--
LmHsp70B 189 AALEWLSSNQE--A----------TKEEYEHRQKELESVCNPIMTKMYQSMGGAAGGMP-GGMPGGMP--
TcHsp70A 189 EALQWLNNNQE--A----------SKEEYEHRQKELENLCTPIMTKMYQGMGA-GGGMP-GGMPGGMPGG
TcHsp70B 189 EALQWLNNNQE--A----------SKEEYEHRQKELENLCTPIMTKMYQGMGA-GGGMP-GGMPGG----
TbHsp70.4 189 AVVNWLDENRD--A----------TKEEYDAKNKELEQVAHPILSAYYVKRAM--EQAP-PAPP------
LmHsp70.4 189 AASSWLEKNRE--A----------TKEEYMEQTKLIEGIAHPILSEFYKKRVM--EAPP-SAGP------
TbHsp70.c 185 HGKEWVGTQLHTYT----------DAASIEAKVAKIERLAKRALKSARREGK--DGWAP-GNEDNG----
LmHsp70.c 186 HTRDWLERQLPSYS----------TVAEVEEKATKIAKLARKAMKKVEKKVR---GGEP-ASKRHR----
TcHsp70.c 185 HGKQWLEQQLPNYT----------ETHAVDVKTTKIMKLVQKAMKTLQRESR--KGTEN-NNES-D-----
TbHsp110 326 EALAWAENEMEKYRKQHKSESPVLTCSTLREKQKEVAEAVRAVVKRPPPPKPK-EA-TP-EAK-------
LmHsp110 332 QAKAWVDQQVAAMLAAPKTQDTMLSKADLEKKASEVEQGIHKVMTKPAPPKPK-ESKKP-ENAEE--D--
TcHsp110 339 EALEWAERFVKKYQQAPKTAAPTITRKDLQEKQVNVTGSVNAVVKRPAPPKPK-ET-KPTEAKNA--P-

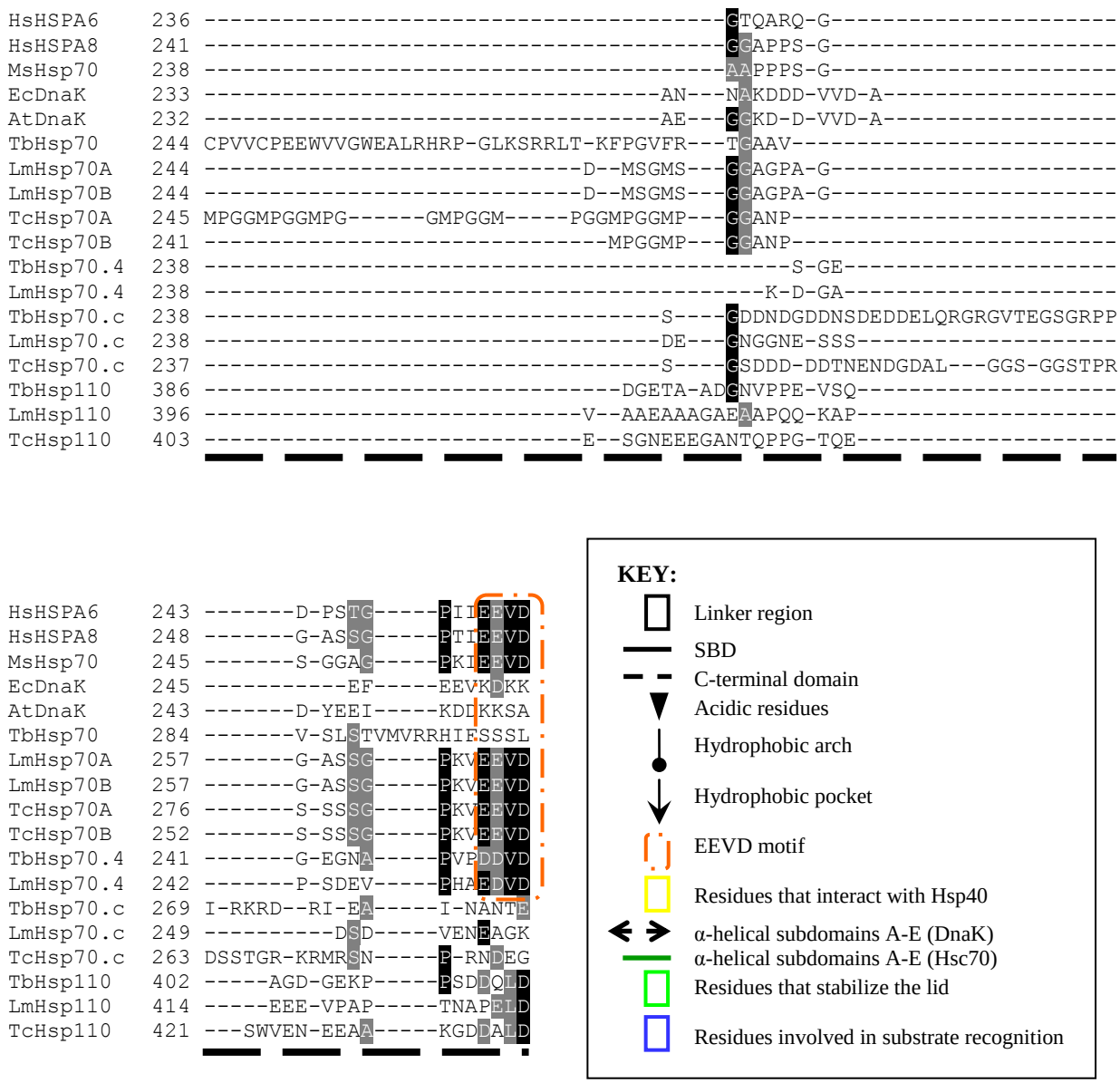


Figure 2.2. Multiple sequence alignment of predicted cytoplasmic Hsp70 proteins from the TriTryps and eukaryotic and prokaryotic canonical Hsp70s.
Multiple sequence alignment of the substrate binding (SBD) and C-terminal domains of Hsp70 proteins. Amino acid accession numbers for the Hsp70 proteins were obtained from GeneDB (http://www.genedb.org/; Hertz-Fowler *et al*., 2004) and NCBI (http://www.ncbi.nlm.nih.gov/; Sayers *et al*., 2008) as recorded in Fig. 2.1. The linker region separates the ATPase domain (Fig. 2.1) from the SBD. MAFFT (Katoh *et al.*, 2005) Boxshade (http://www.ch.embnet.org/software/BOX_form.html) software was used to generate this alignment. Identical residues are white against a black background, and similar residues are against a grey background. Specific features of the Hsp70s have been highlighted and are described in the key.

alignment (Fig. 2.2). The Hsp70.c proteins, and even more so the Hsp110s, show some variation in the SBD from the other aligned proteins, particularly the substituted acidic residues

scattered over the SBD, in the case of the Hsp70.c proteins (Fig. 2.2). The C-terminal domain containing the α-helical subdomains is generally poorly conserved (Fig. 2.2) (Zhu *et al.*, 1996). The five α-helical subdomains of DnaK (A – C) (Chou *et al.*, 2003), and three α-helical subdomains of Hsc70, highlighted with green solid lines, show quite a large degree of variation, especially within the Hsp70.c and Hsp110 groups of proteins. There is no conservation within the Hsp70.c group regarding the residues that form a latch to stabilize the lid, green box; it is, however, well conserved in the Hsp110s (Fig. 2.2) (Ha *et al.*, 1997; Chang *et al.*, 2001). It is interesting to note that the highly conserved EEVD motif (Freeman *et al,*, 1995) is absent from TbHsp70, and the Hsp70.c group of proteins (Fig. 2.2). Not unexpectedly Hsp110 also lacks the EEVD motif (Fig. 2.2). The prokaryotes and Hsp70.4 proteins contain modified EEVD motifs (Fig. 2.2).

2.3.3 Generation and confirmation of the accuracy of a TbHsp70.c homology model

A homology model of the substrate binding domain of TbHsp70.c was generated using the bovine Hsc70 crystal structure as template (Fig. 2.4A). The template shared a sequence identity of 53% with the target TbHsp70.c and the template crystal structure has a resolution of 2.6 Å (Jiang *et al,* 2005). The last 140 residues at the C-terminus of TbHsp70.c were not modelled due to the absence of a suitable template, seen as long unstructured tail when viewed using Pymol (Fig. 2.3F). After generating 100 models using the homology modelling suite Modeller, a model was selected based on overall good structure determined by various validation steps. An absolute global deviation, expressed as RMSD, of > 3.0 Å gives an indication of an unreliable structure. The calculated RMSD for the TbHsp70.c model was 0.257 Å indicating a good quality model (Fig. 2.3E). Early stages of model validation involved computational analysis of the z-score, using the ProSa-web application, which measures the deviation of the total energy of the structure with regard to energy distribution obtained from random conformations (Sippl, 1993; Sippl, 1995). The ProSa-web z-score of bovine Hsc70 (1YUW) was determined to be -10.6 and -9.1 for the model, both within the range of scores typical for proteins of similar size, 70 kDa (Fig. 2.3A,B). ProSa-web also generated an energy plot which gives an indication of the local model quality through plotting energies as a function of residue sequence position *i*, over a 40-residue fragment (Wiederstein and Sippl, 2007). Positive values shown by the energy plot indicate erroneous parts of the model. The energy plot for bovine Hsc70 shows overall negative values

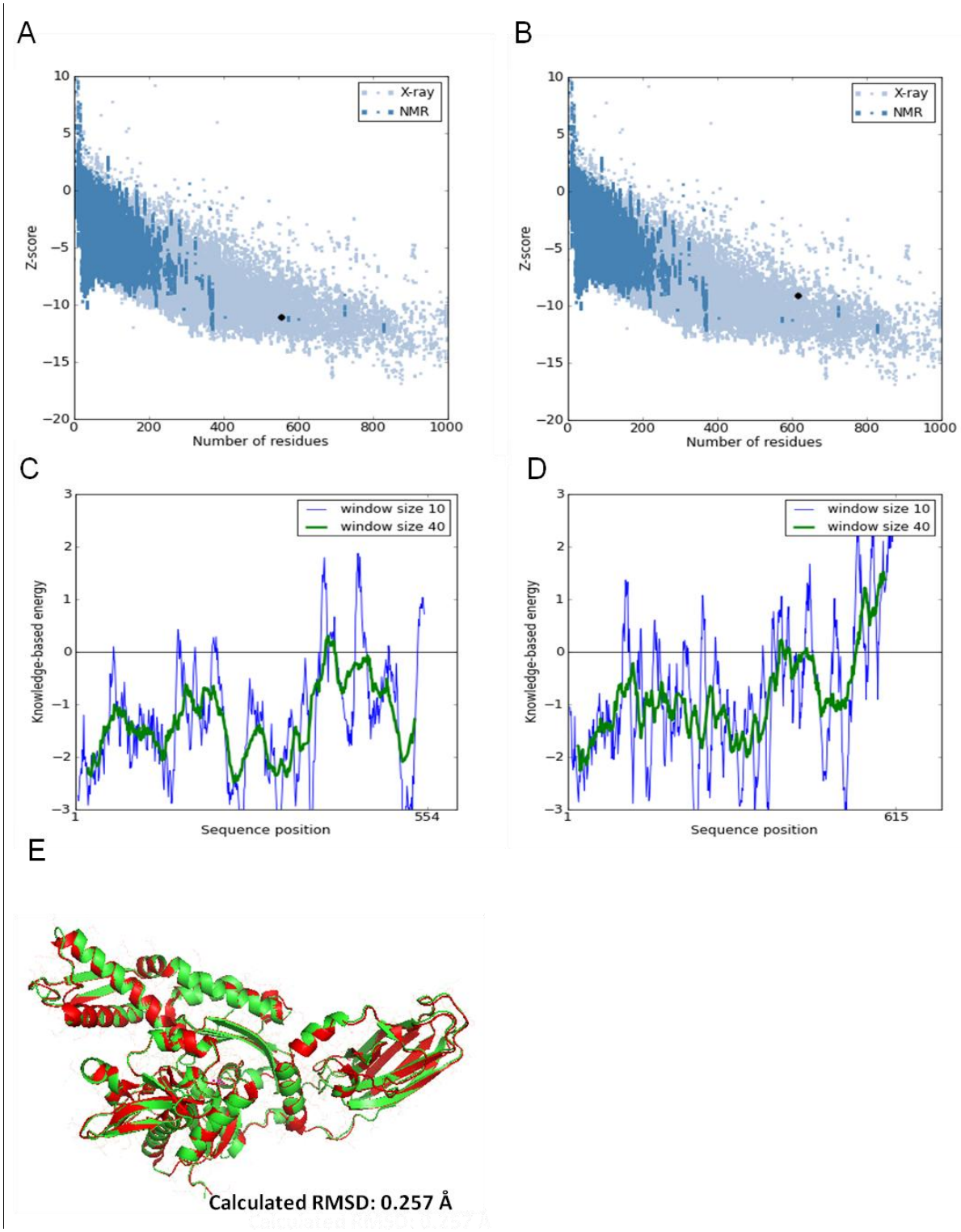


Figure 2.3. Homology model validation steps.
(A) and (B) ProSA plots containing z-scores of all experimentally determined protein chains in PDB. Structures determined experimentally by X-ray crystallography are coloured light blue, and by NMR is coloured dark blue. The ProSa-web z-score of bovine Hsc70 (1YUW) is highlighted as a large black dot and equates to -10.6 (A). The ProSA-web z-score of the TbHsp70.c model equates to -9.1 (B). (C) and (D) represent energy plots of bovine Hsc70 and the TbHsp70.c model, respectively. Residue energies are averaged over a sliding window and are plotted as a function of the middle residue in the window. (E) The root means square deviation (RMSD) of the backbone atoms was calculated to be 0.257 Å, giving an indication of a good quality model.

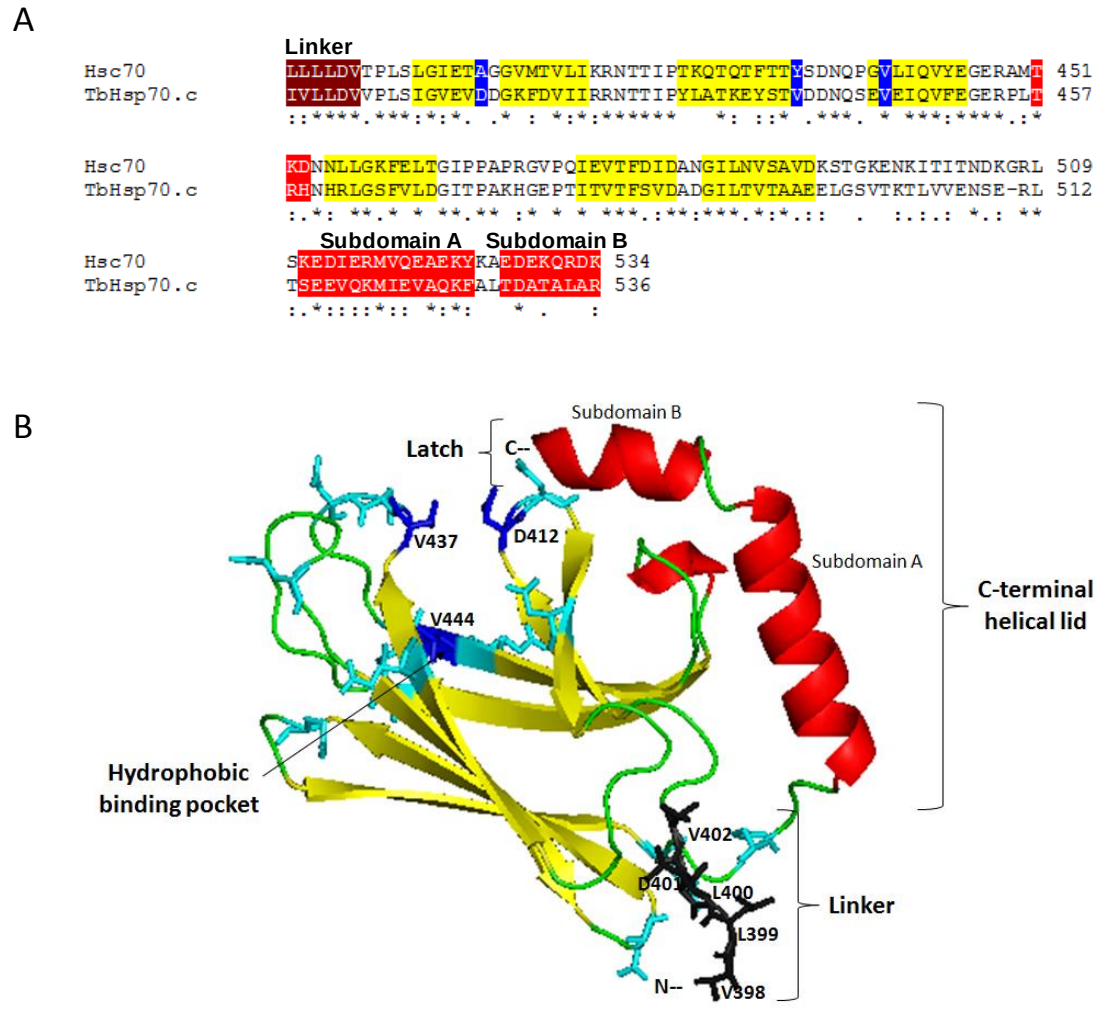


Figure 2.4. A multiple sequence alignment and homology model of the substrate binding domain (SBD) of TbHsp70.c.
A multiple sequence alignment between TbHsp70.c and bovine Hsc70 (A). TbHsp70.c amino acid residues 398 – 536 were aligned with bovine Hsc70, the template against which TbHsp70.c was modelled. Areas highlighted in yellow and red represent predicted secondary structures β-sheets and α-helices, respectively, and coincide with the modelled structure in Panel B. Ribbon representation of the homology model of TbHsp70.c (B). The predicted homology model of TbHsp70.c (amino acid residues 398 – 536) was constructed with Modeller 9v3 software (http://www.salilab.org/modeller) (Eswar *et al.*, 2006) using the crystal structure bovine Hsc70 (1YUW.pdb) as template (Jiang *et al.*, 2005). The linker region is represented by the black sticks along with its residues. Areas highlighted in yellow represent predicted secondary structure β-sheets and red regions indicate predicted α-helices. The arch and pocket residues proposed to participate in substrate binding are highlighted as blue sticks and show deviance from the conserved Y431 (Hsc70) and A406 (Hsc70) residues at the same positions. Acidic residues within the SBD are highlighted as cyan sticks. The root mean square deviation (RMSD) was calculated to be 0.257Å. Pymol was used to visualize the model (DeLano 2002).

(Fig. 2.3C). The resulting energy plot obtained for the model showed negative values up until approximately the last 20 residues, indicating a problem area in that part of the model (Fig. 2.3D). Model validation steps further included colouring of the model using MetaMQAPII, showing predominantly blue α-helices and β-sheets and giving an indication of a reliable

structure (data not shown). The afore-mentioned unstructured tail was coloured red by MetaMQAPII confirming the unreliability thereof (data not shown). To highlight the unique features within the SBD of TbHsp70.c, only the SBD and part of the C-terminus was viewed using Pymol. The dark blue highlighted residues D412, V437 and V444 are involved in substrate recognition and are found in the hydrophobic binding pocket of TbHsp70.c (Fig. 2.4B). Residues D412 and V437 have replaced the residues alanine and tyrosine, respectively, typically found in the orthologues of TbHsp70.c, shown in a pairwise alignment with template bovine Hsc70 (Fig. 2.4A). The third residue highlighted, V444, is well conserved amongst the orthologues and the canonical Hsp70s (Fig. 2.4A). Other residues involved in substrate recognition have been highlighted in light blue.

2.3.4 Secondary structure prediction of TbHsp70.c

Due to the inadequacy of the available templates to generate a complete homology model including the C-terminal 140 residues of TbHsp70.c, an online tool, PROMALS3D, was used to predict the secondary structure of the C-terminal domains of TbHsp70.c aligned with Bovine Hsc70. The conservation of the predicted α-helices separated by coils within the TriTryps Hsp70 proteins could be observed in the structure alignment (Fig. 2.5). TbHsp70.c is predicted to have α-helices separated by coils at the same positions as has been shown for bovine Hsc70 (1YUW.pdb; Jiang *et al.*, 2005) and two additional stretches of α-helices at D644 – R649 and R665 – E669, not observed in the sequence of Hsc70 (Fig. 2.5).

Secondary structure prediction of the substrate binding and C-terminal domains of TbHsp70.c aligned with its proposed orthologues (Section 2.3.2) was performed to further validate putative orthologous relationships of TbHsp70.c. The selected region is not as well conserved as the ATPase domain; a high degree of conservation in the C-terminal domains of TbHsp70.c and its putative orthologues would give a good indication of orthology. The β-sheets and α-helices of the SBD and C-terminus of TbHsp70.c aligned with the Hsp70.c sequences from the TriTryps show a high degree of conservation (Fig. 2.6). Only two stretches of α-helices at residues D644 – R649 and R664 – E668 in TbHsp70.c were not observed to be present in any of the aligned sequences, other than the subspecies TbgHsp70.c (Fig. 2.6). The well conserved secondary structure of TbHsp70.c and its putative orthologues would suggest that these proteins have similar functions.

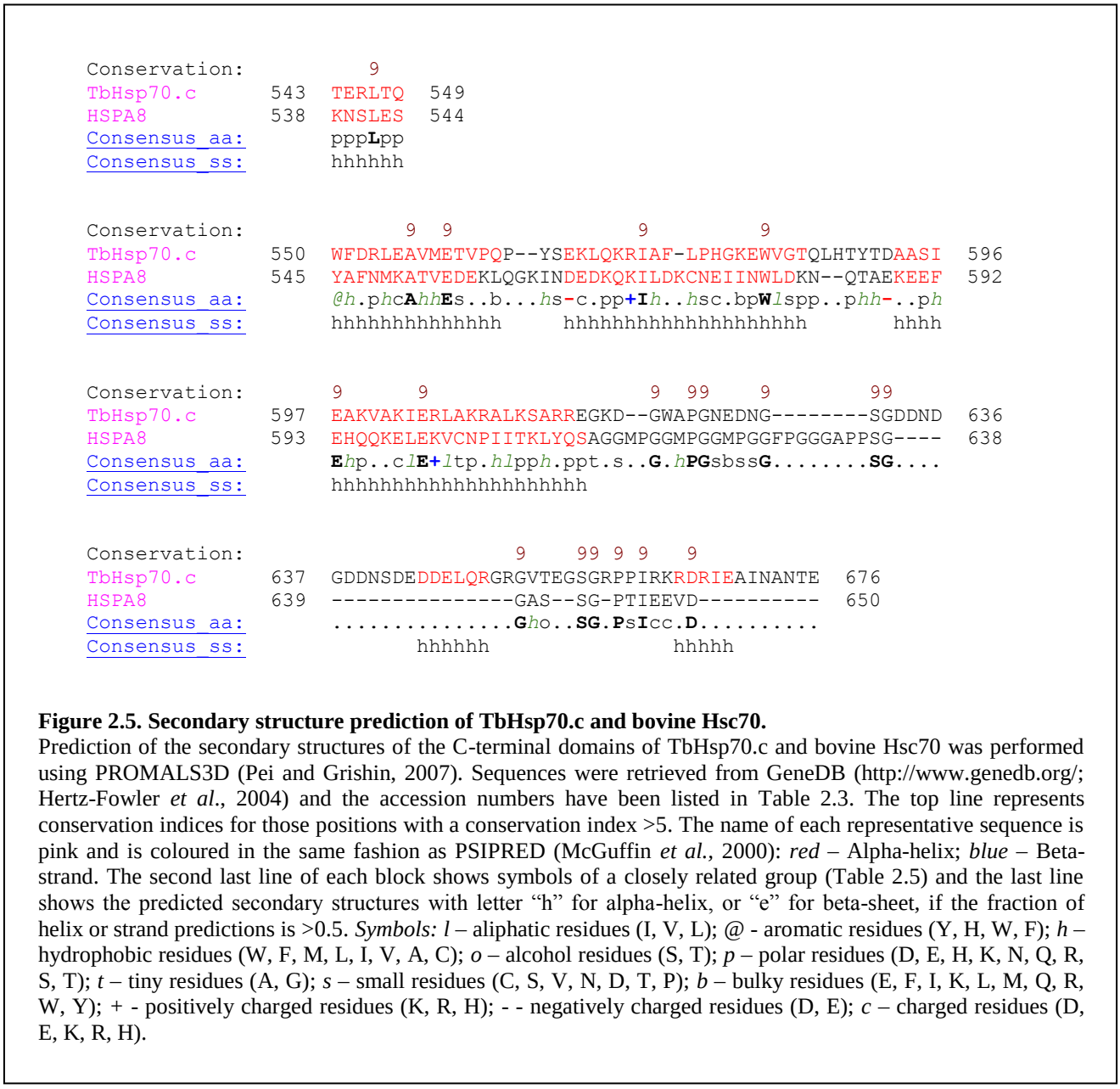
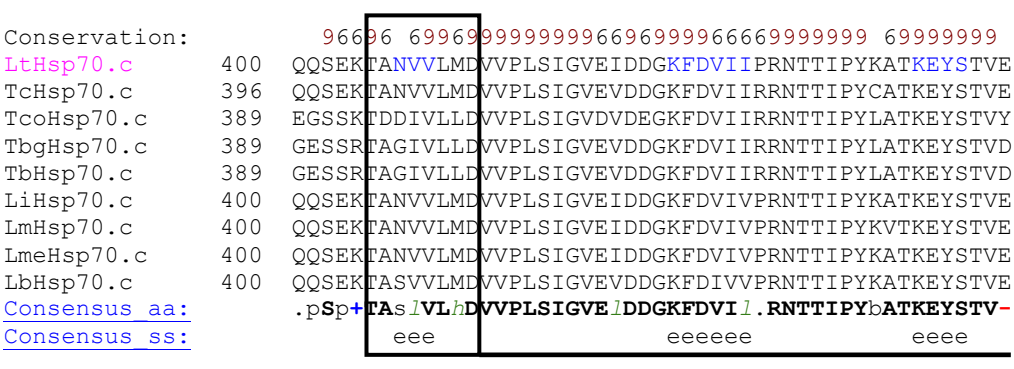
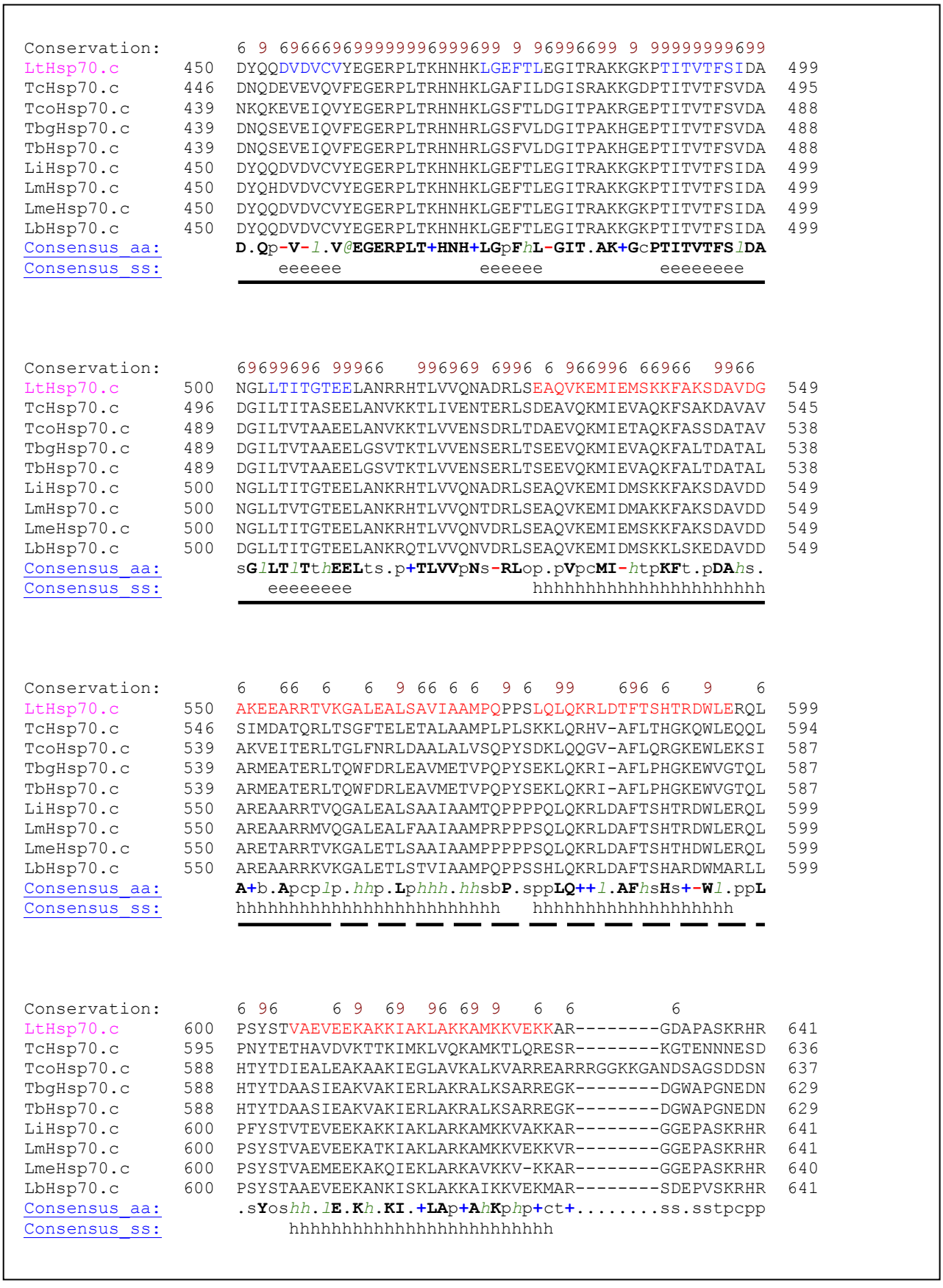


Figure 2.5. Secondary structure prediction of TbHsp70.c and bovine Hsc70.
Prediction of the secondary structures of the C-terminal domains of TbHsp70.c and bovine Hsc70 was performed using PROMALS3D (Pei and Grishin, 2007). Sequences were retrieved from GeneDB (http://www.genedb.org/; Hertz-Fowler *et al.*, 2004) and the accession numbers have been listed in Table 2.3. The top line represents conservation indices for those positions with a conservation index >5. The name of each representative sequence is pink and is coloured in the same fashion as PSIPRED (McGuffin *et al.*, 2000): *red* – Alpha-helix; *blue* – Beta-strand. The second last line of each block shows symbols of a closely related group (Table 2.5) and the last line shows the predicted secondary structures with letter "h" for alpha-helix, or "e" for beta-sheet, if the fraction of helix or strand predictions is >0.5. *Symbols: l* – aliphatic residues (I, V, L); @ - aromatic residues (Y, H, W, F); *h* – hydrophobic residues (W, F, M, L, I, V, A, C); *o* – alcohol residues (S, T); *p* – polar residues (D, E, H, K, N, Q, R, S, T); *t* – tiny residues (A, G); *s* – small residues (C, S, V, N, D, T, P); *b* – bulky residues (E, F, I, K, L, M, Q, R, W, Y); + - positively charged residues (K, R, H); - - negatively charged residues (D, E); *c* – charged residues (D, E, K, R, H).

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TcHsp70.c 446 DNQDEVEVQVFEGERPLTRHNHKLGAFILDGISRAKKGDPTITVTFSVDA 495
TcoHsp70.c 439 NKQKEVEIQVYEGERPLTRHNHKLGSFTLDGITPAKRGEPTITVTFSVDA 488
TbgHsp70.c 439 DNQSEVEIQVFEGERPLTRHNHRLGSFVLDGITPAKHGEPTITVTFSVDA 488
TbHsp70.c 439 DNQSEVEIQVFEGERPLTRHNHRLGSFVLDGITPAKHGEPTITVTFSVDA 488
LiHsp70.c 450 DYQQDVDVCVYEGERPLTKHNHKLGEFTLEGITRAKKGKPTITVTFSIDA 499
LmHsp70.c 450 DYQHDVDVCVYEGERPLTKHNHKLGEFTLEGITRAKKGKPTITVTFSIDA 499
LmeHsp70.c 450 DYQQDVDVCVYEGERPLTKHNHKLGEFTLEGITRAKKGKPTITVTFSIDA 499
LbHsp70.c 450 DYQQDVDVCVYEGERPLTKHNHKLGEFTLEGITRAKKGKPTITVTFSIDA 499
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Consensus_ss: eeeeee eeeeee eeeeeeee
Conservation: 69699696 99966 996969 6996 6 966996 66966 9966
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TcHsp70.c 496 DGILTITASEELANVKKTLIVENTERLSDEAVQKMIEVAQKFSAKDAVAV 545
TcoHsp70.c 489 DGILTVTAAEELANVKKTLVVENSDRLTDAEVQKMIETAQKFASSDATAV 538
TbgHsp70.c 489 DGILTVTAAEELGSVTKTLVVENSERLTSEEVQKMIEVAQKFALTDATAL 538
TbHsp70.c 489 DGILTVTAAEELGSVTKTLVVENSERLTSEEVQKMIEVAQKFALTDATAL 538
LiHsp70.c 500 NGLLTITGTEELANKRHTLVVQNADRLSEAQVKEMIDMSKKFAKSDAVDD 549
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TcoHsp70.c 539 AKVEITERLTGLFNRLDAALALVSQPYSDKLQQGV-AFLQRGKEWLEKSI 587
TbgHsp70.c 539 ARMEATERLTQWFDRLEAVMETVPQPYSEKLQKRI-AFLPHGKEWVGTQL 587
TbHsp70.c 539 ARMEATERLTQWFDRLEAVMETVPQPYSEKLQKRI-AFLPHGKEWVGTQL 587
LiHsp70.c 550 AREAARRTVQGALEALSAAIAAMTQPPPPQLQKRLDAFTSHTRDWLERQL 599
LmHsp70.c 550 AREAARRMVQGALEALFAAIAAMPRPPPSQLQKRLDAFTSHTRDWLERQL 599
LmeHsp70.c 550 ARETARRTVKGALETLSAAIAAMPPPPPSQLQKRLDAFTSHTHDWLERQL 599
LbHsp70.c 550 AREAARRKVKGALETLSTVIAAMPQPPSSHLQKRLDAFTSHARDWMARLL 599
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TbHsp70.c 588 HTYTDAASIEAKVAKIERLAKRALKSARREGK--------DGWAPGNEDN 629
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LmHsp70.c 600 PSYSTVAEVEEKATKIAKLARKAMKKVEKKVR--------GGEPASKRHR 641
LmeHsp70.c 600 PSYSTVAEMEEKAKQIEKLARKAVKKV-KKAR--------GGEPASKRHR 640
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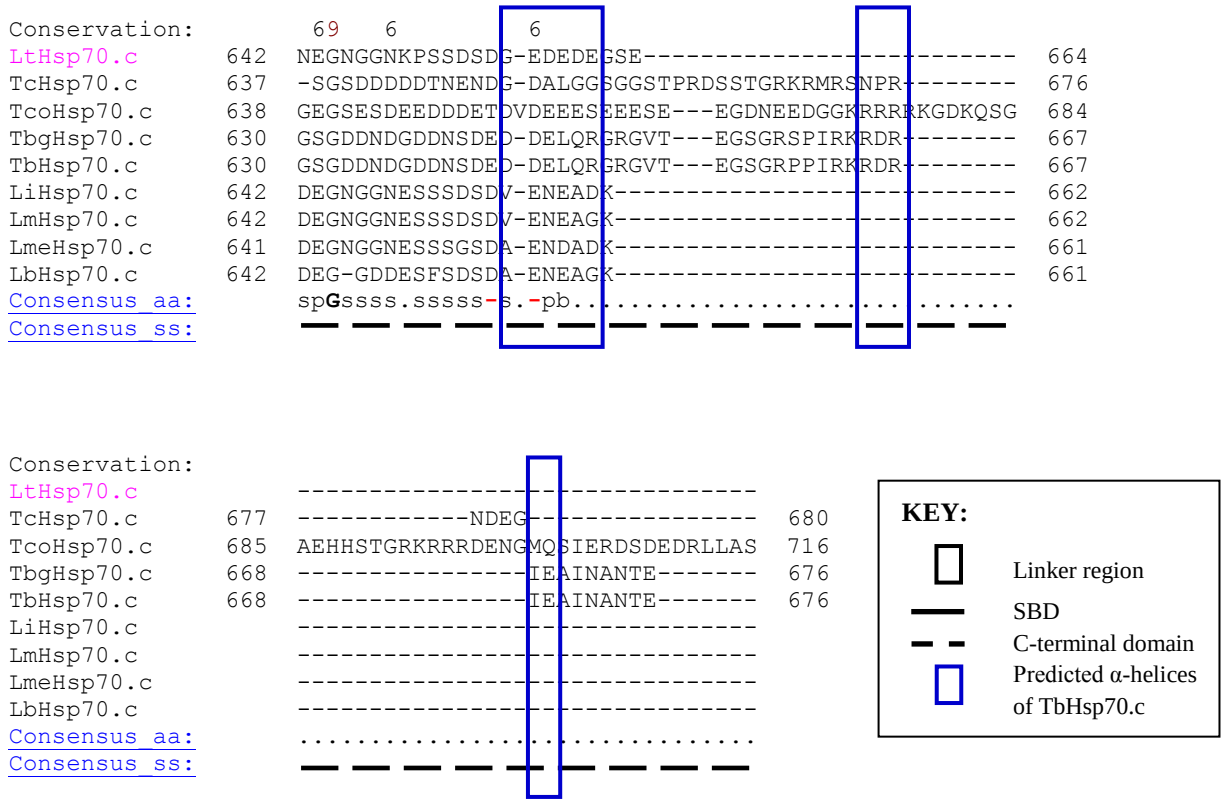


Figure 2.6. Secondary structure prediction of TbHsp70.c and its putative orthologues.
Prediction of the secondary structures of the substrate binding and C-terminal domains of TbHsp70.c and its putative orthologues was performed using PROMALS3D (Pei and Grishin, 2007). Sequences were retrieved from GeneDB (http://www.genedb.org/; Hertz-Fowler *et al*., 2004) and the accession numbers have been listed in Fig. 2.3. The top line represents conservation indices for those positions with a conservation index >5. The name of each representative sequence is pink and is coloured in the same fashion as PSIPRED (McGuffin *et al.*, 2000): *red* – Alpha-helix; *blue* – Beta-strand. The second last line of each block show symbols of a closely related group (Table 2.5) and the last line shows the predicted secondary structures with letter "h" for alpha-helix, or "e" for beta-sheet, if the fraction of helix or strand predictions is >0.5. *Symbols: l* – aliphatic residues (I, V, L); *@* - aromatic residues (Y, H, W, F); *h* – hydrophobic residues (W, F, M, L, I, V, A, C); *o* – alcohol residues (S, T); *p* – polar residues (D, E, H, K, N, Q, R, S, T); *t* – tiny residues (A, G); *s* – small residues (C, S, V, N, D, T, P); *b* – bulky residues (E, F, I, K, L, M, Q, R, W, Y); + - positively charged residues (K, R, H); - - negatively charged residues (D, E); *c* – charged residues (D, E, K, R, H).

2.3.5 Composite analysis of TbHsp70.c and design of peptide for polyclonal anti-peptide antibody production

To enable the design of a peptide for the production of anti-peptide antibodies, knowledge of regions of immunogenicity of the target protein had to be obtained; a composite analysis of TbHsp70.c was performed using Generunner (Fig. 2.7). Three putative epitopes were selected by application of the epitope-finding algorithm, the GenScript Antigen Design Tool, Optimum Antigen™. The first of the 14 amino acid peptides was located in the ATPase domain

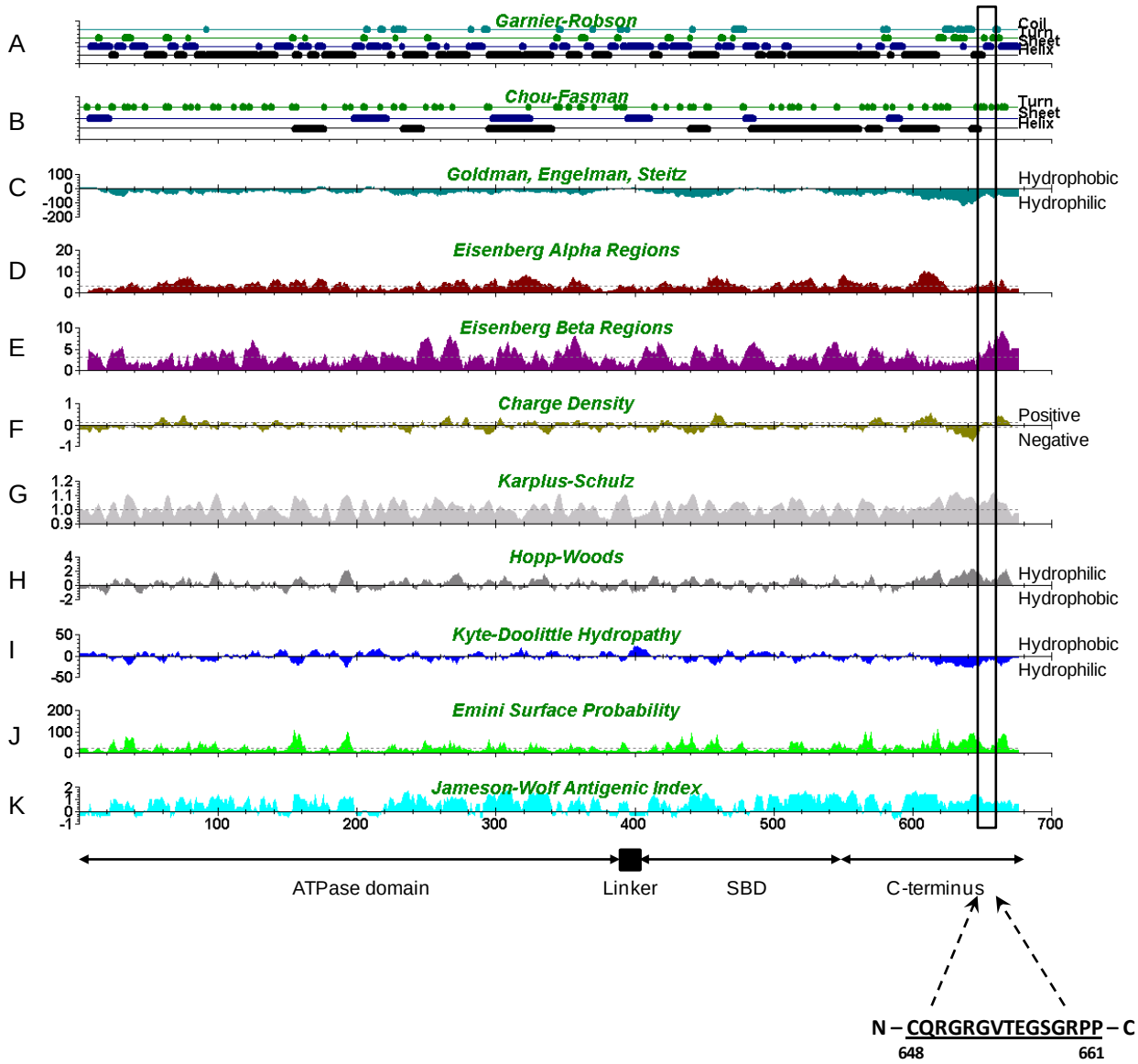


Figure 2.7. Composite analysis of full length TbHsp70.c amino acid sequence.
Antigenicity of TbHsp70.c was analysed through the composite analysis of the amino acid sequence using Generunner. Positioning of residues are specified by numbers along the x-axis. The position of the selected peptide is indicated by a black box, along with the peptide sequence below it. Arrows below the graph indicate the relative postions of the domains of Tbhsp70.c. (A) *Garnier-Robson* – determines the impact residues have on the conformational distance of other residues (Garnier *et al*., 1978); (B) *Chou-Fasman* – predicts secondary structure (Chou and Fasman, 1978); (C) *Goldman, Engelman, Schultz* – predictions of hydrophobicity are plotted as positive, hydrophilicity as negative (Engelman *et al*., 1986); (D) and (E) *Eisenberg alpha and beta regions* – plots of hydrophobic moments as a α-helix, or a β-sheet, respectively (Eisenberg *et al*., 1984); (F) *Charge density* – plots of the average charge distribution based on the sliding window calculation for each residue; (G) *Karplus-Schultz* – predicts chain flexibility and plots enhanced flexibility as a peak (Karplus and Schultz, 1988); (H) *Hopp-Woods* – predicts average local hydropathy, plotting hydrophilicity as positive and hydrophobicity as negative (Hopp and Woods, 1981); (I) *Kyte-Doolittle hydropathy* – plots hydrophilicity as negative and hydrophobicity as positive (Kyte and Doolittle, 1982); (J) *Emini surface probability* – predicts exposed regions of the protein and plots them as peaks (Emini *et al*., 1985; Janin *et al*., 1978); (K) *Jameson-Wolf antigenic index* – computes an overall antigenicity analysis by combining all the previous analyses, epitopic regions are plotted as peaks.

of TbHsp70.c, E24 – T37 (ERVEIIANDQGNRT) and the second and third both in the C-terminal domain, R609 – W622 (RALKSARREGKDGW) and Q648 – P661 (QRGRGVTEGSGRPP). In addition to the information obtained from the composite analysis with the required hydrophilicity (Hopp-Woods analysis), surface exposure (Emini Surface Probability), flexibility (Karplus-Schultz analysis) and antigenicity (Jameson Antigenic Index) (Fig. 2.7), the third peptide Q648 – P661 (QRGRGVTEGSGRPP) was selected based on it showing the lowest degree of sequence conservation. Through the GenScript Antigen Design Tool, OptimumAntigen^{TM} the peptide was determined to have an antigenicity of 2.31, a surface probability of 1.29 and hydrophilicity of 0.73. A cysteine residue was added to the N-terminus of the peptide (**C** QRGRGVTEGSGRPP) to facilitate cross-linking to a carrier protein (limpet keyhole haemocyanin, used to produce the antisera) preceding the injection into the host animal.

Subsequent to the successful design of the antigenic peptide, BLAST analysis was performed on the sequence and no significant similarities to an *E. coli*, trypanosomal (other than *T. b. gambiense* or *T. b. rhodesiense*) or human protein was detected. Anti-TbHsp70.c peptide antibody was synthesized and conjugated by GenScript using the SC1031 Complete Affinity-Purified Peptide Polyclonal Antibody Package (Rabbit) using peptide-KLH conjugate as immunogen. The synthesized peptide was injected into rabbit by GenScript to produce polyclonal antibody. Anti-TbHsp70.c peptide antibody was affinity purified and a yield of 2.734 mg/ml obtained. The antibody was used to detect TbHsp70.c from both bacterial and trypanosomal lysates using western blot analysis (Chapters 3 and 5, respectively).

2.3.6 Conservation level of cytoplasmic Hsp40 proteins from the TriTryps

The level of conservation of the J-domain of the Type I Hsp40s from the TriTryps (J-proteins) that were predicted to be cytoplasmic (Ludewig, 2010, PhD Thesis), was assessed using a multiple sequence alignment, highlighting residues and motifs important for interactions with partner Hsp70 proteins. Putative orthologous relationships between the species aligned in Fig. 2.8 with nomenclatures J2, J3, J4, J45 and J46, respectively, was established (Ludewig, 2010, PhD Thesis).The J-domain was shown to be well conserved in all the species aligned, except for that of Type IV *T. vivax* J47, which showed a low degree of conservation (Fig. 2.8). Many proteins that belong to the Type IV class are Hsp40-like in the sense that the J-domain is

present, but the conserved HPD motif is lacking (Botha *et al.*, 2007). The highly conserved HPD motif was shown to be present in all the other proteins in the alignment (Fig. 2.8). Some variation was observed in the KFK (Fig. 2.8). The crucial residue within the KFK motif required for interaction with Hsp70s, Phe (Kiang *et al.*, 2007), was absent altogether in Linj3, Lbrj45, Tcj45, Tcj46, Tcnj46 and Tvivj47 (Fig. 2.8).

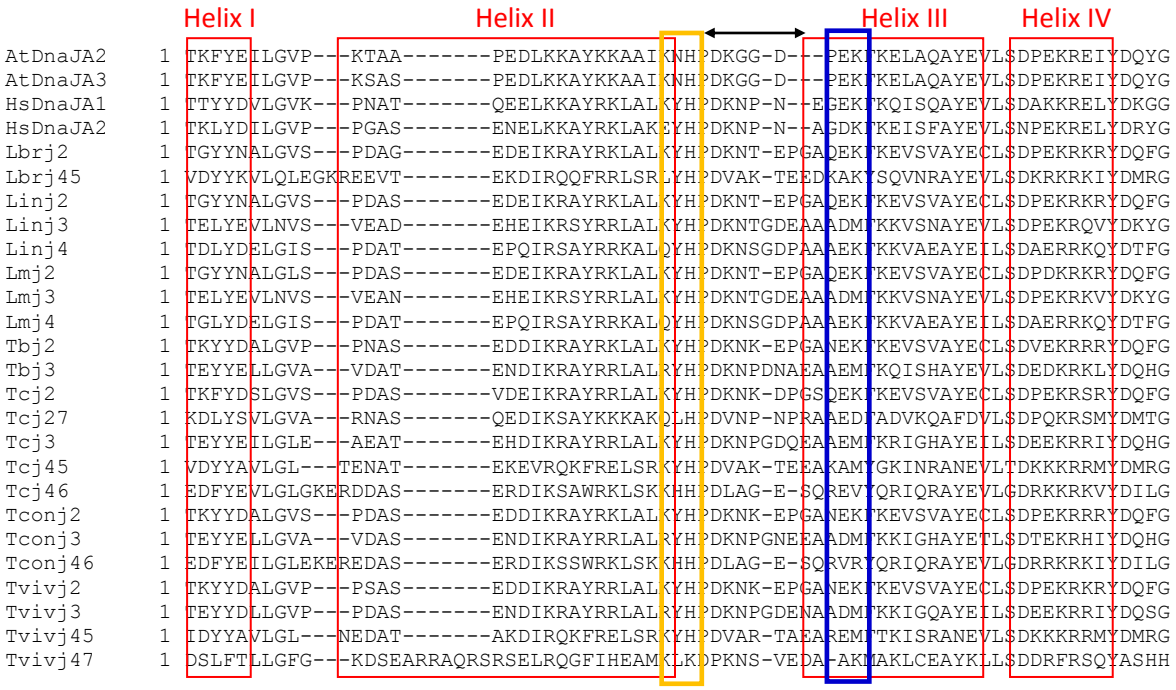


Figure 2.8. Multiple sequence alignment of predicted cytoplasmic J-proteins from the TriTryps.
Multiple sequence alignment of the J domains of Tritryps J-proteins predicted to be cytoplasmic (Ludewig 2010 PhD Thesis). Red boxes represent α-helices; the yellow box indicates the presence of the HPD motif; the blue box the KFK motif; the arrow indicates the loop region between α-helix II and III. Amino acid accession numbers for the Hsp40 proteins were obtained from GeneDB (http://www.genedb.org/; Hertz-Fowler *et al.*, 2004) and NCBI (http://www.ncbi.nlm.nih.gov/; Sayers *et al.*, 2008). *AtDnaJA2 – Agrobacterium tumefaciens* DnaJA2 (GenBank accession no. AAB86799.1); *AtDnaJA3* –(GenBank accession no. AAB49030.1); *HsDnaJA1 – H. Sapiens* DnaJA1 (GenBank accession no. NP_001530.1); *HsDnaJA2* - (GenBank accession no. NP_005871.1); *Lbrj2 – L. braziliensis* J2 (GenBank accession no. LbrM27_V2.2610); *Lbrj45* - (GenBank accession no. LbrM32_V2.3590); *Linj2 – L. infantum* J2 (GenBank accession no. LinJ27_V3.2350); *Linj3* - (GenBank accession no. LinJ21_V3.0550); *Linj4* - (GenBank accession no. LinJ15_V3.1220); *Lmj2 – L. major* J2 (GenBank accession no. LmjF27.2400); *Lmj3* - (GenBank accession no. LmjF21.0490); *Lmj4* – (GenBank accession no. LmjF15.1220); *Tbj2 – T. brucei* J2 (GenBank accession no.Tb927.2.5160); *Tbj3* - (GenBank accession no. Tb10.70.5440); *Tcj2 – T. cruzi* J2 (GenBank accession no. Tc00.1047053511627.110); *Tcj27* - (GenBank accession no. Tc00.1047053510243.30); *Tcj3* - (GenBank accession no. Tc00.1047053511367.138); *Tcj45* - (GenBank accession no. Tc00.1047053511025.100); *Tcj46* - (GenBank accession no. Tc00.1047053509233.80); *Tconj2 – T. congolense* J2 (GenBank accession no. Congo365g12.plk_2); *Tconj3* - (GenBank accession no.Congo541b10.qlk_1); *Tconj46* – Congo1293a06.qlk_); *Tvivj2 – T. vivax* J2 (GenBank accession no. tviv623d01.qlk_23); *Tvivj3* - (GenBank accession no. tviv1100a12.qlk_0); *Tvivj47* - (GenBank accession no. tviv796e07.plk_2). T-Coffee (Notredame *et al.*, 2000) and Boxshade (http://www.ch.embnet.org/software/BOX_form.html) software was used to generate this alignment. Identical residues are white against a black background, and similar residues are against a grey background.

2.4 Discussion and conclusions

The bioinformatics analysis conducted in this study has revealed the novelty of a divergent Hsp70 protein, TbHsp70.c, confirmed by a similar analysis performed by Louw and colleagues (2010). *T. brucei* possesses a complement of 12 Hsp70 proteins (Table 2.3), a number close to that observed in *Homo sapiens*, 13 (Hageman and Kampinga, 2009). Disregarding the C-terminal domain, it is clear that there is a degree of conservation amongst the canonical Hsp70s. Using *E. coli* DnaK as a reference, features of Hsp70 members of Trypanosomatidae, and in particular, TbHsp70.c were highlighted. Some conservation has been lost amongst the divergent and novel group of Hsp70.c proteins, TbHsp70.c and its orthologues, TcHsp70.c and LmHsp70.c (Louw *et al.*, 2010) (Fig. 1). The alignment of this group of Hsp70.c proteins reveals distinct differences from other eukaryotic and canonical Hsp70s, especially in the C-terminal domain, implying a lack of conservation and interestingly they are only present in the trypanosomatids (Simpson *et al.*, 2004). Hsp70 residues involved in the association with Hsp40 in both the ATPase and substrate binding domains were shown to be well conserved, indicating a potential bipartite interaction of TbHsp70.c with its partner TbHsp40, as has been shown to be the case for DnaK and DnaJ (Karzai and McMacken, 1996; Suh *et al.*, 1998). Although the hydrophobic pockets of the Hsp70.c proteins are very well conserved, conservation is lost in the hydrophobic arch and there was a marked loss of conservation in the residues involved in substrate recognition. These were substituted with acidic residues and were also observed to be present in the SBD. These differences would imply that the Hsp70.c proteins possess a unique mechanism of interaction with substrate or that they would show substrate discrimination. Previous observations of loss of conservation of the SBD of *P. falciparum* Hsp70 proteins have led to implications of Hsp70 specificity being directly linked to the poor conservation (Shonhai, 2007). Mammalian Hsp70 has been demonstrated to contain specific binding sites for different cofactors which enables the formation of protein complexes, and mediates specific Hsp70 functions including regulation of the Hsp70 ATPase activity which mediates the affinity of Hsp70 for its substrates (Demand *et al.*, 1998). The EEVD motif, specific to cytoplasmic members of the Hsp70 family, was shown to be essential for interaction with the Hsp70/Hsp90 organizing protein (Hop) and CHIP (Freeman *et al.*, 1996; Demand *et al.*, 1998). Along with the Hsp70 SBD binding site, Hsp40 was also demonstrated to interact with the very well conserved EEVD motif, resulting in decreased/loss of function in its absence; Hop and Hsp40 interact noncompetitively with this motif (Demand *et al.*, 1998). In fact, Hsp40 was demonstrated to still stimulate the ATPase activity of Hsp70 in the absence of the EEVD motif,

but to a lower degree (Demand *et al.,* 1998). This holds interesting implications for TbHsp70.c and its potential interactants. A few TriTryps proteins were shown to lack the EEVD motif, including TbHsp70.c. However, should TbHsp70.c form a functional complex with TbHsp90, a novel mechanism of interaction with structural component TbHop (Accession no. XP 827315.1; Johnson and Brown, 2009) would be an interesting finding. Both Hsp70-interacting protein (Hip) and BAG-1, cofactors of Hsp70, cooperate with Hsp40 in facilitating Hsp70 function; however, BAG-1 and Hip are Hsp70-binding competitors, suggested to be an alternative strategy of Hsp70 regulation (Höhfeld and Jentsch, 1997; Demand *et al.,* 1998).

A study of the evolutionary relationships between kinetoplastid Hsp70 proteins and typical Hsp70 proteins has revealed a novel and divergent group of proteins, the orthologues TbHsp70.c, TcHsp70.c and LmHsp70.c (Louw *et al.,* 2010). Modelling of the SBD of TbHsp70.c visually highlighted the differences in key residues involved in substrate recognition. To compensate for the lack of template for the C-terminus of TbHsp70.c due to unalignable primary structures, secondary structure prediction software was utilized to envisage the rest of the protein. Interestingly TbHsp70.c is predicted to form the same α-helices as bovine Hsc70, implying that this divergent protein would show the C-terminal helical lid structure as can be seen for canonical Hsp70 proteins (Moro *et al.,* 2004). With some debate the helical lid has been suggested to regulate the substrate binding specificity of Hsp70 proteins, even though it shows a great deal of divergence across Hsp70 proteins (Pellecchia *et al.,* 2000, Moro *et al.,* 2004). Due to its immediacy to the linker region, the helical lid has been implicated in interdomain communication (Jiang *et al.,* 2005). The lid is supposed to disturb the α-helical A subunit when Hsp40 partners with Hsp70, thereby manipulating the substrate binding kinetics (Jiang *et al.,* 2005). However, even though the predicted secondary structure is similar to that of Hsc70 (Fig. 2.4), TbHsp70.c has displayed a lack of conservation in residues known to be fundamental for helical lid stabilization (Fig. 2.1).

There are many different Hsp40 proteins relative to Hsp70 proteins (Folgueira and Requena, 2007), and a single Hsp70 may associate with various Hsp40 proteins but these interactions occur with a degree of specificity, such as the inability of an Hsp70 within one organelle to replace the functioning of an Hsp70 within a different organelle (Brodksky *et al.,* 1993). The search for functional Hsp40 partners of TbHsp70.c was narrowed down through *in silico* analysis, taking predicted localizations (Ludewig, 2010, PhD Thesis), the presence of a J-

domain and specific residues known to enable the Hsp70-Hsp40 interaction into consideration (Michel *et al.,* 1999). The presence of the His residue of the HPD motif and the Phe residue of the KFK motif within the J-domain are critical for the Hsp70-Hsp40 interaction, including the ATPase stimulation of Hsp70 (Genevaux *et al.,* 2002; Jiang *et al.,* 2007). These two residues are present in both the *T. brucei* cytoplasmic J-proteins Tbj2 and Tbj3, suggesting the ability to functionally interact with cytoplasmic TbHsp70.c. The focus of this study was not placed on the Hsp40 proteins and so a thorough study of the J-proteins was not performed.

The aim of this study was to utilize available bioinformatic tools to infer the functioning of TbHsp70.c and the potential interactions with partner *T. brucei* proteins, and design and produce TbHsp70.c antibodies. Through various tools such as BLASTs, pairwise alignments, multiple sequence alignments, homology modelling and composite analysis, further knowledge of TbHsp70.c and potential *T. brucei* Hsp40 interactants was gained which gave direction to subsequent characterization. The *in vitro* studies were focused on the ability of TbHsp70.c to function as a canonical Hsp70 protein in its ability to bind substrate and for its ATPase activity to be stimulated by a co-chaperone. The successful design and production of an anti-peptide antibody specific against TbHsp70.c allowed the detection of the protein in both *in vitro* (Chapter 3) and *in vivo* (Chapter 4) analyses, enabling a thorough characterization of this trypanosomal protein.

CHAPTER 3

IN VITRO WORK:

Analysis and optimization of heterologous expression and purification of TbHsp70.c, TcHsp70B and Tbj2

3.1 Introduction

The importance of recombinant proteins in biological research extends from their use in investigating protein interactions, ligand binding and enzyme activity to pharmaceutical drug targets and therapeutic agents, to mention but a few (Sinha *et al.,* 1994; Gallinari *et al.,* 1998; Anderson and Krummen, 2002; García *et al.,* 2002; Howell *et al.,* 2006). *Escherichia coli* (*E. coli*) is a widely used prokaryotic organism for heterologous protein production due to the ease with which it can be grown and genetically engineered (Fields and Johnston, 2005). Advantages such as rapid growth rate, simple nutritional requirements, metabolic diversity and completed genome sequence along with well characterized genetics make it an ideal organism to work with (Neidhardt and Curtiss, 1996). Research based in the *E. coli* system resulted in advancement of knowledge of DNA replication, translation, gene regulation, restriction enzymes and horizontal gene transfer (Gottesman, 1999; Loenen, 2003; Wilson *et al*., 2007). It has been evaluated as a user-friendly and safe system, so much so that it is the most commonly used organism in molecular biology and biotechnology (Hobman *et al.,* 2007). Even though *E. coli* has been the subject of intense investigation for the past 60 years, various challenges such as inclusion body formation, protein aggregation, codon bias, proteolytic degradation, formation of disulphide bonds have complicated the production of complex proteins in their native state from this expression system (Hockeny, 1994; Baneyx and Mujacic, 2004; Birkholtz *et al.,* 2008). In eukaryotes, *Saccharomyces cerevisiae* (budding yeast) has been used as a model organism in the study of genetics and cell biology due to its rapid and easy growth, and the similarity of its life cycle to that of humans (Karathia *et al.,* 2011). Approximately 30% of genes associated with human disease have orthologues in yeast (Foury, 1997), and so the yeast proteome has been utilized in studies of neurodegenerative disorders (Miller-Fleming *et al.,* 2008), aging (Murakami and Kaeberlein, 2009), signal transduction (Hohmann *et al.,* 2007), metabolism and cell cycle (Nasheuer *et al.,* 2002; Brocard-Masson and Dumas, 2006; Lopez-Mirabal and Winther, 2008) and apoptosis (Owsianowski *et al.,* 2008). In addition to its ease of growth, *Drosophila melanogaster* (the fruit fly), has a congenital heart defect and a polytene chromosome that can be inspected using a light microscope, and mutations can easily be induced and observed (Manev *et al.,* 2003). The first multicellular organism to have its entire genome sequenced was the nematode *Caenorhabditis elegans* and has since been used as a model organism for research involving the genetic control of development and physiology (Riddle, 1997).

The sensitivity of proteins to non-optimal conditions at various stages of handling can result in modifications of protein structure leading to protein aggregation and reduced activity

(Kerwin *et al.,* 1998; Kreilgaard *et al.,* 1998; Lu *et al.,* 2001). A constant challenge regarding *in vitro* assays is the requirement for high concentrations of protein, which often results in protein aggregation (Bondos *et al.,* 2000). Aggregation of intermediate forms of protein has been reported to obscure protein folding studies (Goldberg *et al.,* 1991). The rate of aggregation may surpass the protein folding rate, thereby causing inactivation of part of the protein (Goldberg *et al.,* 1991). A variety of co-solvents have been identified that destabilize aggregates and increase protein stability (listed in Bondos and Bicknell, 2003). A few strategies include disruption of protein-protein interactions with either charged co-solvents (Timasheff, 1998; Sharme *et al.,* 2000) or competitive binding with chaotropic species (Hu *et al.,* 2001; Edwin *et al.,* 2002); stabilization of intramolecular protein interactions through the use of ammonium sulphate (Collins and Washabaugh, 1985; Timasheff, 1998) which also inhibits the formation of ice, thereby providing protein protection upon freezing (Kerwin *et al.,* 1998; Timasheff, 1998). Disruption of hydrophobic interactions, hydrogen bonds, van der Waals forces and ionic interactions are required to enhance the solubility of proteins (Rabilloud, 1996; Rabilloud, 2009; Tao *et al.,* 2010). Additives such as the reducing agents dithiothreitol and β-mercaptoethanol can aid protein solubility by preventing disulfide bonds from forming and so reducing protein aggregation (Cappel and Gilbert, 1998). Hydrophobic interactions present within aggregates can be inhibited by detergents (Rajakumari *et al.,* 2006; Tao *et al.,* 2010). Non-ionic detergents such as NP40 and triton X-100 allow purification of native proteins without denaturation (Rajakumari *et al.,* 2006). Many ionic detergents, however, do denature proteins thereby reducing protein function (Frangioni and Neel, 1993; Sunitha *et al.,* 2000). Alkyl anionic sarkosyl with an anionic head group and hydrocarbon straight chain is frequently used to enhance protein solubility through inhibition of hydrophobic interactions and was recently determined to have no significant impact on protein activity (Filip *et al.,* 1973; Burgess, 1996; Sunitha *et al.,* 2000; Park *et al.,* 2010).

Codon "harmonization" refers to closely matching the codon bias within the native host with the target gene sequence expressed in the heterologous host (Angov *et al.,* 2008). This strategy has allowed successful expression of *Plasmodium falciparum* target proteins in *E. coli* (Hillier *et al,* 2005; Angov *et al.,* 2008; Chowdhury *et al.,* 2009). Codon bias has also been accounted for in trypanosomatids and correlates with tRNA gene copy number and levels of expression (Michels, 1986; Alvarez *et al.,* 1994; Necsulea and Lobry, 2006). Atypically, transcription in the kinetoplastids is polycistronic and constitutive, and mRNA

molecules, once mature, are trans-spliced to matching leader sequences (Palenchar and Bellofatto, 2006). Translational selection has been proposed to be responsible for control of the differential expression present in the kinetoplastids, where it assists protein abundance from genes lodged within polycistrons (Horn, 2008).

In vitro biochemical characterization studies require a large quantity of protein that is highly pure. The addition of fusion tags to recombinant proteins has enabled purification of almost any protein, enhancing protein yield and solubility as well as promoting proper folding of the target protein (Arnau *et al.*, 2006; Esposito and Chatterjee, 2006). Unfortunately fusion tags may interfere with protein conformation and biological activity. The most frequently used fusion tags are the affinity tags; exogenous residues that bind strongly to a chemical ligand or antibody. This high affinity enables a high degree of purification of the target protein. Affinity tags include His, STREP II, FLAG, Myc and HA.

Histidine tags (His-tags) consist of a sequence of 6 to 9 histidine residues that are expressed either at the N- or C-terminus of a recombinant protein. The His-tag can bind to several kinds of immobilized metal ions (nickel, cobalt and copper). Even though commonly used, nickel tends to bind non-specific endogenous protein with histidine clusters; a small amount of elution agent (imidazole) in the wash buffer will circumvent the problem. The target protein can be purified or detected without a protein-specific antibody or probe. Affinity purification entails derivatizing beaded agarose supports with chelating groups (nitrilotriacetic acid or iminodiacetic acid) enabling the chosen metal ion to be immobilized. After preparation of the nitrilotriacetic acid (NTA)-agarose resin, the divalent metal is added to produce Ni-NTA agarose. A millilitre of resin will typically allow purification of 1 – 10 milligrams protein. Solubility-enhancing tags are typically large peptides that increase protein expression and solubility. Glutathione S-transferase (GST) and maltose binding protein (MBP) tags act doubly as affinity and solubility-enhancing tags. NusA, thioredoxin (TRX), small ubiquitin-like amplifier (SUMO) and ubiquitin (Ub) need affinity tags for protein purification (Table 1). NusA and MBP are more likely to be expressed as soluble proteins than GST or the smaller affinity tags (Braun *et al.*, 2002; Hammarstrom *et al.*, 2002; Shih *et al.*, 2002; Niiranen *et al.*, 2007).

Various purification strategies have been employed for the purification of trypanosomal proteins and isolation of His-tagged Tbj1, Tcj1 and TcHsp70 involved a denaturing purification in which 8 M urea was used (Edkins *et al.,* 2004; Louw *et al.,* 2010). Urea is used because it disrupts the covalent bonds in proteins, which may increase the solubility of proteins. The molecular basis of protein unfolding by urea involves both direct and indirect mechanisms. Urea forms hydrogen bonds with the polar groups of the protein resulting in solvation of the hydrophobic core through firstly an influx of water molecules and then urea (Bennion and Daggett, 2003). The indirect mechanism involves alteration of water structure and dynamics, which reduces the hydrophobic effect allowing exposure of the residues at the hydrophobic core (Bennion and Daggett, 2003). However, loss of Hsp70 functional capacity has previously been described for proteins denatured with urea as a result of insufficient refolding; the protein's native state is not always achieved subsequent to denaturation (Kathir *et al.,* 2005). A non-denaturing, native protocol has been described for His-tagged Tcj2 and TcHsp70 using nickel-affinity purification (Louw *et al.,* 2010; Ludewig, 2009).

The aim of this study was to heterologously express and purify TbHsp70.c, TcHsp70B and Tbj2 from *E. coli* cells. A high yield and purity of each protein was required for subsequent *in vitro* assays for biochemical characterization. Very few trypanosomal Hsp70 molecular chaperone proteins have been characterised, *T. cruzi* Hsp70B is one of the best characterized (Olson *et al.,* 1994; Krautz *et al.,* 1998; Edkins *et al.,* 2004). TcHsp70B has been included in this study to enable comparative analysis with TbHsp70.c; TcHsp70B is an ideal control due to its cytoplasmic localization and its canonical Hsp70 features and functions.

The aims of the study included:

i. Amplification of the coding sequence of *TbHsp70.c*.

ii. Expression of TbHsp70.c, Tbj2 and TcHsp70B in an *E. coli* system.

iii. Optimization of the purification of TbHsp70.c, Tbj2 and TcHsp70B by nickel-affinity chromatography.

iv. Investigation of the oligomeric states of TbHsp70.c and Tbj2 using size exclusion chromatography.

3.2 Materials and methods

3.2.1 Materials

Reagents used were purchased from Sigma Chemicals Co. (St. Louis, Mo U.S.A.), Merck Chemicals (Darmstadt, Germany), BioRad (U.S.A.) or Roche Molecular Biochemicals (Indianapolis, IN, U.S.A.). The Expand High Fidelity PCR kit was purchased from Roche (Switzerland). Nickel beads were purchased from Pharmacia Biotech (Uppsala, Sweden). Anti-His antibody was purchased from Amersham Pharmacia Biotech (UK). Anti-TbHsp70.c was produced by and purchased from GenScript (U.S.A.). Plasmid TcHsp70B was a gift from Dr. D Engman (Northwestern University Medical School, Chicago, USA) (Table 3.1). The *T. brucei TREU927* strain was a kind donation from Professor George Cross (Rockefeller University, New York, USA). *E. coli* strain BB1994 was kindly provided by Dr. M Mayer (Heidelberg University, Heidelberg, Germany) and the plasmid pET28aTbj2 was kindly provided Dr. M.H. Ludewig (Rhodes University, Grahamstown, South Africa) (Table 3.1). All strains and plasmids used in this study are listed in Table 3.1.

Table 3.1: Description of strains and plasmids used in this study

Strains & plasmids	Description	Source/Reference
Strains		
E. coli XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F´ proAB lacIqZM15 Tn10* (Tetr)].	Bullock *et al.(1987)*
E. coli BL21 (DE3)	F- ompT gal [dcm] [lon] hsdSB λDEs	Studier *et al (1990)*
E. coli BB1994	MC4100 *dnaK52 sidB1::Tc pDMI,1::CmR KanR*	Dr M Mayer
T. brucei brucei SMB	SMB, *T7RNAP::TETR::NEO*	Wirtz *et al., 1999*
Plasmids		
pQE80TbHsp70.c	pQE80 encoding *T. brucei* Hsp70.c, Amp^R	This study [3.2.2.1]
pET28aTbj2	pET28a encoding *T. brucei* Tbj2, Kan^R	Dr MHL Ludewig
pET14bTcHsp70B	pET14b encoding *T. cruzi* Hsp70, Amp^R	Edkins *et al.*, 2004
p2T7tablue/TbHsp70.cRNAi	$p2T7^{TAblue}$ encoding TbHsp70.c as RNAi target	This study [5.2.2.1]

3.2.2 Methods

3.2.2.1 Amplification of *TbHsp70.c* sequence from genomic DNA

3.2.2.1.1 Primer design for PCR amplification of coding region of TbHsp70.c

To amplify the coding region of *TbHsp70.c*, primers were designed to allow polymerization chain reaction (PCR) amplification of the coding region from *T. brucei TREU927* genomic DNA. Primer design was based on the *TbHsp70.c* sequence obtained from the GeneDB database (Accession no. Tb11.01.3080) (www.genedb.org) and the restriction sites were selected so as to not digest the TbHsp70.c coding sequence internally. A forward primer (5' – GGT ACC ATG ACC TAC GAA GGA – 3') with a *Kpn*I restriction site (underlined), and a reverse primer (5' – GTC GAC TTA CTC TGT GTT TGC – 3') with a *Sal*I restriction site (underlined) were used. Primers were synthesized by Inqaba Biotechnical Industries (Pty) Ltd. The PCR reaction parameters were optimized (Stage 1: 96°C for 3 min; Stage 2: 30 cycles of 96°C for 30 s, 55°C for 2 min, and 72°C for 1 min; Stage 3: 1 cycle at 72°C for 10 min). The PCR reaction contained primers to a final concentration of 0.6 µM and 100 ng *T. brucei* genomic DNA.

3.2.2.1.2 Ligation of TbHsp70.c into cloning vector pGEM-T Easy

The *TbHsp70.c* coding region was subsequently ligated into pGEM-T Easy® to produce the pGEM-T-TbHsp70.c construct. A 10 µl ligation reaction included pGEM-T Easy vector (25 ng; 0.5 µl), PCR product (135 ng; 1 µl), 2x Rapid Ligation Buffer (2.5 µl; 60 mM Tris-HCl, pH 7.8; 20 mM MgCl2; 20 mM DTT, 2 mM ATP, 10 % (w/v) polyethylene glycol) and T4 DNA ligase (1.5 U) to initiate the reaction. The ligation reaction was incubated at 4°C overnight followed by transformation into competent *E. coli* JM109 cells. An aliquot of ligation reaction (2 µl) was incubated with competent cells at 4°C for 20 minutes. Cells were heat shocked at 42°C for 45 seconds, returned to 4°C for 2 minutes and incubated with agitation in 2 x YT broth (1.6 % (w/v) tryptone, 1.0 % (w/v) yeast, 0.5 % (w/v) NaCl) at 37°C for 1 hour. Cells harvested by centrifugation (12 000*g*; 1 minute) were plated onto MacConkey agar plates containing ampicillin (100 mg/L) and incubated overnight at 37°C. Colonies retaining the insert were selected based on their inability to utilize the lactose present, producing white colonies. Plasmid DNA was isolated using SMART Buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0, 15% $^{w}/_{v}$ sucrose, 100 mg/L BSA, 200 mg/L DNase free RNase, 2000 mg/L lysozyme). Selected colonies were incubated in 2 x YT broth (5 ml)

with ampicillin (100 mg/L) overnight at 37°C with agitation. An aliquot of cells (1 ml) were harvested (12 000*g*; 1 minute) and the pellet left to dry. After the addition of SMART buffer (50 µl), cells were incubated at 37°C for 30 minutes, boiled for 1 minute, cooled on ice for 10 minutes and centrifuged (12 000*g*; 10 minutes). The resulting supernatant (2 µl) containing the isolated plasmid DNA, was digested by *Eco*RI and analysed by agarose gel electrophoresis (0.8%) to confirm the presence of the insert and pGEM-T Easy vector. Agarose gel electrophoresis was performed as described in [3.2.2.1.4].

3.2.2.1.3 Transformation of TbHsp70.c into expression vector pQE80

Enzymes *Kpn*I and *Sal*I were used to restrict pGEM-T-TbHsp70.c and the insert was gel purified. Upon the addition of plasmid DNA (8 µl) to the restriction digest reaction containing the appropriate 10x NEB restriction buffer (4 µl) and sterile deionized water (26 µl), the reaction was initiated by the addition of enzymes *Kpn*I and *Sal*I and incubated at 37°C for 4 hours. The DNA fragments were resolved on an agarose gel (0.8%) [3.2.2.1.4]. DNA was recovered using the Zymoclean™ Gel DNA Recovery Kit, according to manufacturer's instructions. The resulting DNA was ligated into the pQE80-L expression vector restricted with *Kpn*I and *Sal*I to produce the pQE80TbHsp70.c construct. The ligation and transformation protocol was performed as described in [3.2.2.1.2], using *E. coli* XL1-Blue competent cells. Plasmid DNA was isolated using the peqGOLD Plasmid Miniprep Kit I (peqLAB), according to manufacturer's instructions. A plasmid map of the pQE80TbHsp70.c construct was generated using Vector NTI Advance™ software (Fig. 3.1A).

3.2.2.1.4 Verification of the pQE80TbHsp70.c plasmid by restriction analysis

Restriction analysis and DNA sequencing were employed to confirm the identity of the construct. Purified plasmid DNA (2 µl) was added to the appropriate 10x NEB restriction buffer and sterile deionized water (14 µl). Enzymes *Kpn*I (2 U) and *Sal*I (2 U) were added to initiate the reaction, followed by incubation at 37°C for 4 hours. The resulting digested fragments were resolved using an agarose gel (0.8 %) prepared using Tris-Acetate-EDTA (TAE) buffer (pH 8.0; 40 mM Tris-Acetate, 1 mM EDTA). Prior to casting the gel 20 µg/ml ethidium bromide was added. The restricted DNA samples were prepared for loading with the addition of loading dye (30% glycerol, 0.25% bromophenol blue). The gel electrophoresed for an hour at 100 volts and was viewed under UV light. Plasmid DNA isolated for the

purpose of sequencing was performed as in [3.2.2.1.3]. Plasmid DNA (300 ng) was sequenced by Inqaba Biotechnical Industries (Pty) Ltd. and the sequence of the coding region of TbHsp70.c was determined to correspond to the full-length sequence of TbHsp70.c found in GeneDB database (Accession no. Tb11.01.3080) (www.genedb.org).

3.2.2.1.5 Verification of plasmids pET28aTbj2 and pET14bTcHsp70B by restriction analysis

Restriction analysis of pET28aTbj2 and pET14bTcHsp70B was done to confirm the identity of the plasmids. The restriction digest of pET28aTbj2 and pET14bTcHsp70B was performed as described in [3.2.2.1.4] using *Xho*I and *Eco*RI, respectively. Agarose gel electrophoresis was used to analyse the digests and was carried out as described previously [3.2.2.1.4].

3.2.2.2 Purification of His-tagged TbHsp70.c, Tbj2 and TcHsp70B

3.2.2.2.1 Heterologous expression and purification of TbHsp70.c

An overnight culture was prepared by inoculating *E. coli* BB1994 [pQE80TbHsp70.c] in 25 ml 2 x YT broth supplemented with 100 mg/L ampicillin and 50 mg/L kanamycin at 30°C with shaking. The overnight culture was transferred to 225 ml 2 x YT broth and grown with shaking to mid-log phase (A_{600} 0.6) before inducing TbHsp70.c protein expression by the addition of isopropylthiogalactoside (IPTG) to a final concentration of 1 mM. Levels of protein expression were analysed by removing 1 ml samples pre- and post-induction, every hour for a duration of 5 hours. Samples were harvested (12 000*g*; 1 minute) and resuspended in a volume of phosphate buffered saline (PBS: 16 mM sodium phosphate, 150 mM NaCl, 4 mM potassium phosphate, pH 7.5) as determined by the absorbance reading where 150 µl PBS was added for each 0.5 OD_{600nm} absorbance unit:

$$\text{Volume SDS Sample buffer (µl)} = \frac{(OD_{600nm})}{0.5} \times 150\ \mu l$$

Protein production levels were analysed by SDS-PAGE (10%). SDS-PAGE was performed using the standard procedure (Laemmli, 1970). Samples were prepared for analysis by adding 5 x SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, glycerol, 10 % (w/v) SDS, 0.5 % (w/v) bromophenol blue, 5 % (w/v) β-mercaptoethanol) followed by boiling. A 10 % resolving gel and a 4 % stacking gel was prepared. To view the protein bands, the gels were stained with a Coomassie staining solution [50 % (v/v) methanol, 7.5 % (v/v) glacial acetic acid, 0.24 % (w/v) Coomassie Brilliant Blue] and destained with SDS-PAGE destain (20 %

(v/v) methanol, 7.5 % (v/v) glacial acetic acid). Both *E. coli* strains XL1-Blue and BB1994, the *dnaK* minus strain, were used to express and purify TbHsp70.c. Protein produced in *E. coli* XL1-Blue was grown at 37°C and protein produced in the temperature-sensitive strain BB1994 was grown at 30°C.

Purification of TbHsp70.c from *E. coli* BB1994 entailed harvesting cells 3 hours post IPTG induction. Cells were harvested (5000*g*; 15 min) and resuspended in lysis buffer (100 mM Tris-HCl, pH 8, 300 mM NaCl, 10 mM imidazole). Cells were stored at -80°C overnight and thawed with the addition of 1000 mg/L lysozyme and 1 mM PMSF culminating in lysis. N-lauroylsarcosine sodium salt (sarkosyl) to a final concentration of 7.5% (w/v) was added prior to sonication to enhance the solubility of partially soluble TbHsp70.c. Cellular debris was removed by centrifugation (12 000*g*; 40 min; 4°C) and the supernatant was incubated with nickel-charged Sepharose beads in lysis buffer overnight at 4°C. The bead-suspension was centrifuged (5000*g*; 1 min; 4°C) and three washes were performed with wash buffer (100 mM Tris-HCl, pH 8, 300 mM NaCl, 20 mM imidazole, 10 mM ATP). Bound protein was eluted with 500 mM imidazole. Purified protein was subsequently dialysed overnight [100 mM Tris-HCl, pH8, 100 mM NaCl, 5% (v/v) glycerol, 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM dithiothreitol]. TbHsp70.c purified protein was quantified using Bradford's assay (Bradford, 1976). SDS-PAGE analysis was used to evaluate the solubility and the purity of the purified protein. The protein was also purified in the absence of sarkosyl.

Western analysis was used to verify the production of TbHsp70.c using affinity-purified polyclonal anti-TbHsp70.c peptide antibody. Anti-TbHsp70.c was produced in rabbit using the C-terminal peptide, CQRGRGVTEGSGRPP corresponding to residues 648 – 662, from the TbHsp70.c protein sequence. Proteins separated by SDS-PAGE were transferred onto nitrocellulose in transfer buffer [25 mM Tris, 192 mM glycine, 20 % (v/v) MeOH] for 1 hour at 100 volts. Transferred proteins were visualized by staining the blot in Ponceau S stain [0.5 % (w/v) Ponceau S, 1 % (v/v) glacial acetic acid] and destaining with water. The membrane was washed with TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5). To block the unoccupied sites on the nitrocellulose from non-specific proteins, the membrane was soaked in a protein solution [5 % (w/v) non-fat dried milk in TBS] for 1 hour at 4°C. The membrane was incubated with anti-TbHsp70.c peptide antibody, diluted 1 : 5 000, in 5 % blocking solution overnight at 4ºC and washed twice at 4ºC for 20 minutes in TBSTween 20 [0.1 % (v/v)

Tween 20 in TBS], followed by incubation with goat anti-rabbit secondary antibody (Bio-Rad, USA), diluted 1 : 5 000, in 5 % blocking solution at 4°C. Following 4 x 15 minute washes in TBST, antibodies were detected using the ECL Advance Blotting Detection Kit and viewed using the Chemidoc^{TM} EQ (BioRad, U.S.A.).

3.2.2.2.2 Heterologous expression and purification of Tbj2 and TcHsp70B

Protein production was performed as described for TbHsp70.c [3.2.2.2.1]. *E. coli* BL21(DE3) cells were used for production of Tbj2 and TcHsp70B because pET vectors (T7 promoter) were used and selective pressure was maintained using kanamycin (50 mg/L) and ampicillin (100 mg/L), respectively. The levels of protein expression were assessed using SDS-PAGE as described previously [3.2.2.2.1].

Purification of Tbj2 and TcHsp70B was adapted from the protocol carried out for TbHsp70.c. Both *E. coli* BL21(DE3) [pET28aTbj2] and BL21(DE3) [pET14bTcHsp70B] cells were grown at 37°C and were harvested 5 hours post induction. To remove the presence of co-purified DnaK, Tbj2 and TcHsp70B bound to the Ni-NTA column were washed 5 times with the addition of 20% glycerol and 10 mM ATP to the wash buffer. Western analysis was performed using anti-His antibody to target Tbj2 and TcHsp70B, and anti-DnaK antibody to detect DnaK. As for TbHsp70.c, alterations to the purification protocol included using a starting culture of 1 L 2 x YT broth and purifying protein in the absence of sarkosyl. All other steps of the purification and analysis thereof were performed in the same manner as for TbHsp70.c.

3.2.2.3 Determination of the oligomeric states of TbHsp70.c and Tbj2

Size exclusion chromatography of TbHsp70.c and Tbj2 was performed using an ÄKTA Basic 10 FPLC instrument and a Superdex ^{TM} 200 HR 60/90 size-exclusion column (Amersham Pharmacia Biotech; USA) (60 mm x 90 cm; bed volume 120 ml). FPLC buffer (100 mM Tris-HCl, pH 8; 300 mM NaCl) was filtered, autoclaved and degassed prior to being passed through the column as the mobile phase at a flow rate of 1 ml/min. TbHsp70.c and Tbj2 were injected at a concentration of 0.2 mg/ml, and their presence was determined by the change in absorbance (280 nm). Comparison of the retention volumes of known standards with TbHsp70.c and Tbj2 were used to determine the molecular mass of the proteins. The selected

standards were blue dextran (1000 mg/L; kDa), thyroglobulin (2000 mg/L; 669 kDa), ferritin (240 mg/L; 450 kDa), catalase (2000 mg/L; 225 kDa); alcohol dehydrogenase (2000 mg/L; 150 kDa); bovine serum albumin (5000 mg/L; 66 kDa); ovalbumin (5000 mg/L; 45 kDa) and lysozyme (2000 mg/L; 14.4 kDa).

3.3 Results

3.3.1 Amplification of *TbHsp70.c* from *T. brucei* genomic DNA

Full-length *TbHsp70.c* was amplified from *T. brucei* genomic DNA and confirmation of the pQE80TbHsp70.c construct (Fig. 3.1A) was achieved by restriction digestion analysis (Fig. 3.1B). Digestion at the single *Kpn*I site linearized the plasmid of 6784 bp (Fig. 3.1B). Excision of the TbHsp70.c coding sequence (2031 bp) was achieved through double digestion using *Kpn*I and *Sal*I resulting in fragments of 4753 bp and 2031 bp, corresponding to the pQE80-L expression vector and TbHsp70.c insert sizes, respectively (Fig. 3.1B). The integrity of the construct was confirmed by DNA sequencing and no mutations were identified in the sequence. The insertion of Tbj2 into the pET28a expression vector (Fig. 3.1C) was verified by restriction digestion analysis. The Tbj2 coding sequence (1124 bp) and the pET28a vector (5369 bp) amounts to 6493 bp linearized DNA, confirmed by digestion at the single *Xho*I site (Fig. 3.1D). Similarly, verification of the pET14bTcHsp70B construct (Fig. 3.1E) was obtained by *Eco*RI digestion, linearizing the 6647 bp plasmid (Fig. 3.1F). The coding sequence was thus successfully isolated from genomic *T. brucei* DNA and inserted into an expression vector to generate plasmid pQE80TbHsp70.c.

3.3.2 Analysis of expression, solubility and purification of TbHsp70.c

The expression and purification of TbHsp70.c from *E. coli* XL1 Blue cells was successful, albeit at low levels (Fig. 3.2A). The protein proved soluble but low yields (200 mg/L) were obtained from the purification (Fig. 3.2A, lane 3). In an attempt to increase the protein yield, the 73 kDa protein was purified in the presence of sarkosyl and the resulting protein yield increased to 800 mg/L, confirmed by western analysis (Fig. 3.2A, lane 4). However, western analysis revealed the presence of contaminating *E. coli* DnaK which co-purified with TbHsp70.c (Fig. 3.2A). This problem was overcome by using an *E. coli dnaK*-minus strain to express and purify TbHsp70.c (Fig. 3.2B,C,D). Analysis of the expression levels of

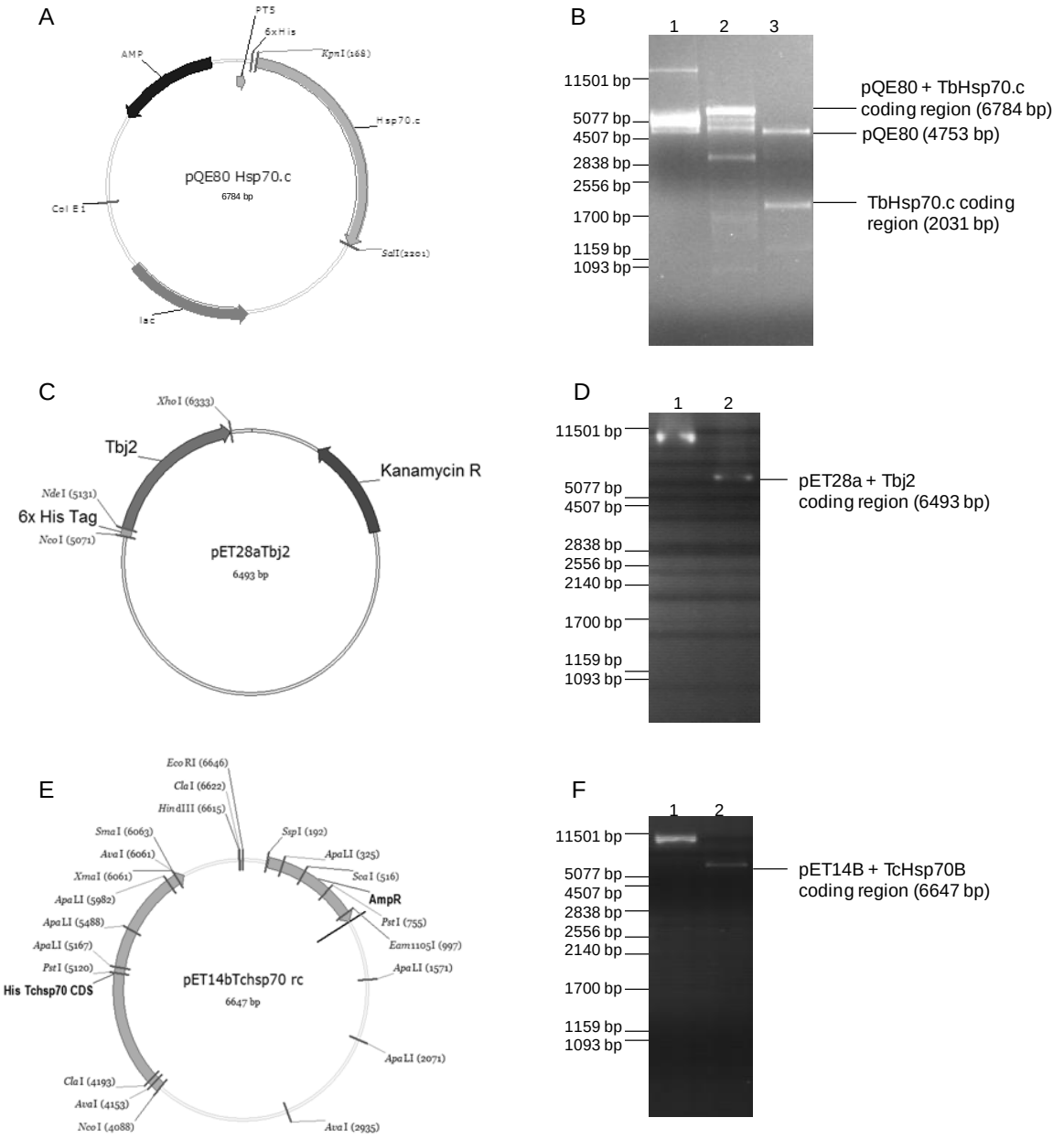


Figure 3.1. Diagnostic restriction analysis of plasmids pQE80TbHsp70.c, pET28aTbj2 and pET14bTcHsp70B.

TbHsp70.c was successfully amplified from *T. b. brucei* genomic DNA. (A) *TbHsp70.c* was inserted into pQE80-L using restriction enzymes *Kpn*I and *Sal*I to generate pQE80TbHsp70.c plasmid (6784 bp), with an N-terminal (His_6)-tag. Restriction analysis of TbHsp70.c (B) using *Kpn*I and *Sal*I verified the integrity of the construct using an agarose gel (0.8%). *Lanes 1* – undigested TbHsp70.c, *2* – single digest with *Kpn*I (6831 bp) linearizing the plasmid DNA, *3* – double digest *Kpn*I and *Sal*I (2031 bp, 4800 bp). (C) The pET28aTbj2 plasmid (6493 bp) contains the coding sequence for full-length *Tbj2* (inserted into pET28a with *Xho*I and *Nde*I) with an N-terminal (His_6)-tag. Restriction analysis of pET28aTbj2 (D) using *Xho*I verified the integrity of the construct using an agarose gel (0.8%). *Lanes 1* – undigested Tbj2, *2* – single digest with *Xho*I (6493 bp) linearizing the plasmid DNA. (E) pET14bTcHsp70B (6647 bp) encodes for (His_6)-tagged full-length *TcHsp70B* in expression vector pET14b. Restriction analysis of pET14bTcHsp70B (F) with restriction enzyme *Eco*RI allowed verification of the plasmid using an agarose gel (0.8%). *Lanes 1* – undigested TcHsp70B and *2* – single digest with *Eco*RI (6647 bp) linearizing the plasmid DNA. Plasmids (A, C, E) were generated using Vector NTI Advance™ software. In (B), (D) and (F), agarose gel analysis where numbers to the left of the gel indicate the *Pst*I restricted lambda DNA molecular marker.

TbHsp70.c produced in the *dnaK* minus strain revealed a similar level of protein production as was seen for TbHsp70.c produced in *E. coli* XL1-Blue cells (Fig. 3.2A, lane 2), and protein levels remained constant over the course of 5 hours post-induction (Fig. 3.2B, lanes 3-7). TbHsp70.c was not over-expressed in *E. coli* BB1994 cells (Fig. 3.2B). TbHsp70.c was also purified from *E. coli* BB1994 cells in the presence of sarkosyl to increase the protein yield. The addition of sarkosyl enhanced the solubility of the protein by approximately 30%, shown by western analysis (Fig. 3.2C, lanes 3,4,6,7). Subsequent nickel affinity chromatography of TbHsp70.c, treated with sarkosyl, resulted in a protein yield of 500 mg/L, lower than what was achieved from *E. coli* XL1-Blue cells, but of high purity (Fig. 3.2D, lane 10). The purification of TbHsp70.c, in the presence of sarkosyl, was up-scaled to further increase the protein yield (Fig. 3.2D, lanes 1-5). Even though an increased protein yield was obtained, SDS-PAGE analysis of the resulting eluted protein indicated the undesired co-purification of numerous contaminants as well as probable protein degradation, as confirmed by western analysis, lowering the protein purity (Fig. 3.2D, lane 5). Purification of TbHsp70.c from *E. coli* BB1994 cells in the presence of sarkosyl was of sufficient purity and quantity (average yields of 500 mg/L) for subsequent *in vitro* assays. The purification of protein from smaller aliquots resulted in greater purity.

3.3.3 Analysis of expression and purification of Tbj2 and TcHsp70B

Analysis of the expression levels of heterologously produced Tbj2 revealed the over-expression of Tbj2 (44 kDa) with maximal production achieved 5 hours post induction (Fig. 3.3A, lanes 3-7). A solubility study of Tbj2 revealed partial solubility and followed by nickel affinity chromatography resulted in a protein yield of 200 mg/L of high purity (Fig. 3.3B, lanes 3,4,8). The addition of sarkosyl enhanced the solubility of Tbj2 and subsequent purification yielded 1000 mg/L protein of (Fig. 3.3B, lanes 6,7,9). The identity of Tbj2 was confirmed by western analysis using anti-His antibodies (Fig 3B). Subsequent Tbj2 purifications were performed in the presence of sarkosyl. Lack of an *E. coli dnaK*-minus strain, compatible with the pET28a vector, gave rise to development of an alternative strategy to reduce the levels of endogenous DnaK co-purified with Tbj2 (Fig 3.3C, lanes 3,4). Modifications to the nickel affinity chromatography protocol included the addition of 20% glycerol and 10 mM ATP to the wash steps. The efficacy of the strategy was investigated through a comparison with Tbj2 purified in the absence of glycerol and ATP (Fig. 3.3C, lanes 5-8 and 1-4, respectively). Purified Tbj2, untreated, resulted in a protein yield of ~1000 mg/L

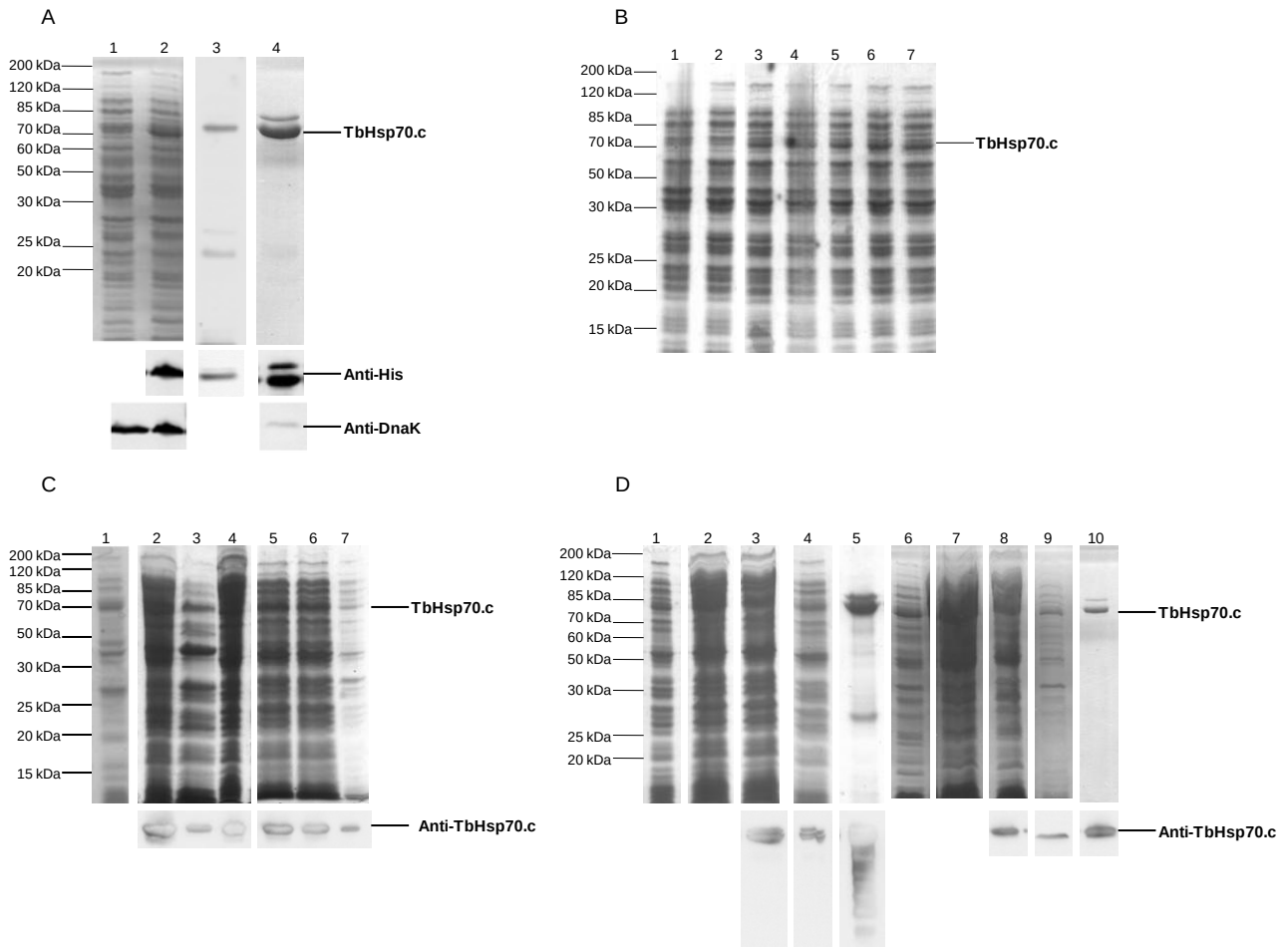


Figure 3.2. Heterologous production and purification of recombinant His-tagged TbHsp70.c.
(A) TbHsp70.c production and purification from *E. coli* XL1-Blue in the presence and absence of sarkosyl using SDS-PAGE (10%) analysis. *Upper panel: Lanes1* - uninduced cells transformed with [pQE80TbHsp70.c], *2* – transformed cells 3 hr post induction, *3* – eluted TbHsp70.c (73 kDa) purified using nickel-affinity purification (500 mM imidazole) in the absence of sarkosyl, *4* – TbHsp70.c elution (500 mM imidazole) in the presence of sarkosyl. *Second panel:* TbHsp70.c was detected at 73 kDa using anti-His antibody. *Third panel:* detection of co-purified DnaK at 70 kDa using anti-DnaK antibody. (B) SDS-PAGE (10%) analysis of protein production in *E. coli* BB1994 [pQE80TbHsp70.c]. *Lanes 1* – untransformed *E. coli* BB1994 cells, *2* – uninduced cells transformed with [pQE80TbHsp70.c], *3* – transformed cells 1 hr after IPTG induction, *4 – 7* – transformed cells 2 to 5 hr, respectively, after IPTG induction. (C) SDS-PAGE (10%) analysis of TbHsp70.c protein in the presence of sarkosyl. *Upper panel: Lanes 1 – E. coli* BB1994 [pQE80TbHsp70.c] whole lysates 3 hr post IPTG induction, *Untreated: 2 - E. coli* BB1994 [pQE80TbHsp70.c] post-sonication fraction, *3* – soluble supernatant fraction post centrifugation, *4* – insoluble pellet fraction, *5 - E. coli* BB1994 [pQE80TbHsp70.c] in the presence of 7.5% sarkosyl post-sonication fraction, *6* – sarkosyl-treated soluble supernatant fraction, *7* – sarkosyl-treated insoluble pellet fraction. *Lower panel:* TbHsp70.c detected at 73 kDa using anti-TbHsp70.c antibody. (D) SDS-PAGE (10%) analysis of a 1 x 250 ml and 4 x 250 ml purification of TbHsp70.c in the presence of sarkosyl. *Upper panel: Lanes 1 - E. coli* BB1994 [pQE80TbHsp70.c] whole lysates 3 hr post IPTG induction from a 4 x 250 ml culture, *2* - soluble supernatant fraction, *3* – fraction unbound to Ni-NTA column, *4* – fraction containing non-target protein removed by 20 mM imidazole and 10 mM ATP, *5* - TbHsp70.c elution (500 mM imidazole), *6* - *E. coli* BB1994 [pQE80TbHsp70.c] whole lysates 3 hr post IPTG induction from a 1 x 250 ml starting culture, *7* – soluble supernatant fraction, *8.* – fraction unbound to Ni-NTA column, *9* – fraction containing non-target protein removed by 20 mM imidazole and 10 mM ATP from the column, *10* - TbHsp70.c elution (500 mM imidazole). *Lower panel:* detection of TbHsp70.c at 73 kDa using anti-TbHsp70.c antibody. Numbers to the left of the panels indicate the protein marker ladder, the Peqlab peqGOLD Protein Marker II.

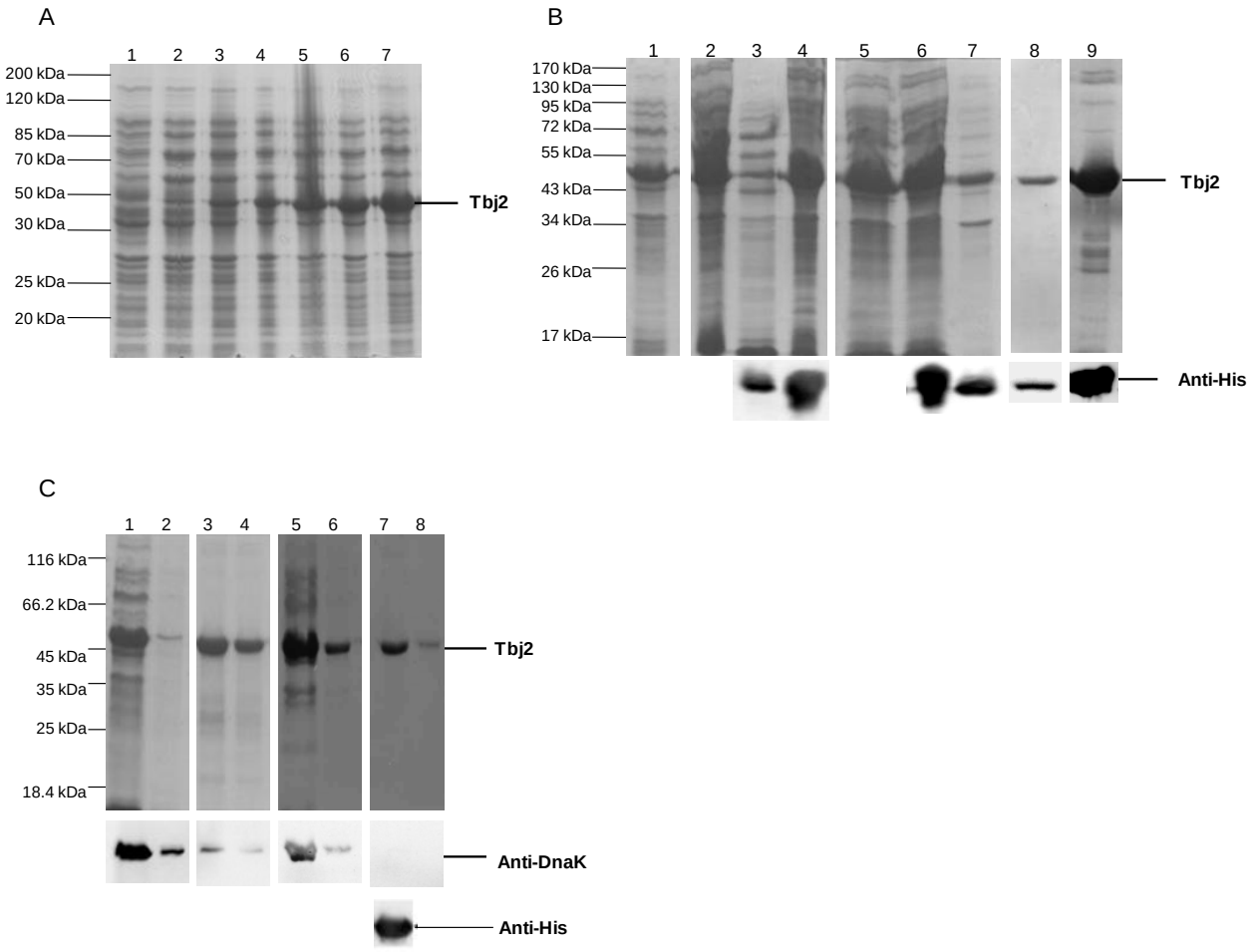


Figure 3.3. Heterologous production and purification of recombinant His-tagged Tbj2 and reduction of DnaK contamination via ATP and glycerol washes.
(A) SDS-PAGE (10%) analysis of optimal protein production of *E. coli* BL21(DE3) [pET28aTbj2]. *Lanes 1* – untransformed *E. coli* BL21(DE3) cells, *2* – uninduced cells transformed with [pET28aTbj2] (44 kDa), *3* – transformed cells 1 hour after IPTG induction, *4 – 7* – transformed cells 2 to 5 hours, respectively, after IPTG induction. (B) SDS-PAGE (10%) analysis of Tbj2 in the presence of sarkosyl. *Upper panel: Lanes 1 – E. coli* BL21(DE3) [pET28aTbj2] whole cell lysates 5 hours post IPTG induction, *2* - *E. coli* BL21(DE3) [pET28aTbj2] post-sonication fraction, *3* – soluble supernatant fraction post centrifugation, *4* – insoluble pellet fraction, *5* - *E. coli* BL21(DE3) [pET28aTbj2] in the presence of sarkosyl post-sonication fraction, *6* – supernatant fraction in the presence of sarkosyl post centrifugation, *7* – sarkosyl-treated insoluble pellet fraction, *8* – Tbj2 eluted from a Ni-NTA column (500 mM imidazole) in the absence of sarkosyl, *9* – Tbj2 elution (500 mM imidazole) in the presence of sarkosyl. *Lower panel:* Tbj2 detected at 44 kDa using anti-His antibody. (C) SDS-PAGE (10%) analysis of the removal of contaminating DnaK by glycerol and ATP, in the presence of sarkosyl. *Upper panel: Lanes 1 –E. coli* BL21(DE3) [pET28aTbj2] soluble supernatant fraction, *2* – fraction from first wash of 5 containing 20 mM imidazole, *3 and 4* - Tbj2 first and second elutions (500 mM imidazole); *5* - fraction unbound to Ni-NTA column, *6* - fraction from first wash of 5 containing 20 mM imidazole, 20% glycerol and 10 mM ATP, *7 and 8* - Tbj2 first (400 mg/L) and second (100 mg/L) elutions (500 mM imidazole). *Second panel:* detection of co-purified DnaK at 70 kDa by western analysis using anti-DnaK antibody showing the removal of contaminating DnaK using glycerol and ATP washes. *Third panel:* Tbj2 detected at 44 kDa by western analysis using anti-His antibody. Numbers to the left of the SDS–PAGE panels (A) and (B) indicate the subunit molecular mass of the Peqlab peqGOLD Protein Marker II, numbers to left of (C) correspond to the Fermentas Pierce Unstained Protein MW Marker.

and western analysis confirmed the identity of undesired co-purified DnaK (Fig. 3.3C, lanes 1-4). Purified Tbj2 washed with glycerol and ATP, in comparison, yielded 600 mg/L purified Tbj2 and removal of contaminating DnaK, the identity of both Tbj2 and DnaK was confirmed by western analysis, using anti-His and anti-DnaK antibodies, respectively (Fig. 3.3C, lanes 5-8). Purification of Tbj2 thus included treatment with glycerol and ATP washes. Purifications of Tbj2 typically resulted in a final protein yield of approximately 600 mg/L (Fig. 3.3); Tbj2 was used in subsequent *in vitro* assays. Study of the expression of TcHsp70B produced in *E. coli* BL21(DE3) showed very low protein levels over the course of 5 hours, confirmed by western analysis (Fig 3.4A). As with Tbj2, an *E. coli dnaK*-minus strain compatible with pET14b was not available and the same purification protocol employed for Tbj2 was utilized for TcHsp70B, with successful removal of contaminating DnaK (Fig. 3.4B). The typical protein yield of TcHsp70B was ~650 mg/L (Fig. 3.4B, lane 3).

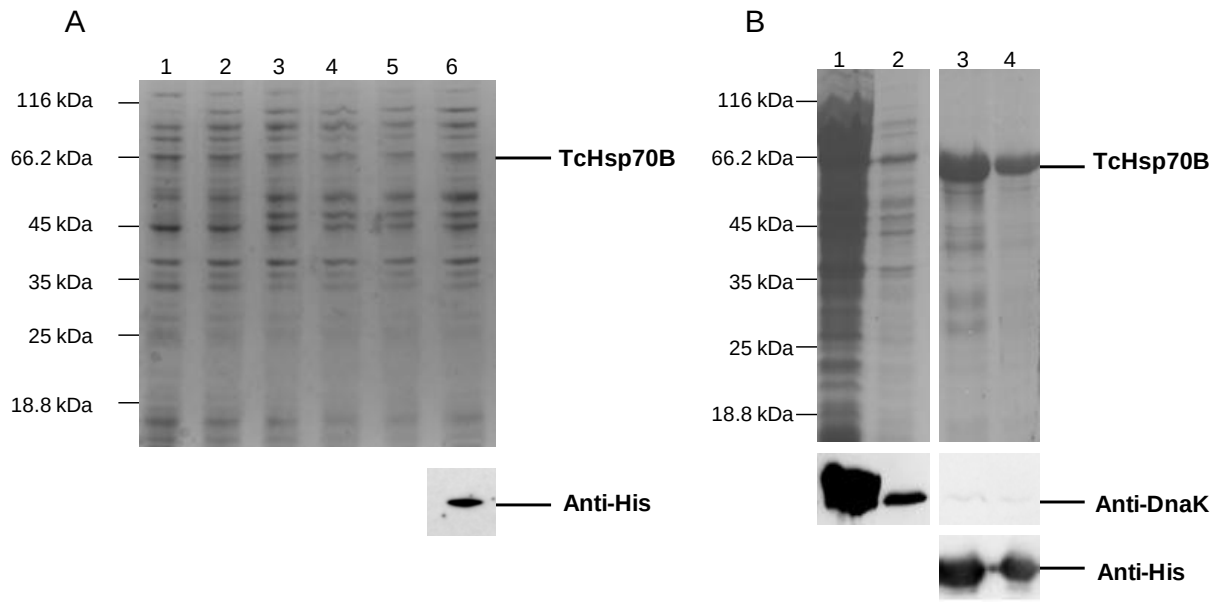


Figure 3.4. Heterologous production and purification of recombinant His-tagged TcHsp70B.
(A) Study of optimal protein production of *E. coli* BL21(DE3) [pET14bTcHsp70B] through SDS-PAGE (10%) analysis. *Lanes 1* – uninduced cells transformed with [pET14bTcHsp70B] (73 kDa), *2* – transformed cells 1 hour after IPTG induction, *3 – 6* – transformed cells 2 to 5 hours, respectively, after IPTG induction. *Second panel:* TcHsp70B detected at 70 kDa by western analysis using anti-His antibody. (B) Analysis of successful denaturing Nickel affinity chromatography purification of TcHsp70B from *E. coli* BL21(DE3) in the presence of sarkosyl and reduction of co-purified DnaK using SDS-PAGE (10%) analysis. *Lanes 1* - fraction unbound to Ni-NTA column from a 1 x 250 ml starting culture, *2* - fraction from first wash of 5 containing non-target protein removed from the column by 20 mM imidazole, 20% glycerol and 10 mM ATP, *3 and 4* – TcHsp70B first and second elutions (500 mM imidazole), 690 mg/L and 450 mg/L, respectively. *Second panel:* detection of co-purified DnaK at 70 kDa by western analysis using anti-DnaK antibody showing the removal of contaminating DnaK using glycerol and ATP washes. *Third panel:* TcHsp70B detected at 70 kDa by western analysis using anti-His antibody. Numbers to the left of the SDS–PAGE panels (A) and (B) indicate the subunit molecular mass of the corresponding protein marker ladder, the Fermentas Pierce Unstained Protein MW Marker.

Western analysis was used to verify the identity of both TcHsp70B and DnaK, using anti-His and anti-DnaK antibodies, respectively (Fig. 3.4B).

3.3.4 Size exclusion chromatography of TbHsp70.c and Tbj2

Size exclusion chromatography (SEC) was performed to determine the oligomeric states of purified TbHsp70.c and Tbj2 as part of their biochemical characterization. TbHsp70.c was found to be monomeric in an extended conformation with higher order oligomers, eluting in two peaks, a minor species corresponding to an apparent molecular mass of 141.57 kDa and a major species with a molecular mass greater than 450 kDa (V_0). Fractions were collected at the void (V_0) and elution volumes (67.29 ml) and the presence of TbHsp70.c was confirmed by western analysis using rabbit anti-TbHsp70.c peptide antibodies (Fig. 3.6B). The addition of ATP to TbHsp70.c did not result in a change of the elution volumes of the two peaks, confirming the TbHsp70.c as a monomer (Fig. 3.7). Two Tbj2 species were detected, the major species corresponding to molecular masses greater than 450 kDa (V_0), representing higher order Tbj2 oligomers (Fig. 3.6C). The minor species correspond to 165.9 kDa and is likely monomeric Tbj2 co-purified with monomeric *E. coli* DnaK in an extended conformation (Fig. 3.6C). Fractions were collected at the void (V_0) and elution volumes (64.28 ml) and the presence of Tbj2 and DnaK was confirmed by western analysis using anti-His and anti-DnaK antibodies (Fig. 3.6B).

3.4 Discussion and conclusions

This is the first report of the successful isolation of the coding region of full-length TbHsp70.c, and the expression and purification of TbHsp70.c from a prokaryotic system. The reproducible native purification protocol was optimized such that contaminating proteins were removed and ideal quantities and purities of TbHsp70.c and Tbj2 were obtained for subsequent *in vitro* biochemical characterisation. TcHsp70B was purified to be utilized in *in vitro* studies as a positive control of a canonical Hsp70 protein. The alkyl anionic detergent sarkosyl has been described to solubilize proteins expressed in bacteria by not only disrupting the self-aggregation of target protein folding intermediates, or preventing aggregation altogether if present in the cell lysis process (Frankel *et al.*, 1991) but disrupts aggregation involving cellular debris such as RNA, liposaccharides and outer membrane proteins (Osborn and Wu, 1980; Trabbic-Carlson *et al*., 2004). In this study TbHsp70.c was expected to be

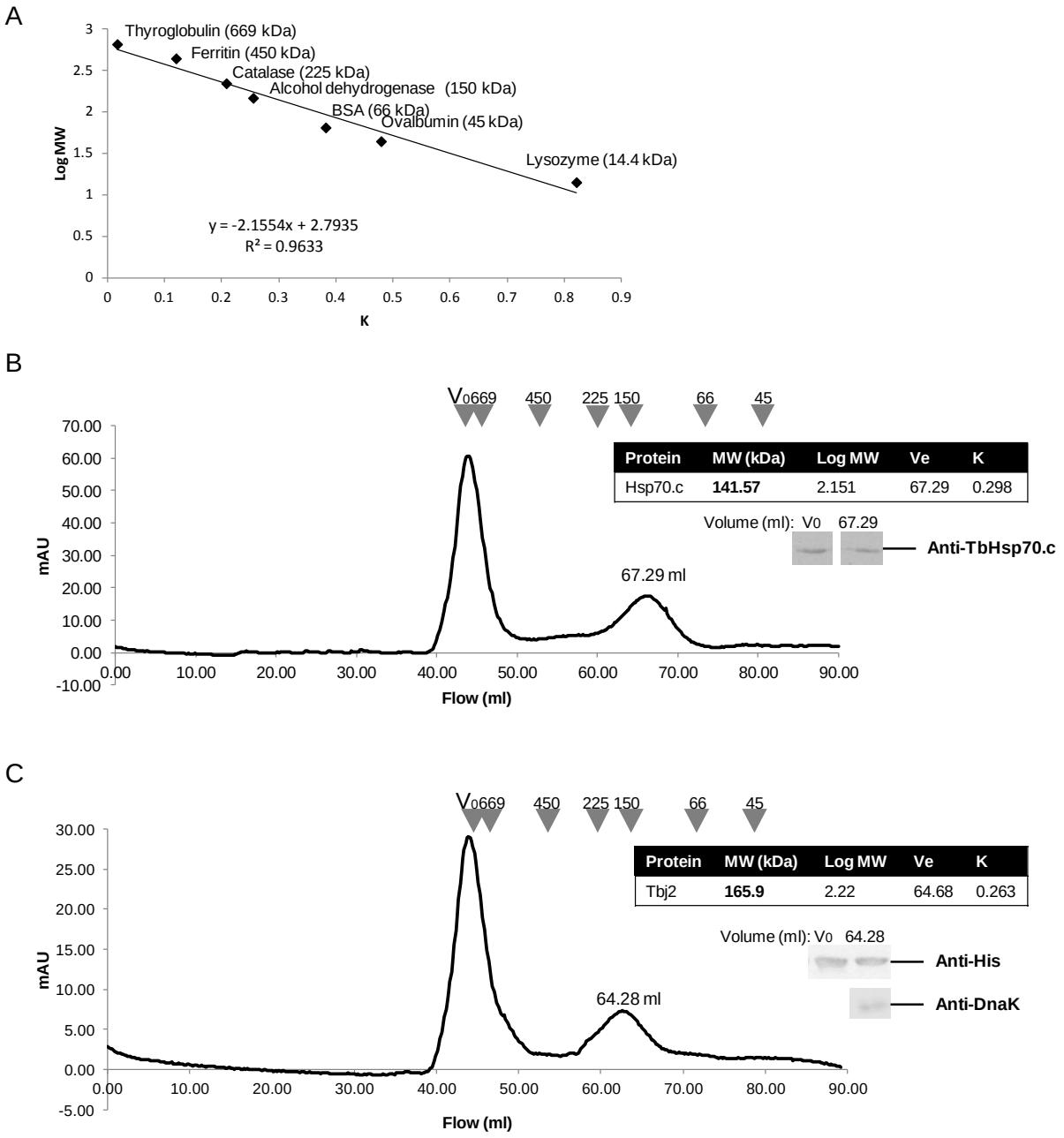


Figure 3.6. Analysis of the oligomeric states of TbHsp70.c and Tbj2 by size exclusion chromatography. (A) Calibration curve of standards thyroglobulin (669 kDa), ferritin (450 kDa), catalase (225 kDa); alcohol dehydrogenase (150 kDa); bovine serum albumin (66 kDa); ovalbumin (45 kDa) and lysozyme (14.4 kDa). The regression line fits the data well, indicated by a coefficient of determination of 0.9633. TbHsp70.c exists as a monomer in an extended conformation in solution (B) and Tbj2 as a dimer in solution (C). (B) TbHsp70.c (300 mg/L) and (C) Tbj2 (300 mg/L) were loaded separately on the column and eluted. Fractions collected from gel filtration were resolved by SDS-PAGE and TbHsp70.c and Tbj2 were identified through western analysis. (B) TbHsp70.c eluted as oligomerized protein (V0) and a monomeric species in an extended conformation (141.57 kDa), as confirmed by western analysis using anti-TbHsp70.c. (C) Tbj2 eluted as oligomerized protein (V0) and a dimeric species (165.9 kDa), confirmed by western analysis using anti-His antibodies. Co-purified DnaK eluted with Tbj2, confirmed by western analysis using anti-DnaK antibodies.

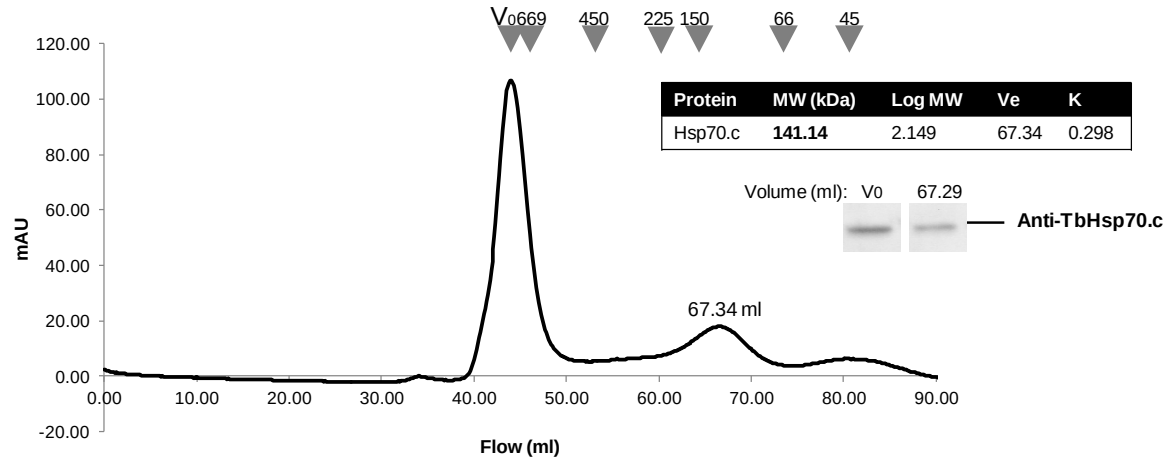


Figure 3.7. Analysis of the oligomeric state of TbHsp70.c and ATP by size exclusion chromatography. TbHsp70.c exists as a monomer in an extended conformation in solution. (B) TbHsp70.c (350 mg/L) and ATP (20 mM) was loaded on the column and eluted. Fractions collected from gel filtration were resolved by SDS-PAGE and TbHsp70.c was identified through western analysis. TbHsp70.c eluted as oligomerized protein (V0) and a monomeric species in an extended conformation (141.14 kDa), as confirmed by western analysis using anti-TbHsp70.c.

soluble, and sarkosyl was used to disrupt protein aggregation involving cellular debris to allow an increased protein yield. The use of sarkosyl did not, however, increase the purity of the elutions. Reports have shown that sarkosyl does not interfere with protein function (Park *et al.,* 2011). A frequent occurrence in the purification of any heterologously produced Hsp70 and Hsp40 in *E. coli* cells is the co-purification of the contaminant DnaK, the bacterial orthologue of Hsp70. DnaK not only binds molecular chaperones but is involved in the maintenance of global protein homeostasis; quantitative proteomics showed ~700 DnaK interactants, predominantly cytoplasmic (Calloni *et al.,* 2012). The presence of DnaK is a major concern due to the possibility that basal expression of bacterial DnaK or co-purified DnaK could mask the chaperone activity of the *T. brucei* heat shock proteins being purified. The co-purification of DnaK is likely as a result of denatured exposed surfaces of the target protein interacting with DnaK by acting as a substrate to the protein. The DnaK contamination levels were greater when purifying Hsp40 than what was observed for the Hsp70 purifications; a similar trend was observed in Louw *et al.* (2010). *E. coli dnaK* minus strain was used to purify recombinant TbHsp70.c, thereby eliminating DnaK (Boshoff *et al.,* 2008). Following protein production, the purification protocol was optimized to obtain a high concentration of pure protein, required for subsequent *in vitro* assays, by the removal of non-specifically bound protein through ATP wash steps. Tbj2 was readily over-expressed after

induction with IPTG. The presence of co-purified DnaK warranted optimization of the purification protocol which involved glycerol (Guo *et al.,* 2007) and ATP washes to reduce contaminating DnaK to insignificant levels. It is probable that not all contaminating DnaK was removed from the Tbj2 purification due to limitations of the western analysis detection. An alternative strategy would be to employ the use of a *dnaK* minus strain compatible with the pET expression vectors.

The oligomerization of TbHsp70.c and Tbj2 were analysed using size exclusion chromatography. Hsp70 proteins typically oligomerize into high molecular mass forms (Kim *et al.,* 1992; Benaroudj *et al.,* 1995; Schönfeld *et al.,* 1995). The balance between different Hsp70 oligomeric forms is dependent on the presence of ATP; ATP binds monomers and ADP binds higher-order oligomers (Kim *et al.,* 1992). TbHsp70.c was shown to be monomeric in an extended conformation and form higher order oligomeric species. The experimentally calculated molecular mass of monomeric TbHsp70.c (141.57 kDa) correlated with what has been reported in literature. *A. tumefaciens* DnaK (Boshoff *et al.,* 2008) and *E. coli* DnaK (Schönfeld *et al.,* 1995), eluting at molecular masses of 140 kDa and 130 kDa, respectively, were both concluded to be monomeric in an extended conformation. The oligomerization of TbHsp70c is not unexpected; it corresponds to its homologue *Agrobacterium tumefaciens* DnaK, which forms higher order associations (Boshoff *et al.,* 2008). Hsc70 also forms multiple species ranging from monomeric up to trimeric forms (Benaroudj *et al.,* 1995). A large number of Hsp40s dimerize in solution, particularly Type I and Type II Hsp40s (Langer *et al.,* 1992; Borges *et al.,* 2005; Shi *et al.,* 2005). They typically have a dimerization domain present at the C-terminus which facilitates their chaperone activity (Langer *et al.,* 1992; Borges *et al.,* 2005; Shi *et al.,* 2005). Dimerization of Type I Hsp40 proteins occurs via interaction of the N- and C-termini of each monomer in mirrored positions, forming a bullet shaped molecule (Borges *et al.,* 2005). Disruption of the motifs required for dimerization has a critical impact on the ability of Hsp40 to function as a chaperone (Sha *et al.,* 2000; Li *et al.,* 2003). The C-terminal dimerization motif of Ydj1 involves a cluster of hydrophobic residues (336 – 378) and disruption of F335D resulted in monomeric Hsp40 (Wu *et al.,* 2005). Tbj2 appeared to form a monomeric species. Unfortunately, even though the purification of Tbj2 had been optimized for the removal of contaminating DnaK, residual levels of co-purified monomeric *E. coli* DnaK, in a likely extended conformation, eluted with monomeric Tbj2. *E. coli* DnaK has previously been

demonstrated to elute as a monomer in an extended conformation with a molecular mass of 130 kDa (Zylicz and Georgopoulos, 1984; Schönfeld *et al.,* 1995). The standard proteins used to produce the log of the standards' molecular masses were all globular proteins. It is possible that the SEC molecular mass estimation for Tbj2 is not accurate as Type I Hsp40 proteins have an elongated shape. Elongated protein standards should thus have been used to more accurately determine the oligomeric state of Tbj2, such as $TNfn_{1-5}$ (50.4 kDa), PR65/A HEAT repeat (60 kDa), $TNfn_{1-8}$ (78.9 kDa), TNfnALL (148 kDa) and Fibrinogen (390 kDa) (Erickson, 2009). The addition of ATP may have provided clarity as to the oligomeric state of Tbj2, in which case Tbj2 and DnaK may have eluted as individual peaks. As Type I Hsp40 proteins do not have ATPase activity, Tbj2 runs with ATP would only serve to remove any residual DnaK not already removed during the ATP and glycerol washes in the purification of Tbj2, and likely not other bound client proteins. Further experimental studies will involve purifying Tbj2 from a *dnaK* minus strain to prevent co-purification of contaminants and using elongated protein standards to determine the oligomeric state of Tbj2. In addition, mutagenesis of the hydrophobic cleft of Tbj2 associated with binding substrate would eliminate its chaperone function and serve as a negative control.

A pairwise alignment of Ydj1 with Tbj2 revealed the conservation of Ydj1 hydrophobic residues L349, I352, L353 and P336 (data not shown) in the Tbj2 amino acid sequence. It is therefore expected that Tbj2, a Type I Hsp40 protein, would dimerize due to the high conservation of the dimerization motif amongst Type I Hsp40 proteins and increases the potential of Tbj2 to act as a cochaperone of TbHsp70.c, as the Hsp40 dimer holds the substrate in an elongated form between its two arms in such a manner that Hsp70 can bind the substrate (Li *et al.,* 2006).

The optimization of heterologous expression of TbHsp70.c and Tbj2 followed by native purification yielded protein of a quality ideal for ensuing *in vitro* protein characterisation assays.

CHAPTER 4

IN VITRO ASSAYS:

Biochemical characterization of TbHsp70.c and Tbj2

4.1. Introduction

A functional interaction between *T. brucei* Hsp70 and Hsp40 proteins has not previously been demonstrated experimentally. These studies set out to biochemically characterize a novel protein, TbHsp70.c, and to investigate the relationship between it and potential co-chaperone, Tbj2, through the use of *in vitro* assays. Knowledge gained through *in vitro* analyses can provide insight into the mechanisms involved in protein functioning and protein-protein interactions. Further *in vivo* analyses would contribute to a more complete characterization. Chaperone function of an Hsp70 protein can be demonstrated through its ability to hydrolyse ATP, regulated by a partner co-chaperone (Mayer *et al.,* 2000; Boshoff *et al.,* 2008) and can be gauged by its interaction with peptide substrates (Schlieker *et al.,* 2002; Nicoll *et al.,* 2006). Insight into the partnerships that TbHsp70.c may form with co-chaperones could provide knowledge of the network that promotes protein homeostasis in the cell.

J-proteins are defined by the presence of the very well conserved J-domain in Hsp40 proteins that interact with the ATPase domain of Hsp70 in an ATP dependent manner (Bukau and Horwich, 1998). J-domain definitive features include four helices, of which the second has a charged surface including a pair of basic residues that are important for association with the Hsp70 ATPase domain (Genevaux *et al.,* 2002). Furthermore, Helix II contains residues that show backbone amide-proton chemical shifts when Hsp70 is in proximity (Greene *et al.,* 1998). The conserved HPD motif is present in the turn between Helix II and III, important for the association with Hsp70 (Greene *et al.,* 1998). J-protein Tbj2 has been demonstrated to localize in the cytoplasm (Ludewig, 2012), making it an ideal co-chaperone as TbHsp70.c has been predicted to be cytoplasmic (Louw *et al.,* 2010). Tbj2 belongs to the Type I class of Hsp40s, which bind substrates and targets them to Hsp70 (Cyr *et al.,* 1994; Bukau and Horwich, 1998; Johnson and Craig, 2001); and has been determined to be essential to the survival of the parasite (Ludewig, 2010). Competitive binding for the ATPase domain of Hsp70 has been demonstrated by co-chaperone heat shock interacting protein (Hip) with nucleotide exchange factor, Bag-1; Hip stabilizes the substrate-targeted-to-Hsp70-by-Hsp40 complex, Bag-1 induces the release of bound substrate (Section 1.3.3) (Höhfeld *et al*., 1995; Höhfeld and Jentsh, 1997). Both Hsp70 cofactors Hip and BAG-1 cooperate with Hsp40 in facilitating Hsp70 function; however, the BAG-1 and Hip are Hsp70-binding competitors which suggest an alternative strategy of Hsp70 regulation (Höhfeld and Jentsch, 1997; Demand *et al.,* 1998).

The well conserved C-terminal EEVD motif, typically present in the cytoplasmic members of the Hsp70 proteins, is required for the association of Hsp70 and Hsp90 through a tetratricopeptide repeat (TPR) domain present in co-chaperone Hop (Section 1.3.1) (Freeman *et al*., 1995; Scheufler *et al.,* 2000). Along with the Hsp70 SBD binding site, Type II Hsp40, Hdj-1, was also demonstrated to interact with the very well conserved EEVD motif, resulting in decreased/loss of function in its absence; Hop and Hsp40 interact noncompetitively with this motif (Demand *et al.,* 1998). Interestingly, Hsp40 demonstrated the ability to still stimulate the ATPase activity of Hsp70 in the absence of the EEVD motif, but to a lesser degree (Demand *et al.,* 1998). The EEVD motif is absent from TbHsp70.c; a functional interaction with probable co-chaperone Tbj2 may still be observed, however, should TbHsp70.c form a functional complex with TbHsp90, a novel mechanism of interaction with structural component TbHop (Accession no. XP 827315.1; Johnson and Brown, 2009) would be an interesting finding.

Hsp70 proteins function both as a holdase, binding and holding onto unfolded polypeptides by withdrawing aggregation-prone proteins, as well as a refoldase, assisting non-native proteins to fold to the native state (Minami *et al.,* 1996; Slepenkov and Witt, 2002). Their ability to prevent aggregation has been exploited in a well-optimized *in vitro* assay. The assay investigates the ability of a chaperone to prevent misfolding of an aggregation prone model thermolabile substrate, malate dehydrogenase (MDH), through suppression of aggregation of MDH (Goloubinoff *et al*., 1999; Basha *et al*., 2004). Model substrates also include rhodanese, denatured by the addition of guanidium-HCl (Allan *et al.,* 2006), reduced carboxymethylated lactalbumin (RCMLA) (Ramya *et al.,* 2006), lactate dehydrogenase and malic dehydrogenase (Manna *et al.,* 2001). Both Hsp70 proteins and Type I Hsp40 proteins can act as chaperones in their own right in their ability to suppress MDH aggregation independently of each other, as has been demonstrated for *Agrobacterium tumefaciens* DnaK and DnaJ, *P. falciparum* Hsp70 and human DNAJB (Borges *et al.*, 2005; Boshoff *et al.,* 2008; Shonhai *et al.,* 2008; Hageman *et al.,* 2010). Some molecular chaperones show substrate discrimination, and do not possess the ability to suppress aggregation of all substrates (Schlieker *et al.,* 2002). *E. coli* IbpA and IbpB are holder chaperones that specifically bind misfolded proteins to prevent their aggregation; *E. coli* DnaK and GroES are folder chaperones that bind unfolded proteins to facilitate ATP-dependent refolding (Schlieker *et al.,* 2002). Co-chaperone of the endoplasmic reticulum Hsp70, BiP, shows discrimination between peptide and protein substrates

(Marcinowski *et al.,* 2011). *In silico* analysis of TbHsp70.c revealed acidic residues in the substrate binding domain and cavity (Fig. 2.1, 2.4); these acidic residues, in conjunction with the ATPase domain that interacts with substrates in the ATP-regulated cycle, may play a role in substrate discrimination in the cell.

The intrinsic ability of Hsp70 proteins to hydrolyse ATP is regulated by Hsp40 co-chaperones (McCarty *et al.,* 1995); independently Hsp70s display weak basal ATPase activity and so are largely reliant on their co-chaperones to stimulate their ATPase activity (Hennessy *et al*., 2000; Gässler *et al*., 2001). This stimulation can be used to detect an *in vitro* functional interaction between Hsp70 and Hsp40. Colorimetric assays have been developed to determine ATPase activity through the detection of inorganic phosphate, released when ATP is cleaved (Chifflet *et al*., 1988; Lanzetta *et al*., 1979). ATP-bound Hsp70 displays an open state of the substrate binding domain in which unfolded substrates are rapidly associated and dissociated (Section 1.3.3). Hydrolysis of ATP results in closing of the substrate binding domain and a subsequent reduced affinity for unfolded substrates (Schmid *et al.,* 1994; Fan *et al.,* 2003). The ATPase activity of *T. cruzi* Hsp70 (TcHsp70) has been demonstrated to be stimulated by a *T. cruzi* Hsp40 Tcj2 (Edkins *et al.,* 2004); a *T. brucei* Hsp40, Tbj1 (Louw *et al.,* 2010), and a *T. cruzi* Hsp40, Tcj1 (Edkins *et al.,* 2004), both Type III Hsp40s, were unable to stimulate the ATPase activity of TcHsp70. None of the 12 Hsp70 proteins of *T. brucei* (Table 2.4), to date, have been characterized in terms of their *in vitro* chaperone activities.

The importance of Hsp70 as a critical chaperone involved in cell survival, signalling and protein homeostasis has drawn attention to it as an emerging drug target (reviewed in Evans *et al.,* 2010). The effect of various small molecule modulators on the activity of molecular chaperones has been assessed with the aim of developing prospective drug targets (Brodsky and Bracher, 2006; Wright *et al.,* 2008; Chang *et al.,* 2011). Hsp70 has been revealed to interact with different classes of small molecules, including spergualin-like compounds, dihydropyrimidines, fatty acids, peptides, ATP mimics, thiophene-2-carboxamides, phenylthynesulfonamide and MKT-077 (reviewed in Evans *et al.,* 2010). The focus on Hsp70 as a potential drug target has intensified; Hsp70 and its co-chaperones have been shown to be associated with cancer and neurodegeneration (Mosser *et al.,* 2004; Brodsky and Chiosis, 2006; Patury *et al.,* 2009). Due to a lack of new treatments against Human African

Trypanosomiasis and emergence of resistance to older drugs, human treatments currently available are connected with high levels of toxicity and resistance (Delespaux and de Koning, 2007). Drugs causing cellular stress resulting in the induction of heat shock proteins have been discovered to ultimately improve cytoprotection (Bhagat *et al.,* 2008). Chemical stress induced by the administration of sodium arsenite was shown to result in the upregulation of Hsp70 in the pancreas which in turn alleviated the severity of acute pancreatitis (Bhagat *et al.,* 2008). The antihepatitis drug, bicyclol, was demonstrated to upregulate both Hsp27 and Hsp70 resulting in the prevention of liver tissue apoptosis and necrosis through inhibition of the nuclear factor NF-κB (Bao and Liu, 2009). Anti-heat shock protein drugs used in combination with current drugs could therefore synergistically improve the effectiveness of available drugs.

Parasitic heat shock proteins have been revealed as drug targets, including malarial drug target *P. falciparum* Hsp90 and trypanosomal *T. evansi* Hsp90 (Banumathy *et al.,* 2003; Pallavi *et al.,* 2010). Amongst the known Hsp90 proteins, PfHsp90 was shown to have the highest ATPase activity, and its inhibition by geldanamycin (GA) was stronger than seen for human Hsp90 (Pallavi *et al.,* 2010). Semi-synthetic inhibitor 17-(allylamino)-17-demethoxygeldanamycin (17-AAG) has been shown to curb growth of the *P. falciparum* parasite and to inhibit the growth of the *T. evansi* parasite by specifically binding and inhibiting Hsp90 (Pallavi *et al.,* 2010). 17-AAG could likely be effective against infection caused by *T. brucei* due to the sequence similarity between TbHsp90 and TeHsp90 (Pallavi *et al.,* 2010). Sera from patients infected with trypanosomes were screened to identify diagnostic antigens, and although TbHsp70 was identified as a candidate, by itself it demonstrated inadequate specificity and sensitivity in diagnosis of trypanosomiasis (Manful *et al.,* 2010). It may however be useful as a diagnostic if used in conjunction with immunogenic proteins (Manful *et al.,* 2010). Research involving *T. cruzi* similarly found the necessity for several antigens in order to function as a diagnostic tool (Cooley *et al.,* 2008). Heat shock proteins have been associated with increased cytoprotection due to knowledge of their upregulation upon administration of anti-malarial drugs and combining anti-heat shock protein drugs with drugs currently available may improve the potency of available drugs (Akide-Ndunge *et al.,* 2009).

Small molecule methylene blue (MB) has been identified as an inhibitor of Hsp70 ATPase activity (Jinwal *et al.,* 2009), and has multiple cellular and molecular targets, such as ion channels and enzymes (reviewed in Oz *et al.,* 2009). The natural flavonoid quercetin (B) has previously been demonstrated to inhibit the biosynthesis of Hsp70 by blocking Hsp70 gene expression (Elia *et al.,* 1994; Hu *et al.,* 2003). *In vivo* analysis has shown small molecule quercetin to effectively inhibit Hsp70 expression at 30 μM in erythroleukaemia K562 cells (Elia *et al.,* 1994), 50 μM in ovarian cancer 2774 cells (Hu *et al.,* 2003) and 100 μM in HeLa and MDA-MB-231 cell lines (Hansen *et al.,* 1997). Quercetin is also an ATPase inhibitor; it has been shown to inhibit hog gastric K(+)-ATPase (Murakami *et al.,* 1992), mitochondrial ATPase (Lang and Racker, 1974) and the interaction between ATP and quercetin was shown by Chávez and Cuéllar (1980).

The broad objectives of this study were to investigate the chaperone activity of TbHsp70.c and Tbj2 using biochemical assays, and to assess the ability of Tbj2 to function as a co-chaperone of TbHsp70.c. Only a small number of Hsp70 proteins from trypanosomes have been characterized in terms of chaperone activity; one of the best studied trypanosomal Hsp70 proteins is Hsp70B from *T. cruzi* (Olson *et al.,* 1994; Krautz *et al.,* 1998; Edkins *et al.,* 2004). TcHsp70B has been included in this study to enable comparative analysis with TbHsp70.c; TcHsp70B is an ideal control protein due to its cytoplasmic localization and its canonical Hsp70 features and functions, including the presence of the C-terminal EEVD motif (Olson *et al.,* 1994; Krautz *et al.,* 1998; Edkins *et al.,* 2004). It may be an orthologue of TbHsp70.4, an Hsp70-like protein within *T. brucei* with a DDVD motif, sharing a 60% sequence identity, but it is more closely related to TbHsp70 (86%), in which the EEVD motif is absent (Section 2.3.1; Fig. 2.2). Even though previous studies have been performed on TcHsp70B, an *in vitro* interaction with Tbj2 has not been shown to date.

The aims of the study included:

i. Investigation of the aggregation suppression activity of TbHsp70.c and Tbj2 using MDH and rhodanese as substrates.

ii. Investigation of the ATPase activity of TbHsp70.c.

iii. Comparison of the basal ATPase activities of TbHsp70.c and TcHsp70, and of the TbHsp70.c-Tbj2 and TcHsp70B partnerships.

iv. Assessment of the effect of small molecule modulators, quercetin, on the chaperone activity of TbHsp70.c and the TbHsp70.c-Tbj2 partnership, and methylene blue on the chaperone activity of TbHsp70.c.

4.2. Methods and materials

4.2.1. Materials

The reagents used in this study are as listed in Section 3.2.1. 3,3′,4′,5,7-Pentahydroxyflavone (quercetin) was obtained from Sigma Chemicals Co. (St. Louis, Mo U.S.A.), and stored as a 1000-fold concentrated ethanolic solution at -20°C. Tetramethylthionine (methylene blue (MB), M9140) was purchased from Sigma Chemicals Co. and suspended in the respective assay buffer as a 1000-fold concentrated solution.

4.2.2. Methods

TbHsp70.c, TcHsp70B and Tbj2 used in the *in vitro* studies described in this chapter were purified in the presence of sarkosyl. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

4.2.2.1. Aggregation suppression assays

4.2.2.1.1. Suppression of rhodanese aggregation

The ability of TbHsp70.c and Tbj2 to suppress aggregation of bovine rhodanese (Sigma-Aldrich) was assessed spectrophotometrically. The assay was performed as described in Allan *et al.* (2006) with a few modifications. Rhodanese was denatured for a duration of 2 hours at 30°C; denatured rhodanese was added to assay buffer to a final concentration of 1.5 μM and the rate of its aggregation, indicated as 100% aggregation (Fig. 4.2) was monitored at 300 nm over a period of 40 min at room temperature using a KC Junior microplate reader (Bio-Tek Instruments, U.S.A.). Molecular chaperone proteins of interest were added at various concentrations to measure their ability to suppress rhodanese aggregation. TcHsp70B (0.5 μM) was added to rhodanese as a control reaction. Absorbance was plotted as percent

rhodanese aggregation over 40 min subsequent to normalizing against assays with rhodanese alone.

4.2.2.1.2. Suppression of MDH aggregation

The ability of TbHsp70.c and Tbj2 to prevent thermal aggregation of MDH was analysed by spectrophotometry. The assay was carried out as previously described by Botha *et al.* (2011). Protein aggregation was assayed using various concentrations of chaperone protein in a Helios Alpha DB spectrophotometer with a Peltier-controlled cell. Alfalfa Hsp70 (0.25 µM) and BSA (0.715 µM) were added separately to MDH as positive and negative control reactions, respectively. TbHsp70.c and Tbj2 are not aggregation prone as no major increase in turbidity was observed when these proteins were assayed in the absence of MDH.

4.2.2.1.3. Analysis of chaperone activity of the size exclusion chromatography protein fractions of TbHsp70.c and Tbj2

Two peaks were detected through size exclusion chromatography (SEC) of TbHsp70.c and Tbj2 (Section 3.3.4.) (Fig. 3.6; Fig. 4.4A). The chaperone activity of each peak produced by size exclusion chromatography was assessed using rhodanese aggregation assays. Protein fractions collected from the SEC (Section 3.2.2.3.) were concentrated using Amicon Ultra-15 Centrifugal Filter Units, 30 kDa (Amicon). The rhodanese aggregation assay was conducted as previously described (Section 4.2.1.1.). Chaperone activity of protein collected from the SEC fractions was compared to Ni-affinity purified protein at a final concentration of 0.5 µM (Section 3.2.2.2.).

4.2.2.1.4. Analysis of chaperone activity of protein purified in the absence and presence of sarkosyl

Sarkosyl was added to protein purifications to increase the overall protein yield (Chapter 3). The effect of sarkosyl on the chaperone activity of TbHsp70.c and Tbj2 was assessed using suppression of aggregation assays. Proteins were purified in the presence and absence of 7.5% sarkosyl (Section 3.2.2.2.). Protein concentrations were kept at 0.5 µM.

4.2.2.2. ATPase activity assays

4.2.2.2.1. ATPase assays

The hydrolysis of ATP by TbHsp70.c was assessed using a modified version of the ascorbic

acid/ammonium molybdate colorimetric assay to measure the release of inorganic phosphate during the reaction (Chifflet *et al.*, 1988). TbHsp70.c (1.6 µM) was equilibrated to 37°C in ATPase buffer (25 mM HEPES, pH 7.4, 2 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT) prior to the addition of ATP at varying concentrations (0 - 800 µM) to initiate the reaction. The assay was performed for 30 minutes before stopping the reaction by the addition of sample (50 µl) to an equal volume of 10% SDS. A calibration curve was prepared using standards from KH_2PO_4 and the phosphate levels within the samples and standards were detected colorimetrically by the addition of ammonium molybdate (50 µl, 1% in 1 M HCl), ascorbic acid (50 µl, 6%) and sodium citrate/acetic acid solution (125 µl, 2%/2%). The reactions were incubated for 1 hour at 37°C after which the absorbance was measured at 850 nm using a KC Junior microplate reader (Bio-Tek Instruments, U.S.A.). Enzyme kinetics were investigated using the Hanes-Woolf plot, and curves fitted to data points using GraphPad Prism (www.graphpad.com).

4.2.2.2.2. Stimulation of the basal ATPase activity of TbHsp70.c by Tbj2

Stimulation of basal ATPase activity by a potential co-chaperone of TbHsp70.c was assessed as described in Section 4.2.2.1. with a few modifications. The TbHsp70.c concentration was maintained at 1 µM, unless otherwise stated and the reaction-initiator concentration, ATP, was kept constant at 600 µM. The ability of Tbj2 to stimulate the ATPase activity of TbHsp70.c was measured at equal (1 µM), submolar (0.5 µM) and concentrations in excess (1.5 µM; 2 µM). Samples were taken in triplicate at regular time intervals (0, 30, 60, 120, 180, 240 minutes). All assays were corrected for the spontaneous breakdown of ATP observed in a control experiment in the absence of protein. Background ATP hydrolysis observed for Tbj2 was corrected for by subtracting accordingly from the reactions containing the chaperone complex.

4.2.2.3. Analysis of small molecule modulators on TbHsp70.c-Tbj2 chaperone activity

4.2.2.3.1. Effect of inhibitor quercetin on the ATPase activity of TbHsp70.-Tbj2 and TcHsp70B-Tbj2

Prior to performing an ATPase activity assay to analyse the effect of quercetin (Fig. 4.1B) on the chaperone activity of TbHsp70.c-Tbj2, a spectral scan of quercetin was conducted using a KC Junior microplate reader at A_{369nm} (Fig 4.6D). Having ensured that the compound was not

detected at A_{850nm} (Fig. 4.1D), the ATPase assay was performed as described in Section 4.2.2.2. The protein concentrations of TbHsp70.c, TcHsp70B and Tbj2 were maintained at 0.4 µM. Quercetin was added to the reaction prior to equilibration at 37°C to a final concentration of 30 µM.

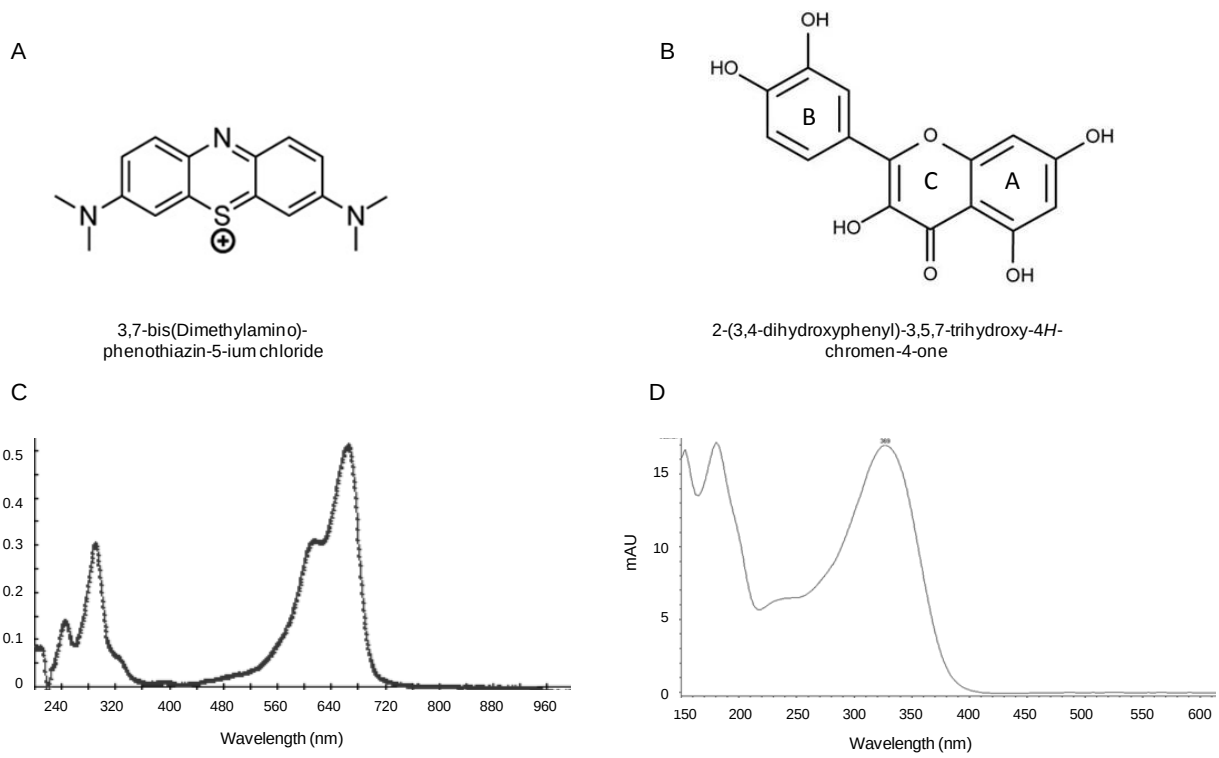


Figure 4.1. Chemical structures of quercetin and methylene blue, small molecule modulators
(A) Methylthioninium chloride, commonly called methylene blue (MB), is a phenothiazine: a heterocyclic aromatic compound consisting of two benzene rings, each with a methylamino group, adjacent to a single thiazine. (B) Natural flavonoid quercetin. (C) A spectral scan of MB (30 µM) suspended in assay buffer revealed a UV visible spectrum at A_{290nm} and another at A_{665nm}. (D) A spectral scan of quercetin resulted in a UV visible spectrum at A_{369nm}.

4.2.2.3.2. Effect of methylene blue on the ATPase activity of TbHsp70.c-Tbj2

As with quercetin, a spectral scan of MB (Fig. 4.1A) was performed prior to analysis of its effect on chaperone activity and had a UV visible spectrum at A_{290nm} and A_{665nm} (Fig. 4.1C). The ATPase activity assay was carried out as described in Section 4.2.2.2. TbHsp70.c and Tbj2 concentrations were not varied (1 µM) and MB (30 µM), suspended in ATPase buffer, was added to the reaction prior to equilibration at 37°C.

4.3. Results

4.3.1. Tbj2 aids TbHsp70.c in suppression of protein aggregation

4.3.1.1. Suppression of denatured rhodanese aggregation

Chemically denatured rhodanese was used to determine the ability of TbHsp70.c to suppress protein aggregation. There was no increase in turbidity due to TbHsp70.c or Tbj2 in the absence of rhodanese signifying no aggregation of the chaperones (data not shown). The addition of TbHsp70.c resulted in a marked increase of aggregation suppression in a dose-dependent manner (Fig. 4.2). Tbj2 resulted in 91.6% rhodanese aggregation. To enable comparative analysis, a fixed concentration of TbHsp70.c was used and Tbj2 concentrations were varied. The addition of Tbj2 at a submolar concentration resulted in 72.2% rhodanese aggregation (Fig. 4.2). Equal concentrations of Tbj2 and TbHsp70.c resulted in 62.5% rhodanese aggregation and Tbj2 in excess caused 59% rhodanese aggregation (Fig. 4.2). Tbj2 in partnership with TbHsp70.c showed increased chaperone activity upon increased concentrations. TcHsp70B was also able to suppress the aggregation of rhodanese (Fig. 4.2).

4.3.1.2. Suppression of MDH aggregation

MDH was used as another substrate to determine whether TbHsp70.c, TcHsp70B or Tbj2 could suppress aggregation of MDH. BSA was used as a negative control and did not suppress aggregation of MDH (data not shown). Alfalfa Hsp70 was used as a positive control and resulted in 58.4% MDH aggregation (Fig. 4.3). TbHsp70.c, TcHsp70B and Tbj2 were assayed in the absence of MDH to determine that the proteins were not prone towards aggregation (data not shown). A dose response was evident upon the addition of various concentrations of TbHsp70.c as turbidity levels decreased with an increase in chaperone concentration (Fig. 4.3).

A similar result was obtained for Tbj2 where increased chaperone concentrations caused decreased turbidity and so a dose-dependent suppression of MDH aggregation (Fig. 4.3). The ability of Tbj2 to act as a co-chaperone of TbHsp70.c was assessed by maintaining TbHsp70.c concentrations and varying those of Tbj2 (Fig. 4.3). TbHsp70.c (0.5 μM) resulted in 38.1% MDH aggregation, and TcHsp70B (0.3 μM) in 46.9% MDH aggregation (Fig. 4.3).

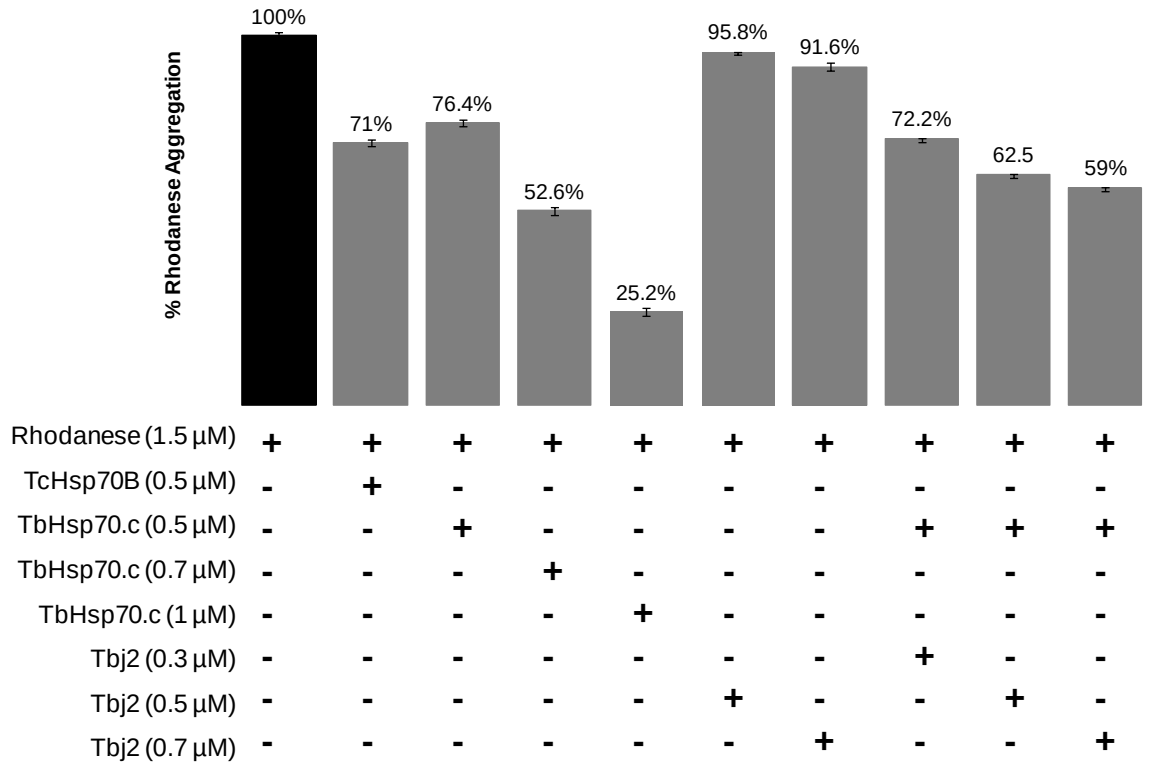


Figure 4.2. Suppression of rhodanese aggregation by TbHsp70.c, TcHsp70B and Tbj2.
The rhodanese concentration was kept constant at 1.5 µM, the aggregation of denatured rhodanese measured at 300 nm over 40 min is indicated as 100% aggregation; the concentrations of all other components are given in the figure. The '+' symbols represent components present in the reaction whilst '-' represent those that were absent. TcHsp70B suppresses aggregation of rhodanese. TbHsp70.c suppresses aggregation of denatured rhodanese in a dose-dependent manner. Tbj2 only slightly suppresses aggregation of rhodanese. The effect of Tbj2 on the ability of TbHsp70.c to suppress the aggregation of rhodanese was investigated. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

The addition of Tbj2 at submolar concentrations (0.1 µM , 0.3 µM) to TbHsp70.c, resulted in 59.7% and 33% MDH aggregation, respectively, and Tbj2 at a concentration in excess (0.7 µM) of that used for TbHsp70.c resulted in 15% MDH aggregation (Fig. 4.3). Submolar concentrations of Tbj2 (0.1 µM) resulted in an inhibition of the chaperone activity of TbHsp70.c; and Tbj2 concentrations of 0.3 µM and 0.7 µM enhanced TbHsp70.c chaperone activity, this result could however be due to an additive effect of Tbj2 rather than Tbj2 co-chaperoning TbHsp70.c. Equal concentrations of Tbj2 and TcHsp70B resulted in 6% MDH aggregation, showing a marked increase in TcHsp70B chaperone activity, and thereby a distinct co-chaperoning effect of Tbj2 on TcHsp70B.

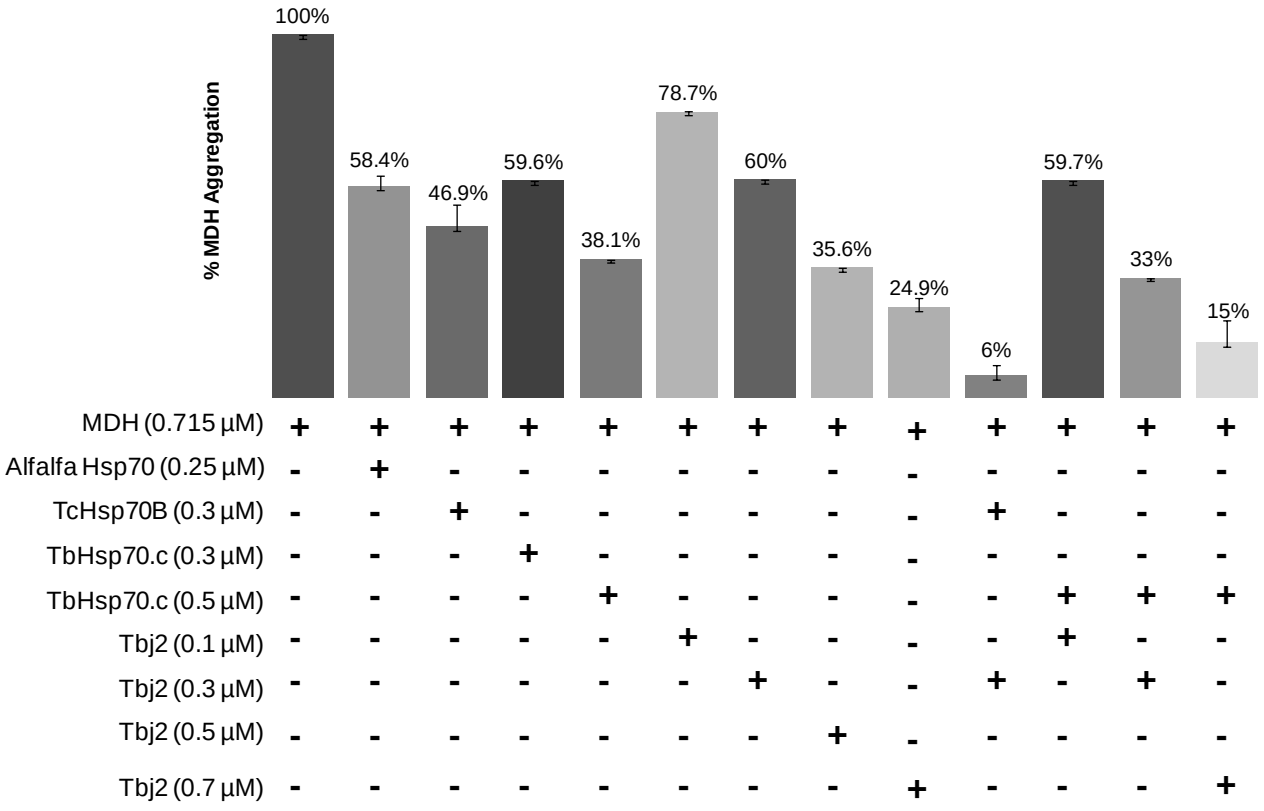
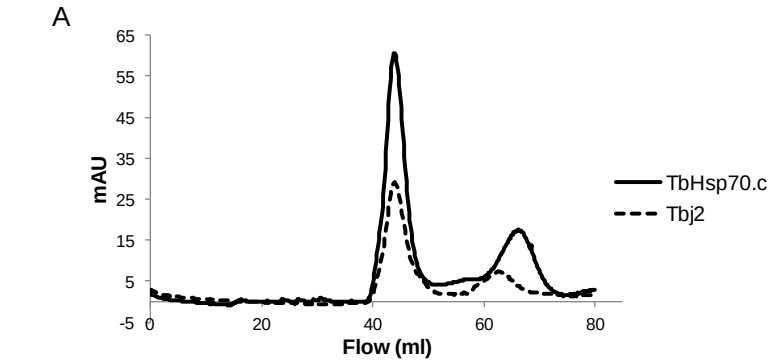


Figure 4.3. Suppression of MDH aggregation by TbHsp70.c, TcHsp70B and Tbj2.
MDH (0.715 µM) was added to the assay buffer to initiate the reaction and produce a bar graph representative of aggregation of MDH alone. The '+' symbols represent components present in a reaction whilst '-' represents those that were absent. Positive controls Alfalfa Hsp70 and TcHsp70B suppress aggregation of MDH. TbHsp70.c and Tbj2 both suppress aggregation of MDH in a dose-dependent manner. The effect of Tbj2 on the ability of TbHsp70.c and TcHsp70B to suppress MDH aggregation was investigated. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

4.3.1.3. Analysis of chaperone activity of size exclusion chromatography protein fractions, and protein purified in the absence and presence of sarkosyl

To assess whether a difference could be observed in the chaperone activity of the different oligomeric states of each protein (Fig. 4.4A), and protein purified in the presence of sarkosyl, fractions collected from SEC were concentrated and assayed. Protein fractions of TbHsp70.c were collected at the void volume and a volume of 67.29 ml; protein fractions of Tbj2 were collected at the void volume and at volume 64.28 ml (Fig. 4.4B). Purified TbHsp70.c resulted in 71% rhodanese aggregation and a 74.3% and 70.4% rhodanese aggregation was observed for SEC volumes V_0 and 67.27 ml, respectively (Fig 4.3C). Purified Tbj2 caused 95.9% rhodanese aggregation and a 98.9% and 93.8% rhodanese aggregation for SEC volumes V_0

and 64.28 ml, respectively (Fig 4.3C). No major change was observed for either TbHsp70.c or Tbj2 upon a comparison of chaperone activity of SEC or sarkosyl. Protein activities were similar to that observed previously (Fig. 4.1).



B

Protein	Peak volume (ml)	Yield (mg/ml)
TbHsp70.c	V0	0.384
	67.29	0.139
Tbj2	V0	0.48
	64.28	0.242

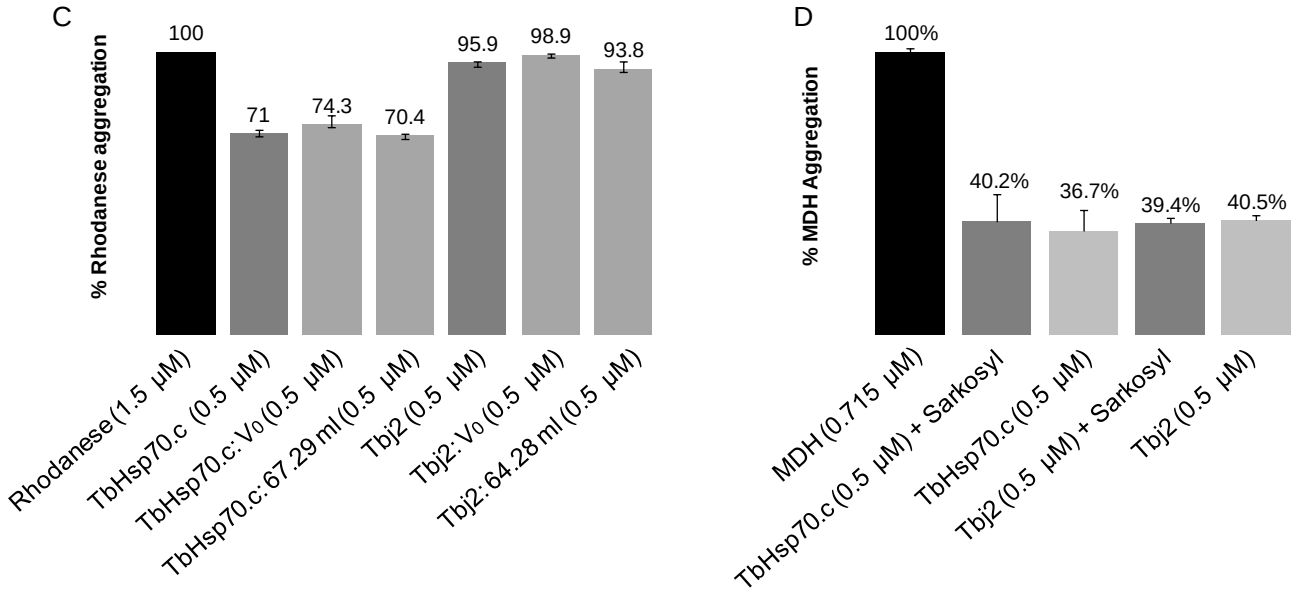


Figure 4.4. Analysis of the chaperone activities of the SEC major and minor peaks of TbHsp70.c and Tbj2, and in the absence and presence of Sarkosyl.

(A) TbHsp70.c oligomerizes in solution and Tbj2 exists as a possible dimer in solution (Fig. 3.6; discussed in Section3.4). (B) Protein yields of TbHsp70.c and Tbj2 obtained after concentration of SEC fractions. (C) Comparison of the chaperone activities of protein eluting as major and minor peaks for both TbHsp70.c and Tbj2 using suppression of rhodanese aggregation assays. Denatured rhodanese was added to the assay buffer to initiate the reaction and produce a bar-graph representative of aggregation of rhodanese alone. The rhodanese concentration was kept constant at 1.5 µM; the concentrations of all other components are given in the figure. The abilities of nickel-affinity purified TbHsp70.c, TbHsp70.c eluting as the major peak, and as the minor peak to suppress aggregation of rhodanese were compared. The same comparison was made for nickel-affinity purified Tbj2, Tbj2 eluting as the major peak, and as the minor peak. (D) Comparison of TbHsp70.c and Tbj2 chaperone activities in the absence and presence of 7.5% Sarkosyl using MDH aggregation assays. MDH was added to the assay buffer to initiate the reaction and produce a bar-graph representative of aggregation of MDH alone. The MDH concentration was kept constant at 0.715 µM; the concentrations of all other components are given in the figure. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

To eliminate the possibility that detergent used in this study influences chaperone activity, TbHsp70.c and Tbj2 was purified in the absence and presence of sarkosyl and activities compared using an MDH aggregation assay. In the presence of sarkosyl, TbHsp70.c resulted in a 40.2% MDH aggregation, its absence in 37.7% MDH aggregation (Fig. 4.4D). A 39.4% MDH aggregation was observed for Tbj2 purified in the presence of sarkosyl and 40.5% MDH aggregation in the absence thereof (Fig. 4.4D). There was thus no major alteration of chaperone activity for either protein, suggesting that sarkosyl does not affect the activity of the proteins. This finding coincides with the observation by Park and colleagues (2011) that no adverse effects on protein activity were evident when purified in the presence of sarkosyl. The chaperone activities for both sets of assays were similar to those previously observed (Fig. 4.1; Fig. 4.3).

4.3.2. ATPase activity assays

4.3.2.1. ATPase assays

The basal ATPase activity of TbHsp70.c was measured by the release of phosphate over time. The Hanes-Woolf plot was generated with the resulting data (Fig. 4.5).

Table 4.1. Kinetic data recorded for steady state ATPase activities of Hsp70 proteins (adapted from Matambo *et al.*, 2004)

Protein	Organism	V_{max} (nmol/min/mg)	K_m for ATP (µM)	Reference
Hsp70.c	*T. brucei*	2.32	59.82	This study
Hsp70	*Plasmodium falciparum*	14.6	616.5	Matambo *et al.*, 2004
Hsp70	*Homo sapiens*	5.5	85	Olson *et al.*, 1994
Hsp70	*H. sapiens*	2	--	Bimston *et al.*, 1998
Hsp70	*T. cruzi*	550	70	Olson *et al.*, 1994
Hsc70	*Bos taurus*	2.9	0.7	O'Brien & McKay, 1998
DnaK	*E. coli*	3.5	20	Liberek *et al.*, 1991
DnaK	*E. coli*	0.43	1	Kamath-Loeb *et al.*, 1995

Using the Hanes-Woolf plot, the V_{max} and K_m were determined to be 2.32 nmol/min/mg and 59.82 µM, respectively, for the basal ATPase activity of TbHsp70.c (Table 4.1). The V_{max} of TbHsp70.c was similar to that seen for values reported for *B. taurus* and *H. sapiens* (O'Brien and McKay, 1998; Olson *et al.*, 1994) (Table 4.1). The K_m of TbHsp70.c was observed to be the closest to that seen for *T. cruzi* Hsp70 (70 µM) (Olson *et al.*, 1994) (Table 4.1).

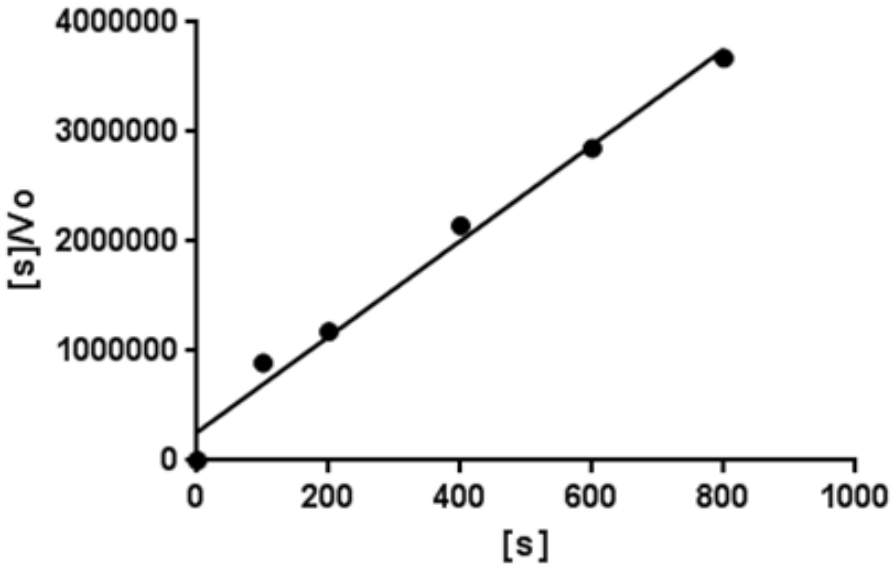


Figure 4.5. Steady-state assay of the basal ATPase activity of TbHsp70.c
Least squares regression analysis of data was used to determine the initial rates and was plotted as Hanes-Woolf plot in which the ratio of initial substrate, ATP, concentration [S] to the reaction velocity (V_0) is plotted against [S]. The *V*max and *K*m were determined to be 2.32 nmol/min/mg of TbHsp70.c and 59.82 μM ATP, respectively. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

4.3.2.2. Stimulation of the basal ATPase activity of TbHsp70.c by Tbj2

The ability of Tbj2 to stimulate the ATPase activity of TbHsp70.c was determined. TbHsp70.c was shown to have a basal ATPase activity of 7.6 nmol Pi/min/mg (Fig. 4.6). At submolarity, the addition of Tbj2 resulted in an ATPase activity of 8.4 nmol Pi/min/mg of TbHsp70.c, thus only a slight stimulation of activity was observed. Equal concentrations of Tbj2 and TbHsp70.c resulted in an ATPase activity of 12.5 nmol Pi/min/mg and Tbj2 in excess (1.5 μM and 2 μM) resulted in an ATPase activity of 13 and 14.2 nmol Pi/min/mg respectively, an approximate 2-fold enhancement of ATP hydrolysis (Fig. 4.6). TbHsp70.c denatured by boiling the sample displayed no activity (data not shown). Tbj2 (0.4 μM) was observed to stimulate the ATPase activity of TcHsp70B (2.5 nmol Pi/min/mg) to 5.3 nmol Pi/min/mg (Fig. 4.7), an approximate 2-fold increase in ATPase hydrolysis.

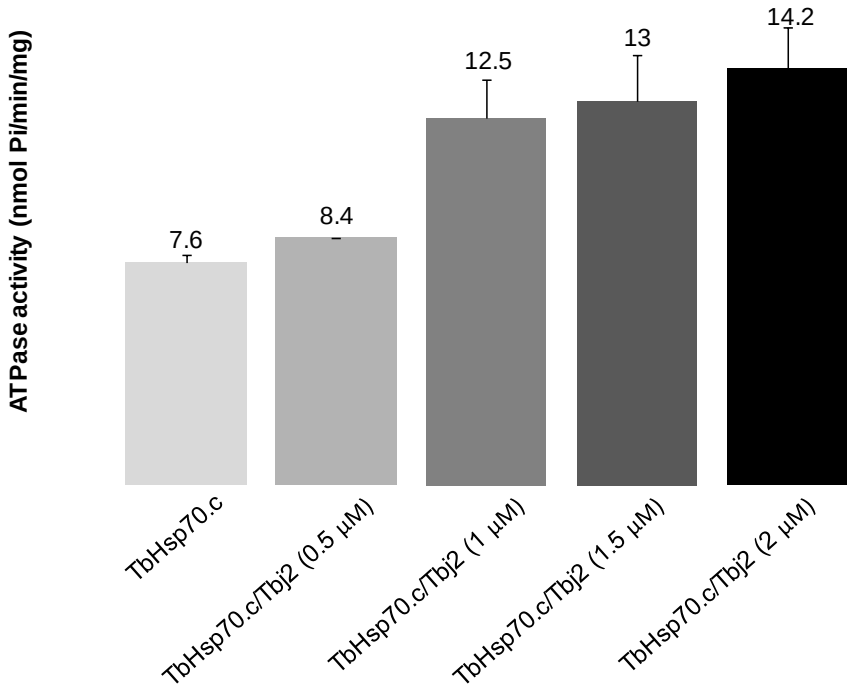


Figure 4.6. The effect of Tbj2 on the ATPase activity of TbHsp70.c.
The TbHsp70.c concentration was kept constant at 1 µM and ATP, present in all reactions, at 600 µM. The concentrations of Tbj2 were varied and are given in the figure. The data points were determined from triplicate ATPase activity measurements for three independent batches of purified protein. The bars represent standard deviations. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

4.3.3. Analysis of small molecule modulators on TbHsp70.c-Tbj2 chaperone activity

4.3.3.1. Effect of inhibitor quercetin on the ATPase activity of TbHsp70.c-Tbj2 and TcHsp70B-Tbj2

Based on their reported ability to inhibit either Hsp70 synthesis or the ATPase activity of Hsp70, two small molecule modulators were selected to analyse their effect on the basal ATPase activity of TbHsp70.c and on the Tbj2 stimulated ATPase activity of TbHsp70.c. Quercetin has been demonstrated to inhibit both Hsp70 expression at concentrations ranging from 30 – 100 µM (Elia *et al.*, 1994; Hansen *et al.*, 1997; Hu *et al.*, 2003); and ATPase activity (Lang and Racker, 1974; Murakami *et al.*, 1992). The natural flavonoid quercetin inhibited the basal ATPase activity of TbHsp70.c (Fig. 4.7).

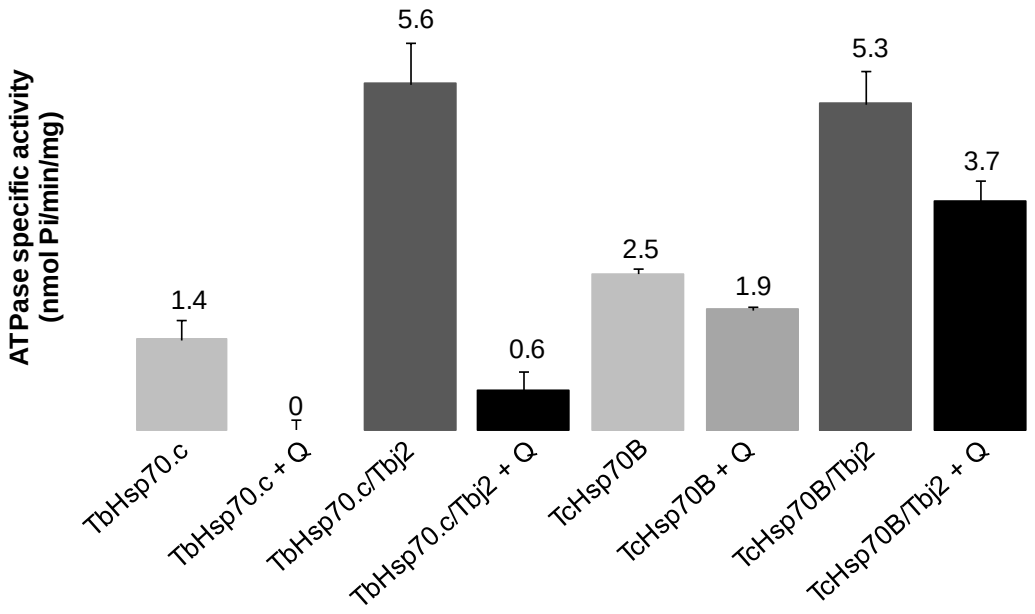


Figure 4.7. The effect of small molecule inhibitor quercetin on the ATPase activity of TbHsp70.c. TbHsp70.c, TcHsp70B and Tbj2 concentrations were kept constant at 0.4 µM and ATP was present in all reactions at 600 µM. The data points were determined from triplicate readings of ATPase activity. Quercetin was present in the reactions as indicated at 30 µM. Each assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

Upon the addition of quercetin to the Tbj2-stimulated ATPase activity of TbHsp70.c, the ATPase activity of the chaperone complex decreased from 5.6 to 0.6 nmol Pi/min/mg, a considerable 9.3-fold inhibition of Tbj2-stimulated ATPase activity of TbHsp70.c (Fig. 4.7; Quercetin induced a 1.3-fold inhibition of the ATPase activity of TcHsp70B and a 1.4-fold inhibition of the Tbj2 stimulated ATPase activity (Fig. 4.7). TbHsp70.c-Tbj2 thus exhibited higher sensitivity to quercetin inhibition than was observed for Tchsp70B-Tbj2 (Fig. 4.7). The ATPase activity of TbHsp70.c (1 µM) was stimulated to a lesser extent by Tbj2 (1 µM) (Fig. 4.6) than was observed for the ATPase activity of TbHsp70.c, stimulated by Tbj2 at 0.4 µM (Fig. 4.7). It is probable that not all of the Tbj2 (1 µM) was in its native state and that unfolded Tbj2 was recognized as substrate by TbHsp70.c, resulting in a reduced hydrolysis of ATP.

4.3.3.2. Effect of methylene blue on the ATPase activity of TbHsp70.c-Tbj2

The effect of the small molecule modulator, methylene blue (MB), was also investigated. MB reduced the basal ATPase activity of TbHsp70.c from 7.6 to 6.4 nmol Pi/min/mg, a 1.2-fold

inhibition (Fig. 4.8A); inhibition of ATPase activity was expected as >80% inhibition of human Hsp70 ATPase activity was previously observed (Jinwal *et al.,* 2010). However, the TbHsp70.c ATPase activity stimulated by Tbj2 (12.5 nmol Pi/min/mg) was enhanced to 18.7 nmol Pi/min/mg in the presence of MB; a 1.5-fold increase of the ATPase activity of TbHsp70.c-Tbj2 (Fig. 4.8A).

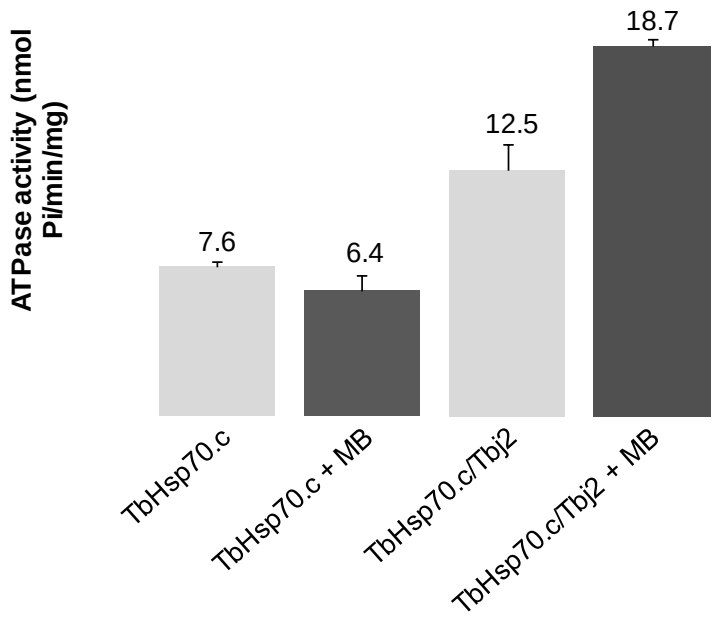


Figure 4.8. Analysis of the effect of small molecule modulator methylene blue on the chaperone activity of TbHsp70.c using an ATPase activity assay.
The effect of methylene blue (MB) on the basal ATPase activity of TbHsp70.c and TbHsp70.c in partnership with Tbj2 was investigated. TbHsp70.c and Tbj2 concentrations were kept constant at 1 µM and ATP was present in all reactions at 600 µM. The data points were determined from triplicate readings of 3 independent protein purifications. MB, as indicated in the figure, was present at a concentration of 30 µM. The assay was conducted in triplicate and three independent experiments on independent batches of protein were conducted.

4.4. Discussion and conclusions

Amongst the various roles of Hsp70 proteins is the ability to act as 'holdases,' preventing aggregation of proteins (Slepenkov and Witt, 2002). Demonstration of the holdase-function, by suppressing protein aggregation, is used to display chaperone activity (Goloubinoff *et al.*, 1999; Basha *et al.,* 2004). The model thermolabile substrate MDH is commonly used for this reason and was used in this study to investigate the ability of TbHsp70.c and Tbj2, in isolation and in partnership, to suppress the heat-induced aggregation of MDH. Suppression

of protein aggregation assays has previously been shown to successfully determine the effect of small molecule modulators on chaperone activity; 15-deoxyspergualin modulates the ability of PfHsp70-1 and PfHsp70-2 to suppress the aggregation of alcohol dehydrogenase (Ramya *et al.,* 2006), and various compounds including lapachol, malonganenones A-C and bromo-β-lapachona were screened for and found to inhibit PfHsp70-1's ability to suppress aggregation of MDH (Cockburn *et al.,* 2011).

A second substrate, rhodanese, was selected for suppression of protein aggregation assays to investigate whether TbHsp70.c would show substrate discrimination due to the presence of the unique acidic substrate binding domain of TbHsp70.c. The novel residues of the hydrophobic binding pocket and the acidity of the SBD did not appear to impact the functioning of TbHsp70.c in its ability, in isolation, to bind and suppress aggregation of both rhodanese and MDH as substrates in a concentration dependent manner, thereby displaying typical chaperone activity. However, TbHsp70.c was able to suppress aggregation of MDH to a greater degree than that of rhodanese. The same trend was evident for TcHsp70B, included in the study as a positive control as being one of the few trypanosomal Hsp70 proteins previously characterized; it is structurally and functionally a typical Hsp70 protein (Olson et al., 1994; Krautz et al., 1998; Edkins et al., 2004). Cytoplasmic TcHsp70B is an orthologue of TbHsp70, sharing 86% sequence identity (Section 2.3.1; Fig. 2.2; Louw *et al.,* 2010). However, TbHsp70 lacks the conserved C-terminal EEVD motif typical of cytoplasmic Hsp70 proteins (Section 2.3.1; Fig. 2.2). TbHsp70.4, an Hsp70-like protein, contains an altered DDVD motif; TcHsp70B shares a lower sequence identity of 60% with TbHsp70.4 (Section 2.3.1; Fig. 2.2). TcHsp70B showed greater activity in suppression of MDH aggregation than for rhodanese, both cytoplasmic substrates, implying that the difference in activity shown between the two substrates is not due to the acidity of the SBD of TbHsp70.c, but most likely due to Hsp70 binding sites that are less well conserved in rhodanese than in MDH. It is also possible that rhodanese is more prone to aggregation than MDH, thus displaying differences in the chaperone activity of TbHsp70.c. Alternatively, once MDH is released from TbHsp70.c, MDH may display a higher degree of auto-refolding than rhodanese.

In addition to the research conducted in this thesis, numerous papers have been published using the MDH aggregation suppression assay without ATP (Boshoff *et al.,* 2008; Botha *et*

al., 2011; Cockburn *et al.,* 2011). Upon ATP binding Hsp70, the affinity of an Hsp70 protein for MDH will be decreased (Shonhai *et al.,* 2008). The addition of ATP to the MDH or rhodanese aggregation suppression assay would have resulted in the release of substrate from TbHsp70.c, as has previously been demonstrated for *P. falciparum* Hsp70 (Shonhai, 2007), *E. coli* Hsc66 and DnaK (Silberg *et al.,* 1998) and bovine Hsp90 (Young *et al.,* 1997). Shonhai (2007) showed the addition of 1 mM ATP to MDH bound to PfHsp70 resulted in the release and subsequent aggregation of MDH. The addition of 400 µM ATP to rhodanese bound to both Hsc66 and DnaK was shown to reduce the ability of the chaperones to suppress aggregation of rhodanese; the addition of ADP showed no effect (Silberg *et al.,* 1998). A similar result was observed using citrate synthase as substrate (Silberg *et al.,* 1998). Using a refolding assay, Young and colleagues (1997) showed that the incubation of ATP with denatured luciferase and Hsp90 resulted in a low activity of luciferase, demonstrating release of substrate. Our current research entails the development of refolding assays, with the addition of ATP, for further characterization of TbHsp70.c.

Tbj2 was selected as a probable co-chaperone of TbHsp70.c due to the presence of its functional J-domain and because it was shown to localize in the cytoplasm. Tbj2 was added to the aggregation suppression assays to firstly determine whether it would recognize rhodanese and MDH as substrates, and secondly whether it could act as a co-chaperone of TbHsp70.c, by testing its ability to assist TbHsp70.c in suppressing protein aggregation. Hsp70s generally function in cooperation with Hsp40s, where Hsp40 either mediates the interaction of Hsp70 with its substrate, or "holds" the substrate and recruits Hsp70 to the unfolded polypeptide (Young *et al.,* 2003). Hsp40 proteins are able to bind unfolded substrates independently of Hsp70 proteins and prevent their aggregation (Cyr *et al.,* 1995; Young *et al.,* 2004). Once bound, the substrate is transferred to Hsp70 by the J-protein, at the same time Hsp40 stabilizes the Hsp70-substrate association by stimulating the ATPase activity of Hsp70 (Young *et al.,* 2004). Tbj2 successfully suppressed aggregation of MDH in a concentration dependent manner. However, Tbj2 showed considerably decreased ability to suppress rhodanese aggregation implying that even though Type I Hsp40 proteins are promiscuous binders, Tbj2 does not bind all its substrates as effectively. In the presence of TbHsp70.c, Tbj2 appeared to enhance the ability of TbHsp70.c to decrease aggregation of rhodanese to a greater degree than shown for MDH and Tbj2 aided TcHsp70B in its ability to suppress MDH aggregation. It should not be forgotten that no ATP was present in the system

and it is possible that the activity observed in the presence of Tbj2 was as a result of Tbj2 binding as substrate to TbHsp70.c, resulting in a release of MDH, and not as co-chaperone. A similar mechanism has been proposed by Edkins and colleagues (2004) in which a *T. cruzi* Type III Hsp40, Tcj1, showed a slight stimulation of the ATPase activity of TcHsp70, even in the presence of model substrate RCMLA. Tcj1 was suggested to be partially unfolded and binding as substrate to TcHsp70 (Edkins *et al.,* 2004). Molecular chaperone function requires the co-operative interactions between Hsp70 and co-chaperones such as Hsp40. The cycle of target substrate binding and release by Hsp70 proteins is modulated by ATP binding and hydrolysis, facilitated by Hsp40 proteins (Palleros *et al.,* 1993; McCarty *et al.,* 1995; Hartl 1996). The aggregation suppression assays do not distinguish between additive and co-operative effects of co-chaperone and chaperone. Hsp70 proteins typically demonstrate refoldase activity; the aggregation suppression assays in this study demonstrate the holdase-function of Hsp70 only; a refoldase assay using luciferase as substrate and where ATP is added in the system may give a better indication of Hsp70 chaperone function, as well as the ability of Tbj2 to enhance the activity of TbHsp70.c (Lu and Cyr, 1998). Also, the stimulation of the ATPase activity of Hsp70 by Hsp40 and the addition of unfolded substrate such as RCMLA, would give a better indication of a co-operative effect.

Alteration of protein structure causing irreversible aggregation can be due to unfavourable conditions during handling, protein production, purification or storage; protein aggregation can negatively impact the activity of the protein (Kerwin *et al.,* 1998; Kreilgaard *et al.,* 1998; Zhang *et al.,* 2001). An obstacle faced upon purification of recombinant proteins from *E. coli* is the often low soluble protein yield, which is often as a result of insoluble aggregates named inclusion bodies. Inclusion bodies form as a result of the auto-catalyzed association between hydrophobic residues of different molecules causing precipitation of the recombinant proteins (Mukhopadhyay, 1997). A constant challenge regarding *in vitro* assays is the requirement of high concentrations of protein, which often results in protein aggregation (Bondos *et al.,* 2000). Aggregation of intermediate forms of protein has been reported to obscure protein folding studies (Goldberg *et al.,* 1991). The rate of aggregation may surpass the protein folding rate, thereby causing inactivation of part of the protein (Goldberg *et al.,* 1991). Purification of TbHsp70.c and Tbj2 from *E .coli* did not result in the formation of inclusion bodies, however, the proteins expressed poorly, justifying the use of the detergent sarkosyl to enhance protein yield. Basic experiments involving size exclusion chromatography using

protein purified in the absence and presence of sarcosyl were performed to ensure that the protein obtained from the sarkosyl purifications were in a native state for subsequent assays. An alternative and more accurate approach would be fourier transform infrared spectroscopy (FTIR) which allows investigation of protein secondary structure and can be used to monitor protein aggregation (Zurdo *et al.,* 2001). FTIR can also be used to determine the state of aggregation in inclusion bodies in intact cells (Natalello *et al.,* 2007).

The major peaks representing high molecular mass species observed during size exclusion chromatography are not aggregated inactive protein but rather oligomerized protein, upon dilution of these proteins in sarkosyl, aggregation suppression assays showed they were active and the degree of activity matched that of undiluted proteins. Sarkosyl was thus shown to have no influence on the chaperoning abilities of either TbHsp70.c or Tbj2, which coincides with what has been demonstrated in literature (Park *et al.,* 2011).

Using a modified ammonium molybdate/ascorbic acid colorimetric assay (Chifflet *et al.,* 1988), evidence of the stimulation of the ATPase activity of TbHsp70.c by Tbj2 increases the probability that Tbj2 is acting as co-chaperone to TbHsp70.c. Enhanced Hsp70 ATPase activity is specific and is modulated through the interaction of an Hsp40 partner. Tbj2 in excess stimulated the ATP hydrolysis activity of TbHsp70.c approximately 2-fold. Tbj2 stimulation occurred in a dose-dependent manner; submolar concentrations of Tbj2 resulted in ATPase activity stimulation of TbHsp70.c; equal concentrations and Tbj2 in excess displayed increased stimulation of ATP hydrolysis by TbHsp70.c. This data would suggest that Tbj2 may act as a co-chaperone by means of its J-domain interacting with the ATPase domain of TbHsp70.c, either recruiting substrates to the Hsp70 or merely mediating the interaction of the Hsp70 with its substrate and stimulating the ATPase activity of the Hsp70. However, the level of ATPase activity stimulation by Tbj2 is low in comparison to what has previously been observed for Hsp70-Hsp40 partnerships. Yeast Hsp40, Ydj1p, was shown to stimulate the ATPase activity of yeast Hsp70, Ssa1p, by 6-8-fold (Cyr *et al.,* 1992; Caplan *et al.,* 1992; Fewell *et al.,* 2004). Hsp40 has been demonstrated to interact with Hsp70 in a bipartite manner, engaging with Hsp70 at both the substrate binding domain as well as the C-terminal EEVD motif (Demand *et la.,* 1998; Aron *et al.,* 2005). The absence of the EEVD motif resulted in a reduced ability of Hsp40 to stimulate the ATPase activity of Hsp70 2.5-fold (Demand *et al.,* 1998). The lowered Hsp40 stimulation coincided with what was

observed in this study – the absence of the EEVD motif in TbHsp70.c may account for the approximate 2-fold stimulation of TbHsp70.c ATPase activity by Tbj2 (Fig. 4.6). Considering how well characterized Hsp70 molecular chaperones are, it is surprising that so little is known about the extreme C-terminal region of Hsp70. The EEVD tetrapeptide typically interacts with the tetracopeptide repeat (TPR) domains of co-chaperones. Smock and colleagues (2011) have proposed that this is not the only function of the extreme C-terminal region of Hsp70. The DnaK residues 624 – 638, containing the conserved EEVD motif, have been suggested to serve as an additional binding site for misfolded or denatured substrates (Smock *et al.,* 2011).

Purification of TcHsp70B was previously reported to be soluble and free of aggregates and demonstrated a basal ATPase activity of 40 nmol Pi/min/mg (Edkins *et al.,* 2004). Louw *et al.,* (2010) reported a basal ATPase activity of 0.5628 nmol Pi/min/mg for TcHsp70B. In this study, the basal ATPase activity of TcHsp70B was shown to be 2.5 nmol Pi/min/mg (Fig. 4.7); a low TcHsp70B protein yield was obtained (data not shown) resulting in the use of sarkosyl (Section 3.3.3). Differences in handling and purification methods may have resulted in the differences in TcHsp70B protein chaperone activity observed between this study and that of Edkins *et al.,* (2004).

To date, very few Hsp70 inhibitors have been identified; the focus has rather been on identifying small molecules that inhibit Hsp90 activity (Whitesell *et al.,* 1994; Chiosis *et al.,* 2003; Zhao and houry, 2005; Hadden *et al.,* 2006; Kang *et al.,* 2009; Kim *et al.,* 2009) due to its increasing significance as a target against cancer (Porter *et al.,* 2010), and potent Hsp90 inhibitors are currently in clinical use (Neckers, 2003; reviewed in Neckers and Workman, 2012). Pyrimidinones have been observed to inhibit the chaperone activity of Hsp70 (Chiang *et al.,* 2009) by associating with a large surface area of the protein, and so large dosages of the compounds are necessary for *in vitro* studies (Wisén *et al.,* 2010). Small molecules have been identified that specifically inhibit the ability of Hsp40 to stimulate the activity of Hsp70 proteins, thereby targeting the Hsp70-Hsp40 partnership (Fewell *et al.,* 2004). Some pyrimidinones have been found to attach to the precise location within the Hsp70 ATPase domain that the J-domain of Hsp40 binds (Wisén *et al.,* 2010).

Amongst the diverse biological activities that have been reported for the >9000 naturally occurring flavonoids (Martens and Mithofer, 2005), a common function has been identified in its ability to bind ATP (Teillet *et al.,* 2008). Chang and colleagues (2011) screened 80 flavonoids against DnaK-DnaJ ATPase activity. The free phenolic groups on ring A (Fig. 4.1B) are essential for the activity of a flavonoid (Chang *et al.,* 2011). Quercetin inhibited the DnaJ-stimulated ATPase activity of DnaK by 67% (IC_{50} = 27.6 μM) (Chang *et al.,* 2011). The effect of quercetin on the chaperone activity of TbHsp70.c and the TbHsp70.c-Tbj2 partnership was investigated. Quercetin is cytoxic by inhibiting the interaction of heat shock factor 1 (HSF-1) to the heat shock element (McMillan *et al.,* 1998). Quercetin was observed to inhibit the basal ATPase activities of both TbHsp70.c and TcHsp70B to a very similar degree at a concentration of 30 μM. However, quercetin showed a stronger inhibitory action in the presence of the TbHsp70.c-Tbj2 partnership; by implication the protein-protein interaction is specifically targeted. The same trend has been observed for a different flavonoid-based inhibitor, myricetin. Myricetin was demonstrated to specifically block the ability of *E. coli* DnaJ to stimulate the ATPase activity of DnaK by preventing contact between chaperone and co-chaperone, an allosteric mechanism (Chang *et al.,* 2011). The TbHsp70.c-Tbj2 partnership displayed a much higher sensitivity to the inhibitory effect of quercetin than was evident for the TcHsp70B-Tbj2 partnership. The differential effects of quercetin on the ATPase activities of the two groups of protein-protein interactants suggest that these partnerships are not identical and that the chaperone machineries between the two kinetoplastids are different. This is the first report of an *in vitro* study demonstrating the inhibitory effect of quercetin on a trypanosomal heat shock 70 protein's ATPase activity.

Phenothiazine class inhibitor, methylene blue, is a known inhibitor of Hsp70 ATPase activity (Jinwal *et al.,* 2009; Wang *et al.,* 2010; Gao *et al.,* 2011). As with the flavonoids, MB binds Hsp70 allosterically, affecting the rate at which ATP is consumed, which is linked to the opening and closing of the C-terminal over the SBD (Jinwal *et la.,* 2009). The lid closes, holding the substrate, upon conversion of ATP to ADP, conversion of ADP to ATP results in opening of the lid and release of substrate. Inhibitors of Hsp70 force the lid to remain open, activators enhance the speed at which the helical lid opens and closes (Jinwal *et al.,* 2009). Interestingly MB was demonstrated to inhibit the ATPase activity of heat-inducible HSPA1A, but not that of constitutively expressed HSPA8. A Cys306 residue, present in HSPA1A, is oxidized by MB which results in decreased binding of ATP due to an alteration

of Cys267 (Miyata *et al.,* 2012). HSPA1A Cys267 and Cys306 mutations resulted in resistance to the effect of MB (Miyata *et al.,* 2012). The Cys267 and Cys306 residues are conserved in TbHsp70.c (Section 2.3.1, Fig. 2.1). Investigation of the effect of MB on the chaperone activity of TbHsp70.c and TbHsp70.c in complex with Tbj2 revealed an interesting finding. As was expected, MB inhibited the basal ATPase activity of TbHsp70.c. However, in the presence of Tbj2, the ATPase activity of TbHsp70.c was further enhanced by the presence of MB, suggesting that the presence of Hsp40 had altered the mechanism of action of MB. MB was also observed to enhance the substrate binding activities of both Alfalfa Hsp70 and TbHsp70.c, to a similar degree, in the absence of co-chaperone. MB, reported inhibitor of Hsp70 ATPase activity, showed stimulation of the ATPase activity of a protein-protein interaction (Fig. 4.8A). This is the first report of an *in vitro* study of the effect of MB on the ATPase activity of a protein-protein interaction. Fundamental to chaperone function is the ability of Hsp70 to hydrolyse ATP. Further analysis of the inhibitory and stimulatory effects of quercetin and methylene blue would entail investigating the chaperone function of TbHsp70.c-Tbj2 with these small molecule modulators using a luciferase refolding assay. Furthermore, *in vivo* assays will be developed to determine the effects of methylene blue and quercetin on TbHsp70.c and the *T. brucei* parasite whilst inducing differentiation from the bloodstream to procyclic form. It is probable that trypanosomal heat shock proteins are more sensitive to small molecule modulators than what has been observed for heat shock proteins from other organisms, including human. Activators from the dihydropyrimidine family increased Hsp70 ATP hydrolysis by ~45%; the activators were demonstrated to preserve tau levels (Jinwal *et al.,* 2009). Together, activators and inhibitors of Hsp70 chaperone activity can be used to provide new mechanistic and therapeutic insights into protein-protein relationships for the development of drug targets. This data provides the first *in vitro* biochemical characterization of TbHsp70.c, providing evidence that co-chaperone Tbj2 likely forms an interaction with TbHsp70.c and that the basal ATPase activity of TbHsp70.c and the chaperone activity of the TbHsp70.c-Tbj2 partnership can be differentially inhibited.

Protein fractions from the major and minor peaks of SEC showed the same level of chaperone activity as displayed by protein purified in the presence of sarkosyl suggesting that protein from the SEC was in its native state. Sarkosyl was demonstrated to have no effect on the chaperone activity of heterologously purified TbHsp70.c or Tbj2. Both TbHsp70.c and

Tbj2 displayed chaperone activity and this study highlighted the fact that Tbj2 has the capability of acting as a co-chaperone of TbHsp70.c. Inhibition of the basal ATPase activity of TbHsp70.c was achieved by both small molecule modulators quercetin and MB. The Tbj2-stimulated ATPase activity of TbHsp70.c as well as the ability of TbHsp70.c to suppress MDH protein aggregation was enhanced by MB. This is the first report that MB enhances chaperone activity. The potential of these and other compounds as modulators of *T. brucei* heat shock proteins needs further investigation to enable identification of likely drug targets against trypanosomiasis.

CHAPTER 5

IN VIVO WORK:

The *in vivo* characterization of TbHsp70.c in the bloodstream form of *Trypanosoma brucei brucei*

5.1. Introduction

The parasitic protozoan *T. brucei* is transmitted periodically between its mammalian host and an insect vector, switching between the respective environments of mammalian blood and tissue fluids, and insect alimentary gut and salivary glands. As the parasite differentiates between the bloodstream and procyclic forms, it needs to adapt to alterations in temperature (28°C and 37°C), pH, nutrients and oxygen tension in order to survive. Some of these stresses could alter the prototypical genetic expression of proteins specifically participating in homeostasis, thereby affecting survival, reproduction and transmission of the parasite. Genes involved in growth are automatically downregulated and proteins associated with cellular survival, such as heat shock proteins, are upregulated (Folgueira and Requena, 2007). Chaperones upregulated under these stress conditions are involved in maintenance of proteostasis, including protecting hydrophobic surfaces exposed in misfolded proteins and during protein refolding, and they are associated with the ubiquitin proteasome system (UPS) and autophagy to facilitate the removal of terminally misfolded polypeptides (Chiang et al. 1989; Dice 1990; Arndt et al. 2007; Kon and Cuervo 2009). Initiation of eukaryotic transcription is augmented through specific heat shock gene factors; other genes undergo selective inhibition of mRNA splicing and nuclear export (Bond, 2006), or transferral of mRNAs to cytoplasmic foci, stress granules, from polysomes (Kedersha *et al.,* 1999); reviewed in Vabulas *et al.*, (2012).

To date very little research has been performed on the heat shock response within *T. brucei*. The focus appears to have rested with investigating the effect of heat stress on *T. cruzi* and/or its vector, *Panstrongylus megistus* (Garcia *et al.*, 2011; Pérez-Morales *et al.*, 2012) as well as on molecular chaperones belonging to the *T. cruzi* parasite (Requena *et al.*, 1992). Heat shock promoters are activated upon cellular stress, the activation of which is dependent on the heat shock element (HSE). Two potential HSE sequences have been identified in the 5´-UTR of *TbHsp70.c* according to the consensus sequence, CNNGAANNTTCNNG of HSE binding site of eukaryotes (Topoi *et al.*, 1985). Both *T. brucei* sequences CACGAAAGTTGTAC and CAGGAAGCATGGTG, 375 bp and 400 bp upstream of the start codon, respectively, have six of the eight HSE conserved nucleotides present, which has been shown to be a sufficient level of conservation (Lee *et al.*, 1990).

However, trypanosomal transcription entails processing of numerous genes on the same transcript into single mRNA segments to which a spliced-leader sequence subsequently gets

attached. Genes are arranged in polycistronic transcription units with over 100 open reading frames. The formation of mature mRNAs occurs through co-transcriptional 5´-*trans* splicing of a capped spliced leader and 3´-polyadnylation (Liang *et al.,* 2003). Through studies of phosphoglycerate kinase, the rate of splicing, however, was shown to not affect mature mRNA concentrations (Haanstra *et al.,* 2007). Information pertaining to the stability of mRNAs post heat stress is thus located in the 3´-UTR (Haüsler and Clayton, 1996; Lee, 1998). A single putative heat shock element was identified in the 3´-UTR 43 bp downstream of *TbHsp70.c* (CCAGAAGTTTCTGA). The expression of *T. brucei* heat shock genes was recently demonstrated to be regulated through RNA-protein interactions (Droll *et al.,* 2013). The *T. brucei* zinc finger CCCH protein, ZC3H11, stabilizes mRNAs encoding for protein refolding chaperones upon heat stressing *T. brucei* procyclic form cells and was also shown to be essential to the survival of bloodstream form cells through RNA interference studies (Droll *et al.,* 2013). ZC3H11 stabilizes mRNA by binding AUU and AUUU repeats in the 3´UTR; *Hsp70* mRNA was observed to decrease upon knockdown of ZC3H11 (Droll *et al.,* 2013). Two AU-rich regions were identified in the 3´-UTR: $(AAT)_2$TTTTTTA [$(AAU)_2$UUUUUUA repeat on RNA), located 29 bp downstream of *TbHsp70.c*, and TATGTTTTTTATAAAAT (UAUGUUUUUUAUAAAAU on RNA), 280 bp downstream of *TbHsp70.c*. Interestingly, no AUU repeats have been indentified in *L. infantum Hsp70* even though the 3´UTR was shown to be required for the stability of mRNA subsequent to heat shock (Quijada *et al.,* 1997; Quijada *et al.,* 2000). Similarly, *L. Mexicana Hsp83* and *L. braziliensis Hsp100* are devoid of AUU repeated in the 3´UTR (David *et al.,* 2009). It is thus evident that the 3´UTR is necessary for mRNA stability; however, due to a lack of conservation of this region, different recognition sequences may be required amongst the kinetoplastids.

A different form of stress to the parasitic cell is depleting it of these heat shock proteins and investigating whether the knockdown of the protein is detrimental to the cell. For instance, the depletion of a yeast Hsp40 protein, Ydj1, was shown to cause a temperature-sensitive phenotype in which *S. cerevisiae* cells grew at a reduced rate at lower temperatures than the usual 30°C (Caplan and Douglas, 1991; Johnson and Craig, 2000). African trypanosomes create death and devastation in both humans and livestock (Simarro *et al.,* 2008) and have had focus placed on them such that they now represent a model for parasite biology, especially being RNAi-proficient (Atayde *et al.,* 2011; Kolev *et al.,* 2011). By definition,

RNAi is a naturally-occurring process in which the presence of cellular double-stranded RNA (dsRNA) initiates the degradation of homologous mRNA. RNAi as a mechanism of gene silencing was "serendipituously" discovered upon finding strange morphological modifications when *T. brucei* cells were transfected with dsRNA of the α-tubulin mRNA 5′ untranslated region (Ngo *et al.*, 1998).

This technology has since been used extensively in *T. brucei*; RNAi and the existing inducible and heritable system, is a major tool in on-going investigations of the trypanosome biology (Atayde *et al.*, 2011; Kolev *et al.*, 2011). For instance, in the past decade, over 500 publications have studied or referred to the usefulness of RNAi in *T. brucei* (Kolev *et al.*, 2011). One of the largest contributions in this regard, was the very recent development of a genome-wide RNAi screen, the RNA Interference Target Sequencing (RIT-Seq) by Alsford and colleagues (2011), providing a cohort of genes shown to be essential to *T. brucei* parasitic survival. Interestingly, *Trypanosoma cruzi* (DaRocha *et al.*, 2004) and *Leishmania major* (Robinson and Beverley, 2003), also from the TriTryps, and malarial parasite *Plasmodium falciparum* (Baum *et al.*, 2009), do not execute RNAi. Yet, the introduction of RNAi into budding yeast suggests that RNAi may still be a possibility in the afore-mentioned RNAi-deficient parasites, through it having provided a proof of principle (Drinnenberg *et al.*, 2009). *L. (Viannia) braziliensis* has been demonstrated to be RNAi-proficient (Peacock *et al.*, 2007; Lye *et al.*, 2010).

The RNAi machinery is typically comprised of an RNase III Dicer endonuclease, processing dsRNAs into small interfering RNAs (siRNAs) with two-nucleotide 3' overhangs; an Argonaute (AGO) "slicer" endonuclease digests target mRNA when complexed with single stranded siRNA and a Dicer cofactor aids the biogenesis of siRNA and transferral thereof into AGO. The AGO-siRNA complex shapes cleaving machinery of the RNA-induced silencing complex (RISC) (Hammond *et al.*, 2000). Present in select organisms with the RNAi pathway is an RNA-dependent RNA polymerase that specifically enhances the RNAi response by generating secondary siRNAs (Cerutti and Casas-Mollano, 2006). The *T. brucei* RNAi components thus far identified, include an AGO slicer, *Tb*AGO1 (Durand-Dubief and Bastin, 2003; Shi *et al.*, 2004c), and two Dicers, *Tb*DCL1 (Shi *et al.*, 2006c) and *Tb*DCL2 (Patrick *et al.*, 2009); knowledge of an RNAi-specific dsRNA-binding protein is absent (Shi *et al.*, 2006b). Nuclear *Tb*DCL2 initiates the process by targeting dsRNAs from retroposons

and satellite-like repeats (CIR147) (Shi *et al.,* 2006a). Cytoplasmic *Tb*DCL1 targets the dsRNA molecules produced by *Tb*DCL2 and any nucleic or cytoplasmic dsRNA still unprocessed (Patrick *et al.,* 2009). siRNA products from both Dicers are channelled into *Tb*AGO1 (Durand-Dubief and Bastin, 2003; Shi *et al.,* 2004c). *Tb*AGO1 targets single-stranded siRNAs (Shi *et al.,* 2004d, Shi *et al.,* 2009). RNA Interference Factor 5 (*Tb*RIF5) was very recently demonstrated to act in partnership with *Tb*DCL1 and to be necessary for cytoplasmic and not nuclear RNAi (Barnes *et al.,* 2012). Duplex siRNAs were shown to increase and avoid association with *Tb*AGO1 in the absence of *Tb*RIF4 (Barnes *et al.,* 2012). The concept of the forward and reverse genetics combined essentially implemented in RNA interference was introduced by Morris and colleagues in procyclic African trypanosomes (2002). RNAi knockdown has been achieved *in vivo* in a mouse model infected with *T. brucei brucei*, in which RNAi of a cathepsin B-like enzyme, TbCatB, was observed to prevent certain death, in which splenomegaly was avoided and no parasites were detected in the blood (Abdulla *et al.,* 2008). Amongst others, RNA interference has been performed on *T. b. brucei* Hsp40 proteins, including Type IV/I Hsp40 Tbj47 (Tb927.1.1230) (Subramaniam *et al.,* 2006) and Type I Tbj2 (Ludewig and Blatch, 2013), both demonstrating a reduced growth rate of cells.

Both heat stress and depletion of heat shock proteins may influence the cell division cycle. The eukaryotic cell cycle involves hundreds of genes expressed at necessary times, and requires cell cycle-regulated transcription factors that arbitrate organized, developing waves of transcription. Conversely, the kinetoplastids were believed to basically lack transcriptional regulation. However, the central importance of post-transcriptional regulation in kinetoplastids was emphasized by the discovery, through transcriptome profiling, that over 430 genes are regulated during the cell cycle; their functions coinciding with cellular processes occurring at times of peak expression (Archer *et al.,* 2011).

Cell division within the kinetoplastids occurs in a typical well-ordered way. Atypical though, is the presence of single-copy organelles and structures such as the mitochondria, endoplasmic reticulum (ER), Golgi and flagellum in G1-phase cells; they are replicated at particular time points in the cell division cycle (McKean, 2003; Ho *et al.,* 2006; Hammarton, 2007). The *T. brucei* cell cycle can be divided into four phases: the early G1 phase - the probasal body matures allowing a new flagellum to form and subsequent replication of the Golgi apparatus (Hammarton, 2007); the late G1 phase – maturation of the probasal body into

a basal body, soon to localize at the base of the new flagellum, and the kinetoplast in S-phase; producing a cell with one nucleus and one kinetoplast (1N:1K). S-phase – elongation of the new flagellum attached to the old one by a mobile flagellar connector (Vaughan, 2010), whilst the basal bodies and kinetoplasts detach. DNA replication occurs and a cell with one nucleus and two kinetoplasts (1N:2K) is produced (Siegel *et al.*, 2008). G2-phase - an intranuclear mitotic spindle is produced subsequent to DNA replication, after which cell undergo mitosis. Prior to cytokinesis the Chromosomal Passenger Complex transfers to the cell anterior from the spindle, and progresses towards the posterior along with the cytokinetic furrow (Li *et al.,* 2008). A cell with two nuclei and two kinetoplasts is produced (2N:2K) (Hammarton *et al.,* 2007). Cytokinesis ensues and the two daughter cells remain attached for a period before the final cell abscission occurs (Robinson and Gull. 1991).

Interestingly, the depletion of Tbj2 by RNAi interference, shown to result in a loss of fitness of *T. b. brucei* bloodstream form cells, and cells stressed at 42°C did not significantly reflect a change in the proportion of cells in the various cell division stages when compared with *T. b. brucei* wildtype cells (Ludewig and Blatch, 2013). Protein synthesis and subsequently the ability of cells to divide would be expected to decrease upon depletion of a protein shown to be essential to the cell. The likelihood of Tbj2 acting as co-chaperone to TbHsp70.c was established as reported in Chapter 4, in which Tbj2 was shown to both enhance the ability of TbHsp70.c to suppress MDH aggregation (Section 4.3.1.2), as well as enhance the ATPase activity of TbHsp70.c (Section 4.2.2.2). TbHsp70.c was predicted to localize in the cytoplasm (discussed in Section 2.1) and Tbj2 has been shown to be cytoplasmic and essential to the survival of the *T. brucei* parasite (Ludewig and Blatch, 2013). This study set out to establish the subcellular localization of TbHsp70.c, determine the effect of knockdown of TbHsp70.c on the *T. b. brucei* cells and investigate the partnership between TbHsp70.c and Tbj2.

The broad objectives of this study were to perform an *in vivo* characterization of TbHsp70.c. The aims of the study included:

1. Investigation of the stress inducible expression of TbHsp70.c at increased temperatures.

2. Investigation of the proposed TbHsp70.c-Tbj2 partnership through binding studies using parasite lysates.

3. Inhibition of TbHsp70.c expression through RNA interference and assessment of the phenotypic effects on *T. brucei brucei* bloodstream form cells.

4. Determination of the subcellular localization of TbHsp70.c.

5.2. Methods and materials

5.2.1. Culturing *Trypanosoma brucei brucei* laboratory strains

Various *T. b. brucei* laboratory strains, all bloodstream stage, were cultured during the course of this study, including the *T. b. brucei 427* strain variants 221 and 3, a kind donation from Professor George Cross (Rockefeller University, New York, USA), and the *T. b. brucei* Single Marker Bloodstream form (SMB, *T7RNAP::TETR::NEO*) strain, which is modified from the *T. b. brucei* Lister 427 cell line by integrating genes for the T7 RNA polymerase and the tetracycline-repressor (Wirtz *et al*., 1999). Cells were maintained in filter sterilized complete Iscoves Modified Dulbeccos Media (IMDM) based HM1-9 medium [IMDM base powder, 3.6 mM sodium bicarbonate, 1 mM hypoxanthine, 1 mM sodium pyruvate, 0.16 mM thymidine, 0.05 mM bathocuprone sulphate acid, 10% (v/v) heat inactivated Foetal Bovine Serum, 1.5 mM L-cysteine, 0.2 mM β-mercaptoethanol, pH 7.5] in a humidified chamber at 37°C with an atmosphere of 5% CO_2. SMB cells were treated with 1 mg/L (final concentration) neomycin (G418) to maintain the selective pressure. Cells were kept at a density below $2x10^6$ cells/ml due to secretion of a cell division inhibitory factor should bloodstream form cells reach high cell densities (Reuner *et al.,* 1997). Growth was measured by using a Neubauer haemocytometer to count the cell number, after which cells were diluted, according to their density, in medium pre-warmed to 37°C in a water bath.

5.2.2. Detection of target protein in *T. b. brucei* lysates using polyclonal rabbit anti-TbHsp70.c peptide antibodies

The ability of rabbit anti-TbHsp70.c peptide antibody to detect target protein was assessed using different trypanosomal cell lines. Whole cell lysates were prepared from *T. b. brucei 427* v.221, *427* v.3 and SMB cell lines by allowing a 20 ml culture of each to grow to a density of ~$1x10^6$ cells/ml at 37°C (5% CO_2) prior to centrifugation at 800*g* for 10 minutes. Cell pellets were washed in 5 ml PBS (10 mM Na_2HPO_4; 2 mM KH_2PO_4; 137 mM NaCl; 2.7 mM KCl) followed by centrifugation at 800*g* for 10 minutes. After removal of the

supernatant, cells were resuspended in SDS-PAGE loading buffer ($5x10^5$ cells/µl). An *E. coli* whole cell lysate and recombinant TbHsp70.c in *E. coli* BB1994 [pQE80-TbHsp70.c] were prepared as negative and positive controls, respectively. Lysates were resolved on 10 % SDS-PAGE gels (Section 3.2.2.2.1), such that equal numbers of parasitic cells were compared ($5x10^6$ cells/lane). To determine the optimal concentration of rabbit anti-TbHsp70.c peptide antibody for western analysis (performed as described in Section 3.2.2.2.1), various dilutions of antibody (1:2000; 1:5000; 1:10 000) were assessed using whole cell lysates prepared from *T. b. brucei 427* v.3, and goat anti-rabbit secondary antibody (1:5000). Pre-immune rabbit serum at a dilution of 1:5000 was used as a negative control.

5.2.3. Detection of TbHsp70.c in *T. b. brucei* lysates under conditions of heat stress

Wild type Lister 427 variant 221 *T. brucei brucei* bloodstream form lysates (10^6 cells/ml) were used for the heat shock inducibility experiment. Separate 25 ml cultures of cells were exposed to heat shock at 42°C for a period of 60 min in plugged flasks, allowing no entry of CO_2. A control experiment was performed under the same conditions except the temperature was kept at 37°C. Cell lysates were harvested by centrifugation at 800*g* for 10 min, washed once in PBS buffer (10 mM Na_2HPO_4; 2 mM KH_2PO_4; 137 mM NaCl; 2.7 mM KCl) and re-pelleted prior to resuspension in SDS-PAGE loading buffer to a final cell count of 5 x 10^5 cells/µl. The lysates (5 x 10^6 cells per lane) were resolved on a 10% SDS-PAGE gel. Differences in TbHsp70.c protein expression were detected using polyclonal rabbit anti-TbHsp70.c peptide antibody (1:5000) and goat anti-rabbit IgG HRP-conjugated secondary antibody (1:5000) in subsequent Western analysis.

5.2.4. *In vivo* assessment of the ability of Tbj2 to bind TbHsp70.c

5.2.4.1 Immunoprecipitation analysis using polyclonal rabbit anti-TbHsp70.c peptide antibodies to target Tbj2

T. b. brucei 427 v.221 cells were cultured in 50 ml medium and pelleted as previously described. Cells were lysed in 1 ml RIPA buffer [50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 1% (w/v) Nonidet P-40, 1 mM sodium deoxycholate, 1 mM phenylmethsulfonylfluoride (PMSF), 2 mg/L protease inhibitor cocktail] at 4°C for 2 hours. Cellular debris was removed by centrifugation at 12 000*g* for 30 minutes followed by incubation with 1:5000 dilution primary TbHsp70.c antibody at 4°C for 2 hours. Coupling of antibody to 20 µl Protein A/G PLUS Agarose beads (Santa-Cruz Biotechnology) was left to

occur for 18 hours at 4°C with gentle agitation. Beads were subsequently washed 4 times with PBS (centrifugation at 1000*g* for 5 minutes at 4°C). Bound protein was assessed by SDS-PAGE (10%) and western analysis using rabbit anti-TbHsp70.c peptide antibody; antibodies were detected using the ECL Advance Blotting Detection Kit and viewed using the Chemidoc™ EQ (BioRad, U.S.A.).

5.2.4.2. Pull-down assay of recombinant Tbj2 to target TbHsp70.c

His-tagged Tbj2 (1µg) purified from *E. coli* BL21 (DE3) was pre-incubated with 10 µl Protein G Agarose (Sigma-Aldrich) flast flow bead suspension (50% slurry) in 100 µl incubation buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl2, 0.01% Nonidet P-40, pH 7.5) at 4°C for 2 hours with gentle agitation. A 50 ml culture of *T. b. brucei 427* v.221 cells were washed and pelleted as previously described and lysed in RIPA buffer at 4°C for 2 hours with gentle agitation. The lysed cells and protein suspension were mixed and incubated for a further hour at 4°C. The beads were collected by centrifugation (1500*g*, 2 minutes), washed 3 times with incubation buffer and analysed by SDS-PAGE and western analysis using rabbit anti-TbHsp70.c peptide antibodies and anti-His antibodies (Amersham Pharmacia Biotech, UK), both at a dilution of 1:5000. Secondary goat anti-rabbit and anti-mouse antibodies were used in the respective immunoblots and antibodies were detected using the ECL Advance Blotting Detection Kit and viewed using the Chemidoc™ EQ (BioRad, U.S.A.).

5.2.5. RNA interference in *T. b. brucei*

5.2.5.1. RNAi target primer design and generation of the RNAi construct

The plasmid used, p2T7^{TaBlue}, containing opposing T7 promoters, allows tetracycline-induced transcription of double-stranded RNA (dsRNA) in transfected parasites (Alibu *et al.*, 2005). Primers for RNA interference target sequences against TbHsp70.c were designed using the RNAit algorithm (Redmond *et al.*, 2003), available as a web-based interface (http://trypanofan.path.cam.ac.uk/software/RNAit.html). Suitable pairs of primers are generated from an input nucleotide sequence for RNA interference targets within the *T. b. brucei* genome sequence. A forward primer (5' – TTG CTG ATA TAA GGA ACC GT – 3') at position 730 bp and a reverse primer (5' – GTT CCG ACG AAT TAT CAC AT – 3') at position 1268 bp were synthesized by Integrated DNA Technologies, Inc. (IDT) with resulting Tm's of 51.1°C and 50.2°C, respectively. The PCR reaction parameters were modified (Stage 1: 96°C for 3 min; Stage 2: 25 cycles of 96°C for 30 s, 45°C for 2 min, and

72°C for 1 min; Stage 3: 1 cycle at 72°C for 10 min); a PCR product of 539 bp was produced using the RNAi primers (0.6 µM) and 100 ng *T. brucei TREU927* genomic DNA (Table 3.3). The PCR product was resolved on an agarose gel (0.8%), gel purified (Section 3.2.2.1.3), ligated into p2T7^{TaBlue} using the A/T overhangs created by *Eam*1105I digestion, and transformed into *E. coli* DH5α with ampicillin selective pressure (Section 3.2.2.1.2). Plasmid DNA was isolated from the transformants and screened by *Not*I digests and resolved on an agarose gel (Section 3.2.2.1.4). The resulting plasmid was named p2T7^{TaBlue}TbHsp70.c.RNAi.1 (Fig. 5.4A). For subsequent transfection into *T. b. brucei* cells, plasmid DNA was isolated using GenElute^{TM} Endotoxin-free Plasmid Midiprep Kit (Sigma-Aldrich) according to manufacturer's directions. DNA was eluted with warmed (50°C) endotoxin-free water.

5.2.5.2. Preparation and transfection of the RNAi target construct

The DNA used for the transfection should be of a high quality, such that it is pure, and free of nicking and denaturation. Linearized DNA was required for transfection into *T. b. brucei* cells; midiprepped DNA (32 µg) was bulk digested with *Not*I (0.1 U/µg DNA) and resolved on an agarose gel (Section 3.2.2.1.4). The remainder of the linearized DNA was phenol:chloroform extracted. The digest was mixed with equal volumes of phenol:chloroform and centrifuged at 12 000*g* for 2 minutes to separate the aqueous from the inorganic phase. The aqueous phase was washed twice with equal volumes of chloroform (12 000*g*, 2 minutes) and followed by an ethanol (EtOH) precipitation. The aqueous layer was mixed with 4 times its volume pre-chilled absolute EtOH and left at -80°C overnight. The DNA/EtOH precipitate was isolated (12 000*g*, 30 minutes, 4°C) and EtOH extracted. The DNA pellet was washed four times with an equal volume of pre-chilled 70% EtOH (endotoxin-free water) (12 000*g*, 15 minutes, 4°C). The precipitated DNA was left to air dry under sterile conditions (laminar flow hood) after which it was resuspended in endotoxin-free water. Upon quantification, a yield of 11 µg DNA was obtained. An overnight culture of *T. b. brucei* SMB cells grown to ~1x10^6 cells/ml were harvested (800*g*, 10 minutes) to yield ~2.5x10^7 cells, the cell density required per transfection. Cells were washed once in 5 ml PBS (800*g*, 10 minutes) in a sterile 15 ml tube, transferred to a 1.5 ml microfuge tube and centrifuged again (800*g*, 10 minutes) to remove all trace of supernatant. Cells were resuspended in 400 µl room-temperature transfection medium consisting of sterile ZFPM [132 mM NaCl, 8 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 1.5 mM $Mg(OAc)_2$, 90 µM $Ca(OAc)_2$] and glucose (50 mM) for

BSF transfections and mixed with 11 µg prepared plasmid DNA. The solution was transferred to a pre-cooled 4 mm gap electroporation cuvette and two square-wave pulses with 10 s between pulses (1.5 kV, 25 µF) were administered using a BioRad Gene Pulser II. The cells were rapidly transferred to 48 ml pre-warmed (37°C) fresh HMI-9 medium and incubated at 37°C for 30 minutes prior to aliquoting out 1 ml per well of 24-well culture plates. The plates were incubated at 37°C (5% CO_2) overnight to allow cells to revive before selective pressure was provided with double strength antibiotic [hygromycin (5 mg/L) and G418 (1 mg/L)] contained in an additional 1 ml of pre-warmed medium added to each well. The cells were incubated at 37°C (5% CO_2) for a further period of 4 days and transfectant populations were identified in the wells in which dense cell populations were observed. Clones were picked by diluting the entire 2 ml from the well into 5 ml medium with appropriate antibiotics for further studies. All stages of the transfection procedure were kept sterile, and handling was conducted in a biosafety level II sterile flow hood system.

5.2.5.3. Assessment of the phenotypic effects of TbHsp70.c knockdown using a growth curve assay

The effect of TbHsp70.c knockdown on the growth rate of *T. b. brucei* cells was investigated by performing a growth curve assay using two clones obtained from the separate transfection events of p2T7^{TaBlue}TbHsp70.c.RNAi.1, isolated from separate wells of the 24 well plates (Section 5.2.5.2). The selective pressure was maintained as described previously. Each clone was divided into two separate cultures, one of which had tetracycline (1 mg/L) added to induce RNA interference. The experiment was initiated by the dilution of each 10 ml culture to 1x10^5 cells/ml once the cell densities had been determined using the haemocytometer. This was achieved by dividing the required cell number (cells/ml) by the current cell number (cells/ml) and multiplying by the total volume:

$$\text{Volume cells to dilute (ml)} = \frac{(1 \times 10^5 \text{ cells/ml})}{\text{(current cell number)}} \times 10 \text{ ml}$$

The resulting volume was then diluted into a final volume of 10 ml medium, and incubated for 24 hours at 37°C. The process was repeated every 24 hours for the duration of 72 hours. The bloodstream form parasites typically produce a factor that initiates arrest of the cell cycle once the cell population reaches above 2 x 10^6 cells/ml (Reuner *et al.,* 1997; Vasella *et al.,* 1997), making dilution of the cultures necessary to obtain an accurate analysis of the growth

curve. Upon completion of the growth curve, cell cultures were harvested and prepared for SDS-PAGE and western analysis (described in Section 5.2.3).

5.2.6. Investigation of the subcellular localization of TbHsp70.c using anti-TbHsp70.c peptide antibodies

Prior to culturing cells, coverslips were coated with poly-L-Lysine (70 – 150 kDa). Coverslips were washed in absolute EtOH overnight and 5 rinses in $ddd H_2O$ were performed before the etching in 50% HNO_3 for 12 hours at room temperature. The coverslips were repeatedly rinsed in $ddd H_2O$ for 30 minutes, after which they were sterilized with absolute ethanol, care being taken that rinses be thorough as a solution of nitric acid and ethanol is highly unstable. The coverslips were allowed to dry in a 24-well plate, after which sterile poly-L-Lysine (0.1 mg/ml) was added to each well, enough to coat, and incubated at 37°C for 24 hours. Coverslips were dried, and re-coated with poly-L-Lysine, repeating the process. After rinsing twice with sterile $ddd H_2O$ and drying, the coverslips were ready for use and each placed in a 24-well culture plate. *T. b. brucei 427* v.221 cells (30 ml) were cultured to a density of ~$1.5x10^6$ cells/ml as in other experiments. Cells were pelleted (800*g*, 10 minutes) and all but a little (~1.5 ml) of the medium was poured off; a half-volume PBS:FBS (1:1) was added to the resuspended cells. The cell suspension was aliquoted (200 µl) into a 24-well culture plate containing poly-L-lysine coated coverslips and incubated at room temperature for 15 minutes. 1% formaldehyde/PBS (1:1) (50 µl) was added to each well to fix the cells, and incubated at 4°C overnight. The medium was removed carefully so as to not dislodge the cells, after which they were permeabilized by the addition of 200 µl 0.5% Triton X-100 in PBS/FBS (1:1) for 30 minutes at room temperature. Once the Triton X-100 solution had been removed, rabbit anti-TbHsp70.c peptide antibody, diluted 1:1000 in 25% FBS/PBS was added and incubated for 1 hour at room temperature. The cells were washed twice with 25% FBS/PBS, each with 10 minute incubations. Cells were allowed to incubate for 1 hour in light-sensitive anti-rabbit Alexa Fluor® 488 Dye (Invitrogen, A11008) (excitation maximum is 495 nm and emission maximum is 519 nm), diluted 1:1000 in 25% FBS/PBS, followed by 3 washes as before. Propidium iodide (1 mg/ml) (excitation maximum is 535 nm and emission maximum is 617 nm) was used as a nuclear counterstain, diluted 1:10 in 25% FBS/PBS, was added to the last wash. The coverslips were mounted on slides using Dako fluorescent mounting medium (DAKO, S3023), allowed to set overnight and sealed with clear nail varnish. The fluorescing cells were viewed under oil immersion with a fluorescence

microscope (100 x objective) with excitation at different wavelengths of light. In addition to determining the localization of TbHsp70.c, cells were analysed for their stage of the cell division cycle, observing either the possession of 1 nucleus: 1 kinetoplast (1N:1K), 1N:2K or 2N:2K.

5.3. Results

5.3.1. TbHsp70.c in *T. b. brucei* lysates

The synthesis of peptides and production of an antibody against a TbHsp70.c peptide allowed for the *in vivo* detection of the target protein in *T. b. brucei* whole cell lysates, using western blot analysis. An *E. coli* whole cell lysate was used as a negative control, and recombinant TbHsp70.c purified from *E. coli* BB1994 cells as a positive control experiment (Fig. 5.1). Anti-TbHsp70.c peptide antibody did not show any cross-reactivity with an *E. coli* whole cell lysate (Fig. 5.1 lane 1) or with purified recombinant Tbj2 (Fig. 5.3B lane 4). A single protein at 73 kDa was detected using purified recombinant TbHsp70.c (Fig. 5.1). Three different strains of *T. b. brucei* were analysed for the presence of TbHsp70.c: probing the SMB whole cell lysate resulted in detection of 3 bands, 73 kDa (TbHsp70.c), one at ~50 and ~60 kDa, as of yet unidentified proteins; probing V221 also produced 3 bands, 73 kDa (TbHsp70.c), one band just below it (~68 kDa) and one band at ~50 kDa; western analysis of V3 produced two bands, again TbHsp70.c at 73 kDa, and the second band at ~50 kDa (Fig. 5.1). Anti-TbHsp70.c peptide antibody thus demonstrated cross-reactivity in the trypanosomal lysates. Probing the V3 strain with anti-TbHsp70.c peptide antibody produced the least number of contaminants but targeted TbHsp70.c to a lesser extent and a 50 kDa band to a greater extent. However, the V3 strain was not used for subsequent experiments as cell survival could not be maintained (data not shown). Different dilutions of antibody were analysed to determine the optimal concentration for future experiments, the anti-TbHsp70.c 1:5000 dilution was used in all subsequent immunoblots (Fig. 5.1).

5.3.2. TbHsp70.c expression is upregulated by heat shock

Under conditions of stress, a cell responds by increasing the synthesis of heat shock proteins to manage the elevated levels of denatured proteins. A heat shock experiment was thus performed to determine whether TbHsp70.c protein levels increased after the cell was exposed to heat stress. Levels of TbHsp70.c were monitored after inducing heat stress at 42°C

using SDS-PAGE and western analysis *T. b. brucei 427* V221 cells were subjected to heat stress for an hour.

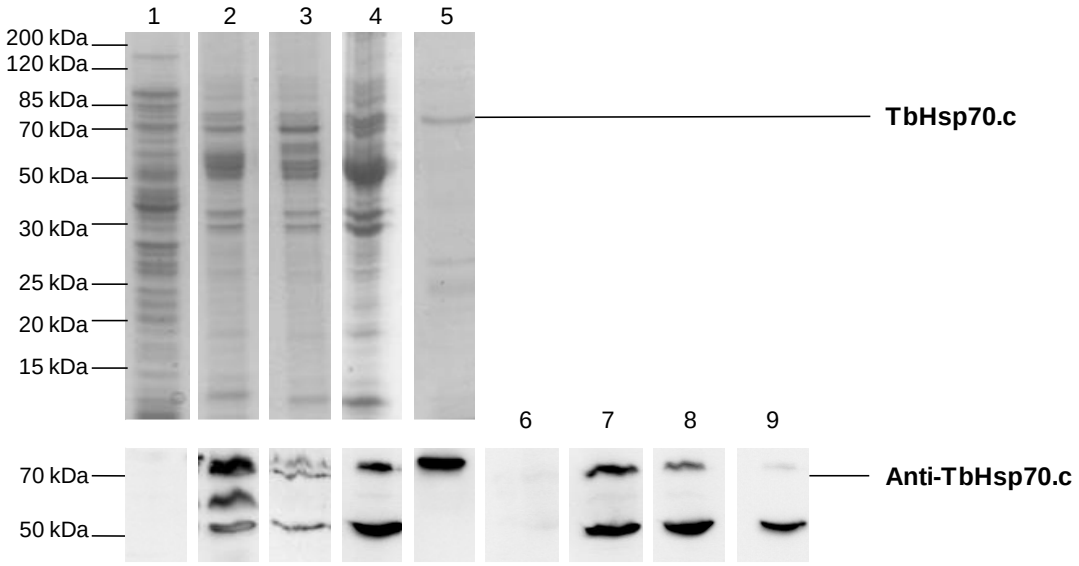


Figure 5.1. Detection of TbHsp70.c in *T. brucei* lysates prepared from different cell lines to assess the rabbit anti-TbHsp70.c peptide antibody.
TbHsp70.c was detected in various *T. brucei brucei* cell lines to assess the specificity of the rabbit anti-TbHsp70.c peptide antibody at a 1:5000 dilution (*lanes 1 – 5*). *Lanes 1 – E. coli* whole cell lysate; *2 – T. brucei brucei* single marker bloodstream form (SMB) whole cell lysate; *3 – T. b. brucei 427* bloodstream form cells variant (v) 221 whole cell lysate; *4 - T. b. brucei TREU927* v.3 whole cell lysate; *5* – recombinant TbHsp70.c in *E. coli* BB1994 [pQE80-TbHsp70.c]. The presence of TbHsp70.c was probed for in *T. b. brucei 427* v.3 whole cell lysates using varying concentrations of rabbit anti-TbHsp70.c peptide antibodies: *Lanes 6* – pre-immune serum at a 1:5000 dilution; *7* – anti-TbHsp70.c at a 1:2000 diltuion; *8* – 1: 5000 diltuion; *9* – 1:10 000 dilution. Goat anti-rabbit secondary antibody (1:5000) was used.

Recombinant TbHsp70.c purified from *E. coli* BB1994 cells was used as a positive control. Even though there was no difference in the TbHsp70.c levels on the SDS-PAGE gel, TbHsp70.c protein expression proved to be inducible by heat stress, as shown by an increase in the top band in the western analysis (Fig. 5.2). Three bands were detected by the anti-TbHsp70.c antibody. The top band 73 kDa increases in intensity from 37°C to 42°C indicating that at 37°C TbHsp70.c is expressed in bloodstream form cells, and upon exposure to heat stress TbHsp70.c was upregulated. Interestingly, the expression level of the 68 kDa band remained constant upon heat shock, which would suggest that a protein not associated with TbHsp70.c is represented (Fig. 5.2). The 50 kDa band appears to decrease upon elevated

temperatures (Fig. 5.2). A loading control such as anti-β-tubulin antibody should have been included.

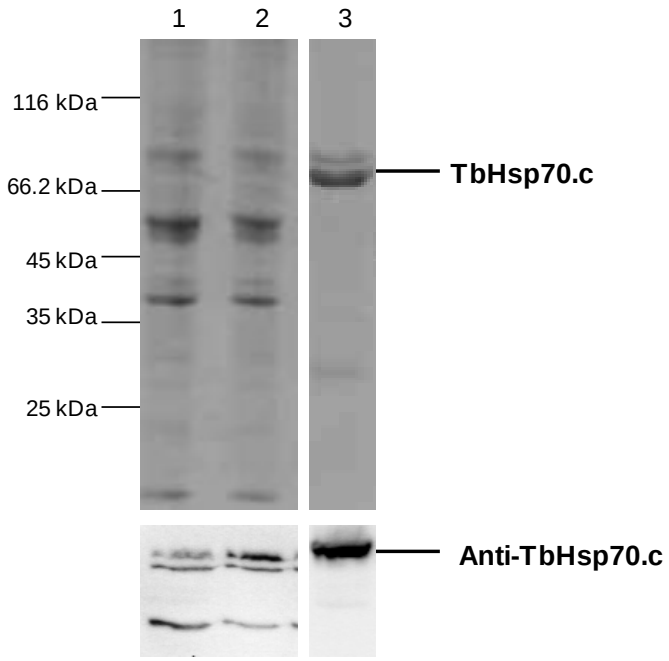


Figure 5.2. TbHsp70.c expression is enhanced under heat stress.
Detection of TbHsp70.c expression levels in a whole cell lysate prepared from Lister 927 *T. b. brucei* v221 cells in the absence (37°C) and presence (42°C) of heat stress by 10% SDS-PAGE (5 x 10^6 cells/lane) and western analysis revealed that TbHsp70.c is heat inducible. *Upper panel: Lanes 1* – cells incubated for 1 hour at 37°C; *2* – cells incubated for 1 hour at 42°C; *3* – purified recombinant TbHsp70.c in *E. coli* BB1994 [pQE80-TbHsp70.c]. *Lower panel: Lanes 1 – 3* - detection of target protein by western analysis using anti-TbHsp70.c antibody, showing increased expression levels of TbHsp70.c under heat stress.

5.3.3. Assessment of the ability of Tbj2 to bind with TbHsp70.c

5.3.3.1. Immunoprecipitation using anti-TbHsp70.c to target Tbj2

The ability of Tbj2 to interact as a co-chaperone of TbHsp70.c was investigated using immunoprecipitation analysis with TbHsp70.c antibodies (Fig. 5.3A). The resulting data led to ambiguous results. It was unclear that Tbj2 had co-precipitated with TbHsp70.c (Fig. 5.3A4) due to the correlation between the molecular mass of Tbj2 (44 kDa) and that of the antibody heavy chain (~50 kDa). Recombinant Tbj2 resolved on SDS-PAGE and Ponceau stained served as a positive control (Fig. 5.3A *lane 2*) Whole cell lysates incubated with agarose beads indicated that Tbj2 did not recognize the beads in the negative control (Fig. 5.3A *lane 3*). A comparison between rabbit anti-TbHsp70.c peptide antibody resolved on

SDS-PAGE and Ponceau stained (Fig. 5.3A *lane 1*) and the immunoblotted precipitated proteins (Fig. 5.3A *lane 4*) showed that TbHsp70.c had been precipitated (~73 kDa). An alternative strategy would have been to use rabbit anti-Tbj2 peptide antibody to co-precipitate TbHsp70.c. However, rabbit anti-Tbj2 peptide antibody is highly non-specific and many proteins are detected when a *T. brucei* whole cell lysate is probed with the antibody (data not shown).

5.3.3.2. Pull-down assay recombinant Tbj2 to target TbHsp70.c

The inconclusive data from the immunopreciptation experiment prompted a further binding study to assess the TbHsp70.c-Tbj2 partnership. A pull-down assay is often performed by incubating recombinant purified proteins with Agarose beads. In this study, purified His-tagged Tbj2 was incubated with beads and a *T. brucei* lysate was passed over the beads in an attempt to pull down TbHsp70.c. Controls of *T. brucei* whole cell lysate and recombinant protein indicated the presence of the target protein within the lysate (Fig. 5.3B *lanes 1,4,5*). The lane containing the *T. brucei* whole cell lysate was overloaded and over exposed (Fig. 5.3B *lane 1*). Lane 4 shows no band because anti-TbHsp70.c does not detect recombinant purified Tbj2 (Fig. 5.3B *lane 4*). Non-specific binding of TbHsp70.c to the Agarose beads was observed in the absence of Tbj2 in the control experiment (Fig. 5.3B *lane 2*), which consisted of lysate incubated with agarose beads. The same endogenous levels of TbHsp70.c were observed in the pull down (Fig. 5.3B *lane 3*). The second protein band (50 kDa) (Fig 5.3B *upper panel: lanes 2,3*) is an artefact of TbHsp70.c antibody, routinely observed with all immunoblots. The pull-down assay thus proved inconclusive as well.

5.3.4. RNA interference in *T. b. brucei*

5.3.4.1. Successful isolation of p2T7^{TABlue}TbHsp70.c.RNAi.1

The RNAi target sequence to *TbHsp70.c* was amplified from *T. brucei* genomic DNA and confirmation of the p2T7^{TaBlue}TbHsp70.c.RNAi.1 construct (Fig. 5.4A) was achieved by restriction digestion analysis (Fig. 5.4B). Digestion at the single *Not*I site linearized the plasmid of 6210 bp (Fig. 5.4B).

5.3.4.2. Partial knockdown of TbHsp70.c by RNA interference

The goal of the RNA interference studies was to validate the *in vivo* effects of RNAi on TbHsp70.c protein expression and the *T. b. brucei* phenotype. Analysis of TbHsp70.c

expression following transfection showed inhibition of TbHsp70.c protein expression upon tetracycline-induced RNAi (Fig. 5.5A). However, the knockdown of endogenous TbHsp70.c

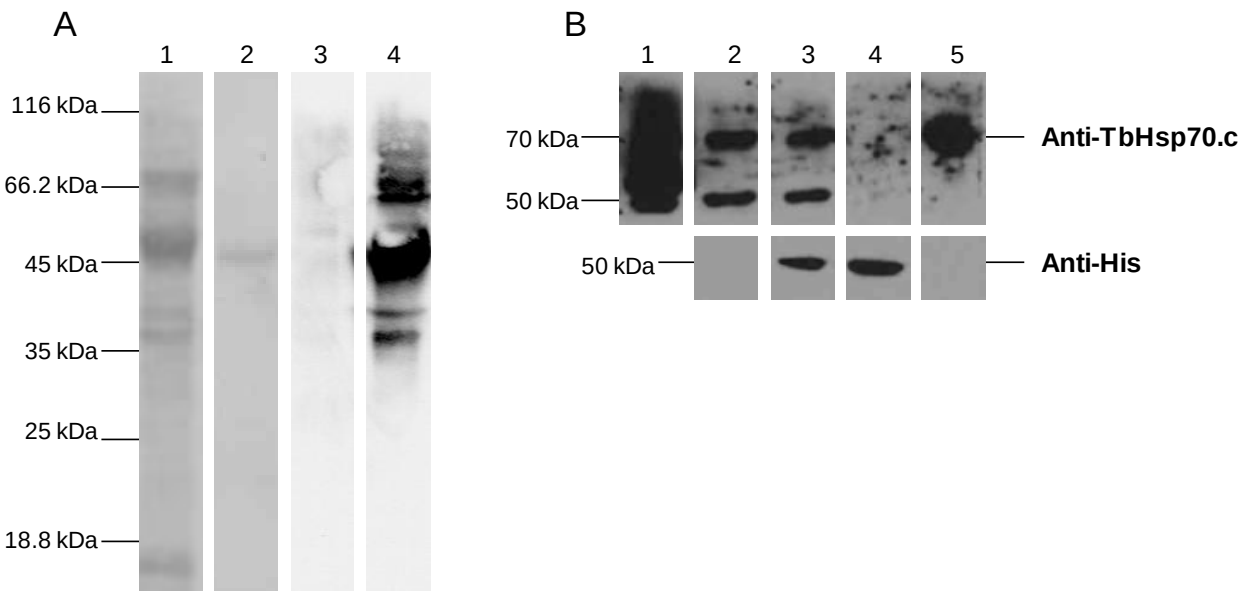


Figure 5.3. *In vivo* binding studies of TbHsp70.c and Tbj2 using immunoprecipitation and pull-down analyses.
(A) Immunoprecipitation with TbHsp70.c antibodies. *T. brucei* wildtype *427* v.221 cells (~1x10^6 cells/ml) were lysed and incubated with anti-TbHsp70.c peptide antibody and protein A/G agarose beads. Precipitated proteins were analysed with anti-TbHsp70.c antibody using western analysis. Experimental controls resolved on 10% SDS-PAGE gels, protein transferred to nitrocellulose membranes and Ponceau-stained: *Lanes 1* – negative control of 5 µg anti-TbHsp70.c peptide antibody, *2* - recombinant Tbj2 in *E. coli* BL21 (DE3) [pET28a-Tbj2]; western analysis of immunoprecipitation of Tbj2 using anti-TbHsp70.c peptide antibody and goat anti-rabbit secondary antibody: *3* – lysate incubated with protein A/G agarose beads, *4* – proteins precipitated from lysate using rabbit anti-TbHsp70.c peptide antibodies, including TbHsp70.c, possibly Tbj2 and the antibody heavy and light chains. (B) Pull-down of TbHsp70.c using recombinant Tbj2. *T. brucei* wildtype *427* v.221 cells (~1x10^6 cells/ml) were lysed and incubated with Tbj2 purified from *E. coli* BL21 (DE3) [pET28a-Tbj2] and protein A/G agarose beads. The presence of a protein interaction was assessed through western analysis using antibodies specific against TbHsp70.c (*upper panel*) and His-tagged recombinant Tbj2 (*lower panel*). *Lanes 1* – whole cell lysates, *2* – lysates incubated with protein A/G agarose beads, *3* – lysates incubated with protein A/G agarose beads and purified Tbj2, *4* - recombinant Tbj2 in *E. coli* BL21 (DE3) [pET28a-Tbj2], *5* - recombinant TbHsp70.c in *E. coli* BB1994 [pQE80-TbHsp70.c].

as a gene target was not tightly controlled, as partial TbHsp70.c protein expression was still evident (Fig. 5.5A *lane 2*). No reduction in protein levels of the 50 kDa band could be observed in the immunoblot (Fig. 5.5A), suggesting the band to be representative of a protein independent of TbHsp70.c, and not a degradation product. It thereby, along with the SDS-PAGE gel, showed loading of similar protein levels (Fig. 5.5A).

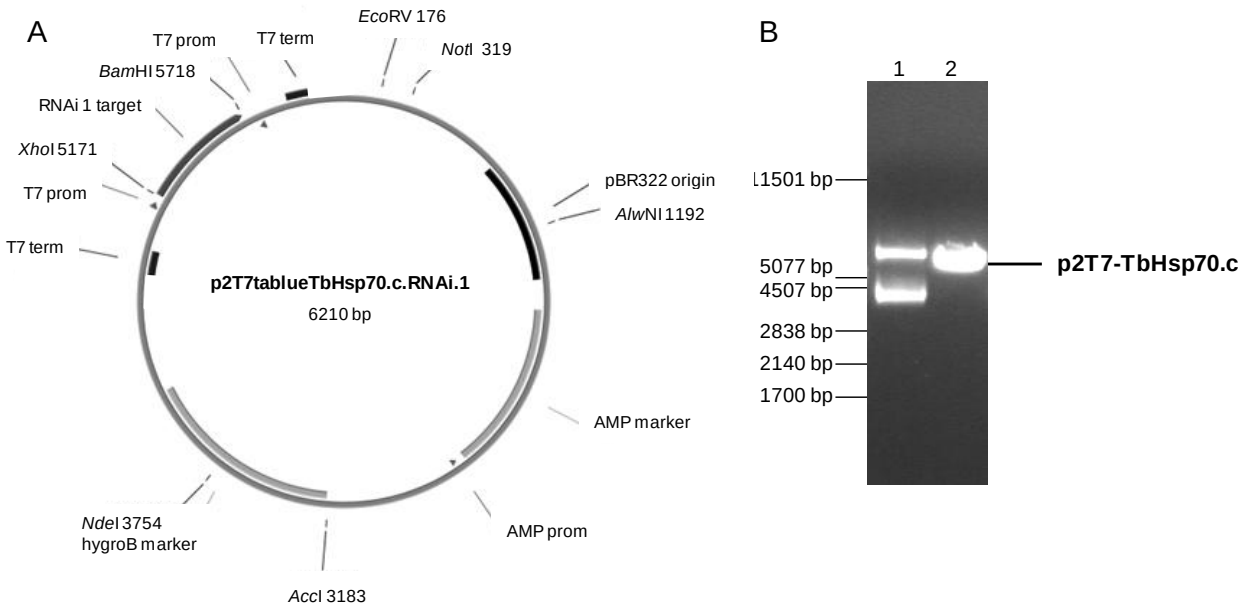


Figure 5.4. Plasmid map and restriction digestion analysis of p2T7^{TABlue}TbHsp70.c.RNAi.1
RNAi interference targets against a region of TbHsp70.c was successfully isolated from *T. brucei* genomic DNA and inserted into the p2T7^{TaBlue}. (A) A map of the resulting p2T7^{TaBlue}TbHsp70.c.RNAi.1 plasmid was generated using PlasMapper Version 2.0 (http://wishart.biology.ualberta.ca/PlasMapper/) (Dong *et al.*, 2004). (B) Restriction analysis of the RNAi target with restriction enzyme *Not*I allowed verification of the integrity of the construct by 0.8% agarose gel electrophoresis. *Lanes 1* – undigested p2T7^{TaBlue}TbHsp70.c.RNAi.1, *2* – single digest with *Not*I linearizing the plasmid DNA (6210 bp).

5.3.4.3. TbHsp70.c partial knockdown does not affect the phenotype of T. b. brucei

The reduction in TbHsp70.c protein levels upon tetracycline-induced RNAi knockdown was moreover analysed using a growth curve assay. Analysis of the population growth of *T. b. brucei* SMB cells by interference with TbHsp70.c expression showed no sign of a growth defect (Fig. 5.5B). The cell growth between RNAi-uninduced and RNAi-induced cultures were compared over a period of 72 hours, in which the cell density was monitored every 24 hours, subculturing cultures to maintain a cell population below 2 x 10^6 cells/ml. The effect of the dilution could be seen in the drop in the growth curve every 24 hours (Fig. 5.5B). Tetracycline and the p2T7^{TaBlue} vector alone do not influence *T. b. brucei* cell growth. A slight lag in growth was evident in the RNAi induced cells in the initial stages of the experiment when compared to the uninduced cells but after 72 hours of tetracycline induction, both induced and uninduced cells showed similar growth populations. A minor

drop in population growth was detected 48 hours after initiation of the experiment in both groups of transfected cells.

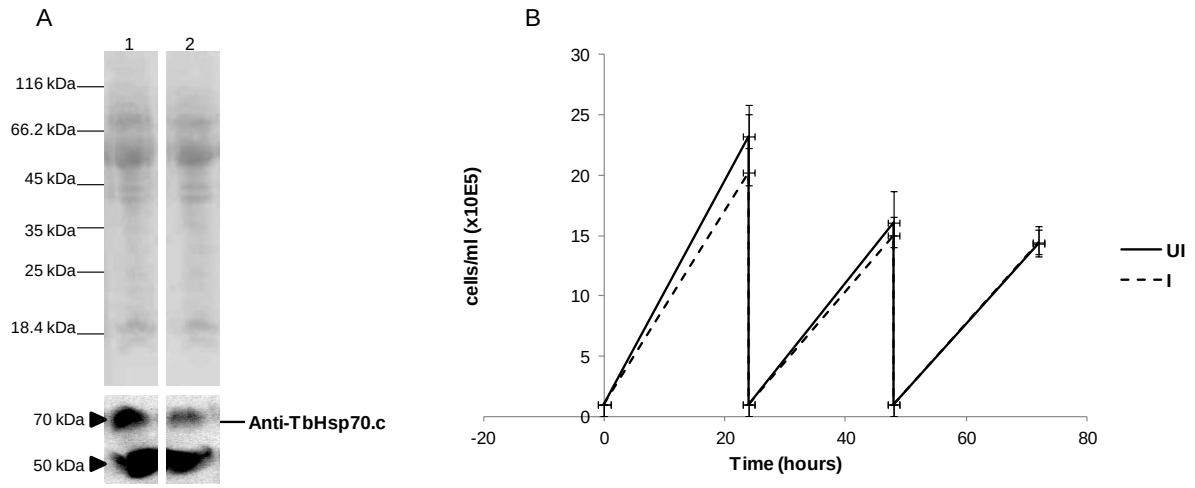


Figure 5.5. Analysis of RNA interference knockdown of TbHsp70.c expression.
(A) RNAi knockdown of TbHsp70.c induced by the addition of tetracyline was analysed by western analysis using TbHsp70.c antibodies (*lower panel*), and a 10% SDS-PAGE gel (*upper panel*) was used to show loading of equal amounts of protein. The analysis represents TbHsp70.c knockdown 72 hours after tetracycline induction. *Lanes 1* – uninduced RNAi knockdown of TbHsp70.c, *2*- induced RNAi knockdown of TbHsp70.c. (B) The phenotypic effect of RNAi knockdown of TbHsp70.c on *T. b. brucei* cells was assessed by means of growth curve analysis. The Y-axis represents 1 x 10^5 cells/ml and the X-axis the duration of the experiment, in hours. Cells were diluted to 1x10^5 cells/ml every 24 hours, demonstrated by the drop in cell population at those time points. *UI* - uninduced RNAi knockdown of TbHsp70.c, *I* - induced RNAi knockdown of TbHsp70.c. Cell densities represent the average of two independent clones.

5.3.5. TbHsp70.c localizes in the cytoplasm of the proliferative bloodstream form of *T. b. brucei.*

Anti-peptide antibodies specific against TbHsp70.c were produced specifically for the purpose of experimentally determining the subcellular localization of TbHsp70.c (Sections 2.3.5; 5.3.1). The anti-peptide antibody appeared to detect protein, but it is unclear whether the cellular distribution of TbHsp70.c or the contaminating proteins (Fig. 5.1, 5.2) is displayed. Immunofluorescence assays of TbHsp70.c produced images showing a cytoplasmic localization, and an apparent perinuclear distribution, in which the image is possibly displaying a part of the cytoplasm over the nucleus which is above another part of the cytoplasm (Fig 5.6); the fluorescence microscope used had no capability of confocal

imaging. This result was consistent with the previously predicted cytoplasmic localization of TbHsp70.c through *in silico* analysis (Louw *et al.,* 2010). However, a partial nuclear localisation cannot be ruled out without confocal imaging.

Observation of kinetoplast and nuclear morphologies stained with an intercalating agent can allow determination of the position of the trypanosome in the cell cycle. The trypanosomal kinetoplast always divides first and the succession of 1 nucleus: 1 kinetoplast (1N:1K) to 1N:2K to 2N:2K in the cell cycle can be observed. Immunofluorescence of *T. b. brucei* cells stained with propidium iodide revealed the different stages of the cell cycle (Fig. 5.7A). Analysis of the percentage cells observed in the stages of the cell division cycle showed the highest percentile (64.5%) to belong to the initial stages of cell division (1N:1K) and the lowest (8.9%) to belong to the final stage of the division cycle (2N:2K) (Fig. 5.7B). The cytoplasmic subcellular localization of TbHsp70.c did not appear to be influenced by the different stages of the cell cycle (Fig. 5.7A).

5.4. Discussion and conclusions

Investigation of the effect of heat shock on kinetoplastid molecular chaperones at the protein level has not really been explored; studies have placed more focus on the mRNA levels (Engman *et al*., 1995; Tibbetts *et al*., 1998; Requena *et al.,* 1992). In fact, very little experimental research has been conducted on molecular chaperones in *T. brucei* at all. In a study of the *T. brucei* parasite, bloodstream form cultures were exposed to heat shock to determine the impact of heat stress on endogenous protein levels of TbHsp70.c. Heat shock promoters are activated upon cellular stress, the activation of which is dependent on the heat shock element (HSE). Two potential HSE sequences have been identified in the 5´-UTR of *TbHsp70.c* according to the consensus sequence, CNNGAANNTTCNNG of HSE binding site of eukaryotes (Topoi *et al.,* 1985). Both *T. brucei* sequences CACGAAAGTTGTAC and CAGGAAGCATGGTG, 375 bp and 400 bp upstream of the start codon, respectively, have six of the eight HSE conserved nucleotides present, which has been shown to be a sufficient level of conservation (Lee *et al.,* 1990). A comparison of cultures grown under normal conditions to those exposed to heat stress revealed expression levels of TbHsp70.c to be

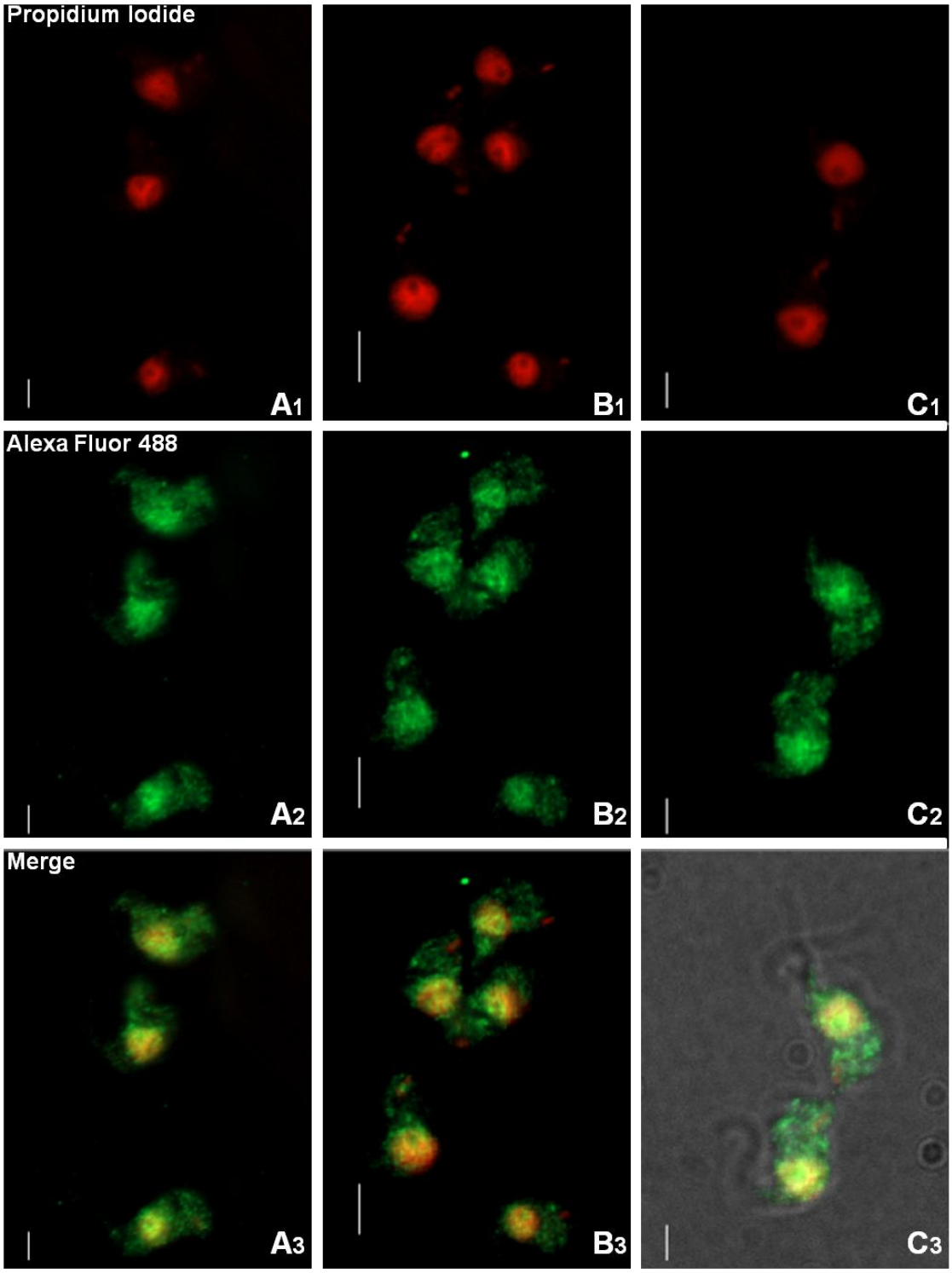
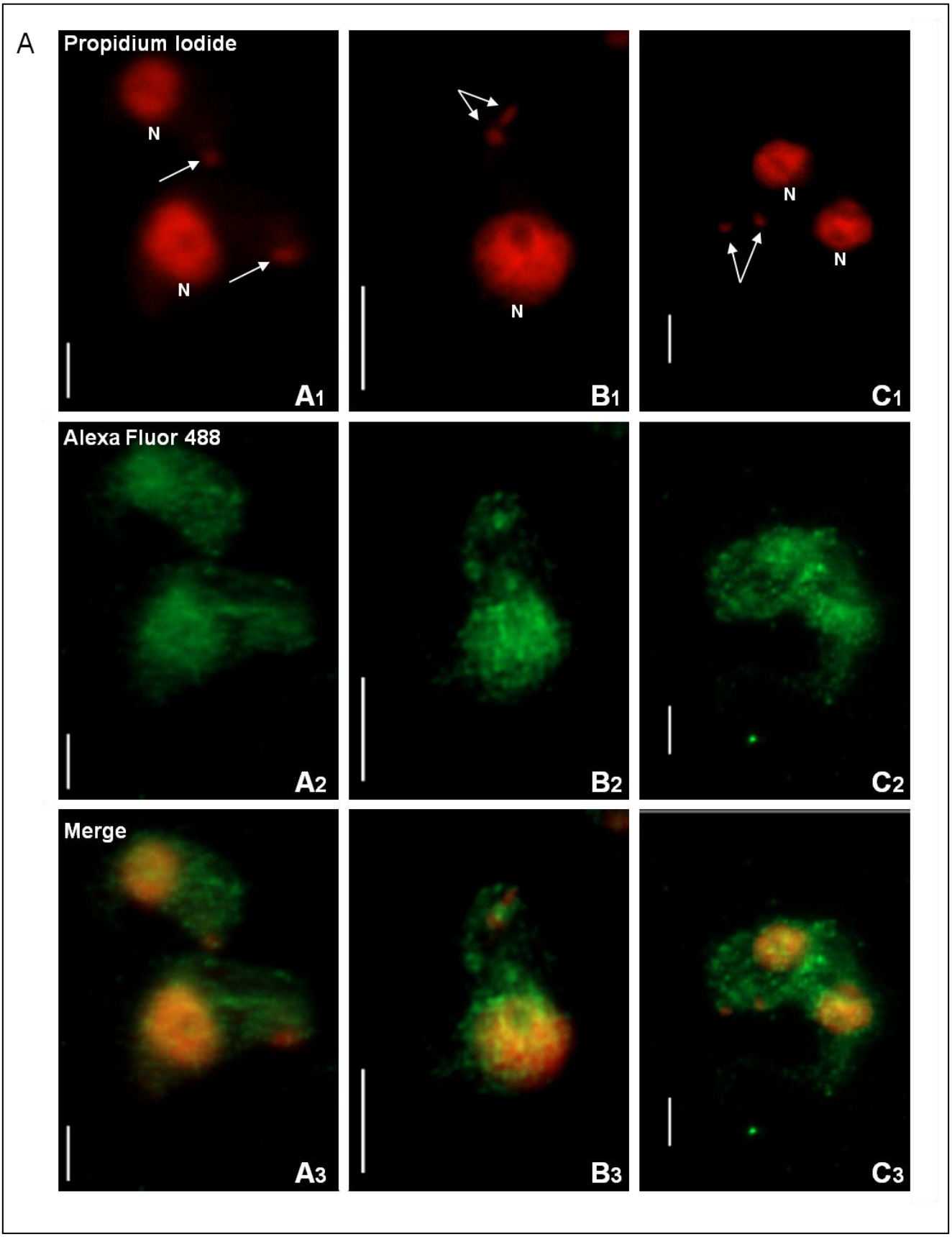


Figure 5.6. TbHsp70.c localization in *T. brucei brucei* bloodstream forms using immunofluorescence staining.
Distribution of TbHsp70.c in *T. b. brucei* cells using TbHsp70.c primary antibody, and Alexa Fluor 488 secondary antibody (A to C). TbHsp70.c appears to localize in the cytoplasm, and shows apparent perinuclear distribution in the parasite cells at various stages of the cell cycle. Parasite DNA was stained with propidium iodide. Upright scale bar, 5 µm. Rows: *Propidium iodide* - parasite DNA, including the nucleus and kinetoplast detected with a UV filter, shown in red; *Alexa Fluor 488* – localization of TbHsp70.c; *Merge* – amalgamated image of DNA staining and TbHsp70.c localization.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Propidium Iodide
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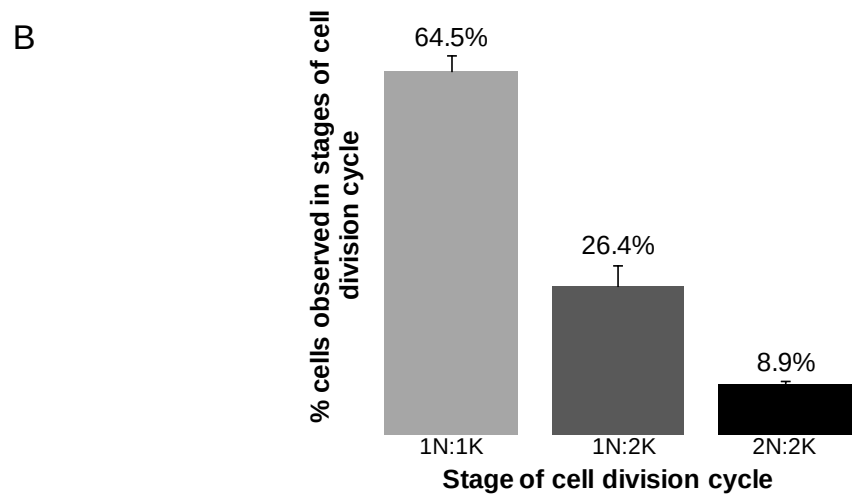

Figure 5.7. TbHsp70.c is cytoplasmic throughout the cell division cycle
(A) Morphology of the kinetoplast at different stages of the *T. brucei* bloodstream form cell cycle. The top row shows the propidium iodide signal in enlarged images of the number and location of nuclei (*N*) and kinetoplasts (*arrow*). TbHsp70.c remains localized in the cytoplasm and perinucleus of the cell through different stages of its cell cycle (A to C). Upright scale bar, 5 µm. Columns: *A* – cells have 1 nucleus and 1 kinetoplast (1N:1K); *B* – 1N:2K; *C* – 2N:2K. Rows: *Propidium iodide* - parasite DNA detected with a UV filter, shown in red; *Alexa Fluor 488* – localization of TbHsp70.c; *Merge* – amalgamated image of DNA staining and TbHsp70.c localization. (B) The percentage *T. b. brucei* cells counted containing 1N:1K, 1N:2K and 2N:2K. Error bars indicate the standard deviation between two replicates.

increased subsequent to heat shock; TbHsp70.c. This finding would imply that TbHsp70.c is required for protein quality control not only under normal conditions, but when the cell is placed under thermal stress upregulation of TbHsp70.c facilitates the survival of the parasite in the mammalian bloodstream. This is the first report of data showing that the expression levels of TbHsp70.c are increased by heat stress. The level of upregulation correlated with what was observed for heat-inducible Hsp40, Tbj2, under the same conditions (Ludewig and Blatch, 2013). Tbj2 was thus also suggested to be necessary for protein quality control under both normal and stressed conditions. Type II Hsp40 from *T. cruzi*, Tcj6, showed a 2-fold protein upregulation upon heat shock (Salmon *et al.,* 2001). TcHsp70 mRNA levels showed a very significant four-fold upregulation upon heat stress at 37°C when compared to mRNA levels at 28°C (Requena *et al.,* 1992). The expression levels of the two non-specific bands detected by TbHsp70.c antibody remained constant upon heat shock, suggesting that they represent separate proteins and are not degradation products of TbHsp70.c. The non-specificity of anti-TbHsp70.c in detecting proteins other than the target presents

complications with regard to using the antibody in immunofluorescence studies. The result obtained would not necessarily be due to TbHsp70.c alone.

Observations from the work reported in the previous chapter implicated Tbj2 as a probable co-chaperone of TbHsp70.c. Binding studies were thus performed to provide conclusive evidence of the TbHsp70.c-Tbj2 partnership. However, both the immunoprecipitation and the pull down assay provided inconclusive data. Similar molecular masses between the antibody heavy chain and recombinant Tbj2 made it difficult to ascertain whether Tbj2 had co-precipitated with TbHsp70.c (Fig. 5.3A). Furthermore, no validation of the partnership was provided by the pull-down assay due to non-specific binding of TbHsp70.c to the Protein G Agarose beads in the absence of Tbj2 (Fig. 5.3B). Both attempts at elucidating the association of TbHsp70.c and Tbj2 proved futile, and the assays require optimization. The association between the chaperones may be too transient to be detected by these two assays in which case an alternative technology would be an *in vitro* biosensor application, surface plasmon resonance (SPR). SPR is utilized for the qualitative and quantitative characterization of specific molecular interactions (Schuck, 1997; Ramakrishnan *et al.*, 2006). The nature of the probable Tbhsp70.c-Tbj2 partnership could thus be investigated. Furthermore, SPR would facilitate characterization of the likely unique interaction of TbHop with TbHsp70.c, and subsequently TbHsp90. A different route to investigating the *in vivo* transient protein-protein interactions between TbHsp70.c and its co-chaperones would be through the use of fluorescence resonant energy transfer (FRET), which involves non-radiative transferral of energy between acceptor and donor fluorophores (Jares-Erijman and Jovin, 2003) and allows tagging the two proteins of interest with different fluorescent proteins (Nguyen and Daugherty, 2005).

dsRNA targets and subsequent knockdown of protein expression leading to altered phenotypes have been demonstrated for various *T. brucei* Hsp70 proteins. RNAi knockdown of two endoplasmic reticulum *T. brucei* Hsp70 proteins (Tb09.160.3090 [TbHsp70.a], Tb09.211.1390 [TbGrp170]), and one cytoplasmic TbHsp70 (Tb11.01.3110 [TbHsp70]) resulted in compromised VSG morphology, biosynthesis and proliferation (Field *et al.*, 2010). Alsford and colleagues developed a high-resolution genomic scale of fitness data delivered upon RNAi knockdown in an RNAi target sequencing (RIT-seq) approach, in which more than 99% of the non-redundant *T. brucei* gene-set was assessed (Alsford *et al.*, 2011). In this study, TbHsp70.c protein expression was successfully reduced through

transcription of dsRNA. Previous studies using the tetracycline-inducible RNAi system have indicated that a partial reduction in protein levels can effectively demonstrate a loss of function (Bonhivers *et al.,* 2008). Cytoskeletal flagellar pocket protein, BILBO1, was shown to be essential for flagellar pocket biogenesis by a mere 50% reduction of protein levels (Bonhivers *et al.,* 2008). Knockdown of TbHsp70.c in the bloodstream form cells resulting in partial reduction of TbHsp70.c levels showed no physiological relevance to *T. b. brucei* bloodstream form cells (Fig. 5.5B). This result was not unexpected as previous studies of depletion of TbHsp70.c by RNAi interference was observed to result in no phenotypic effect on *T. brucei* bloodstream form cells, or procyclic form cells (Alsford *et al.,* 2011). However, a significant loss-of-fitness was observed in cells induced throughout growth as BSF, through differentiation to growth as procyclic forms (Alsford *et al.,* 2011). Testing for the stress-sensitivity of *T. brucei* cells depleted of TbHsp70.c by monitoring growth whilst inducing heat shock, altering pH and exposing the cells to small molecule modulators in differentiation experiments would provide conclusive evidence to the importance of TbHsp70.c to *T. b. brucei* cells during cell differentiation.

That, along with the knowledge that Tbj2 has previously been demonstrated to be essential to the survival of the *T. brucei* BSF cell (Ludewig and Blatch, 2013), can further be investigated to conclusively define the relationship between Tbj2 and TbHsp70.c, and characterize their mechanisms of interaction. Furthermore, the presence of 12 Hsp70 proteins within *T. brucei*, of which five are predicted to be cytoplasmic (Section 2.1), would suggest that another TbHsp70 could complement for the lack of function of TbHsp70.c. RNA interference studies and SPR binding studies would likely reveal knowledge that could be exploited for drug targeting. Furthermore, the relationship TbHsp70.c may have with TbHop, therein the formation of a chaperone complex with TbHsp90, a protein family already well-studied, could allow identification of attractive drug targets. TbHsp90 is essential for *T. brucei* cell survival and has already been implicated as a potential chemotherapeutic target in African Trypanosomiasis (Jones *et al.,* 2008), as well as in Chagas disease (Graefe *et al.,* 2002).

This is the first experimental investigation of the subcellular localization of TbHsp70.c, and resulting data showed an apparent cytoplasmic localization (Fig. 5.6; 5.7). The finding coincided with previous *in silico* analysis performed by Louw and colleagues (2010), which predicted TbHsp70.c to be cytoplasmic through WoLF PSORT (wolfpsort.org; Horton *et al.,* 2007). TbHsp70.c also appeared to maintain its cytoplasmic localization in the different

stages of the *T. b. brucei* cell division cycle (Fig. 5.7). Unfortunately the anti-TbHsp70.c peptide antibody also detected two additional proteins. Detection of TbHsp70.c in *T. b. brucei* lysates using western analysis indicated the presence of a second band at ~50 kDa (Fig. 5.1), which may have been a degradation product of TbHsp70.c. However, upon heat stressing *T. b. brucei* cells, increased expression levels of TbHsp70.c were not accompanied by a subsequent increase in the protein that corresponds to the 50 kDa band (Fig. 5.2). RNAi knockdown of TbHsp70.c furthermore confirmed that the protein represented by the 50 kDa band is not associated with TbHsp70.c as it showed no lowered protein expression levels upon RNA interference (Fig. 5.5). Even though the localization data generated for TbHsp70.c correlated with what had previously been predicted (Louw *et al.,* 2010), it could be argued that anti-TbHsp70.c peptide antibodies are not likely to give an accurate representation of the subcellular localization, due to the cross-reactivity with the second non-identified protein. Further affinity purification using an affinity column with the peptide as the ligand may have provided an antibody in which less cross-reactivity is observed; anti-TbHsp70.c peptide antibody will be affinity purified using Pierce's Sulfolink before conducting any subsequent analysis using this antibody. Further experimental confirmation of the distribution of TbHsp70.c could be obtained by fluorescently tagging an endogenous gene, for which vectors for the expression of the tagged proteins are readily available (Kelly *et al.,* 2007). Confocal laser scanning microscopy, which allows imaging of focal planes at various depths by z-stacking (Pawley, 2006), would likely provide the most conclusive experimental evidence as to the apparent localization of TbHsp70.c (Fig. 5.6).

Future studies involving RNAi in combination with immunofluorescence showing the parasite phenotype upon knockdown of TbHsp70.c could further enhance the work begun in this study. Tbj2 was previously shown to localize in the cytoplasm and to have a crucial impact on the phenotype of the *T. brucei* bloodstream form parasite via RNAi knockdown studies (Ludewig, Pers. Comm., 2010). Its localization increases the likelihood of Tbj2 partnering with TbHsp70.c as its co-chaperone. The results shown in this study support the probability of the TbHsp70.c/Tbj2 partnership but future binding studies such as SPR or FRET would provide conclusive evidence.

This study is the start of a functional characterization of TbHsp70.c, along with its probable partnership with co-chaperone Tbj2. Improved antibodies specific against both TbHsp70.c and Tbj2 would enable further investigation into localization and co-localization, and

immunoprecipitation studies. This research opens up prospects for further studies of the yet unexplored Hsp70/Hsp40 partnerships in *T. brucei*. Characterization of these and other chaperone - co-chaperone interactions could further enhance understanding of the *T. brucei* parasite biology.

CHAPTER 6

FINAL CONCLUSIONS AND FUTURE PERSPECTIVES

Final conclusions

The importance of Hsp70 proteins have been demonstrated in their roles in cell differentiation and cytoprotection. Moreover, the chaperone activity of Hsp70 proteins is often modulated through its co-chaperone partners and associations with substrates. The regulatory effects of binding partners have previously been investigated in the development of drug targets. These studies set out to biochemically characterize a *T. brucei* Hsp70 protein, TbHsp70.c, in terms of molecular chaperone function; and to identify its specific TbHsp70-TbHsp40 partnerships.

Bioinformatic analyses of the *T. brucei* complement of 12 Hsp70 proteins led to the identification of a novel Hsp70, namely TbHsp70.c. Its unusual features warranted its biochemical characterization and identification of potential partner proteins. Only 4 of the 12 Hsp70 proteins, including TbHsp70.c, were predicted to be cytoplasmic. A multiple sequence alignment of the cytoplasmic Hsp70 proteins from the TriTryps revealed some loss of conservation in TbHsp70.c and its orthologues in *T. cruzi* and *L. major*. The distinctive differences detected in this novel group of Hsp70.c proteins included atypical acidic residues within the substrate binding domain and the absence of the well conserved EEVD motif in the C-terminal domain. The importance of the EEVD motif in cytoplasmic Hsp70 members has been demonstrated in its binding with the TPR domains of Hsp40, Hop and CHIP (Freeman *et al.*, 1996; Demand *et al.*, 1998). The absence of an EEVD motif has been shown to result in a reduced ability of Hsp40 to stimulate the ATPase activity of Hsp70 (Demand *et al.*, 1998). Residues shown to be important in the bipartite interaction of DnaJ with DnaK (Karzai and McMacken, 1996; Suh *et al.*, 1998), however, were well conserved across all the aligned proteins. A homology model of the substrate binding domain of TbHsp70.c was generated using bovine Hsc70 as a template. Due to the loss of conservation in the C-terminus of TbHsp70.c, the helical lid could not be modelled. The *T. brucei* complement includes 65 Hsp40 proteins (Folgueira and Requena, 2007), and through bioinformatic analysis probable Hsp40 partners were selected based on predicted localization, the presence of a functional J-domain and residues essential in the Hsp70-Hsp40 interaction. Based on these criteria, both Tbj2 and Tbj3 are feasible candidates for interaction with TbHsp70.c. Furthermore, Tbj2 localizes in the cytosplasm and is essential to the survival of the *T. brucei* parasite (Ludewig, 2012). Tbj3 has been suggested to be paralogous to Tbj2 (Ludewig PhD Thesis, 2010), a pairwise alignment showing a 43% sequence identity. Tbj3, with an

extended C-terminal domain, is 12 residues longer than Tbj2. Tbj2 observed to share a slightly higher percentage sequence identity with the four human Hsp40 proteins (DnaJA1, DnaJA2, DnaJA2b, DnaJA4), as well as with *Arabidopsis thaliana* A2 and A3 than was observed for Tbj3 (Ludewig PhD Thesis, 2010). Tbj2 was selected in preference to Tbj3 for experimentally determining its ability to function as a co-chaperone of TbHsp70.c for two primary reasons: the first is that Tbj2 contains two putative heat shock elements 145 bp and 180 upstream of the start codon (Hertz-Fowler *et al*., 1999), where as Tbj3 does not appear to contain a heat shock element. The second is knockdown of Tbj2 showed a loss of fitness in bloodstream form experiments (Ludewig and Blatch, 2013) and is thus essential to the survival of the bloodstream form parasite. Knockdown of Tbj3 only demonstrated a loss of fitness in procyclic form experiments (Alsford *et al.,* 2011). Our research has been focusing primarily on bloodstream form experiments. Future work would entail investigating whether Tbj3 functions as a co-chaperone of TbHsp70.c. Bioinformatic tools also enabled the design of polyclonal anti-TbHsp70.c peptide antibodies specific against TbHsp70.c, to be used for subsequent biochemical characterization of the target protein.

The successful amplification of the coding region of TbHsp70.c from *T. brucei* genomic DNA allowed optimization of its expression in and purification from *E. coli*. The coding region of TbHsp70.c was inserted into an expression vector with an N-terminal His-tag. Insertion of TbHsp70.c into an additional C-terminally His-tagged expression vector was attempted, but was unsuccessful. The N-terminal tag may influence the ATPase activity of TbHsp70.c, and a C-terminal His-tagged protein would have served as a good control for subsequent protein assays. TcHsp70B and Tbj2 were also successfully expressed and purified. A complicating factor in the purification of heterologous molecular chaperones from *E. coli* is the co-purification of DnaK; its basal expression may mask the chaperone activity of the protein being investigated. TbHsp70.c was purified from an *E. coli dnaK* minus strain, however, inaccessibility to a *dnaK* minus strain compatible with the pET expression vectors compelled optimization of the purification protocol for Tbj2 and TcHsp70B using ATP and glycerol washes to remove contaminating DnaK since Guo and colleagues (2007) employed the use of glycerol for this purpose. However, residual levels of DnaK could still be detected through western analysis, which was corrected for in subsequent ATPase assays. This method was thus not ideal in ridding purified protein of contaminating DnaK and future research would entail the use of an *E. coli dnaK* minus strain for the purification of Tbj2 and

TcHsp70B. Purified protein yields for TbHsp70.c, TcHsp70B and Tbj2 were low and detergent sarkosyl was utilized to enhance protein yield. Solubility studies of all three proteins showed that these proteins were not insoluble but likely trapped in the cellular debris, lowering the overall protein yield. Sarkosyl allowed for increased protein yields sufficient for biochemical characterization.

Investigation of the oligomeric state of TbHsp70.c revealed the formation of monomers in extended conformations and higher order oligomeric species. Hsp70 proteins have been reported to exist in various oligomeric states, including dimers (Boshoff *et al.,* 2008), monomers and trimers (Benaroudj *et al.,* 1995). Tbj2 was shown to form a monomeric species. The successful native purifications of TbHsp70.c, Tbj2 and TcHsp70B, purified for the purposes of a control, enabled subsequent biochemical characterization.

Proteins TbHsp70.c, TcHsp70B and Tbj2 purified in the absence and presence of sarkosyl maintained the same degree of chaperone activity; by implication their native state was not compromised by the addition of the detergent. An effective approach to investigating the secondary structure as well as the aggregated state of purified protein is FTIR (Natelello *et al.,* 2007). FTIR was used to investigate whether purified TbHsp70.c and Tbj2 were in their native states for use in subsequent *in vitro* assays; however, the resulting data was not conclusive as the purified protein concentrations used for both proteins was too low, even when purified in the presence of sarkosyl, to ensure that the proteins were in their native state (data not shown). An alternative tool is circular dichroism (CD) which allows determination of both the protein secondary structure and folding properties (Greenfield, 2006). Chaperone function was demonstrated for TbHsp70.c through its ability to bind and suppress aggregation of both thermolabile MDH and rhodanese as substrates. The unique features of TbHsp70.c including the acidic residues in the substrate binding domain and the lack of a conserved EEVD motif at the C-terminus did not appear to influence TbHsp70.c's holdase-function. TbHsp70.c suppressed MDH aggregation more effectively than what was observed for rhodanese aggregation, a similar trend was observed in the ability of TcHsp70B to suppress aggregation of these two substrates. Tbj2 showed successful suppression of MDH aggregation independent of an Hsp70 protein, coinciding with previous findings that aggregation of unfolded polypeptides is prevented through binding of Hsp40 proteins. *T. brucei* cytoplasmic substrates would have provided a truer reflection of the chaperone activity

of TbHsp70.c and Tbj2. A refolding assay, such as the luciferase refolding assay, would provide a more accurate representation of the ability of Tbj2 to enhance the ability of TbHsp70.c to refold protein; ATP is absent from the MDH aggregation suppression assay and it is likely that some Tbj2 is binding to TbHsp70.c as substrate and not as co-chaperone.

A shortcoming in this study was the absence of a substrate binding assay, particularly because TbHsp70.c contains atypical features in its substrate binding domain. A fluorescence anisotropy peptide substrate binding assay has been used to investigate the binding affinity of various peptides to isolated substrate binding domains of *E. coli* DnaK, Sse1, Sse2 and human Hsp110 and demonstrated the preference of different hydrophobic sequences for each chaperone (Xu *et al.*, 2011). Further research would entail utilizing the fluorescence anisotropy peptide substrate binding assay to elucidate unique differences in the substrate binding of TbHsp70.c.

TbHsp70.c displayed the ability to effectively hydrolyse ATP. Investigation of the protein-protein interaction between TbHsp70.c and Tbj2 revealed the ability of Tbj2 to enhance both the holdase-function of TbHsp70.c in aggregation suppression assays, and the ATPase activity of TbHsp70.c. Multiple contact points between Hsp70 and Hsp40 proteins have been established (Demand *et al.*, 1998; Aron *et al.* 2005). Interestingly, in a study of the *E. coli* proteome, most proteins were predicted to contain numerous binding sites for DnaK and DnaJ, and twice as many predicted for DnaJ, and refolding conducted by the DnaK-DnaJ machinery is inhibited by large DnaJ concentrations (Srinivasan *et al.*, 2012). The frequency of DnaK and DnaJ interaction sites are suggested to be an indication of most favourable stoichiometry necessary for refolding (Srinivasan *et al.*, 2012). Previous research has indicated that catalytic amounts of Tbj2 are required to stimulate the ATPase activity of Hsp70. However, this study has revealed that Tbj2 in excess stimulates TbHsp70.c ATPase activity. It is possible that Tbj2 does not function as a co-chaperone of TbHsp70.c, but is binding as substrate to TbHsp70.c.

Further characterization of the ATPase activity of TbHsp70.c would entail investigating the activation of the ATPase activity of TbHsp70.c by various substrates, including substrate reduced carboxymethylated lactalbumin (RCMLA) and A7 peptide (Arg-Arg-Leu-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly). Maximal stimulation of the ATPase activity of Hsp70

proteins has been demonstrated to take place in the presence of both co-chaperone and substrate (Laufen *et al.,* 1999). RCMLA was reported to have no effect on the ATPase activity of TcHsp70 (Edkins *et al.,* 2004). However, stimulation of the ATPase activity of TcHsp70 by Tcj2 was shown to be further enhanced in the presence of RCMLA (Edkins *et al.,* 2004). The ATPase activity of yeast Hsp70, Ssa1, was shown to be stimulated by both RCMLA and A7 peptide (Needham and Masison, 2008).

Further studies involving the specific binding sites of protein-protein interactions could be investigated using nuclear magnetic resonance (NMR), specifically the more precise cross-saturation (CS) approach (Nishida and Shimada, 2012). The interaction between chaperones is often a weak and transient one (Buyong *et al.,* 2000), and NMR-based approaches can be applied to these particular complex formations more readily than other structural methods (Bonvin *et al.,* 2005). The lack of an EEVD motif promotes interest as to the mechanism of action of TbHsp70.c with cofactors such as TbHop and TbCHIP containing TPR domains.

The effect of two small molecule modulators on the chaperone activity of TbHsp70.c was investigated. Quercetin has previously been reported as an inhibitor of Hsp70 ATPase activity (Lang and Racker, 1974; Murakami *et al.,* 1992). The finding of the inhibition of the basal ATPase activities of both TbHsp70.c and TcHsp70B was therefore not unexpected. Quercetin also demonstrated the ability to significantly inhibit the Tbj2-stimulated ATPase activity of TbHsp70.c, and the Tbj2-stimulated ATPase activity of TcHsp70B, albeit to a lesser degree. The second compound screened, MB, has been documented to result in >80% inhibition of Hsp70 ATPase activity (Jinwal *et al.,* 2010), yet displayed only a slight inhibition of TbHsp70.c ATPase activity. Surprisingly, MB enhanced the Tbj2-stimulated ATPase activity of TbHsp70.c. Furthermore, MB enhanced the holdase-function of both TbHsp70.c and alfalfa Hsp70 in aggregation suppression of MDH. To the best of our knowledge this is the first report of MB stimulating chaperone activity. Puzzling is the fact that the residues Cys267 and Cys306 previously identified to be oxidized by MB resulting in inhibition of activity (Miyata *et al.,* 2012), are conserved in TbHsp70.c. It is possible that the Cys267 and Cys306 residues of TbHsp70.c in partnership with Tbj2 are buried or masked, resulting in a loss in ability of MB to oxidize these key residues thereby displaying enhanced chaperone activity as opposed to inhibition. However, through investigating the chaperone activity of TbHsp70.c and defining an interaction with probable co-chaperone Tbj2, these

initial experiments could be followed by investigations of the specific protein-protein interactions of TbHsp70.c-Tbj2; a functional partnership could subsequently be targeted by small molecule modulators. More recent inhibitors include guanidinium chloride, shown to inhibit the ATPase activity of Hsp104 and the nucleotide binding affinities of Hsp100 (Zeymer *et al.*, 2013), mizoribine was shown to inhibit the folding cycle of Hsp60-Hsp10 and to decrease its ATPase activity (Tanabe *et al.*, 2012), and iminosugar (+)-Lentiginosine was shown to inhibit the ATPase activity of Hsp90 (Piaz *et al.*, 2012). Pyrimidinones have been shown to inhibit the ATPase activity of yeast Hsp70, Ssa1, HsHsp70 and PfHsp70. Sub-classes JAB_{75}, $MAL_{2\text{-}61}$, and $MAL_{2\text{-}215}$ inhibited the ATPase activity of HsHsp70 by 30% (Chiang *et al.*, 2009). $MAL_{3\text{-}39}$ was shown to limit the rate of ATP hydrolysis for Ssa1, HsHsp70 and PfHsp70 (Chiang *et al.*, 2009). DMT_{2264} demonstrated a greater degree of inhibition of ATP hydrolysis of PfHsp70 than Ssa1 or HsHsp70 (Chiang *et al.*, 2009). The immunosuppressant, 15-dexoyspergualin (DSG), has been demonstrated to enhance the ATPase activity of plasmodial Hsp70 proteins PfHsp70-1 and PfHsp70-2 (Ramya *et al.*, 2006). Structural analogues of DSG, the dihydropyrimidines $MAL_{3\text{-}38}$ and $MAL_{3\text{-}90}$, were shown to specifically inhibit the ATPase activity of yeast Hsp70 only if an Hsp40 protein was present (Fewell *et al.*, 2004). These are inhibitors that could be investigated in future studies to determine their effects on TbHsp70.c and its co-chaperones.

TbHsp70.c is expressed in both the bloodstream and procyclic stages of *T. brucei* (Alsford *et al.*, 2011). *In vivo* analysis of TbHsp70.c revealed that protein expression was enhanced upon heat shock, suggesting its importance in protein quality control under stressed conditions in the cell. RNAi of the bloodstream form of TbHsp70.c only showed partial knockdown. Previous studies have revealed that a partial reduction in protein expression may still result in effective loss of function (Bonhivers *et al.*, 2008). However, this was not the case with TbHsp70.c as no detrimental effect was evident in the *T. brucei* cells. A similar result was obtained by Alsford and colleagues (2011); they did, however, show that a deficiency of TbHsp70.c through differentiation from bloodstream to procyclic form does result in a significant loss-of-fitness. By implication, another of the four cytoplasmic *T. brucei* Hsp70 proteins may compensate for the lack of TbHsp70.c function. Should a different *T. brucei* cytoplasmic Hsp70 protein replace its function, TbHsp70.c loses its value as a drug target. This suggests that Tbj2, or any interacting partner of TbHsp70.c would be a better choice for a drug target. The *in vivo* interaction between Tbj2 and TbHsp70.c was investigated using

immunoprecipitation and pull down assays. However, no conclusive data was obtained. Future experiments in which improved antibodies, specific towards Tbj2 and TbHsp70.c, may demonstrate an interaction. Investigation of the subcellular localization of TbHsp70.c showed a cytoplasmic/perinuclear localization. *In vivo* analysis of TbHsp70.c was performed with an anti-TbHsp70.c peptide antibody, which did not appear to be specific for TbHsp70.c since two additional proteins were detected. These two proteins are probably non-specific and not degradation products of TbHsp70.c due to the uniform expression observed upon inducing TbHsp70.c protein expression through heat shock. This was confirmed upon knockdown of TbHsp70.c, when the 68 kDa band was observed to remain constant in its level of protein expression; it most likely do not form part of TbHsp70.c. However, due to the presence of these non-specific reactions, this anti-TbHsp70.c peptide antibody should not be used for any subsequent *in vivo* analysis. It is possible that the approach taken in synthesizing and conjugating the peptide, using the carrier protein keyhole limpet heamocyanin (KLH), resulted in the cross-reactivity of anti-TbHsp70.c peptide antibody with other proteins due to its extreme immunogenicity.

Future perspectives

Preliminary studies using bioinformatics revealed the presence of HIP, CHIP and BAG proteins within the *T. brucei* genome; their numbers within the heat shock protein complement has not yet been ascertained. Further bioinformatic analysis will allow identification and prediction of localization of all the *T. brucei* HIP, CHIP and BAG proteins for subsequent analysis. Both HIP and BAG binds the ATPase domain of Hsp70, however, they affect the ADP-bound state of Hsp70 in opposing directions. Human hHIP is known to stabilize the ADP-bound state of Hsp70 through its substrate binding domain, whilst cofactor hBAG-1 stimulates the release of ADP (Höhfeld *et al.,* 1995). hHIP stabilizes the interaction of Hsp70 with signalling molecules such as hormone receptors and some kinases, allowing recruitment and subsequent complex formation with Hsp90 (Frydman and Höhfeld, 1997). In turn, hHop serves to couple Hsp70 with Hsp90 (Frydman and Höhfeld, 1997). A study of TbHIP, TbBAG and TbHop with TbHsp70.c could provide an interesting biochemical characterization of this functional complex and its mechanism. Only one point of interaction has been demonstrated for Hop and CHIP with Hsp70, it would thus be interesting to investigate if there is any form of interaction of TbCHIP and TbHop with TbHsp70.c. Future research would thus entail isolation, expression and purification of these proteins to enable a

thorough investigation of the protein-protein interactions with TbHsp70.c using alternative approaches such as SPR or NMR to identify specific binding sites. Investigations into the partnerships of TbHsp70.c with its potential co-chaperones, TbHop and TbHsp40s, and cofactors, TbHIP, TbCHIP and TbBAG may reveal the protein network in which TbHsp70.c participates. Once a functional chaperone complex has been identified, subsequent *in vivo* analysis can be performed using antibodies to target these proteins and can be used for co-localization studies which may advance the current understanding of the *T. brucei* parasite biology.

Methylene blue is known to bind ATP and residual levels of ATP present in purified proteins may alter the activity shown by MDH aggregation suppression assays. MB was observed to stimulate the Hsp70 activity in its ability to suppress MDH aggregation. In addition to the potential masking of TbHsp70.c residues recognized and oxidized by MB – it is possible that MB bound the low levels of ATP present in the purified protein and resulted in enhanced suppression of aggregation; the presence of ATP results in the release of bound substrate from Hsp70. Furthermore, MB was expected to inhibit the ATPase activity of TbHsp70.c to a much greater degree than was observed. As before, should residual levels of ATP be present in the purified protein, MB will bind it, suggesting a lowered oxidizing activity (Hsp70 inhibition) will be observed. Future investigations will entail using the same Hsp70 protein as used in the study of Jinwal *et al.*, (2010) to firstly determine that the MB being used is active by achieving similar levels of ATPase inhibition. Secondly, experiments using controls such as dihydropyrimidines 115-7c and SWO2, reported to accelerate the ATPase activity of Hsp70 by ~45% (Jinwal *et al.*, 2010) could provide an indication of whether MB truly functions as an accelerator of the chaperone activity of TbHsp70.c. Furthermore, identification of small molecule modulators with greater affinity and selectivity would be useful in the process of drug discovery against HAT, followed by *in vivo* assays to determine toxicity of the compound to the parasite and human cell lines.

Future work using confocal laser scanning microscopy and improved antibodies specific against TbHsp70.c would allow imaging at various depths of the focal planes to establish the localization (Pawley, 2006). However, a different approach would entail the design and production of an endogenously tagged fluorescent TbHsp70.c protein (Kelly *et al.*, 2007). TbHsp70.c was not predicted to contain any localization signals, so the danger of a

fluorescently-tagged fusion protein masking the signal would not pose a problem. The *in vitro* analysis of the TbHsp70.c-Tbj2 partnership has not provided conclusive evidence that Tbj2 co-chaperones TbHsp70.c. The results obtained from the aggregation assays would suggest that is binding as substrate to TbHsp70.c. However, the ATPase activity assays demonstrated the ability of Tbj2 to stimulate the ATPase activity of TbHsp70.c to a considerable degree. Tbj2 is thus a likely *in vivo* co-chaperone of TbHsp70.c. However, further experimental analysis including luciferase refolding assays, immunoprecipitation assays using improved antibodies specific towards Tbj2 and TbHsp70.c, SPR binding studies and *in vivo* co-localization studies will be conducted to conclusively elucidate Tbj2 as co-chaperone of TbHsp70.c. In addition, Tbj2 is essential to the cell and demonstration of its interaction with TbHsp70.c, both through binding studies and co-localization, may allow further elucidation of the partnerships shared between Hsp70 and Hsp40 proteins. Antibodies specific against both TbHsp70.c and Tbj2 are necessary. Whether TbHsp70.c can function as a canonical Hsp70 cannot be concluded from the content of this study. Further research, such as the fluorescence anisotropy peptide substrate binding assay, the luciferase refolding assay, and the ability of various substrates to enhance the ATPase activity of TbHsp70.c alone, and with Tbj2 will be conducted to conclusively determine if TbHsp70.c can function as a canonical Hsp70; due to its atypical features, TbHsp70.c is likely to have a unique function that has not yet been discovered. The absence of an EEVD motif warrants the investigation of interaction with TbHop as well as Tbj2. A more thorough *in vivo* functional characterization of TbHsp70.c along with its potential partners, TbHop, TbHip, TbCHIP and probable co-chaperone Tbj2 would enable an enhanced understanding of parasite biology. This knowledge would allow identification of potential drug targets in the on-going search for improved drugs against HAT. TbHsp70.c is essential to parasite survival during differentiation from bloodstream to procyclic form; future experiments will investigate partnerships with co-factors and co-chaperones during this process to further identify potential targets for drug development.

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